Cloning, Expression and Purification of Epidermal Growth Factor Receptor for Aptamer Selection for certain Bioassays

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in Partial Fulfilment of the

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Doctor of Philosophy

by

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DECLARATION

This is to declare that the matter embodied in this thesis entitled **"Cloning, Expression and Purification of Epidermal Growth Factor Receptor for Aptamer Selection for certain Bioassays"** is the result of investigations carried out by me under the supervision of **Prof. Utpal Bora**, and is submitted to the Indian Institute of Technology Guwahati, Guwahati-781039, Assam, India for the award of degree of *Doctor of Philosophy in Biosciences and Bioengineering*. This work has not been submitted elsewhere for any degree or diploma of any institute or university to the best of my knowledge and belief.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work of other investigators are referred and copyright licenses have been taken from respective publishers.

Sambhavi

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December, 2016

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CERTIFICATE

This is to certify that the matter embodied in this thesis entitled **"Cloning, Expression and Purification of Epidermal Growth Factor Receptor for Aptamer Selection for certain Bioassays"** is the result of investigations carried out by **Mrs. Sambhavi** (Roll No.: 11610617) under my supervision, and is submitted to the Indian Institute of Technology Guwahati Guwahati-781039, Assam, India for the award of degree of *Doctor of Philosophy in Biosciences and Bioengineering*. This work has not been submitted elsewhere for a degree.

Utpal Bora

Professor
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Guwahati
…………., 2016

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Dedicated

To

My parents, Brother and My entire family

……. Sambhavi

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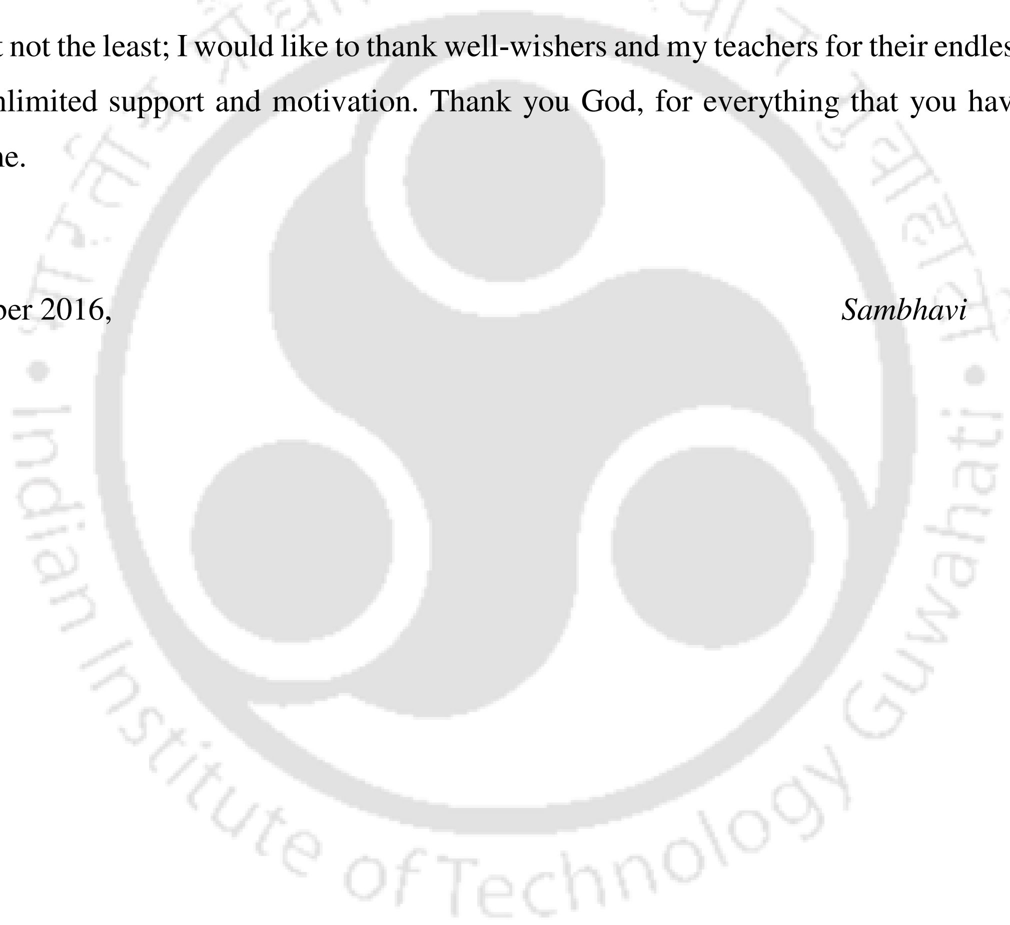

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SYNOPSIS

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase with a wide implication in human malignancies and multiple diseases. The EGFR was the first receptor to be proposed as a target for cancer therapy and after two decades of intensive research, there are several anti-EGFR agents available in the clinic. Aptamers binding to the EGFR with high affinity and specificity could be very useful in cancer diagnosis and therapy and also in other diseases wherever EGFR is overexpressed. The present work demonstrates the cloning, expression and purification of extra cellular domain (ECD) of EGFR for aptamer selection. Further the application of selected aptamers as a molecular probe for the detection of EGFR expression in different cancer cell lines and other bioassays were explored. The thesis is devided into five chapters as described herein.

Chapter 1: Introduction & Review of Literature

Chapter 1 is the General Introduction which embodies the brief review of literature dedicated to the importance of EGFR as one of the molecular marker for human diseases. It also gives an overview of aptamers and the SELEX (Systematic Evolution of Ligands by Exponential Enrichment) methodology. A detailed account of applications of aptamers as an alternative molecular probe in various biomedical and clinical research has also been provided.

Significance of the work has been elaborated in this chapter. EGFR and its ligands are involved in the cell growth of over 70% of all cancer cells. The dysregulation in EGFR expression and its signalling pathways were found to play a critical role in multiple diseases like inflammatory, cardiovascular, kidney, lungs, liver , skin, age related diseases etc (Jost et al., 2000; Komposch and Sibilia, 2016; Makki et al., 2013; Vallath et al., 2014). The oncogenic role of EGFR has been well established and it is widely implicated in the development of various human cancer including breast, colon, head and neck, ovary, kidney, cervical, lung, pancreas, and prostate (Uberall et al., 2008). The patients with altered EGFR activity tend to have a more aggressive disease and are associated with a poor clinical outcome (Holbro et al., 2003). EGFR activation also plays a role in resistance to chemotherapy and radiation treatment in tumor cells. . Over the past few years, EGFR specific agents have received regulatory approval for cancer

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treatment - Cetuximab for metastatic colorectal cancer and squamous cell carcinoma of the head and neck, Erlotinib for advanced or metastatic pancreatic cancer and NSCLC, Panitumumab for metastatic colorectal cancer and Gefitinib for advanced NSCLC (Ennis et al., 1991; Haringhuizen et al., 2004; Lenz, 2007).

Epidermal Growth Factor Receptor also known as ErbB-1 or HER-1 (Human Epidermal growth factor Receptor 1) are membrane spanning proteins belonging to ErbB family of receptor tyrosine kinases (RTKs). Structurally, EGFR consists of an extra cellular domain (ECD), a transmembrane domain, and an intracellular domain consisting of a tyrosine kinase domain and a C-terminal tail (Ogiso et al., 2002). The mutant variants or isoforms of EGFR are generated by either alternative mRNA splicing or via proteolytic cleavage of the receptor (Lafky et al., 2008; Wilken et al., 2011). The soluble EGFR (sEGFR) isoforms (A, B, C, D or mutant variant VII) are comprised solely of ECD portions of the receptor. They are often detected in normal and malignant cells, in tissues and in biological fluids (Perez-Torres et al., 2008; Rose-John and Heinrich, 1994). Thus, for developing a molecular probe for the detection of EGFR, it is very important to know which epitope is recognized by the probe. The probe recognizing intracellular domain can detect only EGFR variant I and VIII. However the probe directed against extracellular domain (specific to domain III and IV) can recognize all the variants of EGFR.

The EGFR status are often studied for determining the choice of treatment for the patients. The expression of EGFR can be evaluated by more than ten different methods designed to detect expression of proteins or gene amplification or gene mutations. There are many studies conducted to determine the EGFR status using many of these assays, but each defines the EGFR over expression a slightly different manner. Furthermore, even when a single technique, such as immunohistochemistry (IHC), was used by several laboratories to evaluate tumor EGFR levels, differences in reagents, detection methods or assay cut-off points led to different results, increasing the variability between studies. As a result, reports from different laboratories cannot be compared to obtain a conclusive result. Most of the assays to study the EGFR protein expression employs the use of antibodies. But, the antibodies suffers from many limitations such as batch to batch variation, poor tissue penetration, immunological reaction, instability, high cost of production etc. Therefore, there is a need to develop a molecular probe that can detect

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EGFR specifically and provides consistent and comparable result. A biorecognition element which can overcome the limitations of antibodies and can be used in biomedical and clinical research. Aptamers have emerged as a new class of nucleic acid ligands that mimics the property of antibodies by binding to its target with very high affinity and selectivity and offers numerous advantages over antibodies. Due to its specific biorecognition property these ligands have received much attention in biomedical and clinical research as a potential multifunctional theranostic probe.

Aptamers are short single-stranded oligonucleotides, either RNA or DNA, that folds into well-defined 3-D structures and have the ability to bind to its target molecules with high affinity and specificity. Aptamers are selected from a randomly synthesized initial library containing up to 10^{13} to 10^{15} different molecules of oligonucleotides through a combinatorial chemistry procedure termed as Systematic Evolution of Ligands by EXponential enrichment (SELEX) consisting of repetitive cycles of selection and amplification (Ellington and Szostak, 1990; Tuerk and Gold, 1990). In last few decades aptamers have been selected against a wide variety of targets, ranging from small inorganic molecules to whole organisms (Blank and Blind, 2005; Nimjee et al., 2005; Proske et al., 2005). Aptamers are analogous to antibodies due to its specific biorecognition property but they have many attributes which makes them superior to antibodies such as smaller size, stability, economic, better tissue penetration, ease of chemical modification and the lack of immune responses. To develop a more sensitive and specific approach for early cancer detection, numerous aptamers were generated against cell surface receptors (Hu et al., 2012; Kasten et al., 2013; Song et al., 2013) and tumor cells (Li et al., 2014; Sefah et al., 2010). Aptamers have been employed in almost every aspect of molecular biology, diagnostics, therapeutics and biosensing, particularly wherever antibodies have been traditionally used. Aptamers have proven to be appropriate tools in many assay forms such as flow cytometry, imaging of cells/tumors, biosensors, ELISA/ELAA/ELONA, IHC, Immunoprecipitation, *in vivo* imaging, therapeutics and drug delivery including siRNA etc.

Objectives: To develop an EGFR specific aptamers that can be used as an alternative to antibodies in various clinical and biomedical assays, following objectives were formulated.

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I. Cloning, Expression and Purification of Extra Cellular Domain of Epidermal Growth Factor Receptor.

II. *In vitro* Selection and Characterization of aptamers against Extra Cellular Domain of EGFR.

III. Exploring the applications of selected aptamers in various bioassays.

Chapter 2: Cloning, Expression and Purification of Extra Cellular Domain of Epidermal Growth Factor Receptor

This chapter describes the cloning, expression and purification of recombinant extra cellular domain of Epidermal Growth Factor Receptor protein in non-denaturing condition for aptamer selection. The full length EGFR cDNA cloned in pBABE vector in DH5α strain was procured from Addgene plasmid repository (Plasmid # 11011). The ECD region of EGFR was PCR amplified from EGFR WT plasmid using ECD_F1 and ECD_R1 primers and cloned into pTZ57R/T vector for TA cloning. The positives clones were screened by colony PCR and confirmed by restriction digestion with *Nde*I and *Xho*I enzyme. The restriction enzyme digested fragment of EGFR ECD was then cloned into pET-28a (+) expression vector and transformed into DH5α strain. The recombinant colonies were confirmed by restriction digestion and also by sequencing using ECD_F1 and ECD_R1 primer. Further for the expression of protein, recombinant plasmid DNA were transformed into *E. coli* BL21 (DE3) strain. The expressed recombinant EGFR ECD protein was successfully purified in non-denaturing condition by methods optimized in this study using Immobilized metal ion affinity chromatography (IMAC). Further proteins were confirmed by western blot analysis using EGFR specific monoclonal antibody and horseradish peroxidase-conjugated Goat Anti-Mouse secondary antibody.

The chapter is further subdivided into separate parts detailing introduction, materials and methods, results, discussions and conclusions.

Chapter 3: *In vitro* Selection and Characterization of Aptamers against the Extra Cellular Domain of Epidermal Growth Factor Receptor.

The study reports the selection of a panel of DNA aptamers against the extra cellular domain of EGFR and its further characterization. The protein SELEX methodology was

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carried out from an initial pool of 10^{13}- 10^{15} ss DNA library with a 40 nucleotide long central block of randomized sequence. A total of eighteen rounds of selection were performed, of which 4 rounds were Negative SELEX and rest 14 rounds were of protein SELEX. The methodology reported here provides the possibility of selecting individual aptamers against any recombinant protein. After 14 rounds of protein SELEX, the enriched aptamer library were cloned and 20 positive clones were sequenced. The sequencing results were subjected to multiple sequence alignment analysis with the Clustal Omega to identify highly conserved motifs in the enriched DNA pool. Secondary structures of the selected aptamers were predicted by the Zuker algorithm (Zuker, 2003) using Mfold (version 3.2). Based on the sequence homology aptamers were grouped in five homologous families. The abundant aptamer sequences or aptamer with unique complex secondary structure representing each homologous family viz EGFR_A1, EGFR_A7, EGFR_A15, EGFR_A13 and EGFR_A16 were synthesized with either FAM or Biotin labelling at 5' end to test their binding interaction with EGFR either by flow cytometry or Enzyme Linked Immunosorbent assay (ELISA). Initially, the affinity of three aptamers viz: EGFR_A1, EGFR_A7 and EGFR_A15 representing family II, III and IV were studied by Flow cytometry and Immunocytochemistry.

The binding of aptamer to its target strictly relies on conformation complementary for target binding. The ECD of EGFR protein used for SELEX was purified from *E. coli* which is a prokaryotic system. For clinical application, the selected aptamer should bind to native conformation of protein. Therefore, to investigate the selectivity and affinity of aptamer candidates, binding of selected aptamers EGFR_A1, EGFR_A7 and EGFR_A15 were performed with various EGFR expressing eukaryotic cancer cell lines (A431, A549, MDA MB 231, U87 MG, Hela and Jurkat) by flow cytometry. All three aptamers were found to recognize EGFR expression in cancerous cell lines *viz* A431, A549, MDA MB 231, and U87 MG. As expected, none of the aptamers binds to Jurkat cell line which do not express EGFR at all. Interestingly the selected aptamers were also binding to U87 MG cancerous cell lines, which is a human glioblastoma cell line and expresses mutant epidermal growth factor receptor variant III (EGFRvIII). Thus, the aptamers were able to recognize EGFRvIII alongwith full length EGFR. Also among the three selected aptamers- EGFR_A1, EGFR_A7 and EGFR_A15; EGFR_A15 had the strongest binding affinity.

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In order to determine the cell surface binding of candidate aptamers on the target cancer cells, flow cytometry was conducted by treating cancer cells with trypsin. EGFRs are receptor proteins which are expressed on the cell membrane. Thus, it was obvious to assume that the selected EGFR specific aptamers will bind to cell surface. The selected aptamers- EGFR_A1, EGFR_A7 and EGFR_A15 significantly lose their binding with cells after trypsin treatment which confirmed that the binding target of aptamers are cells surface protein i.e. EGFR.

Furthermore, the application of selected aptamers as an imaging probe was studied through fluorescence microscopy with A431 and MDA MB 231 cancer cell lines. Intense bright fluorescence of FAM labelled EGFR_A15 was observed at the cell membrane of MDA MB 231 and A431 cell lines. However, the fluorescence of aptamers EGFR_A1 and EGFR_A7 were found to be localized within the nuclei.

It was exciting to note that the initial flow cytometry data correlates with fluorescence microscopy imaging. The aptamer which had the highest signal intensity (EGFR_A15) by flow cytometry also produced brighter fluorescence signal in fluorescence microscopy. Based on the Flow cytometry and Immunocytochemistry data, it was evident that EGFR_A15 had the highest binding affinity. Next, two more aptamers EGFR_A16 and EGFR_A13 belonging to group I and V respectively were chosen alongwith EGFR_A15 for future studies. All three aptamers were synthesized with Biotin modification at 5′ end. Initially Isothermal calorimetry was tried to determine the dissociation constant of selected aptamers. But no conclusive results were obtained. It might be due to the instability of recombinant ECD of EGFR in aptamer binding buffer. Thus we calculated the dissociation constant by an alternative method reported by Li *et al.* with slight modifications (Fu et al., 2014; Li et al., 2011). The dissociation constant of two aptamers EGFR_A15 and EGFR_A13 were 12.31 ± 2.59 nM and 15.59 ± 6.02nM respectively. The aptamer EGFR_A16 exhibited very weak binding with the target.

The chapter is further subdivided into separate parts detailing introduction, materials and methods, results, discussions and conclusions.

Chapter 4: Application of selected Aptamers in various bioassays.

This chapter focuses on the applications of selected aptamers as a biorecognition probe in various bioassays. A dot blot assay was performed to evaluate the specificity and

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affinity of selected aptamers and further investigate the potential application of the selected aptamers. Aptamer EGFR_A15 could detect upto 2.5ng of spotted EGFR ECD proteins while EGFR_A13 could detect 10 fold lower amount of spotted protein (25ng). EGFR_A16 could detect 250ng of protein but with very weak observable signal. Based on all the studies, it was evident that the aptamer EGFR_A16 was a very weak binder and it was not used further for any application studies.

In vitro cell culture model was employed to ascertain the therapeutic potential of the selected aptamers by assessing its anti-proliferative effects by MTT assay and anti-migratory effect by scratch assay against MDA MB 231 cell lines. All the selected aptamers were found to be biocompatible in MTT assay and no effect on the cell proliferation was observed. These aptamers could be used as a vehicle for the delivery of therapeutic drugs, si RNA etc. Further in scratch assay, aptamer EGFR_A15, EGFR_A13 and non-enriched library demonstrated no effect on the migration of cells. However, two aptamer candidates EGFR_A1 and EGFR_A7 exhibited a significant delay in the migration of cells. These two aptamers might be used as an agent for inhibition of migration of cancerous cell. The *in vitro* cell scratch assay is a very preliminary experiment and further experiments like cell invasion assay using trans wells/ boyden chambers and in animal models are required.

Based on all the studies, two aptamers EGFR_A15 and EGFR_A13 were found to have the best recognition ability. Further the usability of the best binding aptamers as a capture agent in microtiter based assay was demonstrated. The results aim towards the development of another detection assay for assessing the EGFR protein expression in cancer. Here, an indirect Enzyme Linked Aptamer assay (iELAA) using best recognising aptamers- EGFR_A15 and EGFR_A13 was developed. Different parameters such as the concentrations of aptamer (1nM, 5nM, 20nM and 50nM) and the amount protein were optimized to find out the optimum concentrations of reagent required. It was observed that EGFR_A15 was more sensitive as compared to EGFR_A13 as it can be used as low in 1nm concentration for the development of detectable signal. Moreover the presence of EGFR specific antibodies and other contaminating proteins of serum did not hampered the performance of aptamers.

The chapter is further subdivided into separate parts detailing introduction, materials and methods, results, discussions and conclusions.

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Chapter 5: Summary and Future Prospect

Conclusively, we hereby demonstrated the cloning expression and purification of EGFR protein and selection and characterization of a panel of DNA aptamer binding specifically to EGFR protein. Also the application of selected aptamers in various biomedical research were explored. The present study convincingly exhibited that the newly developed aptamer EGFR_A15 have the best recognition ability and selectivity as compared to other selected aptamers. It can be used as a bioimaging probe for the detection of EGFR overexpression in cancer cell lines. It can also be used to develop a microtiter based assay for the quantification of EGFR protein. The aptamer EGFR_A15 selected in this study can potentially be used as a recognition probe for the development of any assays for the detection of EGFR overexpression in cancer or other human diseases. Therefore, in future we envisage, EGFR_A15 could be exploited for the development of a non-invasive diagnostic module for detection of cancer or any other disease where EGFR is overexpressed or dysregulated. Furthermore, it can also provide an opportunity to develop a novel cost effective immunoassays and other biosensing platforms for detection of EGFR protein. However, extensive research with animal models are required to evaluate the *in vivo* effect of the aptamers.

Patent Filed:

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LIST OF ABBREVIATIONS

BSA	: Bovine Serum Albumin
DAB	: 3, 3'-Diaminobenzidine
DAPI	: 4', 6-diamidino-2-phenylindole
DMEM	: Dulbecco's Modified Eagle Medium
DMSO	: Dimethyl Sulphoxide
dsDNA	: Double Stranded Deoxyribonucleic Acid
EDTA	: Ethylenediaminetetraacetic acid
EGFR	: Epidermal Growth Factor Receptor
ECD	: Extra Cellular Domain
ELISA	: Enzyme-linked immunosorbent assay
ELAA	: Enzyme Linked Aptamer Assay
FACS	: Fluorescence Activated Cell Sorter
6 FAM	: 6-Carboxyfluorescein
FBS	: Fetal Bovine Serum
FDA	: Food and Drug Administrations
FITC	: Fluorescein isothiocyanate
GBM	: Glioblastoma Multiforme
HRP	: Horseradish peroxidase

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HER 2	: Human Epidermal growth factor Receptor 2
IPTG	: Isopropyl β-D-1-thiogalactopyranoside
ICC	: Immunocytochemistry
IHC	: Immunohistochemistry
IMAC	: Immobilized Metal-ion Affinity Chromatography
K_d	: Dissociation Constant
LB	: Luria Bertani
MTT	: 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide
NC	: Nitrocellulose
OD	: Optical density
PAGE	: Polymerase Agarose Gel Electrophoresis
PEG	: Polyethylene glycol
PBS	: Phosphate Buffer Saline
PBST	: PBS with 1% Tween-20
PCR	: Polymerase Chain Reaction
PMSF	: Phenylmethylsulfonyl fluoride
RNA	: Ribonucleic acid
RT	: Room temperature
RTK	: Receptor Tyrosine Kinase

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SDS	: Sodium dodecyl sulfate polyacrylamide gel
SELEX	: Systematic Evolution of Ligands by Exponential Enrichment
siRNA	: Small interfering RNA
ssDNA	: Single Stranded Deoxyribonucleic Acid
TKI	: Tyrosine Kinase Inhibitor
TMB	: 3, 3′, 5, 5′ TetraMethylBenzidine
TNBC	: Triple Negative Breast cancer
VEGF	: Vascular endothelial growth factor
X-Gal	: 5-bromo-4-chloro-3-indolyl-β-D-galactoside

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

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1.1 Introduction

According to National Institutes of Health, Biomarkers are defined as any "characteristic that can be objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Bethesda MD, 2001). It can be a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease, such as cancer (Henry and Hayes, 2012). Every cell has its own unique molecular signature, referred to as biomarkers, which are identifiable characteristics such as expression of certain proteins. However development of cancer or any other diseases results into change in cellular molecular signature i.e. biomarker status. Detection of such biomarkers not only helps in diagnosing cancer but also proved useful for staging and grading cancer (Ludwig and Weinstein, 2005). In the recent year, knowledge about cancer biomarkers have increased tremendously providing great opportunities for improving the management of cancer patients by enhancing the efficiency of detection and efficacy of treatment (Bhatt *et al*., 2010). Diagnostic and prognostic biomarkers are quantifiable traits that help clinical oncologists at the first interaction with the suspected patients. These particularly aid in (i) identifying who is at risk, (ii) diagnose at an early stage, (iii) select the best treatment modality, and (iv) monitor response to treatment (Ludwig and Weinstein, 2005). With the tremendous increase in knowledge about the biology of cancer and the rapid development of new molecular technology, numerous candidate biomarkers have been discovered. Epidermal Growth Factor Receptor was the first receptor to be proposed as a target for cancer therapy and after two decades of intensive research, there are several anti-EGFR agents available in the clinic (Scaltriti and Baselga, 2006a).

Until the latter part of the 20th century, cancer was diagnosed only when symptoms of tumor growth were manifest. Most of the time cancer was detected at a very advanced stage when the cancer have already spread to other organs. This limits the treatment options as the surgery and radiological treatment won't be much effective Symptomatic presentation is still the predominant route to diagnosis across all cancers, but for some cancer sites, tests have been developed to identify tissue changes that are indicative either

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of cancer precursors or early-stage tumors (Wardle *et al*., 2015). Research shows that the early detection of cancer helps in increasing the survivability of cancer patients (Dsouza *et al*., 2013). The discovery of biomarkers associated with the cancers helps in diagnosing cancer at an early stage.

Treatment regimen of cancer patient mainly depends on the type of cancer and its malignancy status. Surgical removal of cancerous tumors was the first treatment developed in an attempt to cure patients of this disease, and it has remained one of the most effective cancer therapies, particularly for localized solid tumors. However, for non-solid tumors or in cases where the cancer has already metastasized or is difficult to remove, surgery is relatively ineffective. Another most commonly used methods are chemotherapy which involves killing of cancerous cells or to stop their multiplication. It involves taking one or more type of drug that interferes with the DNA of fast-growing cells. These drugs are subdivided into specific classes such as alkylating agents, antimetabolites, anthracyclines and topoisomerase inhibitors. Sometimes radiotherapy, or radiation therapy, are used. It is the clinical use of high energy rays (ionizing radiation) to induce DNA damage in all exposed cells which ultimately kills cancerous cell or prevent cancer growth. Radiotherapy may be used to eradicate some cancers, to reduce recurrence or as palliative treatment. Another strategy is to boost the patient's immune system so that it can fight against cancer known as Immunotherapy. Some cancer immunotherapies consist of antibodies that bind to, and inhibit the function of, proteins expressed by cancer cells. Other cancer immunotherapies include vaccines and T cell infusions. Recently targeted therapy has received much attention as an effort to design a new therapeutic system. In targeted therapy a specific molecules or enzymes or receptor or proteins or signalling pathways are targeted whose functions are disrupted in cancer. Targeted therapies can either block or increase the function of their target in order to treat cancer. Epidermal growth factor receptor is one such marker which is targeted for the treatment of cancer. The oncogenic role of epidermal growth factor receptor is well established and it is widely implicated in the development of various cancers like breast, colon, head and neck, kidney, lung, pancreas, and prostate.

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1.2 Epidermal Growth Factor Receptor

Growth factors are the most essential component for the development, growth and homeostasis of multicellular organisms. Acting through cell surface receptors, growth factors are required for cell-cell communications underlying embryonic tissue induction, fate determination, cell survival, apoptosis, tissue specialization and cell migration. It binds to the cell surface receptor and transduces extracellular signals by activating intracellular messengers or directly by receptor translocation to the nucleus (Wieduwilt and Moasser, 2008). Of the receptor tyrosine kinases (RTKs), the epidermal growth factor (EGF) family of RTKs, also called ErbB or HER receptors, is one of the most extensively studied for its role in in the pathogenesis of several human malignancies and illness? The dysregulation in EGFR expression and its signalling pathways are also found to play a critical role in various inflammatory, cardiovascular, kidney, lungs and liver and age related diseases. The epidermal growth factor receptor (EGFR) family are widely expressed in epithelial, mesenchymal, and neuronal tissues. They regulate cell growth, survival, and differentiation via multiple signal transduction pathways and participate in cellular proliferation and differentiation. Over-activated EGFR can convert a normal to a malignant cell by providing sustained signals for cell proliferation, anti-apoptosis, angiogenesis and metastasis which are the basic properties of cancer. Two key signalling pathways activated by the ErbB family are the RAS/RAF/MAPK pathway, which stimulates proliferation, and the PI3 K/Akt pathway which promotes tumor cell survival (Baselga and Swain, 2009; Jorissen *et al*., 2003).

1.2.1 EGFR family of receptors

The EGFR belongs to a family of receptor tyrosine kinases that includes four distinct members: EGFR/ErbB-1, HER2/ErbB-2, HER3/ErbB-3 and HER4/ErbB-4 (Riese and Stern, 1998). This was the first receptor tyrosine kinases (RTKs) for which ligand binding was studied and for which the importance of ligand-induced dimerization was established (Lemmon *et al*., 2014). These receptors possess intrinsic tyrosine kinase activity. They are found only in metazoans, in contrast to many of the serine/threonine kinase families, which are conserved throughout eukaryotes and are found in both unicellular and multicellular organisms (Stein and Staros, 2000). These receptors derive their name from

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the discovery that the erythroblastosis tumor virus encodes an aberrant form of the human EGF receptor (ErbB) and that there are a family of human EGF receptors (HER) (Arteaga and Engelman, 2014; Yarden and Sliwkowski, 2001).

Epidermal growth factor receptor (EGFR) also known as ErbB1, and HER1 was the first member of the family discovered by Carpenter and coworkers at Vanderbilt University, USA, in 1978 (Carpenter *et al*., 1978; Carpenter and Cohen, 1979). ErbB stands for its origin in the *Erb-b* gene responsible for avian erythroblastosis virus. The *neu* oncogene (also known as HER2, ErbB2, or p185) was discovered by a group of scientists at Massachusetts Institute of Technology, Rockefeller, and Harvard University (Padhy *et al*., 1982; Schechter *et al*., 1984). Interestingly, there is no known ligand for ERBB2, which is believed to undergo ligand-independent activation (Hynes and Lane, 2005). The amplification or overexpression of HER2 occurs in approximately 15-30% of breast cancers (Burstein, 2005) and 10-30% of gastric/gastroesophageal cancers which has both prognostic and predictive implications. HER2 overexpression and/or amplification have also been observed in colon (Schuell and Gruenberger, 2006), bladder (Eltze *et al*., 2005), ovarian (McKenzie *et al*., 1993), endometrial (Hetzel *et al*., 1992), lung (Hirashima *et al*., 2001), uterine cervix (Mitra *et al*., 1994), head and neck (Beckhardt *et al*., 1995), esophageal (Beckhardt *et al*., 1995), and gastric carcinomas (Gravalos and Jimeno, 2008). Trastuzumab (Herceptin™) is a monoclonal antibody which specifically targets HER2 protein by directly binding to extracellular domain of the receptor. Trastuzumab was the first HER2-targeted therapy approved by the United States Food and Drug Administration (FDA) in 1998 for the treatment of HER2-overexpressing metastatic breast cancer (MBC) (Nahta and Esteva, 2007). Trastuzumab enhances survival rates in both primary and metastatic HER2-positive breast cancer patients (Slamon *et al*., 2001; I. Smith *et al*., 2007).

HER3 (ErbB3) is a unique member of the human epidermal growth factor receptor (EGFR) family (ErbB family). It has a defective kinase domain due to substitutions of essential residues such as Cys-721, His-740, and Asn-815 (Carraway *et al*., 1997). Unlike other members of family it cannot form homodimer and functions only through dimerization with other members of the ErbB family modulating the activity and sensitivity to targeted cancer therapies (Berger *et al*., 2004). It was first identified in 1989

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by Kraus *et al*. (Kraus *et al*., 1989). The extra cellular domain of HER3 shares 40–50% identity with EGFR and 40–45% with HER2 (Coussens *et al*., 1985; Ullrich *et al*., 1984). The kinase domain of HER3 shares 60% and 62% similarity with EGFR and HER2, respectively. However, both EGFR and HER2 have 83% amino acid sequence identity in their kinase domains, suggesting they are more closely related to each other than they are to HER3 (Plowman *et al*., 1990). Upregulation of HER3 is commonly seen in various malignancies such as breast cancer, colorectal carcinoma, squamous cell carcinoma of the head and neck (SCCHN), uveal melanoma, and gastric, ovarian, prostate, and bladder cancers (Beji *et al*., 2012; Maurer *et al*., 1998; Mujoo *et al*., 2014; Sithanandam and Anderson, 2008).

The receptor tyrosine kinase, ErBB4/HER4 (referred to here as ErBB4) is the final member of the epidermal growth factor receptor (EGFR) family to be discovered (Elenius *et al*., 1997). ErbB4 has a notable ability to directly transduce extracellular signals to the nucleus through liberation of the intracellular domain by a ligand-dependent dual protease cleavage of the receptor (Schlessinger and Lemmon, 2006). The role of ErBB4 in tumorigenesis remains incompletely understood and may to some extent be tissue dependent. However mutated ErBB4 were found in lung adenocarcinoma, non-small cell lung cancer and colon cancer (Kurppa *et al*., 2016; Williams *et al*., 2015).

1.2.2 Epidermal Growth Factor Receptor/ Her1

EGFR is one of the most investigated RTKs, is constitutively expressed in some normal epithelial tissues, including skin, hair follicle and gastrointestinal tract (Herbst and Shin, 2002; Lemmon and Schlessinger, 2010) and are commonly used as a model for understanding signal transduction pathways. EGFR has been implicated in the development of a wide range of epithelial cancers, including those of the breast, colon, head and neck, kidney, lung, pancreas, and prostate and also in several human diseases.

1.2.3 Ligands of Epidermal Growth Factor Receptor

The mammalian ligands that binds to EGFR includes: Epidermal Growth Factor (EGF) (Brown *et al*., 1994; Domagala *et al*., 2000; Lemmon *et al*., 1997; Odaka *et al*., 1997) Tansforming Growth Factor α (TGFα) (Massagui, 1990), Heparin Binding EGF-like

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growth factor (HB-EGF) (Higashiyamas *et al*., 1992), Amphiregulin (AR) (Shoyab *et al*., 1988), Betacellulin (BTC) (Dunbar and Goddard, 2000; Sasada *et al*., 1993), Epiregulin (EPR) (Shelly *et al*., 1998), and Epigen (Strachan *et al*., 2001). These growth factors are produced as trans-membrane precursors and then processed in soluble molecules by proteolysis (Massague and Pandiella, 1993). All the mature peptide growth factors shares consensus sequences consisting of six spatially conserved cysteine residues (CX7 CX4–5 CX10–13 CXCX8 C) known as "EGF motifs". These motifs are crucial for binding to the members of the HER receptor tyrosine kinase family. HB-EGF, BTC, and EPR also binds to HER4 in addition to EGFR (Harris *et al*., 2003). EGF and TGF-α binds directly only to EGFR, while another ligand neuregulins (also known as heregulins or neu differentiation factors) are specific to ErbB-3 and ErbB-4 (Ogiso *et al*., 2002).

1.2.4 Structure of Epidermal Growth Factor Receptor protein

Unlike other receptor tyrosine kinases EGFR has only one isoform. It is encoded from a single 26 exon gene located across 110kb on chromosome 7p11-13 (Wells, 1999). The protein product of this gene is 1186 amino acid long mature transmembrane glycoprotein. The protein is subdivided into: extracellular domain, transmembrane domain and an intracellular domain (**Fig. 1.1**). A 622 amino acid long from N terminal end is known as Extracellular domain (ECD). EGFR ECD can be further divided into four subdomains: I, II, III and IV. Early binding studies indicate that the major ligand-binding site is located in ECD between amino acids 294 and 543, a fragment restricted primarily to L2 or domain III (Bajaj *et al*., 1987; Lax *et al*., 1988); in addition to domain III, a portion of domain I (or L1) seems to cooperate in growth factor binding (Lax *et al*., 1989). The transmembrane domain consists of a single alpha-helical transmembrane pass. The 542 amino acid long intracellular domain (ICD) is grouped into three domains. The juxta membrane domain (50 amino acids) serves primarily as a site for feedback attenuation by PKC (protein kinase C) and erk MAP kinases (Extracellular signal-regulated kinase, mitogen activated protein kinase). The next domain is a contiguous 250 amino acid tyrosine kinase (SH1, src homology 1) domain also known as intracellular domain. A unique 229 amino acid long carboxy terminal tail contains five autophosphorylation motifs which link to proteins containing SH2 or PTB (phospho tyrosine binding)

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domains. These motifs contain sites for transphosphorylation and proteolytic activation and degradation. This tail also functions as an auto inhibitory substrate; in the absence of either autophosphorylation or removal, ligand-activated EGFR is unable to phosphorylate substrates (Wells, 1999). The intracellular domain of ErbB receptors consists of a highly conserved tyrosine kinase domain (Guy *et al*., 1994). In contrast, the extra-cellular domains are less conserved among the four receptors, suggesting that they have different specificity in ligand-binding (Citri and Yarden, 2006; Yarden, 2001; Yarden and Sliwkowski, 2001).

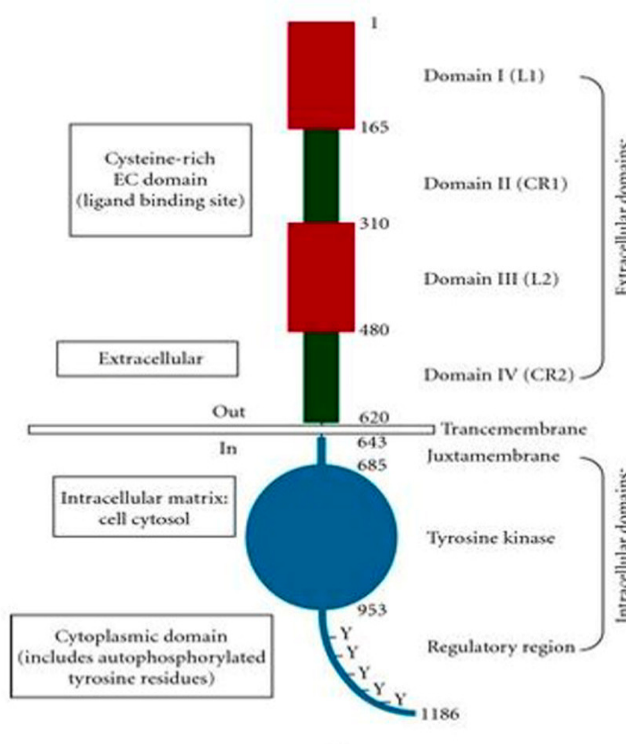

Figure 1.1: Basic Structure of EGFR demonstrating relevant domains. (I) The extracellular domains: (1) domain I: L1; (2) domain II: CR1; domain III: L2; domain IV: CR2. (II) Transmembrane domains. (III) The intracellular domains (1) juxtamembrane domain; (2) tyrosine kinase domain; (3) regulatory region domain. The phosphorylation of several substrates by the tyrosine kinase domain of the EGFR receptor is responsible for activating the various signaling cascades. Reprinted with permission from Wu *et al*., 2009.

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1.2.5 Epidermal Growth Factor Receptor signalling pathways

The EGFR exists in two distinct conformations: (i) a closed, inactive conformation and (ii) an open, active conformation. In the closed conformation, domains II and IV interact with each other thus preventing domains I and III from interacting with their cognate ligand (Ogiso *et al*., 2002). In the open conformation domain II and IV are moved away exposing the ligand binding pockets of domains I and III and thus facilitates its interaction with their corresponding ligand (Dawson *et al*., 2005) (**Fig. 1.2**). The closed conformation is favoured in the absence of a ligand. However, binding of a ligand shifts the equilibrium and stabilizes the open conformation, further enabling the accumulation of active homodimers and maintaining active receptor signaling. In addition to homodimerization, EGFR promotes heterodimerization with other members of the HER family, including HER2, HER3 and HER4.

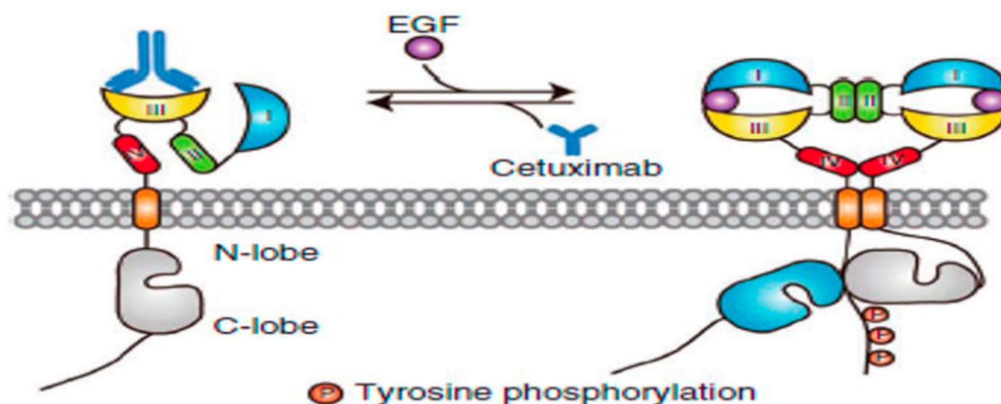

Figure 1.2: Dimerization of EGFR. Unliganded and cetuximab-bound EGFRs exist primarily in the tethered conformation. EGF binding to the ectodomain initiates formation of a specific receptor-mediated dimer and activation of the intracellular kinase domain through formation of an asymmetric dimer. Domain I and III are involved in ligand binding while Domain II is involved in dimerization. The active conformation of the kinase domain is depicted as blue and the inactive conformation is depicted as gray. Cetuximab is shown in light blue and the EGF is shown in purple (not to scale).Reprinted with permission from Wang *et al*., 2011.

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Binding of ligands to the extra cellular domain of EGFR induces conformational changes and receptor dimerization. Further the tyrosine residues present in the intrinsic kinase domain of one receptor cross phosphorylates specific residues in the C-terminal tail of the partnering receptor, thus providing a scaffold for the recruitment of effector proteins (Baselga and Albanell, 2002; Tzahar *et al*., 1996). Subsequently, recruitment of signaling molecules to the phosphorylated tyrosine residues induces the activation of downstream signaling cascades which include the KRAS-BRAF-MEK-ERK pathway, phosphoinositide 3-kinase (PI3K), phospholipase C gamma protein pathway, the anti-apoptotic AKT kinase pathway and the STAT signaling pathway (Citri and Yarden, 2006; Yarden and Sliwkowski, 2001) (**Fig. 1.3**). These often leads to cellular processes such as cell proliferation, angiogenesis, migration, survival, and adhesion which are often deregulated in malignant cells due to the several mutations harbored in various genes involved in these pathways.

Classically, EGFR functions as a plasma membrane bound receptor tyrosine kinase that initiates growth and survival signals (Yarden and Pines, 2012). However, the advances in understanding of EGFR biology have established that the EGFR functions in two distinct signaling pathways: (i) classical membrane-bound signaling and (ii) nuclear signaling. It has been found that EGFR can be localized and function from intracellular organelles, one of which includes the nucleus (Brand *et al*., 2011; Han and Lo, 2012). Previous studies have demonstrated that nuclear EGFR (nEGFR) can enhance resistance to anti-EGFR therapies (Huang *et al*., 2011; Li *et al*., 2009) and is correlated with poor overall survival in breast cancer. Within the nucleus, EGFR can function as a cotranscription factor to regulate genes involved in tumor progression (Brand *et al*., 2011; Han and Lo, 2012), in addition to functioning as a nuclear kinase to enhance DNA replication and repair (Dittmann *et al*., 2005; Hsu *et al*., 2009; S.-C. Wang *et al*., 2006). These nuclear functions have been linked to three parameters of tumor biology: (i) inverse correlation with overall survival in cancer (Hadzisejdić *et al*., 2010; Li *et al*., 2012; Lo *et al*., 2005; Psyrri *et al*., 2005; Traynor *et al*., 2013; Xia *et al*., 2009), (ii) resistance to therapeutic agents including radiation (Dittmann *et al*., 2011, 2010, 2005; Liccardi *et al*., 2011) chemotherapy (Dittmann *et al*., 2005; Hsu *et al*., 2009; Liccardi *et al*., 2011), and anti-EGFR therapies gefitinib and cetuximab and (Huang *et al*., 2011; Li *et al*., 2009) (iii) enhanced tumor growth.

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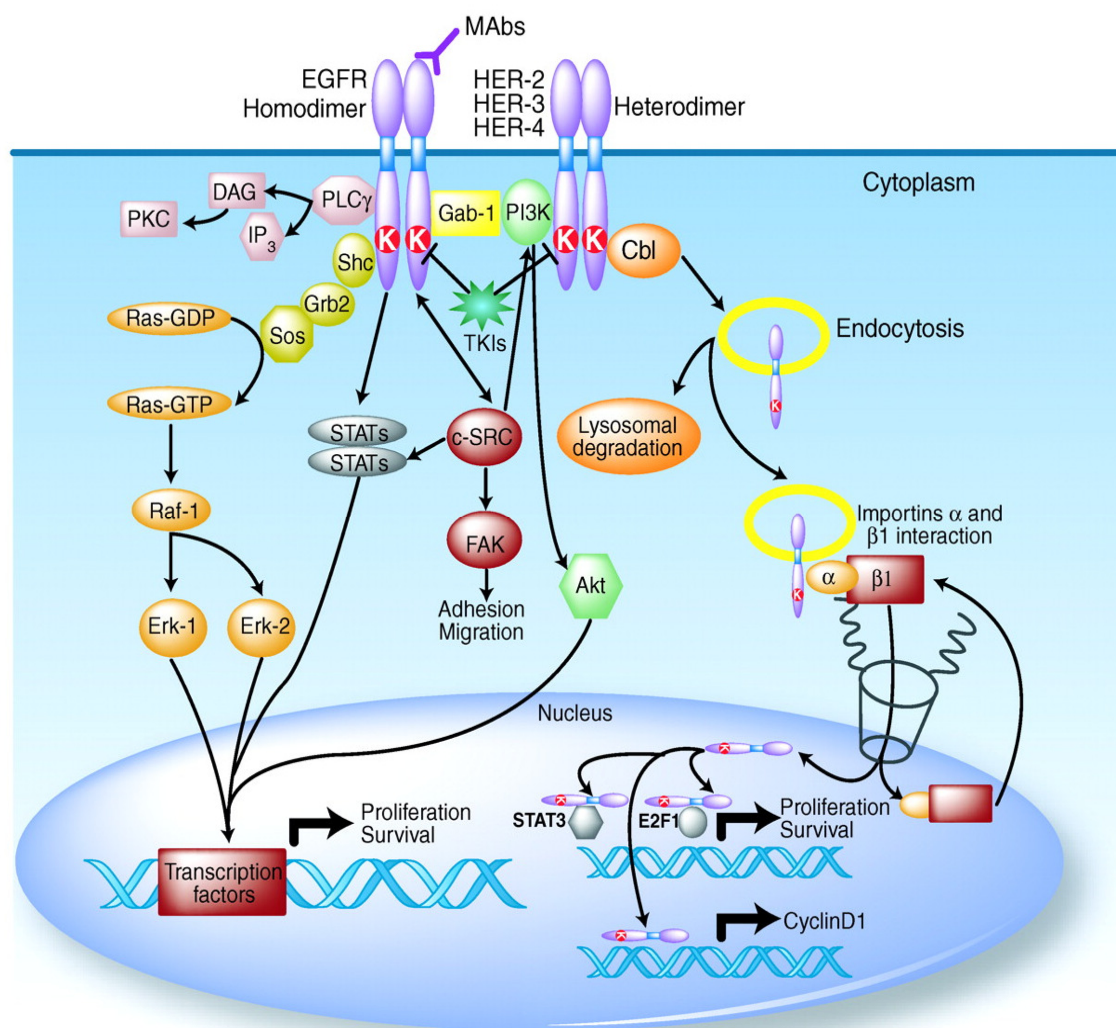

Figure 1.3: Signaling pathways of EGFR. Activation of EGFR leads to homodimerization/ heterodimerization, phosphorylation of specific tyrosine residues, and recruitment of several proteins at the intracellular portion of the receptors. Reprinted with permission from Scaltriti and Baselga, 2006b.

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1.2.6 Variants of Epidermal Growth Factor Receptor protein

Human EGFR is encoded by two transcripts of 10.5 kb and 5.8 kb (isoform A) both of which arise from a single promoter region/gene on chromosome 7 (Ishii *et al*., 1985); the protein product arising from these two transcripts are identical. These two transcripts (EGFR variant 1) encodes full-length EGFR isoform i.e. Isoform A which encodes 170 kDa long protein. The other isoforms of EGFR can be either generated by alternative mRNA splicing events or via proteolytic cleavage of the receptor (Lafky *et al*., 2008; Wilken *et al*., 2011). These soluble EGFR (sEGFR) isoforms comprised solely of ECD portions of the receptor. They are often detected in normal and malignant cells, in tissues, and in biological fluids (Perez-Torres *et al*., 2008; Rose-John and Heinrich, 1994). EGFR gene alternative splicing leads to three more transcripts : EGFR variants 2, 3 and 4 (v2, v3 and v4 respectively) mRNA of 1.8, 2.4, and 3.0 kb in size and encode isoforms C (60 kDa), B (80 kDa) and D (110 kDa) respectively (Flickinger *et al*., 1992; Ilekis *et al*., 1995; Reiter *et al*., 2000; Reiter and Maihle, 1996). Another 110-kDa soluble EGFR isoform known as PI-sEGFR are produced by proteolytic cleavage triggered in part by metalloproteases (Ancot *et al*., 2009; Perez-Torres *et al*., 2008; Rose-John and Heinrich, 1994; Sanderson *et al*., 2008) was also reported. The 1.8 kb transcript results from read-through of an exon (10) intron boundary, the 2.4 and 3.0 kb transcripts, encoding isoforms B and D transcripts diverge from full-length EGFR by incorporating alternate exons 15A or 15B. The 2.4 kb isoform B and its protein product have not been well studied, the 3.0 kb isoform D transcript encodes a 90/110 kDa EGFR isoform that is associated with the cell membrane through an unidentified mechanism (Reiter and Maihle, 2003) and also can be detected in human serum (Baron *et al*., 2002; Lafky *et al*., 2008) . sEGFR/sHER receptors also have been reported to modulate EGFR/HER tyrosine kinase activity (Basu *et al*., 1989; Lee *et al*., 2001). The 1.8 kb isoform C transcript codes for a secreted 60/80 kDa soluble EGFR protein that contains only subdomains I, II and half of subdomain III of the EGFR extracellular region followed by a unique carboxy-terminal Leu-Ser and 3' UTR. EGFRvIII, lacks part of the extracellular ligand-binding domain due to deletion of exons 2–7, which results in a constitutively active receptor (Huang *et al*., 1997). This variant is frequently expressed in various tumour types, including glial, breast, ovarian and non-small cell lung tumours (Moscatello *et al*., 1998).

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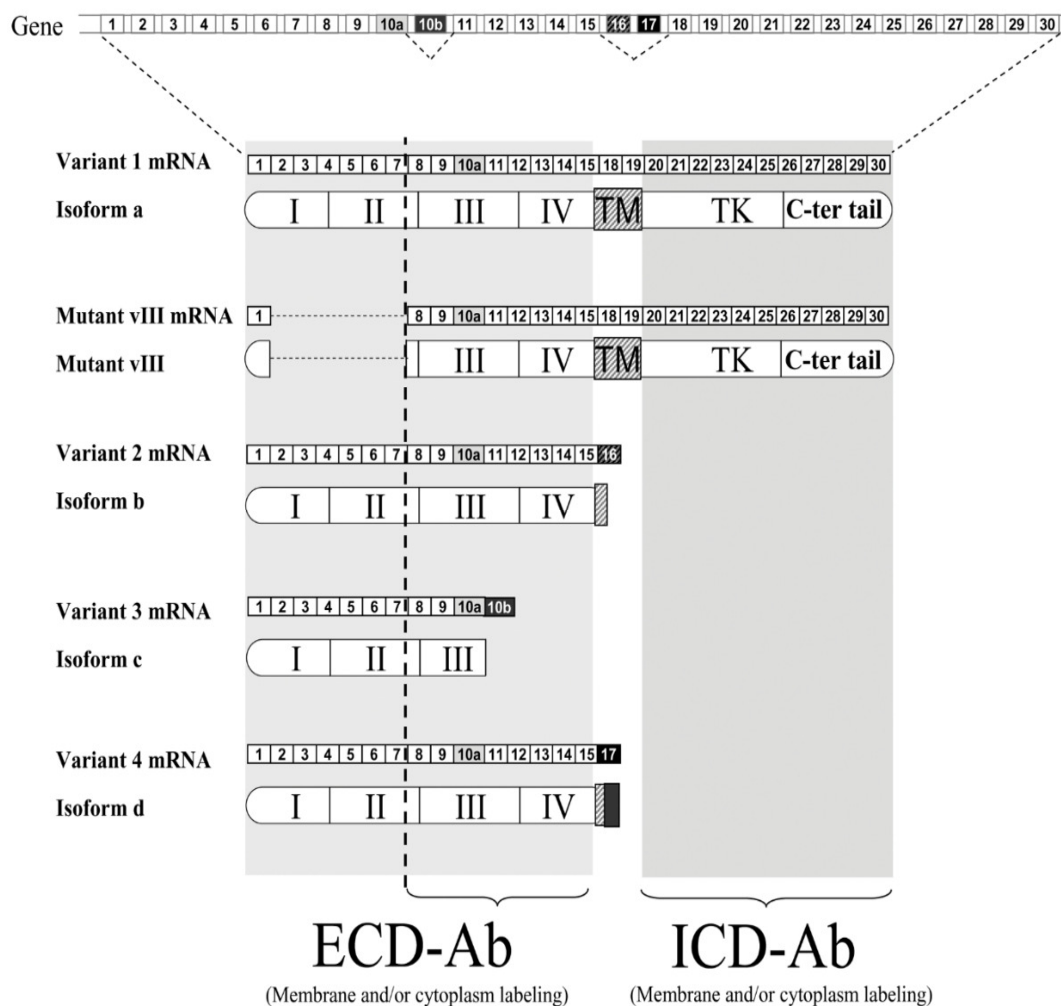

Figure 1.4: ECD-Ab and ICD-Ab targeting. EGFR gene contains 30 exons and generates 5 different mRNAs. Variant 1 mRNA encodes the whole EGFR isoform a. Alternative splicing generates variant mRNAs 2, 3, and 4 that encode sEGFR isoforms B, C and D respectively. EGFRvIII mutant mRNA with a 801 bp (exons 2–7) deletion produces EGFR vIII that lack amino acids 2 to 273. ECD-Ab targets EGFR extracellular domain and recognizes sEGFR and vIII mutant, whereas ICD-Ab targets EGFR intracellular domain and recognizes EGFR isoform A and vIII mutant. Reprinted with permission from Guillaudeau *et al*., 2012.

Circulating sEGFR level have been used as prognosis and theragnosis predictive markers in the serum of patients with cervical (Oh *et al*., 2000), colorectal (Zampino *et al*., 2008), ovarian and breast (Baron *et al*., 2009a, 2009b, 2001; Volkmar Müller, Isabell Witzel,

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Klaus Pantel *et al*., 2006) cancer. The predictive value of sEGFR was also studied directly in tumor tissues from cervical or lung cancer (Halle *et al*., 2011; Maramotti *et al*., 2012).

The profiling of EGFR status plays very significant role in determining treatment regimen for cancer patients. Currently antibodies are used for the detection of EGFR expression. It is very critical to know which epitope is recognized by the antibody when studying protein expression. Since different EGFR isoforms are produced due to alternative splicing, the same antibody did not serve all the purposes. Antibody recognizing intracellular domain can detect only EGFR variant I and VIII. However antibodies directed against extracellular domain (specific to domain III and IV) can recognize all the variants of EGFR (**Fig. 1.4**).

1.2.7 Role of Epidermal Growth Factor Receptor in cancer

EGFR plays key role in essential cellular functions including proliferation and migration. However, its aberrant expression can lead to the development of cancer. The epidermal growth factor receptor (EGFR) is a rational target for antitumor strategies. High levels of EGFR activity results in increased cell proliferation and invasiveness. Increased expression of wild type and mutant EGFR is a widespread feature of diverse types of cancer. EGFR signaling in cancer has been the focus of intense investigation for decades primarily for two reasons. First, aberrant EGFR signaling is likely to play an important role in the pathogenesis of cancer, and therefore, the mechanisms of EGFR-mediated oncogenic signaling are of interest. Second, the EGFR signaling system is an attractive target for therapeutic intervention (Hatanpaa *et al*., 2010). EGFR has been implicated in the development of a wide range of epithelial cancer including those of the breast, colon, head and neck, kidney, lung, pancreas, and prostate. Deregulation of EGFR often correlates with decreased disease-free and overall survival of the patients.

In Non-Small Cell Lung Cancer (NSCLC), EGFR overexpression is observed in 40–80% of patients and is related to low grade of differentiation, increased tumor growth, and high metastatic rate (Hirsch, 2003; Pavelic *et al*., 1993). EGFR has been shown to be over-expressed in colon cancer cell lines and is detectable by immunohistochemistry (IHC) in 65-75% of colorectal cancer tumors (Ciardiello *et al*., 1991). The EGFR was

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found to act as a strong prognostic indicator in head and neck, ovarian, cervical, bladder and oesophageal cancers. In these cancers, increased EGFR expression was associated with reduced recurrence-free or overall survival rates in 70% (52/74) of studies. In gastric, breast, endometrial and colorectal cancer, the EGFR provided more modest prognostic information, correlating to poor survival rate in 52% (13/25) of studies (Nicholson *et al*., 2001). EGFR is a well-established treatment target for colorectal cancer, non-small cell lung cancer, and squamous cell carcinoma of the head and neck.

In breast cancer, EGFR overexpression has been reported in up to 70-80% of triple-negative breast cancer, suggesting that EGFR is a potential therapeutic target for triple-negative breast cancer (Choi *et al*., 2012; Gumuskaya *et al*., 2010; Hwangbo *et al*., 2013; D. Liu *et al*., 2012; Martin *et al*., 2012; Nakajima *et al*., 2014; Rakha *et al*., 2007; Reis-Filho *et al*., 2006; Shao *et al*., 2011; Tan *et al*., 2008; Teng *et al*., 2011; Toyama *et al*., 2008; Viale *et al*., 2009). Triple negative breast cancers (TNBC) are defined as tumors that lack overexpression of estrogen receptor (ER), progesterone receptor (PR) and epidermal growth factor receptor 2 (Her2/cerbB2/EGFR2) expression. Due to lack of expression of hormone receptors and Her 2 it is resistant to targeted therapies and hormone antagonists such as tamoxifen or aromatase inhibitors, nor trastuzumab, a monoclonal antibody against HER 2. Thus, the TNBC patients are left with the only option of chemotherapy and radiation therapy. Till date there is no FDA approved targeted therapy available for TNBC patients. EGFR/ HER-1 plays most important role as a prognostic marker in TNBC. Despite its well-studied role in TNBC pathogenesis, many of the Phase II study of EGFR tyrosine-kinase inhibitor in metastatic breast cancer failed and has at most 5% response rate. Although inhibition of EGFR activity has yielded modest clinical success inTNBC, substantial gains in clinical response rates have not been achieved. However, a major breakthrough in EGFR-targeted therapy was reported at the European Society for Medical Oncology meeting in October 2010. In a randomized phase II study consisting of 173 patients with metastatic TNBC, receive either cetuximab, an anti-EGFR antibody, plus up to six 3-week cycles of cisplatin (N=115) or cisplatin alone (N=58). An overall response rate of 20% was seen in patients who received the cetuximab/cisplatin combination, compared with a response rate of 10.3% in the cisplatin-alone arm. Adding cetuximab to cisplatin doubled the progression-

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free survival duration from 1.5 to 3.7 months (HR 0.675, P = 0.032). These exciting clinical data suggest that EGFR is an important target in TNBC (Baselga *et al*., 2013).

EGFR gene amplification and overexpression are observed in approximately 40% of tumors of glioblastoma (GBM). GBM is the most common primary malignant tumor of the central nervous system in adults. In approximately 50% of tumors with EGFR amplification, a specific EGFR mutant (EGFRvIII, also known as EGFR type III, de2-7, ΔEGFR) can be detected (Ekstrand *et al*., 1991; Wong *et al*., 1992). This mutant is highly oncogenic and is generated from a deletion of exons 2 to 7 of the EGFR gene, which results in an in-frame deletion of 267 amino acids from the extracellular domain of receptor. EGFRvIII is unable to bind ligand, and it signals constitutively. EGFRvIII is usually coexpressed with the wild type (wt) receptor in GBM (Biernat *et al*., 2004; Ekstrand *et al*., 1991).

Table 1.1: Current drugs targeting EGFR on market or under clinical trial. Reprinted with permission from Chen *et al*., 2016.

Brand Name	**Generic Brand**	**Action**	**Status**	**Application**
Iressa	Gefitinib	EGFR TKI	On market (64 countries)	Metastatic NSCLC, breast cancer and other cancers
Tarceva	Erlotinib	EGFR TKI	On market (U.S.)	NSCLC, pancreatic cancer and other cancers
Tykerb/Tyverb	Lapatinib	EGFR/HER2 dual TKI	On market (multiple countries) Phase III	Breast cancer, gastric cancer, other solid tumors,
Gilotrif/Giotrif	Afatinib	EGFR/HER2 dual TKI	On market (multiple countries)	Metastatic NSCLC with EGFR mutations

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Icotinib	Icotinib	EGFR TKI	On market (China only)	Metastatic NSCLC with EGFR mutations
Vectibix	Panitumumab	EGFR mAbs (IgG2)	On market (multiple countries)	EGFR expressing metastatic CRC with disease progression, refractory EGFR-expressing metastatic CRC with WT KRAS
Erbitux	Cetuximab	EGFR mAb (IgG1)	On market (multiple countries)	Metastatic CRC and NSCLC, head and neck cancers
TheraCIM*	Nimotuzumab	EGFR mAbs	On market (multiple countries)	Glioma, SCCHN, nasopharyngeal cancer
CO-1686	Rociletinib	Mutant EGFR TKI	Phase III	NSCLC with EGFR-T790M
AZD9291		Mutant EGFR TKI	Phase III	NSCLC with EGFR-T790M
ASP8273		Mutant EGFR TKI	Phase I/II	NSCLC with EGFR-T790M
CimaVax-EFR		EGF vaccine	Phase II	NSCLC
LY3016859		TGF-α, EPR mAbs	Completed Phase II	Diabetic nephropathy

EGFR TKI, epidermal growth factor receptor tyrosine kinase inhibitor; NSCLC, non-small cell lung cancer; CRC, colorectal cancer; SCCHN, squamous cell carcinoma in head and neck; EGFR-T790M, EGFR with T790Mmutation. *BIOMab EGFR in India, TheraCIM in Canada, and CIMAher in Europe.

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1.2.8 Role of Epidermal Growth Factor Receptor in human diseases

The epidermal growth factor receptor and its ligands plays a very important role in the regulation of multiple cellular processes. It is involved in the proliferation and development of epithelial cells in multiple organs. EGFR activation is crucial for embryogenesis and organogenesis and impairment of EGFR signaling during organogenesis may result in respective defect in different organs **(Fig. 1.5)**.

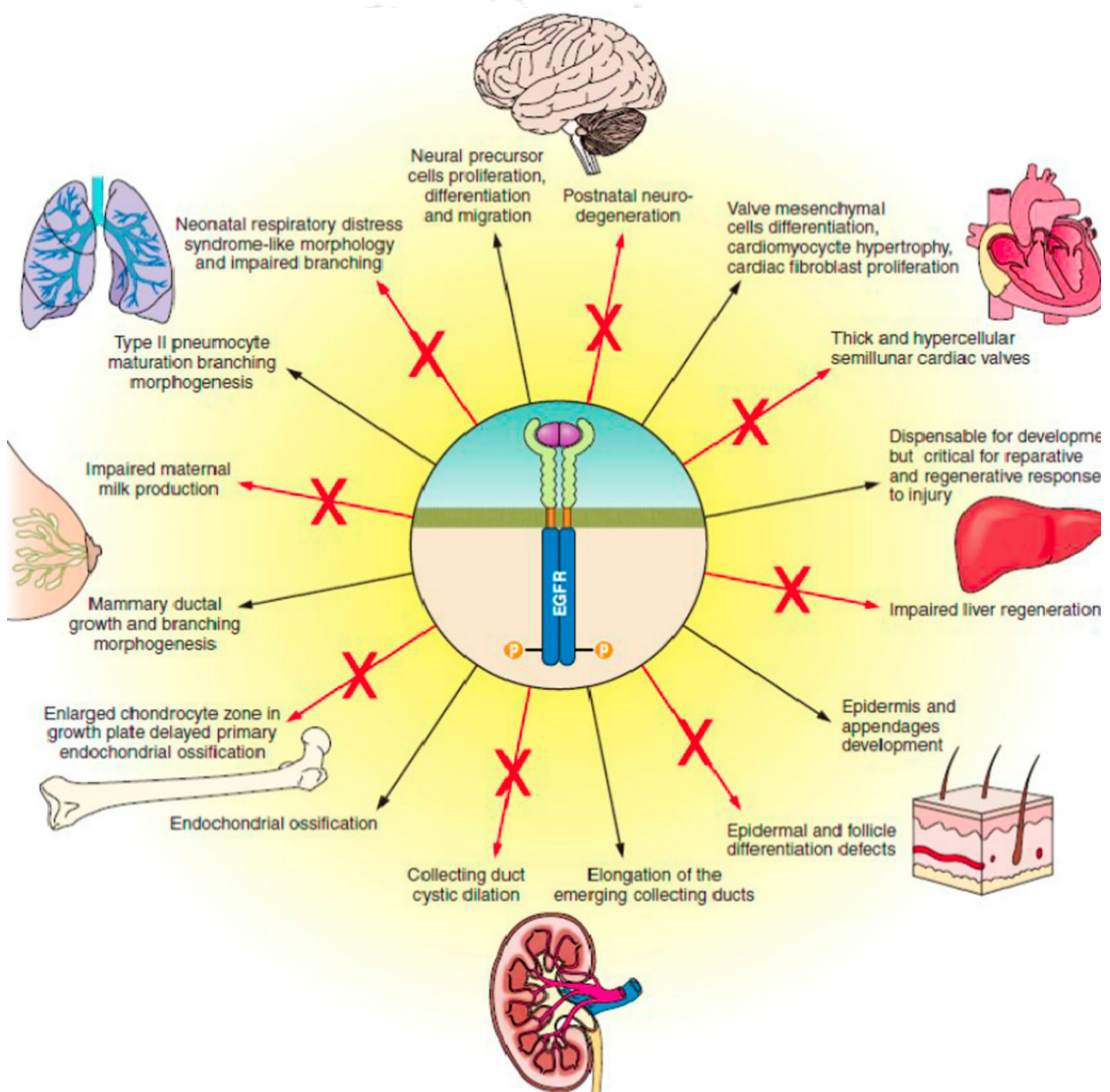

Figure 1.5: Potential role of EGFR signaling in multiple organs and the potential defects resulting from impairment of EGFR signaling in respective organ. Reprinted with permission from Chen *et al*., 2016.

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The EGFR-deficient mice suffers from several abnormalities in the skin, kidney, brain and gastrointestinal tract (Kornblum *et al.*, 1998; Miettinen *et al.*, 1995; Sibilia *et al.*, 1998). The dysregulation in EGFR expression are often related to multiple diseases like inflammatory, cardiovascular, kidney, lungs, liver , skin, age related diseases etc (Jost *et al.*, 2000; Komposch and Sibilia, 2016; Makki *et al.*, 2013; Vallath *et al.*, 2014). However its role in such diseases is still ill defined. The researchers have been concentrating on exploring the role of EGFR and its signalling pathways in various human diseases.

Healthy individuals have few goblet cells in their airways, but in patients with hypersecretory diseases often have goblet-cell upregulation which results in mucus hypersecretion, airway plugging, and death. Multiple stimuli produce hypersecretion via epidermal growth factor receptor (EGFR) expression and activation, causing goblet-cell metaplasia from Clara cells by a process of cell differentiation. These cells are also believed to be the cells of origin of non-small-cell lung cancer, but this occurs via cell multiplication (Nadel *et al.*, 2001). Deregulation of the EGFR pathway causing aberrant EGFR signalling is associated with the early stage pathogenesis of lung fibrosis and numerous airway hypersecretory diseases, including chronic obstructive pulmonary diseases (COPD) , asthma and cystic fibrosis (Vallath *et al.*, 2014). Despite the fact that the EGFR appears to be involved in goblet cell hyperplasia and metaplasia in COPD and other diseases, its value as a potential therapeutic target for treating COPD or other airway inflammatory diseases has yet to be demonstrated (Voelkel and MacNee, 2008).

Among all tissues, EGFR is highest expressed in hepatocytes of adult liver (Carver *et al.*, 2002), indicating an important role in maintaining liver function. The EGFR signaling axis has been shown to play a key role during liver regeneration following acute and chronic liver damage, as well as in cirrhosis and hepatocellular carcinoma (HCC) highlighting the importance of EGFR in the development of liver diseases (Komposch and Sibilia, 2016). Natarajan *et al* deleted EGFR in livers of adult and fetal mice. Perinatal deletion of EGFR in hepatocytes resulted in decreased body weight, whereas deletion in adult liver did not affect body mass. Following partial hepatectomy, adult mutant mice showed impaired liver regeneration, and the regenerating livers displayed an impaired stress response (Natarajan *et al.*, 2007).

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The epidermal growth factor receptor (EGFR) pathway has a critical role in renal development, tissue repair and electrolyte handling. In the kidney, EGFR is expressed by tubular cells throughout the nephron (Breyer *et al.*, 1990) but also in the glomerulus including the podocytes. As EGFR is expressed throughout the whole epidermis, it contributes to epidermal proliferation, differentiation and hair growth. It plays key roles in the innate immune responses and inhibition of EGFR signaling will inevitably compromise skin integrity (Ehmann *et al.*, 2011) Many genetic animal models with an increase or decrease in EGFR ligands or EGFR have abnormal skin phenotypes (Luetteke *et al.*, 1994).

Aberrant EGFR signaling has been reported in neonatal inflammatory skin and bowel diseases (Brooke *et al.*, 2014). Campbell *et al.* describe a unique patient with an inflammatory bowel, lung and cutaneous syndrome that presented within the first year of life and persisted until death at the age of 2.5 years due to infection. Whole-exome sequencing (WES) genetic analysis, in conjunction with immunohistochemistry and in vitro cell-based studies, support a homozygous missense mutation in the EGFR gene as the underlying cause of this syndrome (Campbell *et al.*, 2014). The skin lesions involve perioral and perianal erythema, psoriasiform erythroderma, with flares of erythema, scaling, and widespread pustules. Gastrointestinal symptoms include malabsorptive diarrhea that is exacerbated by intercurrent gastrointestinal infections. The hair is short or broken, and the eyelashes and eyebrows are wiry and disorganized.

The EGFR signaling appears to be very important for some biologic processes such as skin repair, wound healing etc (Pastore *et al.*, 2008; Repertinger *et al.*, 2004). On the other hand, excessive EGFR signaling may participate in processes that are ultimately destructive to skin, such as in the skin's carcinogenic response to ultraviolet (UV) exposure (El-Abaseri *et al.*, 2013, 2006, 2005; Knebel *et al.*, 1996; Saeed and Salmo, 2012).

Alterations in EGFR expression levels can be also observed during neurodegeneration. Studies performed by Repetto and co-workers demonstrated that presenilin 1 (PS1) is a critical regulator of the EGFR pathway (Repetto *et al.*, 2007). Mutations in PS1 and PS2

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genes are responsible for the vast majority of early onset familial Alzheimer disease (Sherrington *et al.*, 1995).

The EGFR activity is essential for normal cardiac development and its function in the vasculature. However its role in cardiovascular disease are only beginning to be elucidated. Activation of EGFR has been implicated in blood pressure regulation, endothelial dysfunction, neointimal hyperplasia, atherogenesis and cardiac remodeling. Although EGFR inhibitors are currently being used clinically for the treatment of cancer, the role of EGFR in vascular disease is poorly defined and additional studies are necessary to determine whether abrogation of EGFR signaling is a potential strategy for the treatment of cardiovascular disease (Makki *et al.*, 2013).

1.2.9 Inhibitors of Epidermal Growth Factor Receptor

EGFR represents an attractive target for anticancer therapies in a variety of malignant neoplasms, including colorectal cancer, non-small-cell lung cancer (NSCLC), head and neck carcinomas, breast cancer and gliomas. The first anti-EGFR drugs were developed in the 1980s (Masui *et al.*, 1984). Currently two distinct therapeutic approaches are employed for targeting EGFR in various human malignancies: monoclonal antibodies and small molecule tyrosine kinase inhibitors. Anti EGFR monoclonal antibodies binds to the extra cellular domain of EGFR when it is in inactive state and thereby blocks binding of ligands to its receptor. It further induces receptor internalization and its degradation, resulting in downregulation of surface EGFR expression (Hynes and Lane, 2005; Li *et al.*, 2005; Normanno *et al.*, 2003). Cetuximab is the most extensively studied anti-EGFR mAb. Small-molecule inhibitors (gefitinib, erlotinib and lapatinib) are either reversible or irreversible by nature, competes with ATP for binding to the intracellular tyrosine kinase domain of the receptor and thus, inhibit EGFR autophosphorylation and downstream signalling.

Anti-EGFR monoclonal antibodies are highly specific and selective as they exclusively recognize this receptor. But various small-molecule EGFR tyrosine kinase inhibitors can block different growth factor receptor tyrosine kinases, including other members of the EGFR family, or the vascular endothelial growth factor receptor. Various irreversible EGFR tyrosine kinase inhibitors are now in early stages of clinical development. The

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mechanism (or mechanisms) of action, pharmacologic effects, and spectrum of activity of anti-EGFR monoclonal antibodies and small-molecule EGFR tyrosine kinase inhibitors have differences that may be relevant for clinical activity. Different monoclonal antibodies and tyrosine kinase inhibitors are summarized in **Table 1.2 and 1.3**. The patients receiving these treatments often develops numerous side-effects such as severe dermatologic toxicity and anaphylactic or allergic reactions. Additionally these treatment results into primary or acquired resistance to the inhibitors (Ciardiello and Tortora, 2008).

Table 1.2: EGFR family tyrosine kinase inhibitors (TKIs) under investigation for the treatment of breast cancer. Adapted with permission from Masuda *et al*., 2012.

Tyrosine Kinase Inhibitors (TKI)	**Target**	**Class of Action**
Gefitinib	EGFR	Reversible TKI
Erlotinib	EGFR	Reversible TKI
Aderbasib	EGFR	Reversible TKI
AE37	EGFR	Reversible TKI
AZD4769	EGFR	TKI
Lapuleucel-T	EGFR	Designed to stimulate cellular immune responses against HER2/neu
CL-3877785	EGFR	Irreversible TKI
Lapatinib	EGFR, ErbB2	Reversible TKI
BIBW2992	EGFR, ErbB2	Irreversible TKI
S222611	EGFR, ErbB2	Reversible TKI
TAK285	EGFR, ErbB2	TKI
AV412	EGFR, ErbB2	Irreversible TKI
PKI-166	EGFR, ErbB2	TKI

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Varlitinib(ARRY-334543)	EGFR, ErbB2, ErbB4	Reversible TKI
BMS-599626	EGFR, ErbB2, ErbB4	Reversible TKI
EKB-569	EGFR, ErbB2, ErbB4	Irreversible TKI
PF299804	EGFR, ErbB2, ErbB4	Irreversible TKI
AZD8931	EGFR, ErbB2, ErbB3	Reversible TKI
Vandetanib	EGFR, VEGF, RET	TKI
CUDC101	EGFR, ErbB2, HDAC	Irreversible TKI
Neratinib (HKI-272)	Pan-EGFR	Irreversible TKI
Canertinib (CI-1033)	Pan-EGFR	Irreversible TKI
BMS690514	Pan-EGFR, VEGFR2	Irreversible TKI
XL647	EGFR, ErbB2, EphB4,VEGF	Reversible TKI
AEE788	EGFR, ErbB2,VEGF	Reversible TKI
ARRY380	ErbB2, AKT	Reversible TKI

Table 1.3: Monoclonal antibodies (MAbs) against epidermal growth factor receptor for the treatment of breast cancer. Adapted with permission from Masuda *et al.*, 2012.

Monoclonal Antibody (Mab)	**Type**
Cetuximab	Chimeric Mab
Panitumumab	Humanized MAb
GA 201	Mab
Nimotuzumab	Humanized MAb
Matuzumab	Humanized MAb

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Cetuximab (IMC-C225 / Erbitux) is an FDA approved human-murine chimeric anti-EGFR monoclonal antibody (Ennis *et al*., 1991; Lenz, 2007). It binds to the L2 domain of EGFR ECD thereby blocking its downstream signaling by prompting receptor internalization and encumbering ligand-receptor interaction. It has been found that cetuximab treatment is effective in 60% of the patients expressing a KRAS wild-type tumor (van Krieken *et al*., 2008). In 2008, the Committee for Medicinal Products for Human Use (CHMP) approved Cetuximab for patients with advanced colorectal cancer who had 75% EGFR positive expression and wild-type KRAS in their tissues. Cetuximab was approved by FDA in 2004 and by CHMP in 2008 in combination with platinum-based therapy for the treatment of patients with squamous cell carcinoma of the head and neck with metastatic disease and in combination with radiation therapy for locally advanced cancer. The available clinical data on cetuximab in advanced NSCLC are contradictory and cetuximab is currently undergoing phase III trials in advanced NSCLC. Apart from EGFR inhibition, cetuximab also down regulate VEGF, IL-2 and bFGF (Galizia *et al*., 2007; Mendelsohn *et al*., 2000; van Krieken *et al*., 2008).

Panitumumab formerly known as ABX-EGF is the first FDA (2006) approved fully human monoclonal antibody used for the treatment of EGFR-expressing metastatic colorectal cancer. It is a human monoclonal antibody specific to human EGFR, developed by immunizing transgenic mice (XenoMouse) that are capable of producing light and heavy chains of human immunoglobulin (Yang *et al*., 2001). It acts as an antagonist by binding to the ECD of EGFR, thereby preventing binding of the endogenous ligands- epidermal growth factor (EGF) or transforming growth factor-alpha and induces internalization of EGFR (Dubois and Cohen, 2009). It was found effective for colorectal cancer patients carrying KRAS mutation. However patients undergoing treatment suffers from side effects such as eye, skin and gastrointestinal problems. Hypomagnesaemia is another common problem which is due to its effect on tubular reabsorption of filtered magnesium in the kidney and on magnesium absorption in the gastrointestinal tract (Dubois and Cohen, 2009).

Gefitinib is an anilinoquinazoline derived EGFR tyrosine kinase inhibitor and was first characterized in the year 1996 (Wakeling *et al*., 1996; Woodburn, 1999). It is an orally active low-molecular-weight EGFR inhibitor with selective tyrosine kinase activity but

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does not inhibit serine-threonine kinase activity. Gefitinib has a 200-fold greater affinity for EGFR relative to the other ErbB family members (Thomas and Grandis, 2004) . Gefitinib has been approved by FDA as monotherapy for patients with locally advanced or metastatic nonsmall-cell lung cancer (NSCLC) (Haringhuizen *et al*., 2004).

Erlotinib (OSI-774; Tarceva) is another FDA-approved low molecular weight molecule similar to gefitinib, available in the form of an orally potent and selectively reversible inhibitor of EGFR tyrosine kinase. Like gefitinib, erlotinib functions as an ATP analogue by competing with ATP binding pockets within the RTKs. Also, it exerts anti-proliferative effects, cell-cycle arrest and apoptosis (Moyer *et al*., 1997; Wakeling, 2002). It is most commonly used in combination with other chemotherapeutic agents, including Capecitabine and Docetaxel.

Lapatinib (GW-572016) Lapatinib is an orally active, reversible and specific RTK inhibitor of both EGFR and HER2. It was also found to exhibit activity against AKT overexpressing human tumor xenografts (Rusnak *et al*., 2001). It is used as a broad spectrum anti tumor agent due to its nonselective nature of EGFR inhibition. Its mechanism of action is similar to that of gefitinib and erlotinib (Konecny *et al*., 2006).

Table 1.4: Comparison between EGFR specific monoclonal antibodies vs. Tyrosine Kinase Inhibitors. Adapted with permission from Seshacharyulu *et al*., 2012.

Characteristic features	**Anti-EGFR monoclonal antibodies**	**Anti EGFR TKI**
Nature of the molecule and size	Biological, recombinant immunoglobulin either of type IgG2 (Panitumumab) or IgG1 (Cetuximab) and large proteins approximately 150 kDa.	Synthetic chemicals, Small molecules and approximately 500 Da.
Half life	3.1 to 7.8 days.	Less as compared to mAbs (Gefitinib 48 hrs and erlotinib 36 hrs).
Specificity	Binds to ectodomain with high specificity.	Lesser as compared to mAbs, either selective to

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		specific nucleotide binding site or dual or multi selective.
Mode of action	Therapy suited for extracellular targets, activates endocytosis and apoptosis.	Effective against both intracellular and extracellular targets, inhibits phosphorylation and downstream proliferative signal and induces apoptosis.
Inhibition	Achieved at lower concentrations.	Higher concentration is required and dependent on the cell type.
Toxicity, mode of administration and dosage	Less toxic, intravenous and weekly once or bi-weekly.	Mild or highly toxic, oral and daily.
Success rate in clinical studies	Higher (18% chimeric and 24% humanized).	Lesser as compared to mAbs (5%).
Immune response to the therapy	Immune-antibody response which will render antibody therapy ineffective.	No such reaction.
Response to therapy	Less or ineffective against EGFRvIII. Fails to recognize the extracellular ligand binding domain.	EGFR independent constitutively activated K-RAS signaling will impair inhibitor response to the therapy.
Cost and advantage	Expensive and in therapeutics it is highly specific and selective.	Inexpensive and Specific to tyrosine kinase domain.
Adverse effect	Severe acne like rash, rash, fevers, chills, dyspnea, wheezing, dizziness, hypotension,	Acneiform skin rash, serum transaminase elevations, stomatitis,

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	anaphylaxsis reaction, bronchospasm, cardiac arrest, photosensitivity, hypomagnesemia, cardiac toxicity, nausea, weight loss and abdominal pain.	bone pain, dyspnea, alveolitis, pneumonitis and interstitial pneumonia.

1.2.10 Evaluation of Epidermal Growth Factor Receptor

The EGFR status are often studied for determining the choice of treatment for the cancer patients. EGFR can be evaluated in different ways: quantitation of the receptor at the DNA, RNA or protein level, or assessment of the degree of signalling from the receptor through analysis of receptor activation or activation of downstream markers.

The EGFR gene amplification, its mutation or deletion are quantified by analysing the DNA. However, this does not necessarily gives an idea about the amount of protein produced. The gene copy number are measured by fluorescence *in situ* hybridization (FISH) and the presence of specific mutations within the TK domain of the EGFR are identified by mutational analysis (Cappuzzo *et al*., 2005; Lynch *et al*., 2004; Paez *et al*., 2004; Pao *et al*., 2004; Tokumo *et al*., 2005). Northern blotting or quantitative reverse transcription polymerase chain reaction (PCR) are used to assess the mRNA levels. But the techniques based on mRNA estimation are associated with the problems of RNA degradation and contamination. Moreover posttranslational modifications might affect the quantity of protein produced; therefore, it may not be appropriate to make assumptions about EGFR levels based on mRNA measurement (Ciardiello and Tortora, 2003).

Activation of the EGFR signalling network can be estimated by measurement of EGFR phosphorylation, for example, IHC or immunoprecipitation, or assessment of downstream targets of EGFR, such as proliferation and maturation markers (Albanell *et al*., 2002, 2001). EGFR protein levels may also be quantified by western blot analysis and enzyme immunoassay. These methods measure total receptor protein and provide no information on cellular localisation. EGF binding assays with radioactive ^{125}I-EGF use autoradiography to determine the localisation of accessible, unoccupied cell surface

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receptors, but considerable interassay variability has been reported (Robertson *et al.*, 2002).

The EGFR protein expression level are commonly evaluated by Immunohistochemistry (IHC) which is the most convenient and widely practised method for analysing clinical samples (Nicholson *et al.*, 2001). This technique have an advantages of determining the subcellular localisation of protein, evaluating the levels of activated (phosphorylated) EGFR and also helps in comparing the samples. However, this method is not strictly quantitative as there is no uniform scoring system and the interpretation of staining intensity is highly subjective. In general, samples are scored as EGFR-positive when either the percentage of stained cells or the staining intensity is above a specified threshold level and are considered EGFR-negative when below this threshold. Different labs are using different threshold level for assessing a tumor sample as positive or negative. In addition, variations in protocols, such as in fixation procedures and antibodies, are likely to affect the sensitivity of these assays, making comparison of results from different laboratories difficult (Nicholson *et al.*, 2001). There is still a lack of agreement on a standard assay or cutoff for EGFR expression levels with respect to prognosis.

In addition to this antibodies suffers from other limitations such as batch to batch variation, poor tissue penetration, immunological reaction, instability, high cost of production etc. Thus intense research were fuelled in search of new molecular probe for the detection of cancer biomarkers. Research is being done to develop targeted molecules that are aimed at proteins overexpressed by cancerous cells or produced by specific gene mutations in cancer cells. Recently oligonucleotide probes i.e. aptamers emerges as a new class of nucleic acid ligands that binds to its target with very high affinity and selectivity. In last few years, aptamers have dramatically impacted nearly every branch of medicine and clinical research as a promising alternative to antibodies. Aptamers exhibit significant advantages relative to protein therapeutics in terms of size, synthetic accessibility and modification by medicinal chemistry.

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1.3 Aptamers

1.3.1 Introduction

Earlier it was believed that nucleic acids are the storage house of genetic information. But with the advancement of new technology many applications of nucleic acids were explored such as siRNA, miRNA etc. Recently it was found that nucleic acids can not only hybridize to one another to form double stranded structure, but can also be folded into a myriad of complex three dimensional shapes that are specific to protein and small molecule targets. Thereafter, a series of technological advances aided the development of in vitro evolutionary methods for the discovery of additional, non-biological oligonucleotides that can bind to protein targets.

Aptamers are short single-stranded oligonucleotides, either RNA or DNA that fold into well-defined 3-D structures and bind specifically to their ligand by complementary shape interactions (**Fig. 1.6**). Aptamers are selected from a randomly synthesized initial library containing up to 10^{13} to 10^{15} different molecules of oligonucleotides through a combinatorial chemistry procedure termed as Systematic Evolution of Ligands by EXponential enrichment (SELEX) consisting of repetitive cycles of selection and amplification (Ellington and Szostak, 1990; Tuerk and Gold, 1990). In last few decades aptamers have been selected against a wide variety of targets, ranging from small inorganic molecules to whole organisms (Blank and Blind, 2005; Nimjee *et al.*, 2005; Proske *et al.*, 2005). Aptamers are analogous to antibodies due to its specific biorecognition property but they have many attributes which makes them superior to antibodies. Most commonly either DNA or RNA aptamers are used. A third group of aptamers represented by short peptide sequences has also been described. Peptide aptamers are molecules in which a peptide moiety, with affinity for a given target protein, is displayed from an inert scaffold protein (Hoppe-Seyler *et al.*, 2001). Recently, a novel class of chemically derived nucleic acids, called xeno nucleic acids (XNAs) has emerged (Herdewijn and Marlière, 2009). These are oligonucleotides with an unnatural sugar backbone that were shown to possess similar features as DNA and RNA.

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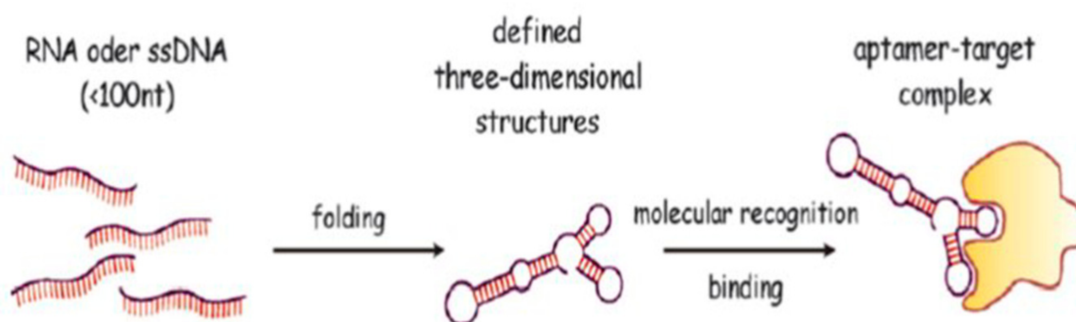

Figure 1.6: Molecular recognition of targets by aptamers with defined three dimensional structures. Reprinted with permission from Stoltenburg *et al*., 2007.

1.3.2 History of aptamers

In 1990, two research groups demonstrated that the large libraries of RNAs could be screened *in vitro* for RNA ligands that binds to T4 DNA polymerase (Tuerk and Gold, 1990), and a variety of organic dyes respectively (Ellington and Szostak, 1990). This selection process was termed SELEX (Systematic Evolution of Ligands by EXponential enrichment) by Tuerk and Gold (1990), and RNA ligands were named aptamers by Ellington and Szostak (1990). The term aptamer is derived from the Latin word “aptus”- which means fitting and the Greek word “meros” - which means particle (Ellington and Szostak, 1990). Aptamers folds into a complex three-dimensional shape characterized by stems, loops, bulges, hairpins, pseudoknots, triplexes, and/or quadruplexes. Based on their three-dimensional structures, aptamers can bind to a wide variety of targets. Binding of the aptamer to the target molecule results from structure compatibility: stacking of aromatic rings, electrostatic, van der Waals interactions, hydrogen bindings, or from a combination of these effects (Hermann and Patel, 2000).

1.3.3 Aptamer library

The central region of an aptamer molecule consists of a random DNA or RNA oligonucleotide sequence. This sequence must be flanked by 5′ and 3′ constant region that provide primer hybridization sites for Klenow extension, a primer-annealing site for cDNA synthesis, PCR amplification and T7 RNA polymerase transcription all of which are essential in the SELEX protocol (Fitzwater and Polisky, 1996). The length of random

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region varies in between 30 to 120 nucleotides and the primer region are generally 15 to 20 nucleotides in length. Currently, libraries containing both ssDNA and RNA molecules are widely used for the selection of aptamers. The complexity of an aptamer library can be calculated very easily. For example in a library which consists of oligonucleotides of N nucleotides in length, generated from 4 different nucleotides, the complexity of library will be 4^{35}. Therefore the maximum, referred to as the "sequence space" is around 10^{21}. However, practically the maximum diversity that can be introduced in a library varies from 10^{13}–10^{15} (Sampson, 2003). The library could be labelled with a fluorophore for monitoring aptamer enrichment during SELEX rounds.

1.3.4 The general principle of SELEX methodology

Systematic Evolution of Ligands by EXponential enrichment (SELEX) is a combinatorial chemistry procedure that allows rapid selection of the oligos that have strong binding affinity to a given molecular target, starting from a large initial library of oligonucleotides, (Tuerk and Gold, 1990). The initial aptamer library are obtained via the conventional methods for the chemical synthesis of oligodeoxyribonucleotides using a mixture of all four monomers when synthesizing a randomized fragment. In case of DNA SELEX the DNA derived from solid phase synthesis can be used directly. However in RNA SELEX an additional step is introduced. The primer region are incorporated with promoter sequence for T7 RNA polymerase. The DNA oligonucleotide population generated synthetically serves as a transcription template to generate RNA pools via *in vitro* transcription for the RNA SELEX process. The general scheme of the SELEX method comprises of repetitive cycles of incubation, separation and elution is represented in **Fig. 1.7**. The library is incubated with the desired target molecule under conditions suitable for binding. Next, the unbound nucleic acids are partitioned from those bound specifically to the target molecule, which are then eluted from the target molecule and amplified by PCR. This selection procedure is reiterated for several rounds until the resulting sequences are highly enriched. For the selection of RNA aptamers, SELEX also comprises the following additional stages: production of the RNA library on the DNA matrix, reverse transcription of the bound RNA molecules to produce DNA and DNA amplification.

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The number of rounds necessary depends on a variety of parameters, such as target features and concentration, design of the starting random DNA oligonucleotide library, selection conditions, ratio of target molecules to oligonucleotides, or the efficiency of the partitioning method. Generally five to twenty rounds of selection are typically performed to obtain aptamers. Additional steps can be introduced into the SELEX process particularly with regard to the specificity of the oligonucleotides. Negative selection steps or subtraction steps are strongly recommended to minimize an enrichment of unspecifically binding oligonucleotides or to direct the selection to a specific epitope of the target (Marshall and Ellington, 2000).

The affinity of the oligonucleotides to their target can be influenced by the stringency of the selection conditions. Typically, the stringency is progressively increased in the course of a SELEX process. This can be achieved by reducing the target concentration in later SELEX rounds or changing the binding and washing conditions (buffer composition, volume, time) (Marshall and Ellington, 2000). The detection of an enrichment of target-specific oligonucleotides indicates that the SELEX process is finished. The last SELEX round is stopped after the amplification step and the PCR products are cloned to get individual aptamer clones from the selected pool. These individual aptamers are sequenced and analysed. Potential aptamers obtained are then evaluated by various methods. Mutation and truncation experiments can be performed to narrow down the minimal binding region within the aptamer sequence. Generation of aptamers using this method lasts from few weeks up to a month. Conventional SELEX is a well-established and effective method but due to its large time and labor consumption, continuous development of alternative methods for aptamer selection has been inevitable.

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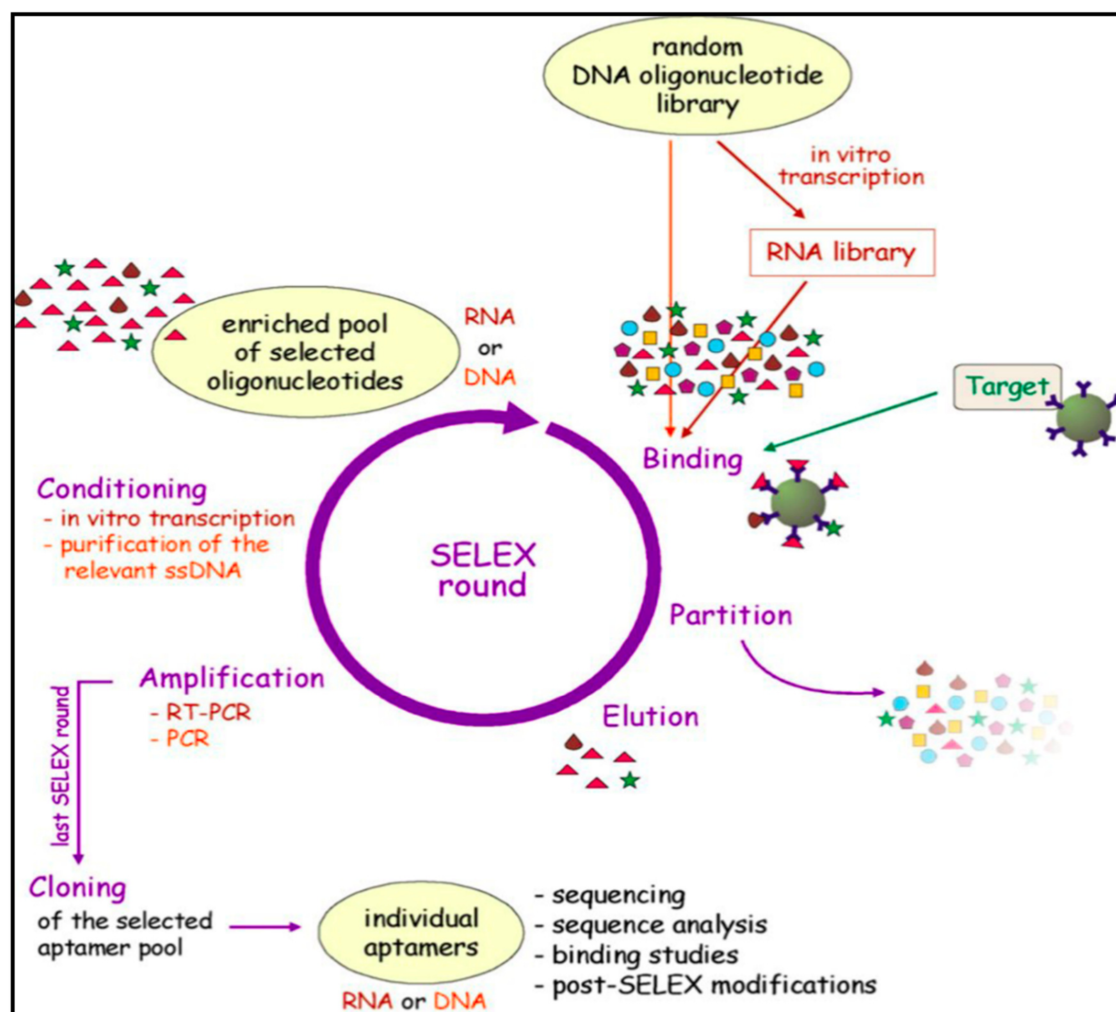

Figure 1.7: Schematic representation of SELEX process. Reprinted with permission from Stoltenburg *et al*., 2007.

This SELEX methodology is often modified to select aptamers for different applications. A large number of variations of the procedure originally established by Tuerk and Gold (1990) were described during last few years. Some of these methods were developed to increase affinity or specificity of the selecting aptamers, others to optimize the procedure. Some of the SELEX variants described in literature are Blended SELEX, Cell SELEX, Counter-SELEX or subtractive SELEX, Negative SELEX, SELEX-SAGE, Chimeric SELEX, Multi-stage-SELEX, Deconvolution-SELEX, Covalent SELEX or cross-linking SELEX, Photo-SELEX, SPIEGELMER Technology, Tailored-SELEX, Genomic SELEX or cDNA-SELEX, Toggle-SELEX, Indirect selection, *in vivo* selection, Tissue

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SELEX, TECS-SELEX, FluMag-SELEX, CE-SELEX, Non-SELEX, On-chip selection etc (Djordjevic, 2007; Stoltenburg *et al*., 2007; Xi *et al*., 2014).

1.3.5 Cell SELEX

It is the most widely used method for selection of aptamers for cancer related proteins. The usage of cells as targets for in vitro selection was first described by K. Morris *et al* (Morris *et al*., 1998). Almost all the methods of SELEX are based on knowledge of the target for aptamer selection. The only exception is cell-SELEX. This well-developed method does not require prior target knowledge. Whole cells, both eukaryotic and prokaryotic, can be used as a target to select highly specific aptamers using this technique. The use of Cell-SELEX for the generation of aptamers has many advantage compared to the purified proteins as target. Most importantly, using this process aptamers can be generated specific to those proteins even without its detail knowledge. Also, there is no need to purify specific molecular targets, and cell-surface proteins remain in their native conformations throughout the selection process. Moreover aptamers can also be selected for proteins with cancer derived post-translational modifications (Phillips *et al*., 2008). The difference in the molecular signature of target cell line and control cell line are exploited for aptamer selection. To successfully implement cell-SELEX, there should be evidence that the target cancer cell has some up-regulated or completely different protein expressed on its surface compared to the control cell line. Most commonly Cell SELEX technology are used to select aptamers against cancerous cell line. This also opens a novel method for discovering new cancer biomarkers- AptaBid (Aptamer based biomarker discovery). By the end of selection process a panel of aptamers were generated which can bind several different biomarkers. Finally, the ability to purify and sequence target proteins led to the discovery of a new biomarker.

1.3.6 Targets for aptamer selection

Since its discovery different classes of targets *viz* inorganic molecules, small organic molecules, peptides, proteins, carbohydrates, antibiotics, and complex targets as whole cells, bacteria, and viruses used as a target in aptamers selection. Several aptamers have been developed against cancer-related proteins, such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), tenascin-C, nuclear factor kappa-

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light-chain-enhancer (NFκB) of activated B cells, and prostate-specific membrane antigen (PSMA), Vascular Endothelial Growth Factor(VEGF), tenacin C, Human epidermal receptor 2 (Her2) and Human Epidermal Receptor 3 (HER3) (Chen *et al.*, 2003; Hasegawa *et al.*, 2008; Hicke *et al.*, 2001; Ireson and Kelland, 2006; Z. Liu *et al.*, 2012; Lupold *et al.*, 2002; Mori *et al.*, 2004).

Cell SELEX method has been successfully employed to obtain a series of aptamers against various cancer cell types. Aptamers selected against cancer related protein is summarized in **Table 1.5.** A substantial number of aptamers were also produced against bacterial cells, such as *Trypanosoma cruzi* (Ulrich *et al.*, 2002), *Vibrio parahaemolyticus* (Duan *et al.*, 2012) *Salmonella typhimurium* (Dwivedi *et al.*, 2013), *Escherichia coli* (Kim *et al.*, 2013) using cell-SELEX technology. Besides the above-mentioned, aptamers were also developed against baby hamster cell lines (BHK-21) infected by rabies virus. Such types of aptamers, capable to suppress viral replication (Liang *et al.*, 2012) could have a great impact on medicine.

Numerous aptamers have been selected against the therapeutic targets, such as IgE (Immunoglobulin E), IFN-g (Interferon gamma), alpha-thrombin, PTPase, and they have shown great efficacy in tissue culture experiments and animal models. In earlier years, an antithrombin aptamer was used in place of heparin for anticoagulation during heart bypass surgery in canines (DeAnda *et al.*, 1994), whereas aptamers against inflammation factor human neutrophil elastase (hNE) were shown to significantly reduce lung inflammation in rats and had better specificity for their target than an antielastase IgG control (Bless *et al.*, 1997).

Table 1.5: Aptamer selected against cancer related biomarkers.

Target	Selection technique	Aptamer	Reference
Brain tumor gliosarcoma	Cell SELEX using K308 cell line	DNA	Wu *et al.*, 2016
Metastatic colorectal cancer	Cell SELEX	DNA	W.M. Li *et al.*, 2014
Prostate cancer	Cell SELEX using PC-3 cell line	DNA	Y. Wang *et al.*, 2014

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CD 44 exon v10	Peptide-SELEX	DNA	Iida *et al.*, 2014
Metastatic breast cancer	Cell SELEX using MDA MB 231 cell line	DNA	Li *et al.*, 2014
Matrix metalloproteinases (MMPs)	Protein SELEX	DNA	Han *et al.*, 2014
Siglec-5	Cell SELEX using NB4 cell line	DNA	Yang *et al.*, 2014
Epidermal Growth Factor Receptor (EGFR/Her1)	Protein SELEX Cell SELEX using A549 cell line	DNA RNA DNA	D.-L. Wang *et al.*, 2014 Esposito *et al.*, 2011 Xu *et al.*, 2013 Li *et al.*, 2011
Interferon-Gamma	Protein SELEX	DNA	Cao *et al.*, 2014
CD44, Cancer stem cell marker	Protein-SELEX	RNA	Ababneh *et al.*, 2013a
Hepatocarcinoma	Cell SELEX using HepG2 cell line	DNA	Ninomiya *et al.*, 2013
Epidermal growth factor receptor variant III	Cell SELEX using U87 glioma cell line	DNA	Tan *et al.*, 2013
c-kit	Cell SELEX using murine BJAB cell line	DNA	Meyer *et al.*, 2013
Epithelial cell adhesion molecule (EpCAM)	Protein SELEX	DNA RNA	Song *et al.*, 2013 Shigdar *et al.*, 2011
Metastatic hepatocellular carcinoma	Cell SELEX using HCCLM9 cell line	DNA	Wang *et al.*, 2013

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Hodgkin Lymphoma	Cell SELEX using HDLM2 cell line	DNA	Parekh *et al.*, 2013
Heparanase	Recombinant Protein	DNA	Simmons *et al.*, 2012
Breast cancer	Cell SELEX using MCF 10A T1 cell line	DNA	Zhang *et al.*, 2012
Urokinase-type plasminogen activator (uPA)	Protein SELEX	RNA	Botkjaer *et al.*, 2012
Lung cancer	Cell SELEX using H23 cell line	DNA	Jimenez *et al.*, 2012
Primary cultured mouse tumor endothelial cells	Cell SELEX	DNA	Ara *et al.*, 2012a
Her 2	Peptide-SELEX	DNA	Liu *et al.*, 2012
MUC1	Peptide SELEX Protein SELEX	DNA	Hu *et al.*, 2012 Ferreira *et al.*, 2006
Small Cell Lung Cancer	Cell SELEX	DNA	Kunii *et al.*, 2011
Glioblastoma multiforme	Cell SELEX using A172 cell line	DNA	Bayrac *et al.*, 2011
HPV-16 E7 Oncoprotein	Recombinant Protein	RNA	Toscano-Garibay *et al.*, 2011
Colorectal cancer	Cell SELEX using DLD-1 cell line	DNA	Sefah *et al.*, 2010
Ovarian cancer	Cell SELEX using TOV-21G cell line	DNA	Simaeys *et al.*, 2010

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Human Epidermal Growth Factor receptor 2 (HER-2)	Cell SELEX using SK-BR-3 cell line	RNA	Kang *et al.*, 2009
Unglycosylated EGFRvIII ectodomain	Recombinant Protein	RNA	Liu *et al.*, 2009
Acute myeloid leukemia	Cell SELEX using HL60 cell line	DNA	Sefah *et al.*, 2009
U87MG glioma cells	Cell SELEX	RNA	Cerchia *et al.*, 2009
Vascular endothelial growth factor (VEGF)	Protein SELEX	DNA	Burmeister *et al.*, 2005
Protein tyrosine kinase 7 (PTK7)	Cell SELEX using CCRF-CEM	DNA	Shangguan *et al.*, 2006
Tenascin C	Cell SELEX using U251 glioblastoma cells	DNA	Daniels *et al.*, 2003
Human epidermal growth factor receptor-3 (HER3	Protein SELEX	RNA	Chen *et al.*, 2003
Prostate-specific membrane antigen (PSMA	Protein SELEX	DNA	Lupold *et al.*, 2002

1.3.7 Chemical modifications of aptamers

For therapeutic purposes as well as a probe for *in vivo* imaging, a durable performance of aptamers in bio fluids are required. Most targets for aptamer are either in solution, in the blood plasma or displayed on the surface of cells that are accessible from the blood plasma, such as on the surface of the vasculature. Aptamers in this medium are subjected to nuclease-mediated degradation by serum nucleases, renal filtration, and uptake by the

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liver and other tissues such as the spleen. With the advancement of chemical biology, numerous attempts have been made to overcome these obstacles.

In general, wild-type RNA and DNA molecules are too susceptible to nuclease-mediated degradation. Different modifications are introduced in aptamers to increase their stability in biological media, as well as their functionality. Endogenous serum nucleases have higher degradation rates when cleaving at pyrimidine residues (Uhlmann *et al.*, 2000). The introduction of different substituents at the 2' position of ribose (the most common modification used to increase an aptamer's stability) helps to prevent the cleavage of aptamers by endonucleases. Nucleotides can be modified by replacing the 2′ position with either a fluoro-(F), amino- (NH_2) or O-methyl (OCH_3) group for enhanced nuclease resistance (**Fig. 1.8**). These type of modifications are generally used for RNA aptamers. 2 ' F and 2 ' NH_2 modified RNA aptamers are at least 1,000-fold more resistant to degradation in plasma than their unmodified RNA counterparts.

In serum 3′-exonuclease activity is much higher than 5′-exonuclease activity. Thus the capping of oligonucleotide termini, especially at the 3′-terminus, increases stability to endogenous serum nucleases (Hicke *et al.*, 2001; Schmidt *et al.*, 2004). This is often accomplished by inverting the nucleotide at the 3′-terminus so that the oligonucleotide has two 5′-termini and no 3′-termini. The phosphodiester backbone can also be modified to phosphorothioated linkage by replacing one of the non-bridging oxygen by sulfur. However it imparts resistance to nuclease degradation, but in general hybridize to the target sequences with lesser affinity than the phosphodiester counterpart. The introduction of 2'-O, 4′-C methylene-linked bicyclic nucleotides (LNA – locked nucleic acids) is another way of increasing the stability of aptamers of a known sequence. This modification is used both in RNA (Schmidt *et al.*, 2004) and DNA aptamers (Shangguan *et al.*, 2007).

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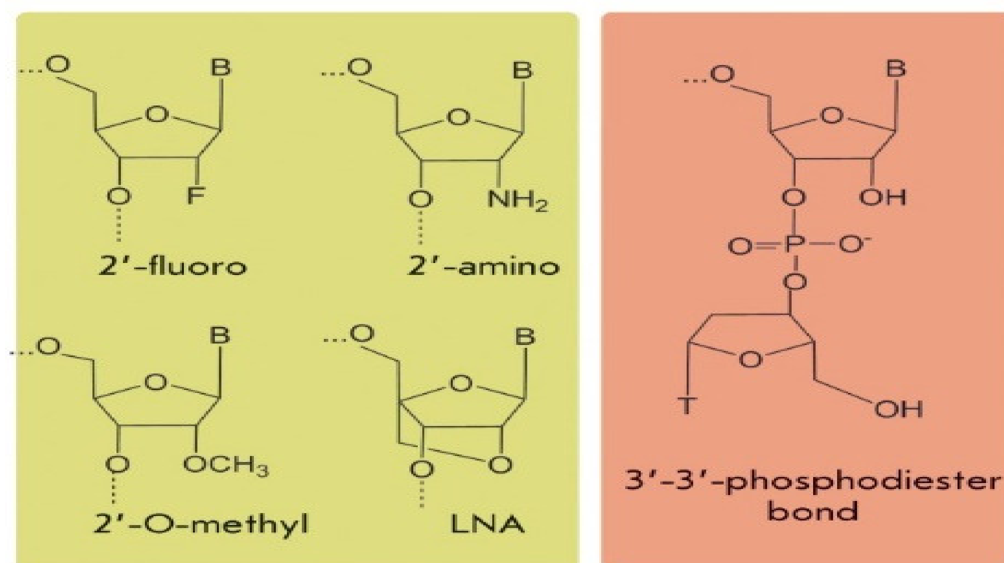

Figure 1.8: Chemical modifications of aptamers to increase the resistance of aptamers in biological media. Adapted with permission from Davydova *et al*., 2011.

Short blood residence time is another challenge with in vivo aptamer applications, which is due to fast removal of aptamer from the circulation by renal filtration. Generally aptamers are of size 5-15 kDa and are therefore susceptible to renal filtration as the molecular mass cutoff for the renal glomerulus is 30-50 kDa. Thus the aptamers are conjugated to high molecular weight molecules like PEG, cholesterol etc for reducing renal filtration rates. For example 40 kDa PEG conjugated to a fully 2′-O-methyl aptamer persisted in circulation with a half-life of 23 hours in mice (Burmeister *et al*., 2005). In another example, Rusconi *et al*. reported that the conjugation of cholesterol to a factor IXa-specific aptamer resulted in an increase half-life from 5-10 minutes to 1-1.5 hours in swine (Rusconi *et al*., 2004).

1.3.8 Aptamers vs Antibodies

Antibodies are the most popular and widely used class of molecules as a molecular probe in basic research as well as in clinical practice. However, there are certain limitations associated with antibodies. Aptamers have many attributes that make them superior to antibodies as a molecular probe. They have high binding affinity for its target with dissociation constants in the order of μM to pM. Also, aptamers recognize their targets with high specificity, distinguishing between highly homologous proteins or peptides

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with few amino acid differences (Blank and Blind, 2005, Jayasena, 1999) . Aptamers can discriminate between epitopes on related proteins. For example Aptamer against protein kinase C (PKC) can distinguish between its isozymes that are 96% identical (Conrad *et al*., 1994). The advantages of aptamers compared with antibodies are described below:

- It is well known that proteins are easily denatured and lose their tertiary structure at high temperatures or pH, while oligonucleotides are highly stable over a wide range of temperature and pH. They have long-term stability as dry powders or in solution and can easily be re-natured by one cycle of heating and cooling. Aptamers recover their native conformation and can bind to targets after re-annealing, whereas antibodies easily undergo irreversible denaturation. Thus, aptamers can be used over a wide range of assay conditions. Moreover it can be transported at room temperature. Hence it is economic contrary to antibodies.
- The production of antibodies are laborious and very expensive process as it involves screening of large number of colonies and mammalian cell culture or organism for large scale production. Only skilled persons are able to handle organisms. Moreover, antibodies suffers from batch to batch variations. Unlike traditional methods for producing monoclonal antibodies, no organisms are required for the in vitro selection of oligonucleotides. Aptamers are produced chemically in a readily scalable process with extreme accuracy and reproducibility. These chemical processes are more cost effective than the production of antibodies.
- The chances of modifying an antibody are very limited due to its proteinous nature. The structures of aptamer can be fine-tuned easily with various reporter molecules, fluorophores, nanomaterials, toxic agents, and so on, without affecting their binding capabilities (Jayasena, 1999). Furthermore, aptamers can be easily modified by various chemical reactions to increase their stability and nuclease resistance (E. Wang *et al*., 2011).
- Aptamers are usually biocompatible and lacks any toxicity or immunogenicity, because nucleic acids are not typically recognized by the human immune system

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as foreign agents (Cerchia *et al*., 2002). However, antibodies are significantly immunogenic, which precludes repeat dosing.

- Antibodies are saddled with a fundamental disadvantage of their large size (~155 kDa) which results in slow tissue penetration and long blood residence. Truncated aptamers are 25 to 40 nucleotide long and weigh 5 to 15 kDa (**Fig. 1.9**). Due to small size they have faster tissue penetration with shorter blood residence time and distribute in organs more efficiently, making them suitable for clinical use.

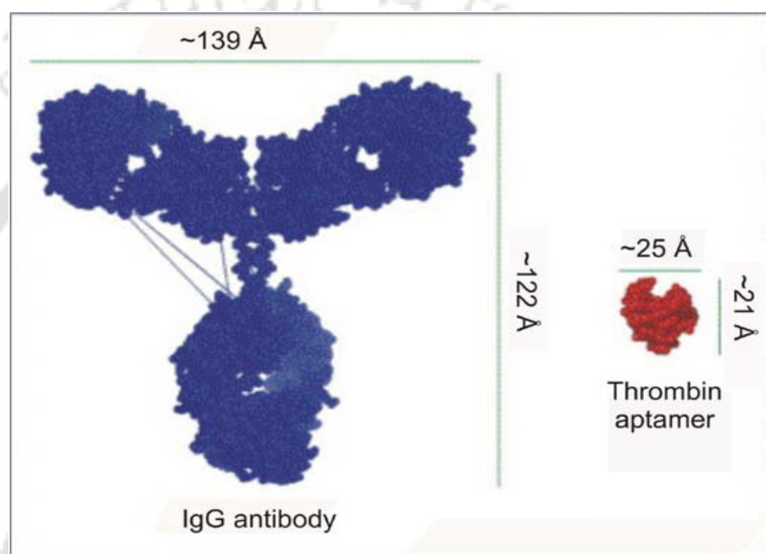

Figure 1.9: Antibody-aptamer size comparison. An estimated comparison of the size difference between an antibody (human IgG) and a selected aptamer (anti-thrombin DNA aptamer) is shown with space-filling models of both. The anti-thrombin aptamer is only 17 residues in length. Reprinted with permission from Lee *et al*., 2006.

- The binding of aptamers with their targets usually relies on specific conformations, such as G-quaduplex, hairpin. The conformational variations before and after the formation of aptamer-ligand complexes offer a great possibility and feasibility for the construction of aptamer-based assays or biosensors (Han *et al*., 2010).
- Nucleic acid aptamers can hybridize with their complementary sequences, which can be used to create the antidotes (Han *et al*., 2010).

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Conclusively, aptamers act similar to antibodies by recognizing their target with very high affinity and specificity. Thus they are often termed as "chemical antibodies" or "magic bullets". However aptamers offer many advantages over antibodies which make them an ideal candidate for the diagnosis and therapy of diseases.

1.3.9 Applications of Aptamer

Despite our understanding of genetics and molecular aspects of diseases, development of a diagnostics with high sensitivity and specificity and a therapeutics with better efficacy and lower toxicity still remains a challenge for the researchers. Some of the current challenges encountered by clinical researchers are (1) to develop a sensitive molecular diagnostic which can discriminate even a minute difference in the expression profile of analytes/markers between a diseased and a normal cell or tissues (2) targeted drug delivery specifically to the diseased cells or tissues in a controlled manner to avert its nonspecific toxic effects to the normal cells. In recent years, as a rapidly emerging field, molecular imaging has received much attention in biomedical research and clinical diagnoses. The traditional imaging technologies were based on morphological information, while the molecular imaging typically utilizes specific molecular probes to study molecular-level abnormalities particular biological events. These probes should be designed and fabricated to develop a non-invasive, real-time and in-situ way of diagnoses of diseases like cancers. Among the various reported molecular imaging probes, such as antibodies, ligands, and enzyme substrates and so on, nucleic acid aptamers are relatively new and promising.

Aptamers have been employed in almost every aspect of molecular biology and biosensing, particularly wherever antibodies have been traditionally used (**Fig. 1.10**). Aptamers have proven to be appropriate tools in many assay forms such as flow cytometry, imaging of cells/tumors, biosensors, ELISA/ELAA/ELONA, IHC, Immunoprecipitation, *in vivo* imaging, therapeutics and drug delivery including siRNA etc.

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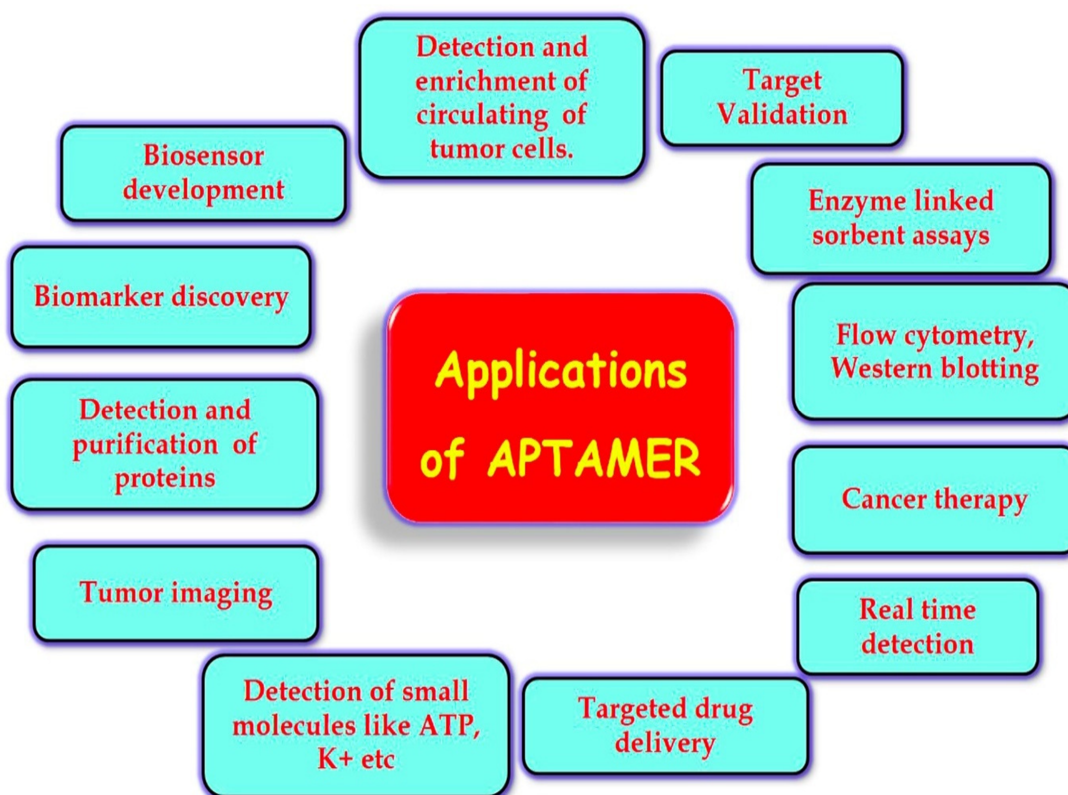

Figure 1.10: Applications of Aptamer.

1.3.9.1 Molecular imaging

An accurate and specific diagnosis of carcinoma to identify its type or even subtype is especially crucial and directly determines the treatment design from doctors. In recent years, as a rapidly emerging field, aptamers as an imaging probe has received much attention in biomedical research and clinical diagnoses. Small oligonucleotide aptamers have clear superiority over antibodies for in vitro or in vivo bioimaging applications. The antibody-based bioimaging agents suffer from poor tissue penetration, high immunogenicity, and long blood residence time that can result in unwanted side effects (Kobayashi and Choyke, 2011). Using aptamers as imaging agents has the advantage of their being non-toxic, because oligonucleotide moieties are present in the human body. Additionally, as aptamers have high specificity for their target, accurate targeting and rapid diffusion through the blood circulation, use of these molecules can increase the certainty of the results obtained during diagnosis or clinical analysis. Aptamers can be chemically synthesized and easily modified, thus they can be modified with fluorescent

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groups or quantum dots differential dyes or colorimetric reporter molecules, and used for imaging. Based on these advantages, aptamers have been considered as an imaging agents for cell imaging as well as single-protein imaging (Song *et al*., 2012). Shi *et al* were the first group to report the imaging of tumors in mice with high specificity using Ramos lymphoma cells specific Cy5-labeled TD05 aptamer.(Shi *et al*., 2010).

Fluorescence imaging of cancerous cell or tumors with aptamers has been well documented in the literature. The aptamer probes were labelled with fluorescent molecules like Cy5, FITC, 6-FAM etc and are used for imaging as well as tracking cancerous cells *in vitro* and *in vivo*. In one study, an RNA aptamer that binds to recombinant integrin $\alpha_v\beta_3$ was labeled with Cy5 and tested for its ability to bind to endogenous integrin $\alpha_v\beta_3$ on cell surface, as well as to subsequently affect cellular responses (Mi *et al*., 2005). In another study, aptamers generated against Epithelial cell adhesion molecule (EpCAM) were labelled with FITC and used for imaging MDA-MB-231, Kato III, and HEK 293T cells (**Fig. 1.11**) (Song *et al*., 2013). These are one step detection method where aptamers were directly conjugated to fluorescent molecule. Another approach involves two step detection strategy where capture aptamers were biotinylated and the detection molecules were fluorescent tagged. For instance Bayrac *et al* labelled GMT 8 aptamers (specific to A172 cell line) with biotin group and streptavidin-PE were used for signal generation. Sometimes nanorods were used as a scaffold for multivalent binding of multiple aptamers to enhance both the signal intensity and binding affinity for cancer cell recognition. In one example aptamer, KK1HO8 with relatively weak binding affinities to its targets on conjugation with nanorod showed a 300 fold increase in fluorescence signal on binding to K-562 cells (Huang *et al*., 2008).

Quantum dots (QDs) are inorganic fluorescent semiconductor nanoparticles with many desirable properties for imaging applications such as high quantum yields, high molar extinction coefficients, strong resistance to photobleaching and chemical degradation, narrow emission spectra and large effective Stokes shifts (Cai *et al*., 2007; Li *et al*., 2007). A biotin labeled ssDNA aptamer TLS9a specific to mouse liver hepatoma cell line BNL 1ME A.7R.1 (MEAR) was fabricated on the streptavidin-modified quantum dots (SA-QDs) to obtain a biocompatible probe for live cell imaging. In another example, multifunctional Quantum dot aptamer approach was devised where quantum dot (QD)-

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aptamer(Apt)-doxorubicin (Dox) conjugate [QD-Apt(Dox)] was used for cancer imaging as well as targeted delivery vehicle (Bagalkot *et al.*, 2007).

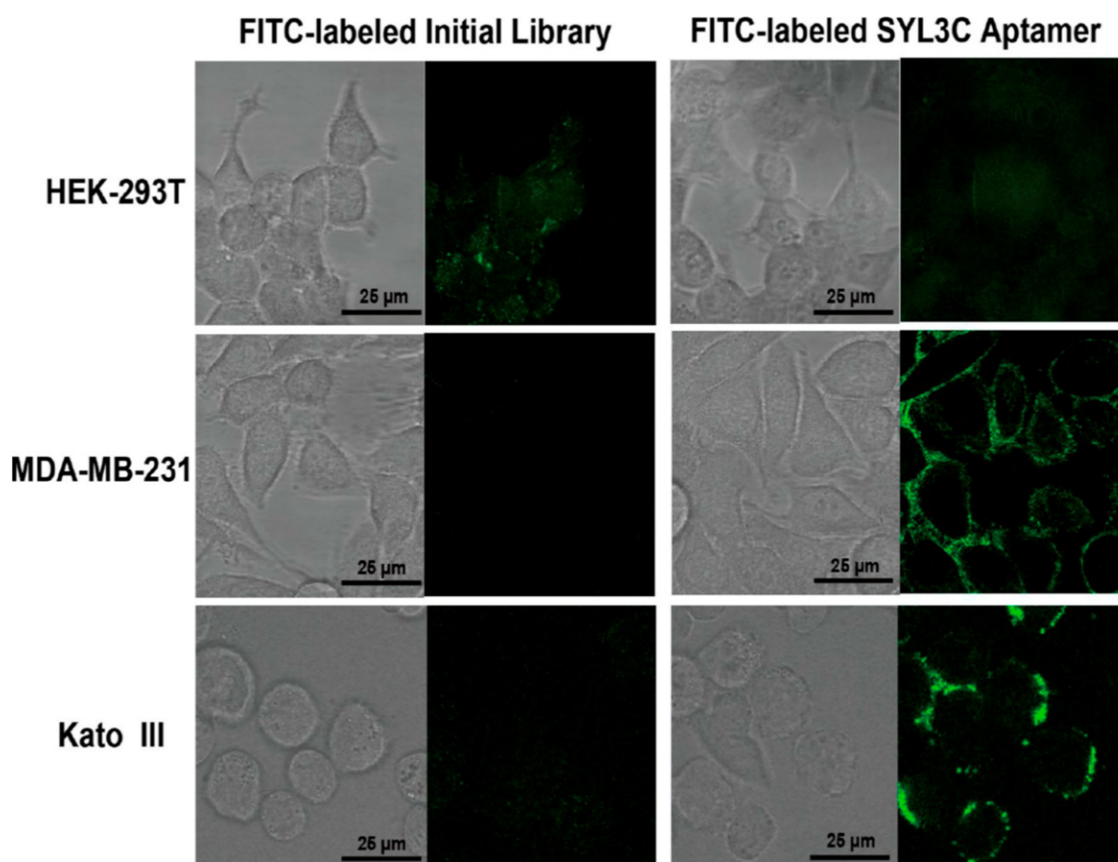

Figure 1.11: Confocal images of cultured HEK-293T, MDA-MB-231, and Kato III cells stained with the FITC-labelled initial library and SYL3C aptamer. "Reprinted with permission from Song et al., 2013. Copyright 2013 American Chemical Society."

Gold nanoparticles possess unusual optical and electronic properties, high stability and biological compatibility, controllable morphology and size dispersion, and easy surface functionalization (Daniel and Astruc, 2004; Grzelczak *et al.*, 2008; Rosi *et al.*, 2006; Sperling *et al.*, 2008; Storhoff *et al.*, 2004). Aptamer-conjugated gold NPs (Apt-AuNPs) provide a new platform to facilitate targeted recognition and detection. . The Mirkin group pioneered the use of AuNP-DNA conjugates, that is, AuNPs modified with thiolated oligonucleotide probe, which led to development of a series of novel assay methods for the ultrasensitive detection of DNA and proteins (Mirkin *et al.*, 1996; Rosi and Mirkin, 2005). Apt-AuNP conjugates were also used to develop colorimetric assays for the detection of cancerous cells (Medley CD *et al.*, 2008) , biomolecules such as

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platelet-derived growth factors (PDGFs) (Huang *et al*., 2005) and small molecules like k+ ions (L. Wang *et al*., 2006), cocaine (Zhang *et al*., 2008), ATP (Wang *et al*., 2007) etc. Nanospheres were also used for fabricating aptamers. A labeled anti-human epidermal growth factor receptor antibody or aptamer was conjugated with hollow gold nanospheres for in vivo imaging of head and neck cancer. Interestingly, the tumor uptake of aptamer-guided imaging probes was much higher than that of the antibody-guided imaging probes (Melancon *et al*., 2014).

1.3.9.2 Therapeutic applications

Aptamers can be used for therapeutic purposes in much the same way as monoclonal antibodies. As the aptamers are chemically synthesized, it has an advantage of easy manipulation, whereas in antibodies it is difficult or impossible to manipulate as organisms were involved for production. In addition, aptamers have a unique niche relative to other oligonucleotide therapeutics. For antisense oligonucleotides or siRNAs, the therapeutic target should be intracellular, whereas aptamer therapeutics can be developed for intracellular, extracellular or cell-surface targets (Keefe, 2010). Most of the therapeutic aptamers functions by inhibiting protein-protein interactions, such as receptor-ligand interactions, and thereby function as antagonists. Aptamers selected against the cancer related proteins often exhibit inhibiting effect on cellular proliferation and tumor development. For example aptamer generated against PDGF (Green *et al*., 1996), $\alpha v\beta 3$ integrin (Mi *et al*., 2005) , Tenascin C (Hicke *et al*., 2001) , PSMA (Lupold *et al*., 2002), Gonadotropin-releasing hormone 1 (Leva *et al*., 2002), E2F transcription factor (Martell *et al*., 2002), Cytotoxic T-lymphocyte-associated protein 4 (Santulli-Marotto *et al*., 2003), Cytohesin 2 (Theis *et al*., 2004), Mucin 1 (Ferreira *et al*., 2008), Epidermal growth factor receptor variant III (Liu *et al*., 2009) and Lymphocyte function-associated antigen 1 (Blind *et al*., 1999) inhibits tumor development. However, some aptamers have been shown to have agonist-like activities. For example, aptamers isolated against the extracellular domain of the protein human epidermal growth factor receptor 3 (HER3; also known as ERBB3) can promote oligomerization (although this does not result in inhibition of downstream phosphorylation) (Chen *et al*., 2003). Another example includes a DNA aptamer isolated against an isoleucyl tRNA synthetase enhanced editing activity (Hale and Schimmel, 1996).

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The most desirable applications of aptamers are tumor targeted drug delivery. Due to its various properties such as stability, adjustable pharmacokinetics, very low immunogenicity and toxicity, and their variety of targets, aptamers have become a new class of targeting ligands for a wide array of tumor-targeted drug delivery methods Aptamers that are internalized inside the cell have been exploited to deliver drugs and a variety of other cargo into cells and are often referred as "Escort Aptamers". As the aptamers are chemically synthesized, they can be easily uploaded with small chemicals, especially gene-therapy drugs. There are mainly two strategies for targeted delivery: (i) aptamer-drug conjugates, in which aptamer is directly conjugated to the drug molecules via either a connector or a physical intercalation, and (ii) the aptamer-nanomaterials system, in which the aptamer is decorated on the surface of nanocarriers for targeted drug delivery.

A simple, but effective, strategy is to non-covalently intercalate small chemical drugs, such as doxorubicin, into aptamer three-dimensional structures. Doxorubicin (Dox) have an inherent property to intercalate in GC rich sequences. A series of studies have shown the effectiveness of this simple aptamer-doxorubicin conjugation that results in improved therapeutic indices and decreased side effects. An aptamer HB5 conjugated with doxorubicin (Apt-Dox) could selectively deliver doxorubicin to HER2-positive breast cancer cells while reducing the drug intake by HER2-negative cells (Z. Liu *et al.*, 2012). Similarly Doxorubicin was physically conjugated to the double stranded region of the A10, a 2′-fluoropyrinidine-modified anti- PSMA RNA aptamer. Compared with free Dox, a 3.5-fold increase in aptamer-Dox intake was observed in LNCaP cells that expresses PSMA (Bagalkot *et al.*, 2006). Huang *et al.* proposed the delivery of Dox to the CCRF-CEM cells by conjugating it with sgc8c aptamer via a hydrazine linker. The Dox–sgc8c conjugate retained aptamer's binding properties and specifically interacted with the CCRF-CEM cells with high affinity (Huang *et al.*, 2009). As the conjugate enters the cell, the covalent bond between Dox and sgc8c was hydrolysed in the acidic environment of the endosome (where the construct localizes after internalization) and releases Dox. (**Fig. 1.12**). They found that the construct was less cytotoxic than free doxorubicin. Free Dox is membrane permeable, while the sgc8c-Dox internalization was demonstrated to occur through receptor-mediated uptake. Therefore, the aptamer-

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conjugation strategy, simultaneously decreases non-specific internalization of Dox and greatly enhances its uptake by target cells.

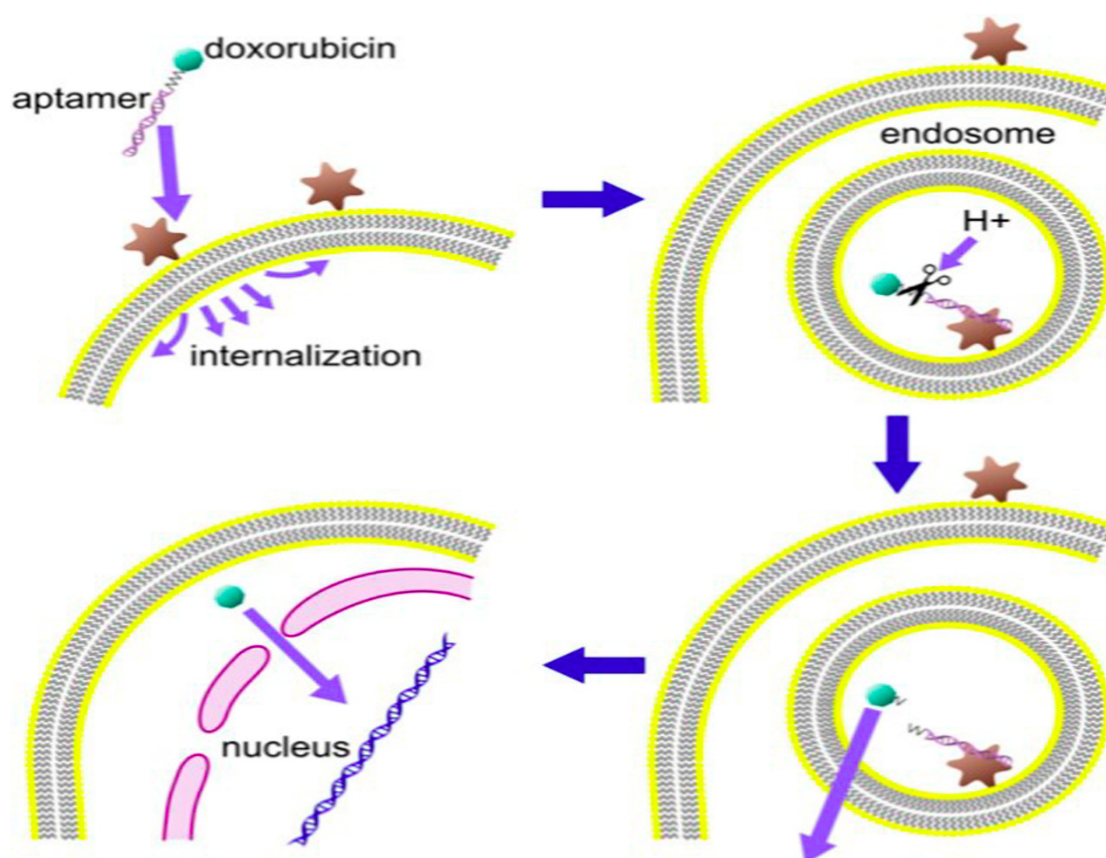

Figure 1.12: Delivery of doxorubicin using specific cytotoxic aptamer-doxorubicin (Dox) conjugate. The construct is internalized via endosome, where the acidic environment favours the cleavage of the bond between the aptamer and Dox molecule. The antibiotic diffuses through the endosome membrane, penetrates the nucleus and intercalates into genomic DNA, causing cytotoxic effects. Reprinted with permission from Radom *et al*., 2013.

In addition to small chemotherapeutic agents, cell-type specific aptamers are also functionalized with proteins (enzymes or toxins) for targeted delivery. A ribosomal toxin gelonin is an N-glycosidase protein that causes cell death. Chu *et al*. generated RNA aptamer- gelonin conjugate for its efficient delivery to the cells expressing PSMA. This conjugate had an IC_{50} of 27 nmol/l when used on PSMA-overexpressing cells, and when compared with PSMA-negative cells, the conjugate's toxicity was at least 600-fold higher (Chu *et al*., 2006). The linking of aptamers with siRNAs have been described

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recently to achieve targeted siRNA delivery, enhance RNAi potency, and to reduce unwanted side-effects (Zhou and Rossi, 2010). The first success of delivering of anti-human immunodeficiency virus (anti-HIV) siRNAs was accomplished by fusing it to an anti-gp120 aptamer which inhibits HIV replication (Zhou *et al*., 2008). Using anti-PSMA aptamers, two studies were conducted to study the successful delivery of siRNA into tumor cells by aptamer-siRNA chimera. In one study, the anti-PSMA aptamer (known asA9) was conjugated to an anti-lamin A/C siRNA using streptavidin as a modular bridge (Pagratis *et al*., 1997) to deliver siRNA to the prostate tumor cells. . In another study, aptamer-siRNA chimeras were used for targeted delivery of siRNA to two survival genes: polo-like kinase 1 (PLK1) and BCL2, overexpressed in most human tumors (McNamara *et al*., 2006).

Recently applications of aptamer-nanoparticle conjugates as a model for biorecognition in molecular biochemistry as well as in therapy were actively investigated. To enhance the efficiency of aptamers, they were conjugated with various nanoparticles (NPs) to improve its binding avidity and targeting efficacy. Nanoparticles provide large surface for multivalent aptamer binding and enhanced drug loading. Moreover uniform size and shape helps in excellent biodistribution. The integration of nanoparticles with aptamers has an added advantage as it increases both the half-life and the drug payload capacity of aptamer-mediated drug delivery. For example, copolymers and liposomes are biodegradable, while metal materials offer exceptional Photothermal and magnetic performance. Thus, NPs are used extensively in drug delivery and controlled release systems. Most commonly aptamers are conjugated with gold nanoparticles, silver nanoparticles, magnetic nanoparticle, quantum dots, nanorods or SWNT's. Farokhzad group were first to report the usage of nanoparticle-aptamer bioconjugates for the targeted drug delivery to cancerous cell. The D,L-lactide and OH-PEG3400-COOH were used to synthesize poly (D,L-lactic acid)-block-polyethylene glycol-COOH copolymer (PLGAPEG-COOH) and rhodamine-labeled dextran (as a model drug) were encapsulated within these nanoparticles. These drug loaded nanoparticle were further conjugated to PSMA specific RNA aptamer A10 to examine its efficacy for targeted delivery of chemotherapeutic drugs to prostate cancer cells. They found a 77-fold increase in drug intake in aptamer nanoparticle bioconjugates treated LNCaP epithelial

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cells, which express the prostate-specific membrane antigen protein as compared to control cells (Farokhzad *et al*., 2004).

1.3.9.3 Aptamer-facilitated Biomarker Discovery

Different types of cancers are associated with different biomarker protein status which plays very critical role in diagnosis, prediction of disease progression, and monitoring the efficacy of treatment. It is very crucial to detect and measure differential protein expression associated with cancer. Although there have been many attempts to identify specific disease biomarkers using a variety of technologies, the effective use of cancer-specific biomarkers is still not routine. Through Cell SELEX, aptamers can be selected against the overexpressed proteins on the cancerous cell and can even detect small differences among cell-surface proteins. Combination of Cell-SELEX and aptamer-based purification of proteins gave rise to a procedure called **AptaBiD** — Aptamer-facilitated Biomarker Discovery (Berezovski *et al*., 2008). This technique was utilized to isolate and determine (by mass spectrometry) biomarker proteins. Such strategy was utilized by Shangguan group to identify a biomarker for T-cell acute lymphoblastic leukemia (T-ALL). First, a panel of aptamers were selected for a T-cell acute lymphoblastic leukemia (T-ALL) cell line, CCRF-CEM cells (Shangguan *et al*., 2006). One of the selected aptamer sgc8, displayed high selectivity and affinity for its target on most of T-ALL and acute myeloid leukemia (AML) cells, as well as some B-cell acute lymphoblastic leukemia (B-ALL) cells. However, sgc8 did not show a detectable level of binding to either lymphoma cells or normal human bone marrow cells. Then, sgc8 was conjugated with magnetic beads and used to capture and purify the binding targets on the leukemia cell surface. Afterward Protein tyrosine kinase 7 (PTK7) was identified as the target protein of sgc8 on the cell surface, and was thereby established as a identified as a potential biomarker for T-ALL (Shangguan *et al*., 2008).Another example of biomarker discovery using cell-SELEX is the discovery of tenascin-C aptamers using glioblastoma cell line, U251 (Daniels *et al*., 2003).

Alongwith discovery of new biomarkers, aptamers also help to differentiate the stage of carcinogenesis within one subtype of cancer. In one study DNA aptamer with very specific affinity for protein binding revealed 44 different biomarkers able to distinguish

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among Stage I–III lung cancer (Ostroff *et al*., 2010). Most recently, Ray *et al.* compared human pancreatic cell secretomes by *in vitro* aptamer selection and identified cyclophilin B as a candidate pancreatic cancer biomarker (Ray *et al*., 2012). Thus aptamers opens up a new arena of early of cancer detection as well as staging of cancer which enhances the treatment options for patients.

1.3.9.4 Applications of aptamers in laboratory techniques

In most of the laboratory techniques aptamers can successfully compete with the universally used antibodies. ELISA (Enzyme Linked Immunosorbent Assay), one of the major clinical diagnostic tests available, is a versatile technique to detect almost any protein or peptide with high sensitivity. The first report of aptamer application in ELISA was written by Drolet in 1996 (Drolet *et al*., 1996). Flow cytometry is commonly used for detection and characterization
of cells. Many reports have shown the specific detection of target cells using aptamers in flow cytometry (Ara *et al*., 2012b; Kim *et al*., 2014; Sefah *et al*., 2010; Y. Wang *et al*., 2014). Combined with cell-sorting (Fluorescence Activated Cell Sorting; FACS) it can serve for separation of particular cell type from mixture (Davies, 2012). In one example, cyanin (Cy5)-conjugated RNA aptamer to mouse CD30 protein was used to isolate the CD30-overexpressig cells from a mixture of multiple lymphoma and bone marrow cells (Zhang *et al*., 2009). Another standard laboratory technique in which aptamers have been demonstrated as a worthy alternative to antibodies is Western blotting. Murphy *et al.* used a specific DNA anti-TTF1 aptamer conjugated with biotin to stain the protein in the membrane. Then Streptavidin-horseradish peroxidase was added to the reaction for colorimetric detection. Amazingly the whole aptamer based detection system turned out to be on a par with an antibody-based one (Murphy *et al*., 2003).

Immobilized Metal Ion Affinity Chromatography (IMAC) is one of the most common technique for protein purification based on affinity between the histidine and nickel or cobalt ions. The histidine tag was attached to either the N- or C-terminus of a recombinant protein. The histidine's ability to bind nickel or cobalt ions enables isolation of polyHis-conjugated molecules on ion-coated columns. As an alternative approach aptamers specific to hexahistidine tag can be immobilized on solid surfaces or on

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nanoparticles to produce the aptamer-based His-specific column. One of the proposed design involves replacing the IMAC column with iron oxide magnetic nanoparticles (MNPs) conjugated with aptamers. Using such techniques purification of two proteins expressed in *Escherichia coli* (PFEI and BSTE) was reported. The yield obtained with aptamer specific column was found to be comparable with a standard IMAC cobalt column (Kökpinar *et al.*, 2011).

Antibodies are routinely used to achieve upto 1000 to 10,000 fold purification from complex mixtures (Harlow and Lane, 1988). However, there are constraints that reduce the effectiveness of antibody based purification especially for large scale or industrial applications. These constraints include: (1) cross reactivity of antibody with closely related molecules; (2) linkage of antibodies to columns that often result in couplings that are not uniform, leading to reduced capacity and/or affinity and that can allow leaching of the antibody from the column; (3) inability of antibodies to survive sanitation procedures common to manufacturing scale separations due to denaturation of the antibody; (4) elution conditions that can be harsh, requiring extremes of pH, detergents, organic solvents or chaotropic salts leading to denaturation of the target as well as the antibody. In fact some elution conditions, such as reducing agents, are not feasible because they cleave the disulphide bonds between the immunoglobulin heavy and light chains (Bill *et al.*, 1995; Harlow and Lane, 1988; Huang *et al.*, 1996; Li *et al.*, 1998; Maclennan, 1995). Aptamers specific for particular proteins may be utilized for its purification from a complex mixture. To date, successful purifications of proteins (Connor and Mcgown, 2006; Javaherian *et al.*, 2009; Kanoatov *et al.*, 2011; Romig *et al.*, 1999) and small molecules (Deng *et al.*, 2001) have been demonstrated. Notably, Romig *et al.* managed to purify L-selectin upto 1500-fold in a single step (Romig *et al.*, 1999).

1.3.9.5 Biosensor Development

The development of biosensors using aptamers (**Aptasensor**) has attracted significant attention in recent years as a promising alternative to traditional diagnostic methods. Biosensors offers certain advantages over other conventional methods such as rapidity, ease-of-use, cost, simplicity, portability, and ease of mass manufacture. In last few decades numerous aptasensor have been devised exploiting the properties of aptamers

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such as target-binding affinity and ligand-induced structural rearrangement in aptamer. Aptamers generally undergo a change in conformation on binding with its target. This property provides an advantage of designing unique switchable aptasensors which cannot be achieved by antibody. Aptamers are synthesized chemically, thus there is not much lot to lot variation. Moreover, they can be modified with various reporter molecules like fluorophores, quantum dots, methylene blue etc. without affecting its affinity. It gives flexibility of designing various detection schemes for aptasensor. Aptasensors are more stable as compared to immunosensors and can be easily regenerated for reuse. Among various aptamer-based transduction techniques, electrochemistry has been extensively investigated because of their high sensitivity, simple instrumentation, low production cost, fast response and portability (Xu *et al*., 2009). The detection schemes employed in the aptasensors are either label-free methods, such as surface plasmon resonance (SPR) and quartz crystal microbalance (QCM) measurements, Raman spectroscopy, electrochemical impedance spectroscopy etc or Labelled such as fluorescence, chemiluminescence, electrohemiluminescence (ECL), field effect transistors, nanoparticles etc.

Binding induced conformation change is one of the promising approach for the development of electrochemical aptasensor. The aptamers are labelled at both ends, one end moieties for fabrication on electrode surface and other end with reporter molecule for signal generation. In the absence of target electron transfer takes place between reporter and electrode. However on binding with target aptamer forms a beacon-like conformation drawing reporter molecule faraway from electrode resulting in decrease in electron transfer. For instance, Liu *et al*, developed an electrochemical aptasensor based on this principle. The IFN γ specific DNA aptamer were modified with thiol group and methylene blue. As shown in **Fig. 1.13** aptamer forms a hairpin like structure in the absence of IFN γ which facilitate the transfer of electron between methylene blue and electrode surface. But, the binding of IFN-γ molecules disrupts the hairpin structure, thus decreasing the efficiency of electron transfer between MB and the electrode which was quantified by square wave voltammetry (SWV). The limit of detection for optimized biosensor was 0.06 nM with linear response extending to 10 nM. (Liu *et al*., 2010).

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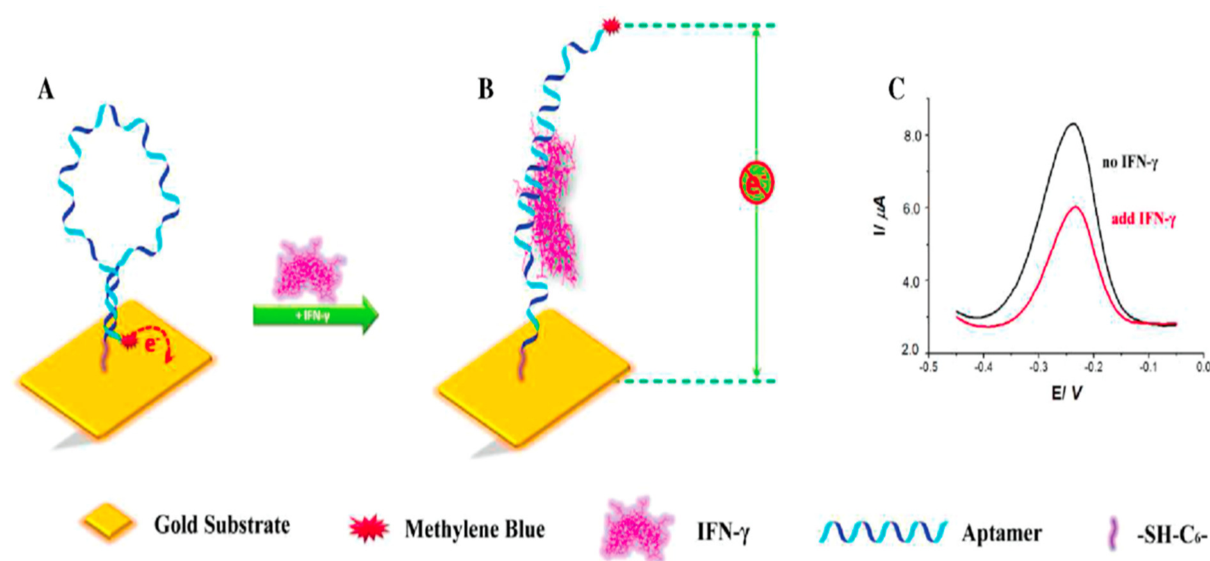

Figure 1.13: Schematic of aptamer-based electrochemical sensor for detection of IFN-γ. Reprinted with permission from ref. Liu *et al.*, 2010. Copyright © 2010, American Chemical Society.

1.3.9.6 Two nanoparticle Assay

During cancer progression tumors have tendency to exfoliate cancer cells spontaneously into biological fluids like blood and sputum or even into various effusions (Wolf *et al.*, 2003) due to the weakening of cell-to-cell and cell-to-extracellular matrix contacts as a consequence of up-regulation and activation of extracellular matrix–degrading enzymes (Birkedal-Hansen, 1995; Friedl and Bröcker, 2000).The exfoliated cells are generally far outnumbered by normal cells in bodily fluids. These exfoliated cells may possibly serve as an important signature to identify precancerous or very early stage cancers. A method should be developed that can accurately differentiate and detect the low number of abnormal cells among the millions of normal cells in biological fluid. The aptamers generated by Cell-SELEX targeting various cell line could be used to recognize these rare cancerous cells. A novel two-nanoparticle assay was developed in which aptamers were conjugated to two separate nanoparticles - one magnetic and one fluorescent. The magnetic nanoparticles (MNPs) permitted the collection and enrichment of the cancer cells, whereas the fluorescent nanoparticles (FNPs) clearly marked the cell for fluorescence detection. For example, aptamer specific to CCRF CEM cells were modified with iron oxide-doped nanoparticles for selective leukemia cell extraction from

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complex clinical samples or biological fluid by MACS (magnetic activated cell sorting) technique whereas it was also attached to fluorescent rubpy dye doped nanoparticles to provide enhanced signal and a sensitive means for cell detection which was not possible by using either particle alone. The recently developed two-particle assay is very fast and requires as little as 5-min incubation for sufficient nanoparticle binding, and the entire method can easily be performed in less than 1h (J. E. Smith *et al*., 2007). Further multiple aptamers could also be used to increase the sensitivity without impacting the selectivity of the NPs against the control cells. The LOD of the most sensitive and selective ACNP system using multiple aptamer was found to be as low as 6.6 cells (Medley *et al*., 2011).

1.3.10 Aptamers in clinic

A number of aptamers are currently in different stages of clinical trials. The first aptamer which was FDA approved (2004) was an RNA aptamer selected against vascular epidermal growth factor (VEGF) for the treatment of neovascular (wet) age-related macular degeneration (AMD) disease. It is currently marketed by Pfizer and Eyetech as Macugen (Pegaptanib). Recently last year an aptamer based assay known as "OTA-Sense" was approved for the detection of ochratoxin in food sample. Some of the aptamers that have undergone clinical trials are listed in **Table 1.6.**

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Table 1.6: Aptamers in the clinic. Adapted with permission from Keefe, 2010.

Name (company)	Composition	Target	Indication	Current phase	Reference
Pegaptanib sodium/ Macugen (Pfizer/ Eyetech)	2′-O-methyl / 2′-fluoro conjugated to PEG, 3′ inverted dT	VEGF	AMD	US & EU	Gragoudas *et al.*, 2004; Ng *et al.*, 2006
AS1411/ AGRO001 (Antisoma)	G-rich DNA	Nucleolin	AML	Phase II	Bates *et al.*, 2009, 1999; Teng *et al.*, 2007
REG1/ RB006 RB007 (Regado Bioscience)	2′-ribo/ 2′-fluoro (RB006)/ 40 kDa PEG 2′-O-methyl antidote (RB007)	Coagulation factor IXa	Percutaneous coronary intervention	Phase II	Cooper *et al.*, 2008; Yu *et al.*, 2009
ARC1779 (Archemix)	DNA & 2′-O-methyl conjugated to 20 kDa PEG, 3′ inverted dT	von Willebrand factor	Thrombotic microangio-pathies and carotid artery disease	Phase II	Krieg, 2008, 2006
NU172 (ARCA biopharma)	Unmodified DNA aptamer	Thrombin	Cardiopul-monary by pass	Phase II	Sheehan *et al.*, 1998
ARC1905 (Ophthotech)	2′-ribo/ 2′-fluoro conjugated to	Compleme-nt	AMD	Phase I	Goebl *et al.*, 2007

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	40 kDa PEG, 3′ inverted dT	component 5			
E10030 (Ophthotech)	DNA and 2′-O-methyl 5′-conjugated to 40 kDa PEG, 3′ inverted dT	PDGF	AMD	Phase I	Crooke, 2008
NOX-A12 (NOXXON Pharma)	L-RNA with 3′-PEG	CXCL12	MM & NHL	Phase I	Sayyed *et al.*, 2009
NOX-E36 (NOXXON Pharma)	L-RNA with 3′-PEG	CCL2	Type 2 diabetes, diabetic nephropathy	Phase I	Kulkarni *et al.*, 2009; Maasch *et al.*, 2008

AMD, Age related macular degeneration; AML, Acute Myeloid leukemia; MM, Multiple myeloma; NHL ,non-Hodgkin's lymphoma; PDGF, Platelet-derived growth factor; CCL2, chemokine (C-C motif) ligand 2 (also known as MCP1); CXCL12, chemokine (C-X-C motif) ligand 12 (also known as SDF-1α); EU, European Union; PEG, polyethylene glycol; US, United States.

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1.4 Rational of the Study

Analysis of the existing literature on EGFR and prognosis clearly indicates that elevated levels of EGFR are correlated with poor patient outlook in many different cancer types such as breast, colon, head and neck, kidney, lung, pancreas, and prostate cancer. Over the past decade, the landscape of cancer has changed. Earlier diagnosis coupled with advances in treatment has led to better outcomes and longer survival of cancer patients. Although EGFR is considered as one of the prognostic marker for cancer, still there are lack of agreement on the true predictive significance of the EGFR. This is mainly due to inconsistencies in the assay methods used and the heterogeneity of the patient populations between studies. The role of EGFR has also been highlighted in liver regeneration and skin repair mechanism. However its role in other diseases like cardiovascular, lung, kidney, or other airway inflammatory diseases is still at its infancy and its value as a potential therapeutic target for treating such diseases has yet to be demonstrated.

The lack of a standardised assay for determining tumour EGFR status is particularly problematic. Tumour EGFR status can be evaluated by more than ten different methods designed to detect gene amplification, gene mutation or elevated levels of either mRNA transcripts or protein. There are many studies conducted to determine the tumour EGFR status using many of these assays, but each defines the EGFR overexpression in a slightly different manner. Furthermore, even when a single technique, such as immunohistochemistry (IHC), was used by several laboratories to evaluate tumour EGFR levels, differences in reagents, detection methods or assay cut-off points led to different results, increasing the variability between studies. As a result, reports from different laboratories cannot be compared to obtain a conclusive result. Most of the assays to study the EGFR protein expression employs the use of antibodies. There are loads of antibodies with varying sensitivity are available in market. Additionally antibodies are very costly and suffers from batch to batch variations. Thus there is a need to develop a molecular probe that can detect EGFR and provides consistent and comparable result. A biorecognition element which can overcome the limitations of antibodies and can be used in biomedical and clinical research. Aptamers have emerged as a new class of nucleic acid ligands that mimics the property of antibodies by binding

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to its target with very high affinity and selectivity and offers numerous advantages over antibodies. Due to its specific biorecognition property these ligands have received much attention in biomedical and clinical research as a potential multifunctional theranostic probe. The goal of this research project is to obtain and characterize DNA aptamers against extra cellular domain of Epidermal Growth Factor Receptor (EGFR) and to evaluate potential applications of these aptamers. The following objectives were formulated:

1.5 Objectives of the study

- Cloning, Expression and Purification of Extra Cellular Domain of EGFR (Epidermal Growth Factor Receptor).
- *In vitro* Selection and Characterization of aptamers against Extra Cellular Domain of EGFR.
    - Selection of aptamer against Extra Cellular Domain of EGFR by SELEX.
    - Cloning, Sequencing and Characterization of selected aptamer.
    - Binding studies of selected aptamers with Epidermal Growth Factor Receptor.
- Exploring the application of selected Aptamers in various bioassays.

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CHAPTER 2

CLONING, EXPRESSION & PURIFICATION OF EXTRA CELLULAR DOMAIN OF EPIDERMAL GROWTH FACTOR RECEPTOR

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2.1 Introduction

The Epidermal Growth Factor Receptor is a 170 KDa protein containing an extracellular domain, a transmembrane region and an intracellular tyrosine kinase domain. The ligands of EGFR eg. EGF binds to the extracellular region within the amino-terminal of 622 amino acids. The Extra cellular region/ domain (ECD) is further sub devided into four domains I–IV, also known as the L1, S1, L2, and S2 domains, respectively (Bajaj *et al*., 1987). Domains I and III share 37% amino acid identity, while domains II and IV are homologous Cys-rich domains, CR1 and CR2, respectively (Ward *et al*., 1995). The ligand-binding domain of the epidermal growth factor receptor is separated from the cytoplasmic protein tyrosine kinase domain by a single transmembrane domain of 24 amino acids (Carpenter *et al*., 1991). The intracellular portion of the epidermal growth factor receptor consists of a tyrosine kinase domain of 541 amino acids (Hanks *et al*., 1988) which is further subdivided into a core kinase domain of approximately 290 amino acids and a COOH-terminal domain of approximately 230 amino acids. The COOH-terminal domain contains all five sites of autophosphorylation (Cadenas *et al*., 1994; Downward *et al*.; Margolis *et al*., 1989; Walton *et al*., 1990) which plays an important role in the assembly of substrates involved in signal transduction through SH2 domain (Cantley *et al*., 1991; Koch *et al*., 1991). The COOH terminus also contains sequences required for ligand-induced endocytosis and down-regulation of cell surface receptors (Chang *et al*., 1993, 1991; Chen *et al*., 1989; Wiley *et al*., 1991).

Fabricant *et al* established the expression of EGFR protein in the placenta and A431 carcinoma cell lines (Fabricant *et al*., 1977). Initially A431 cell lines were chosen for the isolation of EGFR because they expresses abnormally high levels of EGFR *viz* 10-50 times more than any other cell lines (Fabricant *et al*., 1977; Wrann and Fox, 1979). In the pioneering studies by Cohen, intact detergent solubilized membrane were isolated from A 431 cell line which retain the ability to bind to ligand EGF (Cohen *et al*., 1981). Their group established the biochemical mechanisms of EGFR signaling by working with detergent solubilized preparation of EGFR. Furthermore, partial purification of endogenously expressed EGFR from A 431 cells was achieved by immunoaffinity

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chromatography and other means (Cohen *et al*., 1982, 1981; Hock *et al*., 1980; Weber *et al*., 1984; Yarden *et al*., 1985). While these approaches provided sufficient quantities of wild-type protein preparations for biochemical characterization, but they do not provide sufficient quantities or a means to isolate mutant constructs for biophysical characterization of intact receptor molecules. In addition, the stability of highly purified detergent-soluble EGFR preparations has not been characterized in detail. Later on for the production of EGFR protein in large quantity, EGFR expressing cell lines such as A 431, CHO cell line or placenta were used for the construction of EGFR cDNA. For example the cDNA from placenta and A431 cell line were used for the cloning of EGFR using λg10 vector system (Ullrich *et al*., 1984). Sato *et al* isolated full-length cDNA encoding EGFR by PCR from a human placenta and cloned into mammalian expression plasmid pcDNA3.1/Zeo (+) (Sato *et al*., 2000). Lin *et al* constructed prokaryotic expression plasmid pET30a (+) carrying intracellular domain of EGFR from the mRNA of A 431 cell line. The recombinant protein was purified using Ni^{2+}-NTA agarose for the generation of monoclonal antibodies (Lin *et al*., 2004). In another study the recombinant intracellular domain were produced as a glutathione S-transferase (GST) fusion in *Escherichia coli* using pGEX6-P-1 vector, and the solubilisation was performed by sarkosyl addition during extraction (Elloumi-Mseddi *et al*., 2013). The ectodomain of EGFR was cloned, expressed and purified using polycistronic approach. This expression system yielded 20 fold higher than that of A 431 cell line (Cadena and Gill, 1993).

Generally for the purification of recombinant eukaryotic protein mammalian expression system or baculovirus system are used. Many studies were reported where mammalian expression system (Cadena and Gill, 1993; Chen *et al*., 2008; Mi *et al*., 2008; Ogiso *et al*., 2002) or insect cell lines (Ferguson *et al*., 2003; Greulich *et al*., 2005) were used for the expression and purification of extra cellular domain of EGFR, which is more expensive compared to *E. coli*. The advantages of *E. coli* as an expression host include well studied physiology, genetics and availability of advanced genetic tools, rapid growth, high-level protein production rates achieving up to 10–30% of total cellular protein, ease of handling in a standard molecular biology laboratory, low cost and the ability to multiplex both expression screening and protein production. There are however several disadvantages, particularly for eukaryotic proteins, of expression in a prokaryotic

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system. The lack of eukaryotic chaperones, specialised post-translational modifications, ability to be targeted to sub-cellular locations or to form complexes with stabilising binding partners can result in protein mis folding and aggregation.

In the present study EGFR was chosen as a target for the development of oligonucleotide probes that can be used for the detection of EGFR. Structurally EGFR is devided into three domains, *viz* Extra cellular Domain, Transmembrane domain and cytoplasmic intracellular domain. The extra cellular domains plays most significant role as it is involved in binding of its ligands and further stimulation of various signalling pathways. Thus extra cellular domain i.e. ECD of EGFR was cloned, expressed and purified for aptamer selection. The full length EGFR gene cloned in pBABE vector was procured from Addgene plasmid repository. The ECD region of EGFR was subcloned into pET 28a (+) vector and expressed in *Escherichia coli* BL21 strain. The expressed proteins were further purified by immobilized metal ion affinity chromatography (IMAC) and confirmed by western blot analysis. The ECD region of EGFR proteins were purified for the selection of EGFR specific aptamers.

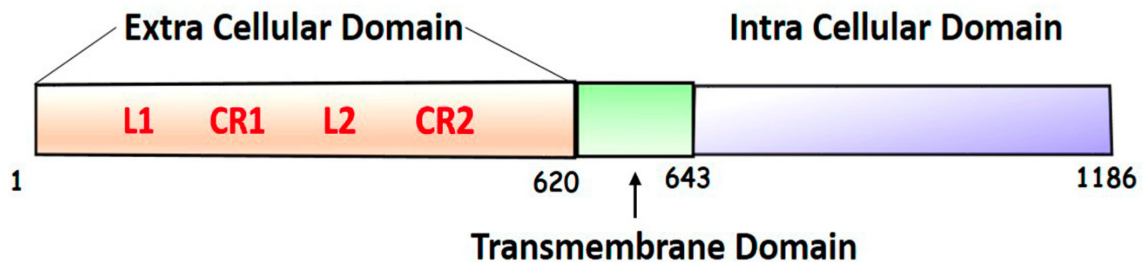

Figure. 2.1: Molecular architecture of EGFR showing boundaries and designation of different domains.

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2.2 Materials and Methods

2.2.1 Chemicals, reagents and kits

The oligonucleotide primers for PCR amplification of ECD of EGFR were purchased from ILS, India. BIOTAQ DNA polymerase was supplied by Biolone, UK. The PCR mix containing dNTPs and $MgCl_2$ were obtained from Sigma-Aldrich Pvt. Ltd., USA. pTZ57R/T vector system for TA cloning was purchased from Thermo Scientific,USA. Restriction enzymes *Nde*I, *Xho*I, T_4 DNA ligase and DNA 1 kb ladder were purchased from New Englands Biolabs, UK. The expression vector pET-28a, was purchased from Novagen, Germany. RNAse solution (20 mg/ml), glacial acetic acid (99.9 % pure) Trizma base (Tris free base), ethidium bromide, agarose, nuclease free water (pH 8.0) and components of polyacrylamide gel electrophoresis were obtained from Sigma-Aldrich Pvt. Ltd. USA. The GenElute plasmid miniprep isolation kit and GenElute gel-extraction kit was from Sigma-Aldrich Pvt. Ltd. India. Medium range protein marker was purchased from Merck Millipore, USA. Disodium ethylenediamine tetra acetate salts (EDTA), glucose, sodium hydroxide, sodium dodecyl sulphates (SDS), LB medium, Agar, ampicillin and kanamycin were supplied by Sigma-Aldrich Pvt. Ltd. India.

2.2.2 PCR amplification of Extra Cellular Domain of EGFR from EGFR WT plasmid (Addgene, Plasmid # 11011)

The full length EGFR cDNA cloned in pBABE vector in DH5α strain was procured from Addgene plasmid repository (Plasmid # 11011). The EGFR cDNA was 3639 bases long and vector backbone was 5169 bases long (**Fig 2.2**).

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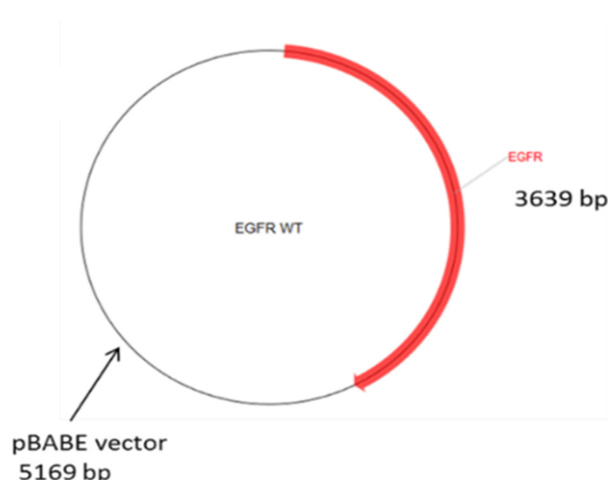

Figure 2.2: Architecture of EGFR WT plasmid (Plasmid # 11011) procured from Addgene.

The primers complementary to 5′ and 3′ ends of Extra Cellular Domain of human EGFR gene were designed. These primers were prefabricated with *Nde1* and *Xho1* restriction enzyme site to enable efficient cloning into pET28a (+) vector. The primers used to amplify the extra cellular domain of EGFR gene from EGFR WT plasmid were -

ECD_F1: 5′ GCCATATGATGCGACCCTCCGGGACGG 3′ (Extra Cellular Domain Forward primer) and

ECD_R1: 5′ GCCTCGAGGGACGGGATCTTAGGCCCA 3′ (Extra Cellular Domain Reverse primer).

PCR amplification was carried out in a final reaction volume of 50µl containing Mg^{2+} ions (2.5mM), dNTPs (0.2mM); Primers (1.5µM), 1.0 µl of Taq DNA polymerase (1µl of 1Unit/µl) and 1 µl of EGFR- pBABE plasmid DNA. PCR amplification was performed in a thermal cycler (Applied Biosystems, Gene Amp PCR System 9700). The PCR cycles for amplification are detailed in **Table 2.1**. The amplicons were run on a 0.8 % (w/v) agarose gel in presence of 1 kb DNA ladder as mentioned in Section 2.2.2.1

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Table 2.1: Conditions for PCR thermal cycles for amplification of ECD of EGFR from EGFR WT plasmid.

Steps		Time
I. Denaturation at 94°C		4 min
II. 25 cycles of	i) Denaturation at 94°C	30 sec
	ii) Annealing at 61°C	45 sec
	iii) Extension at 72°C	50 sec
III. Final extension at 72°C		10 min

2.2.2.1 Agarose gel electrophoresis of PCR amplified EGFR ECD amplicons

The PCR amplicons were run on 0.8% agarose gel prepared in 1x TAE buffer. A stock solution of TAE buffer was prepared according to Sambrook and Russell, 2001 (Sambrook and Russell, 2001) keeping the concentrations of components to 10x (400 mM Tris-acetate, 10 mM EDTA pH 8.0). The gel was prepared by dissolving 400 mg of agarose in 50 ml of 1x TAE buffer by heating in a microwave oven to get a clear solution. Then 5.0 µl of ethidium bromide (5.0 mg/ml) was added when the solution temperature was around 50°C. The solution was mixed well and poured on the casting apparatus, combs were placed and the gel was allowed to solidify. 1x TAE (Tris-acetate-EDTA) buffer was used as an electrophoresis running buffer. The DNA sample and DNA loading dye were mixed in 4:1 ratio and the gel was run at 60 Volts for 70% migration of the leading dye. The bands were then visualized under UV illumination in a gel documentation system (BioRad XR Gel documentation system).

2.2.2.2 Extraction of PCR amplified EGFR ECD DNA from agarose gel

The PCR amplified DNA were purified from agarose gel using Sigma Gel Extraction Kit, according to the manufacturer's instruction. The extracted DNAs were eluted in 30 µl of elution buffer supplied with the kit (Sigma-Aldrich Pvt. Ltd.).

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2.2.3 Cloning of gel purified ECD amplicons into pTZ57R/T Vector

PCR amplicons of EGFR ECD after gel extraction was subjected to TA cloning. Detail description of pTZ57R/T vector construct from Thermo fisher Scientific, USA is given in section 2.2.3.1. Ligation reaction was setup following the protocol mentioned in section 2.2.3.2.

2.2.3.1 Description of pTZ57R/T vector

The pTZ57R/T Vector Systems are convenient system for the cloning of PCR products. The pTZ57R/T cloning vector is linearized and ddT tailed for direct use in cloning of PCR products, generated with Taq, Tth, Tfl or other DNA polymerases or polymerase mixtures. The map and the MCS region of the vector are presented in **Fig.2.3**. These T overhangs at the insertion site greatly improves the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases. These polymerases often add a single deoxyadenosine, in a template-independent fashion, to the 3´-ends of the amplified fragments. The high copy number pTZ57R/T vector contain T7 RNA polymerase promoters flanking a multiple cloning region within the alpha-peptide coding region of the enzyme beta-galactosidase. Hence successful insertion of the PCR amplicons of EGFR ECD will interrupt the coding sequence of β-galactosidase and the recombinant clones can be identified by colour screening on indicator plates. Clones containing PCR products genearates white colonies, whereas blue colonies usually do not contain any clone. Rarely the blue colonies may contain positive clone, which happens when the PCR fragments are cloned in-frame with the lacZ gene.

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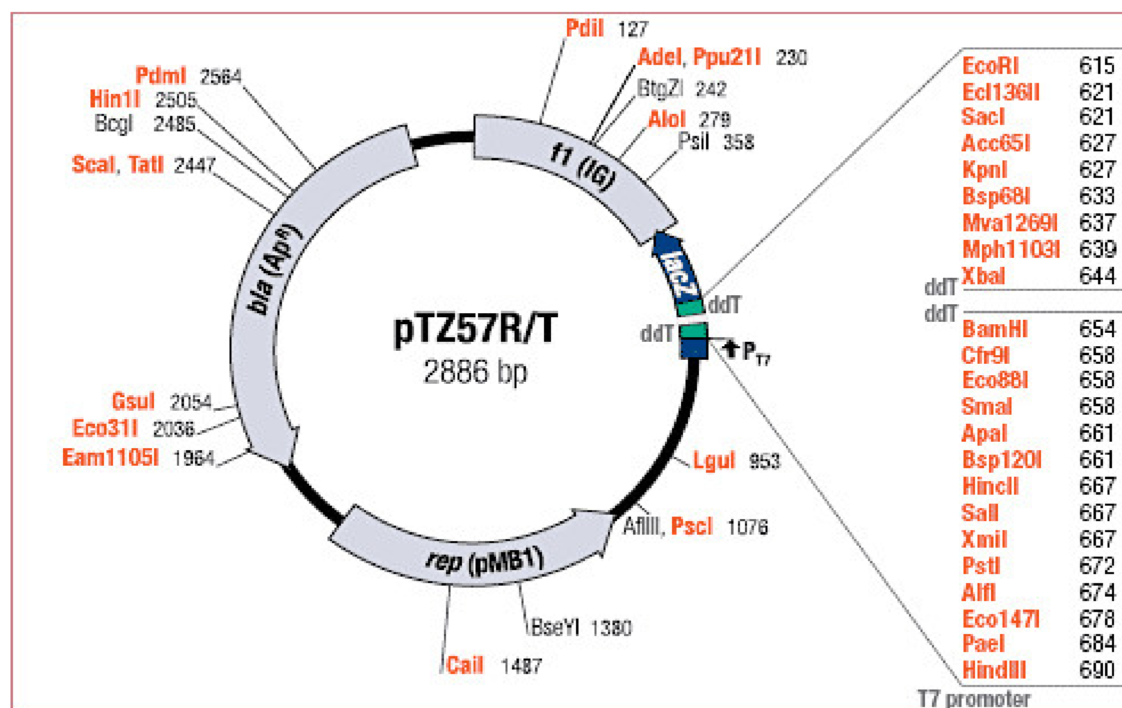

Figure. 2.3: Detailed vector map of pTZ57R/T vector.

2.2.3.2 Ligation of gel purified EGFR ECD amplicons into pTZ57R/T vector

The gel eluted PCR amplicons of EGFR ECD were ligated to pTZ57R/T vector following the components of ligation reaction as mentioned in **Table 2.2.** The ligation reactions were setup in 0.5 ml microcentrifuge tubes and incubated at 4°C overnight to get maximum number of transformants. The reactions were setup at an insert: vector molar ratio of 3:1, where the amount of insert required in a reaction is calculated using the following formula

$$\frac{\text{Amount of vector (ng)} \times \text{Size of insert(kb)}}{\text{Size of vector (kb)}} \times \text{Insert :Vector molar ratio} = \text{Amount of insert (ng)}$$

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$$\frac{55\ (\text{ng}) \times 1.935\ (\text{kb})}{2.886\ (\text{kb})} \times \frac{3}{1} = 110.628\ \text{ng}$$

Table 2.2: Components for ligation reaction setup for TA cloning of PCR amplified ECD amplicons.

Reaction components	**Volume (µl)**
10X Ligation Buffer	1.0
pTZ57R/T Vector (50ng)	1.0 (55ng)
PCR product	2.7 (110 ng)
T4 DNA Ligase (3 units/µl)	1
Nuclease-free water	4.3
Total reaction volume	10.0

2.2.3.3 Transformation of ligated products after TA cloning

After 16 hours of incubation, ligated construct was transformed into competent *E. coli* (XL10 Gold) cells.

2.2.3.3.1 Preparation of E. coli (XL10 Gold) competent cells

Day 1

1. 50 µl of culture of *E. coli* (DH5α) from glycerol stocks were inoculated into 5.0 ml LB medium and incubated overnight at 37ºC and 180 rpm.
2. 0.1 M $CaCl_2$ solution was filter-sterilized by passing through 0.22 µm filter in laminar air flow and kept in refrigerator.

Day 2

3. 1.0 ml culture from day 1 was inoculated into 100 ml LB medium and incubated at 37ºC with 180 rpm till cell OD reached 0.4-0.6 at 550 nm.

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4. The cultures were transferred aseptically to round bottom centrifuge tubes and centrifuged at 4ºC with 4000g for 10 min.
5. The cell pellet was first suspended in 3-4 ml sterile, ice-chilled 0.1 M $CaCl_2$ solution followed by making up the final volume to 20 ml. The suspension in centrifuge tubes was kept on ice for 10 min.
6. The tubes were centrifuged again at 4000g at 4ºC for 10 min. The supernatant was carefully removed and the pellet was resuspended in 3.0 ml of sterile ice chilled 0.1 M $CaCl_2$ solution.
7. 200 µl of competent cells were aliquoted into each 1.5 ml microcentrifuge tube containing 10- 15 (%, v/v) glycerol (final concentration) and kept at -80ºC for further use.

2.2.3.3.2 Transformation of ligated products after TA cloning

The *E. coli* (XL10 Gold) competent cells were transformed with the ligated product. The aliquots of competent cells (200 µl) was taken out from -80°C and kept on ice for 5 min, followed by addition of 10 µl of ligation mixture. The tube was gently tapped 4-5 times and kept on ice for 30 min. Then the cells were given a heat shock at 42°C for 90 secs. The cells were immediately transferred to ice for 2-3 min. 800 µl of super optimal medium with catabolite repression (SOC) was added to the transformed cells. The transformed cells were incubated at 37°C in a shaking incubator at 180 rpm for 1h (Hanahan, 1983). The cells were harvested by centrifugation at 2000g at 25°C for 5 min. 800 µl supernatant was discarded and the cell pellet was resuspended in remaining 200 µl supernatant. The 200 µl transformed cells were spread plated on LB agar plates supplemented with antibiotics ampicillin, IPTG (Isopropyl β-D-1-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) at a final concentration of 50 µg/ml, 0.2 mM and 80 µg/ml, respectively. The LB agar plates were incubated overnight at 37°C.

2.2.3.4 Screening of positive TA clones of EGFR ECD

Positive TA clones were identified by blue white screening. Overnight grown plates were observed for blue white colonies. White colonies containing the positive clones were

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picked up in a laminar air flow and grown overnight in 5 ml LB media supplemented with ampicillin (50 µg/ml). The positive TA clones produce white colonies are unable to produce β-galactosidase because of the interrupted *lacZ* gene induced by the presence of the insert DNA. In absence of β-galactosidase the cells cannot degrade X-Gal and produce blue colour colonies.

2.2.3.5 Isolation of plasmid DNA from positive colonies of TA clones by NID method

Plasmid DNA was isolated from fully grown recombinant *E.coli* (XL10 Gold) cells which were picked up from the LB plates after transformation of the ligated TA cloned products of EGFR ECD. NID method as described below was employed for isolation of plasmid (Lezin *et al*., 2011).

Table 2.3: Composition of NID extraction buffer for plasmid isolation.

Components	**Final Concentration**
Tris pH 8.0	50mM
Sucrose	5%
EDTA	50 mM
NH_4Cl	75mM
Triton X-100	0.5(%v/v)
Lysozyme	100 µg/ml
RNase A	25 µg/ml
$CaCl_2$	50mM

1. 1.5-2 ml of bacterial cultures were pelleted at 6000-7000 rpm for 1 min.
2. After drawing 150 µl extraction buffer into a pipette tip, the pellet was loosened off the tube wall with the tip without releasing the buffer. Then the extraction buffer was added and the pellet was resuspended.
3. The bacterial suspension was incubated at 65°C for 5 min.
4. Suspensions were centrifuged at maximum rpm for 10 min or until a tight bacterial pellet was formed. The pellet was removed with a toothpick.

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5. 100-120 µl isopropanol was added, followed by mixing and centrifugation of the solution at 7000 rpm for 10 min at RT.
6. DNA usually forms film-like precipitates that adhere well to tube walls and are invisible in isopropanol solutions. After discarding the supernatant, the DNA was centrifuged after adding 70% ethanol. Ethanol was removed, and the DNA pellet was dissolved in 20-50 µl TE buffer.

A 100x enzyme stock containing 10 mg/ml lysozyme and 2.5 mg/ml of RNase A prepared in 50% glycerol and 50 mM Tris pH 8 was stored at −20°C and used repeatedly.

2.2.3.6 Screening of recombinant plasmid DNAs for identification of positive TA clones by colony PCR and Restriction digestion

1 µl plasmid DNA isolated from individual colonies were subjected to PCR amplification using ECD_F1 and ECD_R1 primers as described in section 2.2.2. The PCR amplicons were run on 0.8% agarose gel.

5µl of the recombinant plasmid DNA of PCR positive clones EGFR ECD were taken in a fresh sterile microcentrifuge tube for restriction enzyme digestion analysis. The recombinant plasmid DNA was digested with restriction enzymes, *Nde*I and *Xho*I, to check for positive clones following a 20 µl reaction mixture set up as mentioned in **Table 2.4**. The reaction mixtures were incubated at 37ºC in a water bath for 90 min. The digested products were then run on 0.8% agarose gel as described in Section 2.2.2.1. The digested vector and the respective insert DNA of above mentioned recombinant derivatives were visualized by placing the gel under UV transilluminator. The digested fragments of expected size were taken as positive TA clones. Glycerol stocks of *E. coli* (XL 10 Gold) cells containing the positive TA clones were prepared in glycerol (15-20 % v/v) and stored at -80°C.

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Table 2.4: Restriction enzyme digestion set up of recombinant plasmid DNA of EGFR ECD.

Digestion set up	**1x (µl)**
10x buffer	2.0
DNAse free water	11.0
Recombinant plasmid DNA (approx. 75 ng)	5.0
*Nde*I (10 U/µl)	1.0
*Xho*I (10 U/µl)	1.0
Total reaction volume	20.0

2.2.4 Subcloning of EGFR ECD into pET-28a (+) vector

The pET-28a (+) vector is commonly used for cloning and expression of recombinant proteins in *E. coli.* It is a modified form of pBR322 with a strong T7 promoter system originally developed by Studier and colleagues (Studier *et al*., 1990; Studier and Moffatt, 1986). The pET-28a (+) vector is incorporated with an N-terminal His_6-Tag/thrombin/T7-Tag configuration in addition to an optional C-terminal His_6-Tag sequence. The thrombin tag can be used to remove the His_6-Tag, during structural and western blotting detection of the recombinant protein. The location of His-Tag, T7 coding sequence, T7 terminator, kanamycin coding region and f1 origin are depicted in the **Fig. 2.4**.

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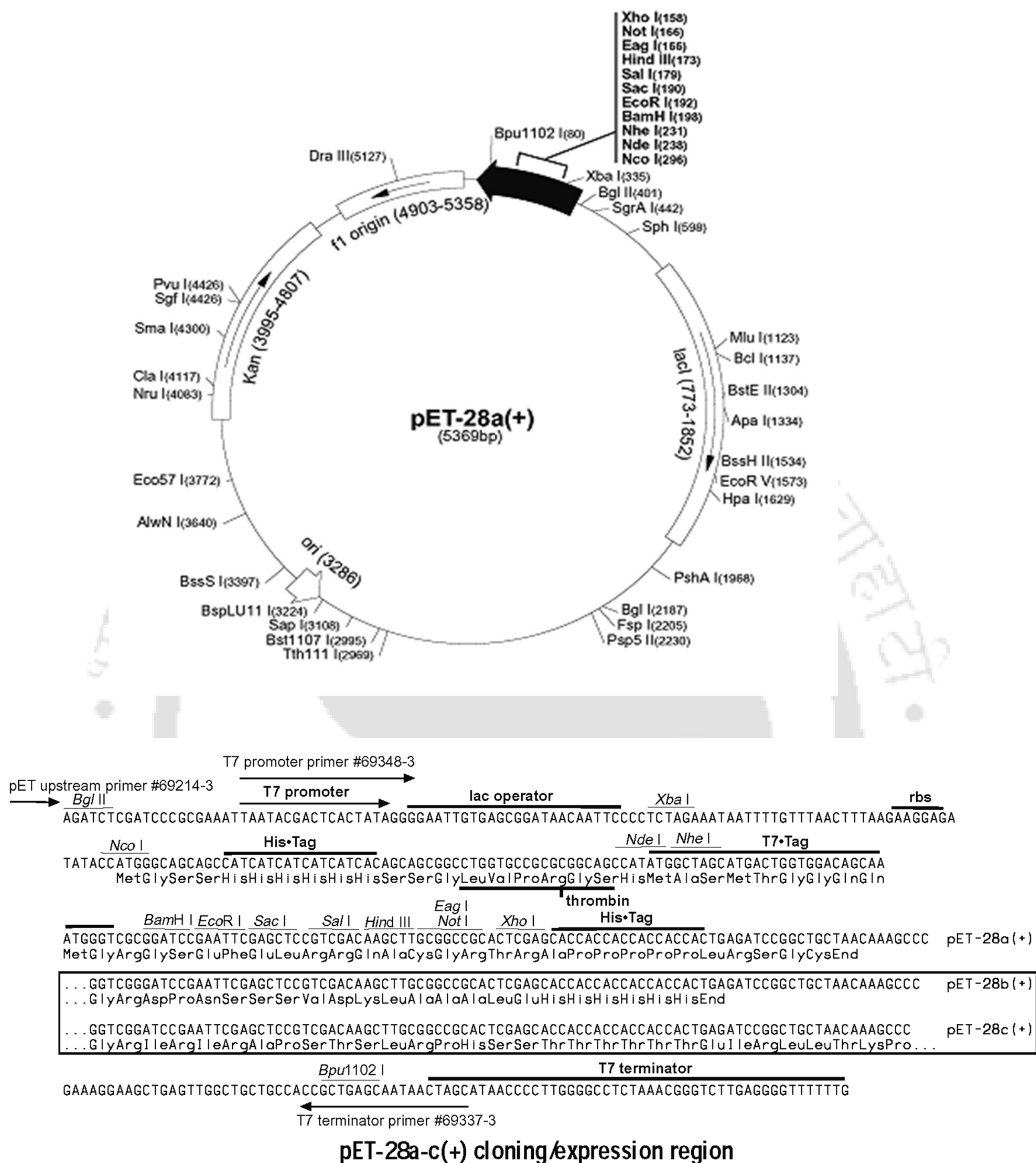

Figure. 2.4: Restriction map of the pET-28a(+) expression vector showing multiple cloning site (158-203 bp), restriction enzyme sites, N-terminal His_6-Tag coding sequence (270-287 bp), C-terminal His_6-Tag coding sequence (140-157 bp), T7 promoter (370-386), T7 terminator (26-72 bp), pBR322 origin (3286 bp), kanamycin marker (3995-4807 bp) and a f1 origin (4903-5358). *Nde*I cuts at 238 and *Xho*I at 158.

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The pET-28a (+) vector was digested with *Nde*I-*Xho*I to prepare them for cloning of EGFR ECD fragment as obtained in section 2.2.3.6. The restriction enzyme digestions of pET vectors were then carried out as described above (**Table 2.4**). The digestion mixtures were incubated at 37°C in a water bath for 90 min. The *Nde*I-*Xho*I digested pET vectors were purified from agarose gel as described in Section 2.2.2.2

2.2.4.1 Ligation of EGFR ECD inserts to pET-28a (+) vector

The *Nde*I-*Xho*I digested fragments of EGFR ECD obtained from TA clones discussed in Section 2.2.3.6 were cloned into pET-28a (+) vector, which were also digested with same restriction enzymes as described above. The ligation reactions were setup using the reaction components mentioned in **Table 2.5** and incubated at 4°C overnight to get maximum number of transformants. Again the reactions were setup at an insert: vector molar ratio of 3:1 (section 2.2.3.2) where the amount of insert is calculated as mentioned below.

$$\frac{50\ (\text{ng}) \times 1.935\ (\text{kb})}{5.369\ (\text{kb})} \times \frac{3}{1} = 54.05\ \text{ng}$$

Table 2.5: Components for ligation reaction setup for subcloning of EGFR ECD into pET-28a (+) expression vector.

Reaction components	**Volume (µl)**
10X Ligation Buffer	1
pET-28a (+) Vector (100ng)	0.5 (50 ng)
TA clone digested product	2.5 (54 ng)
T4 DNA Ligase (3 units/µl)	1
Nuclease-free water	5
Total reaction volume	10.0

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2.2.4.2 Transformation of recombinant pET-28a (+) into E. coli (XL10 Gold) competent cells

The *E. coli* (XL10 Gold) competent cells were transformed with recombinant pET-28a (+) vector carrying EGFR ECD fragment. Transformation protocol was followed as mentioned in Section 2.2.3.3. The transformed recombinant XL 10 Gold cells were plated on LB plates supplemented with kanamycin (25 µg/ml) and grown overnight at 37°C.

2.2.4.3 Isolation of plasmid DNA from recombinant pET-28a (+) vector carrying EGFR ECD

Colonies preferably from the centre of the plate were randomly picked in a laminar air flow and grown overnight in 5 ml LB medium supplemented with kanamycin (25 µg/ml). The plasmid DNA from this 5 ml culture was isolated by NID method following the protocol mentioned in Section 2.2.3.5.

2.2.4.4 Screening of recombinant pET-28a (+) clones by colony PCR and Restriction digestion

1 µl plasmid DNA isolated from individual colonies were subjected to PCR amplification using ECD_F1 and ECD_R1 primers as described in section 2.2.2. The PCR amplicons were run on 0.8% agarose gel.

5µl of the recombinant plasmid DNA of colony PCR positive clones of EGFR ECD were subjected to restriction digestion with *Nde*I and *Xho*I restriction enzyme in a 20 µl reaction mixture set up as described in section 2.2.3.6. Glycerol stocks of *E. coli* (XL 10 Gold) cells containing the positive recombinant pET-28a (+) clones were prepared in glycerol (15-20% v/v) and stored at -80°C.

2.2.5 Sequencing of positive recombinant EGFR ECD- pET 28a (+) clones

The plasmid of recombinant clone carrying EGFR ECD in pET 28a (+) vector was isolated using Sigma miniprep kit according to the manufacturer instructions. The isolated recombinant plasmid were sent for sequencing to Scigenome Pvt Ltd, India. The

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sequencing was performed from both directions with ECD_F1 and ECD_R1 primers. The obtained sequences were then aligned with human EGFR sequence of NCBI database (NM_005228.3).

2.2.6 Expression of EGFR ECD protein

The constructed pET 28a (+) vector carrying EGFR ECD region were transformed into competent BL 21 (DE3) cells for the expression of protein.

2.2.6.1 Transformation of recombinant pET 28a (+) plasmids containing genes encoding EGFR ECD into E. coli (BL21) cells for expression

10 µl of recombinant plasmid of positive pET-28a (+) clones isolated in Section 2.2.5 were used for transformation of competent *E. coli* (BL-21) following the protocol described in Section 2.2.3.3.2 The transformed BL 21 cells were plated on LB agar plates supplemented with kanamycin (25 µg/ml) and grown overnight at 37°C.

2.2.6.2 Expression profiling of recombinant EGFR ECD protein

The overnight grown plates were observed and colonies preferably from the centre of the plate were randomly picked within a laminar air flow and grown in 5 ml LB media supplemented with kanamycin (25 µg/ml) at 37°C and 180 rpm. The cells were grown till mid-exponential phase or till cell O.D. reached $A_{550} \approx 0.6$ (San-Miguel *et al*., 2013). 1 ml of this culture, containing uninduced cells was used for sample preparation for SDS-PAGE analysis and stored for glycerol stock preparation. The remaining 4 ml culture was then induced with isopropyl--D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM for expression of recombinant proteins and analysed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). Cultures were placed at varying temperature and for different incubation periods (6-24 h). The following expression conditions were employed in this study:

1. Incubation at 37°C, 180 rpm for 6 hours.
2. Incubation at 25°, 140 rpm for 16 hours.
3. Incubation at 16°C, 140 rpm for 16-18 hours.

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2.2.6.3 SDS-PAGE analysis of recombinant EGFR ECD protein

SDS PAGE was used to separate components of a protein mixture based on their size (Laemmli, 1970; Sambrook and Russell, 2001). The analysis of expression of EGFR ECD protein was done in 12% SDS-PAGE. The SDS-PAGE was run in 1X Tris-Glycine (pH 8.3-8.5) running buffer at constant voltage of 50 to 100 V. The expressed and purified protein samples were viewed after staining the gel with staining solution containing Coomassie Brilliant Blue (CBB) R-250 dye prepared by dissolving (0.25% w/v) in 50 ml deionized water, 40 ml methanol and 10 ml glacial acetic acid. The gels were destained by immersing the gel in destaining solution containing 50 ml deionized water, 40 ml methanol and 10 ml glacial acetic acid with gentle shaking and periodic change of buffer, until the protein bands became clearly visible.

2.2.7 Purification of recombinant EGFR ECD protein

The *E. coli* BL-21(DE3) cells harboring recombinant plasmids were grown in 1.2 L LB medium supplemented with kanamycin (25 µg/ml) in 1L flask. The recombinant proteins containing a His_6-tag at the N-terminal were purified through an immobilized metal-ion affinity chromatography (IMAC) as described in section 2.2.7.1. His-tag purification of these recombinant proteins was done in 5.0 ml sepharose columns (GE Healthcare, HiTrap chelating HP). The compositions of binding as well as elution buffers used for affinity column purification are mentioned in **Table 2.6**.

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Table 2.6: Composition of buffers required for purification recombinant proteins by affinity purification (IMAC).

Buffers	**Composition**
Equilibration buffer	50mM Sodium Phosphate buffer, 400 mM NaCl, 30mM Imidazole, pH 7.2
Wash buffer	50mM Sodium Phosphate buffer, 400 mM NaCl, 60mM Imidazole, pH 7.2
Elution buffer	50mM Sodium Phosphate buffer, 400 mM NaCl, 400mM Imidazole, pH 7.2
Cleaning buffer	50 mM Tris-HCl, pH 8.0 500 mM NaCl, 50 mM EDTA

2.2.7.1 IMAC purification protocol for recombinant protein

The bacterial cells (1.2 L culture) were harvested by centrifugation at 8,000 rpm, 4°C for 10 minutes. The cell pellet was resuspended in Lysis Buffer (50mM Sodium Phosphate buffer, 400 mM NaCl, Lysozyme (0.2 mg/ml) and PMSF (1mM), pH 7.25). Cells were lysed by sonication on ice (8secs ON, 20 secs OFF pulse for 30-45 cycles, with 33% amplitude) and centrifuged at 11,000g at 4ºC for 30 min to get the crude cell free extract. The HisTrap crude column were prepared by washing with 5 column volumes of filtered and degassed water to remove the alcohol. It was then charged using 2.0 ml of 0.1 M $NiSO_4$ solution and the unbound nickel was washed away with 2-5 volumes of water. Then the column was equilibrated with 5-10 volumes of equilibration buffer (**Table 2.6**).The cell free extract of recombinant protein was loaded on to the column at a flow rate of 1 ml/min. The column was then washed with 50-60 column volumes of wash buffer to remove the unbound proteins. The retained protein of interest were then eluted with elution buffer and 1 ml fractions were collected. The column was cleaned using cleaning buffer. Then the column was washed with 2-5 column volumes of water and finally stored in 20% (v/v) ethanol at 4°C.

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The concentration of eluted proteins were quantified using Bradford reagent. The purity and molecular mass of recombinant proteins were verified by SDS-PAGE. The eluted fractions containing protein of interest were then concentrated using Millipore Amicon Filter of 30 KDa cut off. The concentrated sample was again purified using 1 ml sepharose column (HisTrap FF crude) following the above mentioned protocol.

2.2.8 Western Blot analysis of purified recombinant ECD of EGFR protein

The purified ECD protein was run on 12% SDS PAGE and then electroblotted onto a nitrocellulose membrane. The electroblotting was carried out at 4° C for 5-6 hrs. The transfer of protein was confirmed by Ponceau staining. The membrane was then blocked with 5% Skim milk for 2h at room temperature. After blocking, membrane was extensively washed with PBST buffer (10mM Na_2HPO_4, 1.8mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl and 0.05% Tween 20) thrice for 5 min. The membrane was incubated with the EGFR specific primary antibody (Abcam) at 4°C overnight. The membrane was again washed with PBST for removing unbound EGFR antibodies. Then membrane was incubated with horseradish peroxidase-conjugated Goat Anti-Mouse secondary antibody (Abcam). Finally after washing with PBST, membrane was developed using Chemiluminescent Peroxidase Substrate kit (sigma). Water was taken as negative control.

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2.3 Results

2.3.1 PCR amplification of ECD of EGFR from EGFR WT plasmid (Addgene, Plasmid # 11011)

EGFR ECD were amplified from EGFR WT plasmid (plasmid # 11011) procured from Addgene plasmid repository using the conditions as mentioned in Section 2.2.2. The PCR amplified amplicons were detected by 0.8% agarose gel electrophoresis as shown in **Fig. 2.5**. An expected band of size 1.9 kb confirmed the amplification of ECD region of EGFR. The PCR amplicons were purified from gel using Sigma Gel Extraction kit as mentioned in Section 2.2.2.2 and stored at -20°C for subsequent TA cloning.

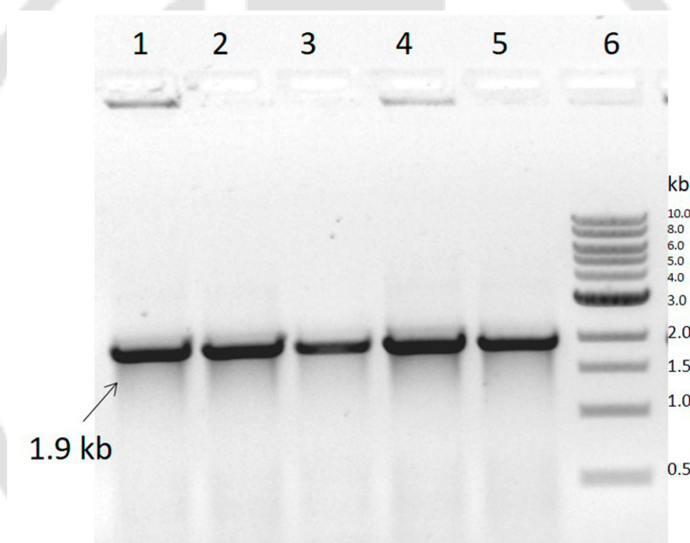

Figure 2.5: 0.8% agarose gel depicting PCR amplification of Extra Cellular Domain of EGFR from full length EGFR. Lane 1 to 5: PCR amplified EGFR ECD (1935bp), Lane 6: NEB 1kb ladder.

2.3.2 TA cloning of PCR amplified ECD of EGFR into pTZ57R/T vector

The PCR amplified product of EGFR ECD with 3′-A overhang was ligated to pTZ57R/T vector following the method described in Section 2.2.3. The ligated products were transformed into *E.coli* (XL 10 Gold) competent cells and positive clones were selected by blue-white colony selection as described in Section 2.2.3.4.

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2.3.2.1 Screening of recombinant plasmid DNAs for identification of positive TA clones by colony PCR and Restriction digestion

Plasmid DNA from white colonies were successfully isolated by NID method as described in Section 2.2.3.5. The isolated plasmids were checked on 0.8% agarose gel. The positive TA clones were further screened by colony PCR (**Fig 2.6**) and confirmed by restriction digestion with *Nde*I and *Xho*I enzyme (**Fig 2.7**). An insert release of 1.9 kb from vector backbone of 2.8 kb confirmed the cloning of EGFR ECD in pTZ57R/T vector. The digested fragments of EGFR ECD were gel eluted and prepared for their cloning into pET-28a (+) vector. The expression vector was also linearized after restriction digestion by *NdeI* and *XhoI*, and used for ligation with the insert.

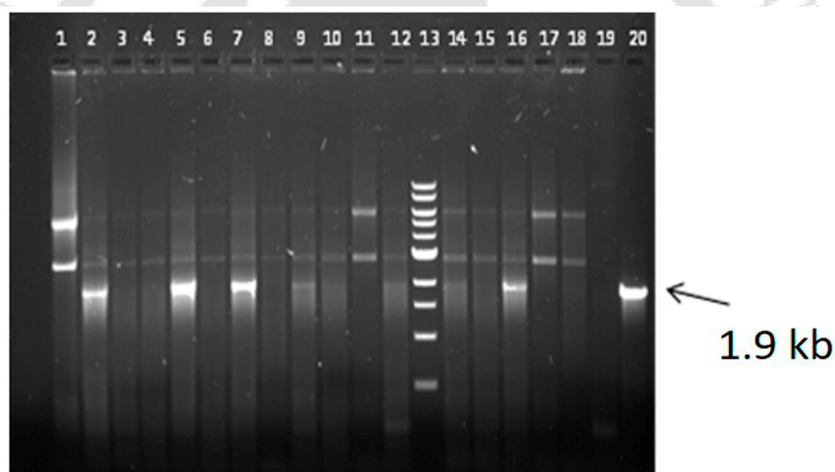

Figure. 2.6: 0.8 % Agarose gel of colony PCR of plasmid DNA isolated from TA clones of EGFR ECD. Lane 1-12 & 14-20: PCR amplified DNA, Lane 13: NEB 1kb ladder.

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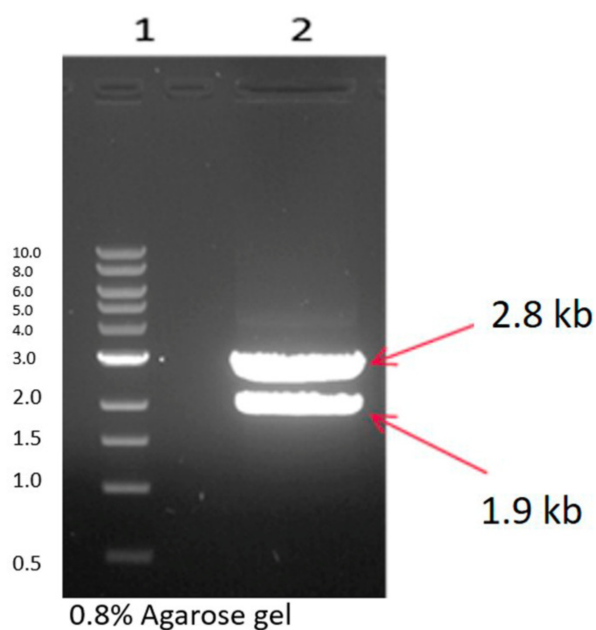

Figure 2.7: Agarose gel (0.8%) showing *Nde*I-*Xho*I digested TA clone plasmid DNA. Lane 1: NEB 1kb ladder. Lane 2: Insert release of 1935 bp from a vector backbone of 2886 bp.

2.3.3 Subcloning of restriction digested EGFR ECD into pET-28a (+) vector

The gel purified restriction enzyme digested fragments of EGFR ECD were ligated with the linearized fragments of pET28a (+) vector following the protocol mentioned in Section 2.2.4.1. The ligated product was transformed into *E. coli* (XL10 Gold) competent cells and grown overnight at 37°C on LB agar plates under stationary condition.

2.3.3.1 Screening of recombinant plasmid DNAs for positive pET28a (+) clones by colony PCR ad Restriction digestion

After transformation, colonies were grown in LB broth media and plasmid DNA were isolated by NID method as described in Section 2.2.3.5. The plasmid isolated from colonies were subjected to PCR amplification using ECD_F1 and ECD_R1 primer. The PCR amplicons were checked by running on 0.8% agarose gel (**Fig 2.8**). The clones showing positive results in colony PCR were selected and digested with *Nde*I and *Xho*I enzyme. The plasmid DNA after restriction digestion was run on 0.8% agarose gel. The

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insert release of 1.9 kb from vector backbone of 5.3 kb confirmed the cloning of EGFR ECD in pET-28a (+) vector (**Fig 2.9**).

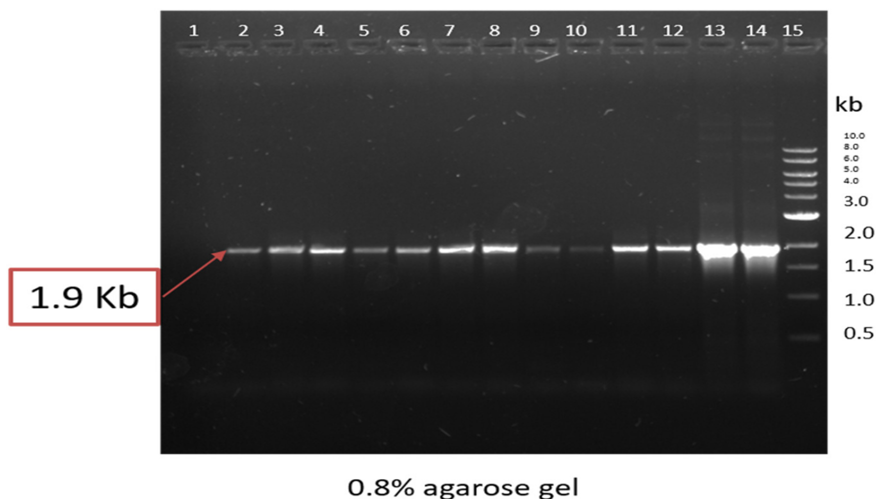

Figure 2.8: 0.8 % agarose gel of Colony PCR of recombinant pET-28a (+) vector carrying EGFR ECD. Lane 1: Negative control, Lane 2 to 12: PCR amplification of plasmid isolated from colonies of ECD-pet28a transformed into DH5α. Lane 13- 14: Positive Control, Lane 15: NEB 1Kb Ladder.

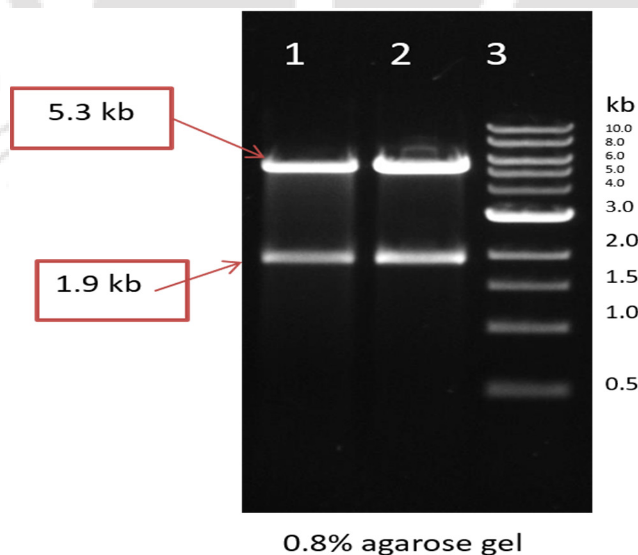

Figure 2.9: 0.8 % Agarose gel showing *Nde*I-*Xho*I digested recombinant pET-28a plasmid DNA. Lane 1 & 2: 1.9 kb EGFR ECD insert fragment and 5.3 kb pET-28a (+) vector, Lane 3: NEB 1Kb Ladder .

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2.3.4 Sequencing of recombinant pET 28a vector carrying ECD of EGFR

The subcloning of EGFR ECD in pET 28a (+) vector was further confirmed by sequencing from both direction using ECD_F1 and ECD_R1 primer. The obtained sequences were then aligned with the reference EGFR sequence, retrieved from NCBI database (NM_005228.3). The pairwise alignment depicts that the sequences were 99.9% similar as shown in **Fig 2.10**.

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```
                   *      1080         *      1100         *      1120         *      1140         *      1160
Reference : cgaatattaaacacttcaaaaactgcacctccatcagtggcgatctccacatcctgccggtggcatttaggggtgactccttcacacatactcctcctctggatcc : 1166
Reverse   : -------------TTCAAAAACTGCACCTCCATCAGTGGCGATCTCCACATCCTGCCGGTGGCATTTAGGGGTGACTCCTTCACACATACTCCTCCTCTGGATCC :   92
                         TTCAAAAACTGCACCTCCATCAGTGGCGATCTCCACATCCTGCCGGTGGCATTTAGGGGTGACTCCTTCACACATACTCCTCCTCTGGATCC

              *      1180         *      1200         *      1220         *      1240         *      1260         *
Reference : acaggaactggatattctgaaaaccgtaaaggaaatcacagggttttgctgattcaggcttggcctgaaaacaggacggacctccatgcctttgagaacctagaa : 1272
Reverse   : ACAGGAACTGGATATTCTGAAAACCGTAAAGCAAATCACAGGGTTTTGCTGATTCAGGCTTGGCCTGAAAACAGGACGGACCTCCATGCCTTTGAGAACCTAGAA :  198
            ACAGGAACTGGATATTCTGAAAACCGTAAAG AAATCACAGGGTTTTGCTGATTCAGGCTTGGCCTGAAAACAGGACGGACCTCCATGCCTTTGAGAACCTAGAA

                1280         *      1300         *      1320         *      1340         *      1360         *      13
Reference : atcatacgcggcaggaccaagcaacatggtcagttttctcttgcagtcgtcagcctgaacataacatccttgggattacgctccctcaaggagataagtgatggag : 1378
Reverse   : ATCATACGCGGCAGGACCAAGCAACATGGTCAGTTTTCTCTTGCAGTCGTCAGCCTGAACATAACATCCTTGGGATTACGCTCCCTCAAGGAGATAAGTGATGGAG :  304
            ATCATACGCGGCAGGACCAAGCAACATGGTCAGTTTTCTCTTGCAGTCGTCAGCCTGAACATAACATCCTTGGGATTACGCTCCCTCAAGGAGATAAGTGATGGAG

            80         *      1400         *      1420         *      1440         *      1460         *      1480
Reference : atgtgataatttcaggaaacaaaaatttgtgctatgcaaatacaataaactggaaaaaactgtttgggacctccggtcagaaaaccaaaattataagcaacagagg : 1484
Reverse   : ATGTGATAATTTCAGGAAACAAAAATTTGTGCTATGCAAATACAATAAACTGGAAAAAACTGTTTGGGACCTCCGGTCAGAAAACCAAAATTATAAGCAACAGAGG :  410
            ATGTGATAATTTCAGGAAACAAAAATTTGTGCTATGCAAATACAATAAACTGGAAAAAACTGTTTGGGACCTCCGGTCAGAAAACCAAAATTATAAGCAACAGAGG

                 *      1500         *      1520         *      1540         *      1560         *      1580         *
Reference : tgaaaacagctgcaaggccacaggccaggtctgccatgccttgtgctcccccgagggctgctggggcccggagcccagggactgcgtctcttgccggaatgtcagc : 1590
Reverse   : TGAAAACAGCTGCAAGGCCACAGGCCAGGTCTGCCATGCCTTGTGCTCCCCCGAGGGCTGCTGGGGCCCGGAGCCCAGGGACTGCGTCTCTTGCCGGAATGTCAGC :  516
            TGAAAACAGCTGCAAGGCCACAGGCCAGGTCTGCCATGCCTTGTGCTCCCCCGAGGGCTGCTGGGGCCCGGAGCCCAGGGACTGCGTCTCTTGCCGGAATGTCAGC

                  1600         *      1620         *      1640         *      1660         *      1680         *
Reference : cgaggcagggaatgcgtggacaagtgcaaccttctggagggtgagccaagggagtttgtggagaactctgagtgcatacagtgccacccagagtgcctgcctcagg : 1696
Reverse   : CGAGGCAGGGAATGCGTGGACAAGTGCAACCTTCTGGAGGGTGAGCCAAGGGAGTTTGTGGAGAACTCTGAGTGCATACAGTGCCACCCAGAGTGCCTGCCTCAGG :  622
            CGAGGCAGGGAATGCGTGGACAAGTGCAACCTTCTGGAGGGTGAGCCAAGGGAGTTTGTGGAGAACTCTGAGTGCATACAGTGCCACCCAGAGTGCCTGCCTCAGG

            1700         *      1720         *      1740         *      1760         *      1780         *      1800
Reference : ccatgaacatcacctgcacaggacggggaccagacaactgtatccagtgtgcccactacattgacggcccccactgcgtcaagacctgcccggcaggagtcatggg : 1802
Reverse   : CCATGAACATCACCTGCACAGGACGGGGACCAGACAACTGTATCCAGTGTGCCCACTACATTGACGGCCCCCACTGCGTCAAGACCTGCCCGGCAGGAGTCATGGG :  728
            CCATGAACATCACCTGCACAGGACGGGGACCAGACAACTGTATCCAGTGTGCCCACTACATTGACGGCCCCCACTGCGTCAAGACCTGCCCGGCAGGAGTCATGGG

                  *      1820         *      1840         *      1860         *      1880         *      1900
Reference : agaaaacaacaccctggtctggaagtacgcagacgccggccatgtgtgccacctgtgccatccaaactgcacctacggatgcactgggccaggtcttgaaggctgt : 1908
Reverse   : AGAAAACAACACCCTGGTCTGGAAGTACGCAGACGCCGGCCATGTGTGCCACCTGTGCCATCCAAACTGCACCTACGGATGCACT-------------------- :  813
            AGAAAACAACACCCTGGTCTGGAAGTACGCAGACGCCGGCCATGTGTGCCACCTGTGCCATCCAAACTGCACCTACGGATGCACT

              *      1920         *
Reference : ccaacgaatgggcctaagatcccgtcc : 1935
Reverse   : --------------------------- :    -
```

Figure 2.10: Pairwise alignment of sequencing data with the reference sequence available in NCBI database (NM_005228.3). Forward and reverse represents the sequences obtained from sequencing of recombinant pET28a vector with ECD_F1 forward primer and ECD_R1 reverse primer respectively.

2.3.5 Expression and purification of recombinant EGFR ECD protein

The *E. coli* (BL-21) competent cells were transformed with the constructed recombinant pET-28a (+) vector carrying EGFR ECD DNA. The colonies were picked randomly and grown in 5 ml LB medium supplemented with kanamycin (25 μg/ml). The cells were induced for hyperexpression of protein at mid exponential stage. Hyper-expression of protein was analysed on SDS-PAGE by loading uninduced as well as the induced cells of recombinant protein on adjacent wells, as depicted in **Fig. 2.11**.

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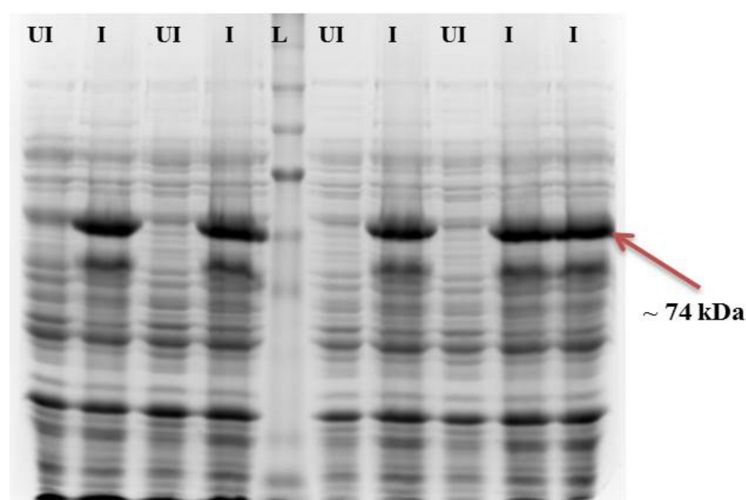

Figure 2.11: SDS-PAGE (12%) gel showing hyper expression of recombinant EGFR ECD protein in *E. coli* BL-21 cells on induction with 0.1M IPTG. UI: Uninduced cell extract, I: Induced cell extract, L: NEB prestained protein Ladder.

The parameters required for expression of EGFR ECD protein in soluble fraction were optimized to 16 hours of incubation at 16°C after induction with 0.1M IPTG. The hyperexpressed recombinant EGFR ECD protein were purified by immobilized metal ion affinity chromatography as described in Section 2.2.7. Initially after first round of purification the eluted fraction were run on 12% SDS PAGE. The purified EGFR ECD protein showed an expected band of molecular size 74 kDa. However the elute fractions were found to contain some non-specific proteins (**Fig 2.12 A**). The eluted fractions were pooled and concentrated using Millipore Amicon Filter of 30 KDa cut off. The buffer exchange was also performed for removal of imidazole and salts. The concentrated sample was again purified using 1 ml (HisTrap FF crude) sepharose column using the same protocol (Section 2.2.7) The eluted fractions after second round of purification were found to be free from any non-specific protein on 12% SDS PAGE (**Fig 2.12 B**).

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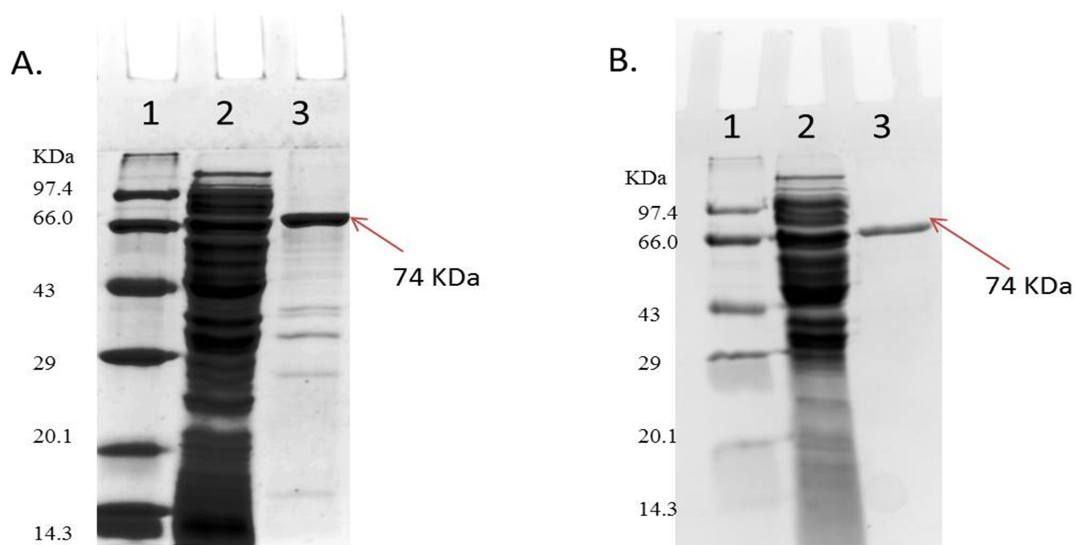

Figure 2.12: A. 12 % SDS-PAGE analysis of EGFR ECD protein after first round of purification. B. 12 % SDS-PAGE analysis of EGFR ECD protein after second round of purification. The purified EGFR ECD showed molecular size of 74 kDa. Lane 1: Ladder, Lane 2: Crude bacterial extract, Lane3: Purified EGFR ECD protein.

2.3.6 Western Blot analysis of recombinant EGFR ECD protein

The EGFR ECD protein after second round of purification was run on 12% SDS PAGE and successfully electroblotted onto NC membrane. The membrane was blocked followed by incubation with EGFR specific primary antibody and horseradish peroxidase-conjugated Goat Anti-Mouse secondary antibody and developed using Chemiluminescent Peroxidase Substrate kit.

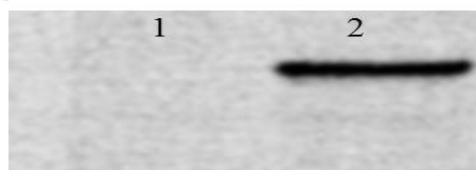

Figure 2.13: Western Blot analysis of purified recombinant EGFR ECD protein. Lane 1: Negative control, Lane 2: EGFR ECD protein.

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2.4 Discussions

The extra cellular domain of EGFR plays most significant role as it binds to ligand and induces the activation of downstream signaling pathway. Also these domains are responsible for the formation of either homodimers or heterodimers that amplifies the signalling cascades. Mostly alongwith the full length EGFR, mutant variants of EGFR are also detected in normal and malignant cells, in tissues and in biological fluids of cancer patients (Perez-Torres *et al*., 2008; Rose-John and Heinrich, 1994). These isoforms/variants are comprised solely of extra cellular domain portions of the receptor. It is very important that any probe developed for the detection of EGFR should be directed to extra cellular domain of EGFR so that it can detect all the EGFR variants alongwith the full length EGFR. Thus in the present study the extra cellular domain of EGFR protein was cloned, expressed and purified for aptamer selection.

The main objective of this chapter was to subclone EGFR Extra cellular Domain into pET28a vector and purify the EGFR ECD protein in non-denaturing condition for aptamer selection. The full length EGFR cDNA cloned in pBABE vector in DH5α strain was procured from Addgene plasmid repository (Plasmid # 11011). Then the ECD region of EGFR was PCR amplified from EGFR WT plasmid using ECD_F1 and ECD_R1 primer. The primers were prefabricated with restriction enzyme site. This strategy is simple and still the most common, method for cloning PCR products. It allows direct, directional cloning of the insert into the vector after restriction digestion (Kaufman and Evans, 1990). Further to improve the efficiency of cloning of ECD into pET28a vector, the PCR amplicons were first cloned into pTZ57R/T vector for TA cloning. The TA cloning method takes advantage of the terminal transferase activity of Taq polymerase. This enzyme adds a single, 3'-A overhang to each end of the PCR product. This makes it possible to clone the PCR product directly into a linearized cloning vector with single 3'-T overhangs. Also the colony screening for positive clones can be easily performed by blue-white screening. The colonies carrying recombinant vector produces white colonies, whereas blue colonies usually do not contain any clone.

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For the cloning and expression of recombinant ECD of EGFR protein, pET-28a (+) vector system was selected. pET28a (+) vector is a most commonly used vector for cloning and expression of recombinant proteins in *E. coli.* It is a modified form of pBR322 with a strong T7 promoter system originally developed by Studier and colleagues (Studier *et al*., 1990; Studier and Moffatt, 1986). The desired genes to be cloned in pET plasmids are under the influence of strong bacteriophage T7 transcription and the expression is induced by T7 RNA polymerase in the host cell. Although this system is extremely powerful, it allows the user to control the expression levels simply by manipulating the concentration of inducer. Another advantage with pET system is its ability to maintain target genes transcriptionally silent in the uninduced state. Also the His_6-Tag are incorporated at the ends of recombinant protein. The rationale for the choice of an N-terminal hexahistidine is manifold. An N-terminal tag ensures that the bacterial transcription and translation machineries always encounter 5′ and N-terminal sequences that are compatible with robust RNA synthesis and protein expression, respectively. The incorporation of oligo histidine-tagged dramatically aid in proteins purification using a relatively simple protocol of immobilized metal affinity chromatography (IMAC). Further, histidine tags rarely affect the characteristics, which distinguishes it, for example, from glutathione S -transferase (GST), which itself is a dimer that then imposes dimerization on the recombinant protein. Also hexahistidine tags do not have a consistent impact on the N-terminal structure of the target protein.

The recombinant pET-28a (+) vector carrying EGFR ECD were transformed into *E. coli* (BL21) cells for the production of recombinant protein. The *E. coli* as a host is the most widely used system for protein overproduction, both on a laboratory and industrial scale. It has the advantage of producing large quantities of recombinant proteins in a short time. A simple and inexpensive bacterial cell culture and well-known mechanisms of transcription and translation facilitate the use of these microorganisms. Over the years, much effort has been put into optimizing *E. coli* as an expression host for production of stably folded proteins from higher organisms (Peti and Page, 2007). BL21 (DE3) strain of *E. coli* is an appropriate *E. coli* strain for high-level protein production purposes. It has the advantage of being deficient in both *lon* and *ompT* proteases and it is compatible with the T7 *lacO* promoter system (William Studier *et al*., 1990). For eukaryotic

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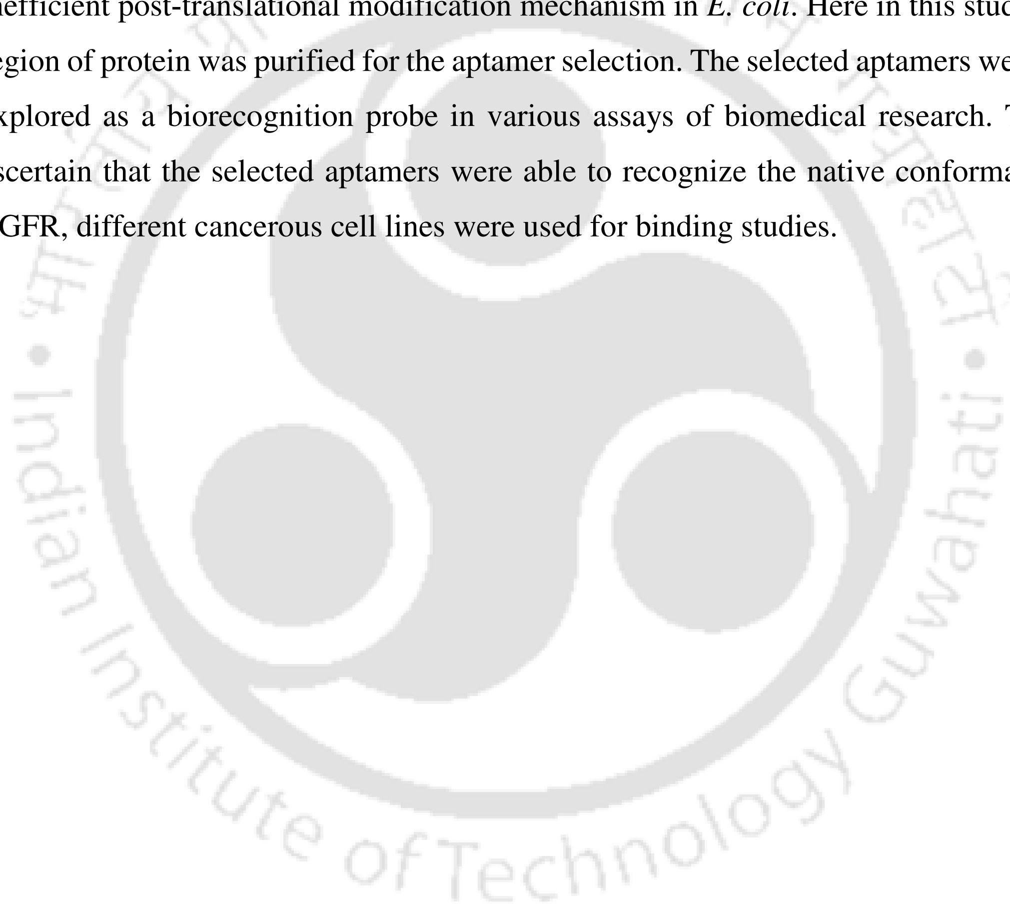
proteins, it is often important to use BL21 (DE3) derivatives carrying additional tRNAs to overcome the effects of codon bias. Another crucial factor of protein production is the temperature during induction. Generally in T7 system many recombinant proteins often precipitate when expressed at 37 °C, but are soluble when the temperature during induction is 15–25 °C, presumably because slower rates of protein production allow newly transcribed recombinant proteins time to fold properly (Vera *et al*., 2007). Thus, lower temperature was used in our study for expression of ECD region of EGFR protein so that the recombinant protein folds properly. Still the major drawback of this system is inefficient post-translational modification mechanism in *E. coli*. Here in this study ECD region of protein was purified for the aptamer selection. The selected aptamers were used explored as a biorecognition probe in various assays of biomedical research. Thus to ascertain that the selected aptamers were able to recognize the native conformation of EGFR, different cancerous cell lines were used for binding studies.



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2.5 Conclusions

The extracellular domain of EGFR was successfully cloned into pET28a vector from EGFR WT plasmid DNA procured from Addgene (plasmid # 11011). The cloning of EGFR ECD in pET-28a (+) vector was confirmed by restriction digestion and sequencing. Further the recombinant vector were expressed in *E. coli* BL21 (DE3) system and recombinant EGFR ECD protein were purified using Immobilized metal ion affinity chromatography (IMAC) for aptamer selection. Finally the recombinant EGFR ECD was confirmed by Western blot analysis using EGFR specific antibody.

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CHAPTER 3

IN VITRO SELECTION AND CHARACTERIZATION OF APTAMERS AGAINST THE EXTRA CELLULAR DOMAIN OF EPIDERMAL GROWTH FACTOR RECEPTOR

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3.1 Introduction

Early detection, accurate diagnosis and effective treatment could led to better outcome and longer survival of cancer patients. Currently the EGFR based diagnostic and therapy were mainly based on EGFR specific antibodies. However the antibodies suffers from many limitations such as batch to batch variation, poor tissue penetration, immunological reaction, instability, high cost of production etc. In last few years, oligonucleotide probes i.e. aptamers have dramatically impacted nearly every branch of medicine and clinical research as a promising alternative to antibodies. Aptamers are short single-stranded oligonucleotides, either RNA or DNA, that fold into well-defined 3-D structures and have the ability to bind to its target molecules with high affinity and specificity. Numerous aptamers have been selected against a wide variety of targets, ranging from small inorganic molecules to whole organisms (Nimjee *et al*., 2005; Proske *et al*., 2005). To develop a more sensitive and specific approach for early cancer detection, numerous aptamers were generated against cell surface receptors (Hu *et al*., 2012; Kasten *et al*., 2013; Y. L. Song *et al*., 2013) and tumor cells (Li *et al*., 2014; Sefah *et al*., 2010). Its biorecognition property have been extensively exploited in the field of cancer as a theranostic module. Proteins constitute by far the largest class of aptamer targets. The high stability of aptamer-protein complexes, frequently characterized by a Kd in the low nanomolar range, combined with an exquisite specificity of interaction make aptamers valuable tools for many applications: as therapeutics, as diagnostic, as imaging probe, for target validation and for high throughput screening (Ni *et al*., 2011).

Each aptamer consists of a central region of random sequence, which is flanked by two regions of fixed sequence that enable its amplification by PCR. The length of random region is typically between 30 and 250 nucleotide, while each flanking region is typically 15-25 bases long. Aptamers are generated by a molecular directed evolution approach from a library of 10^{13}-10^{15} oligonucleotides. This technique is usually named as **SELEX** (**S**ystematic **E**volution of **L**igands by **EX**ponential enrichment) which consists of repetitive cycles of selection and amplification. During each cycle, oligonucleotides with affinity for a desired target are retained and amplified, leading to their enrichment in the pool which is finally cloned and sequenced to identify the candidate aptamers. Since

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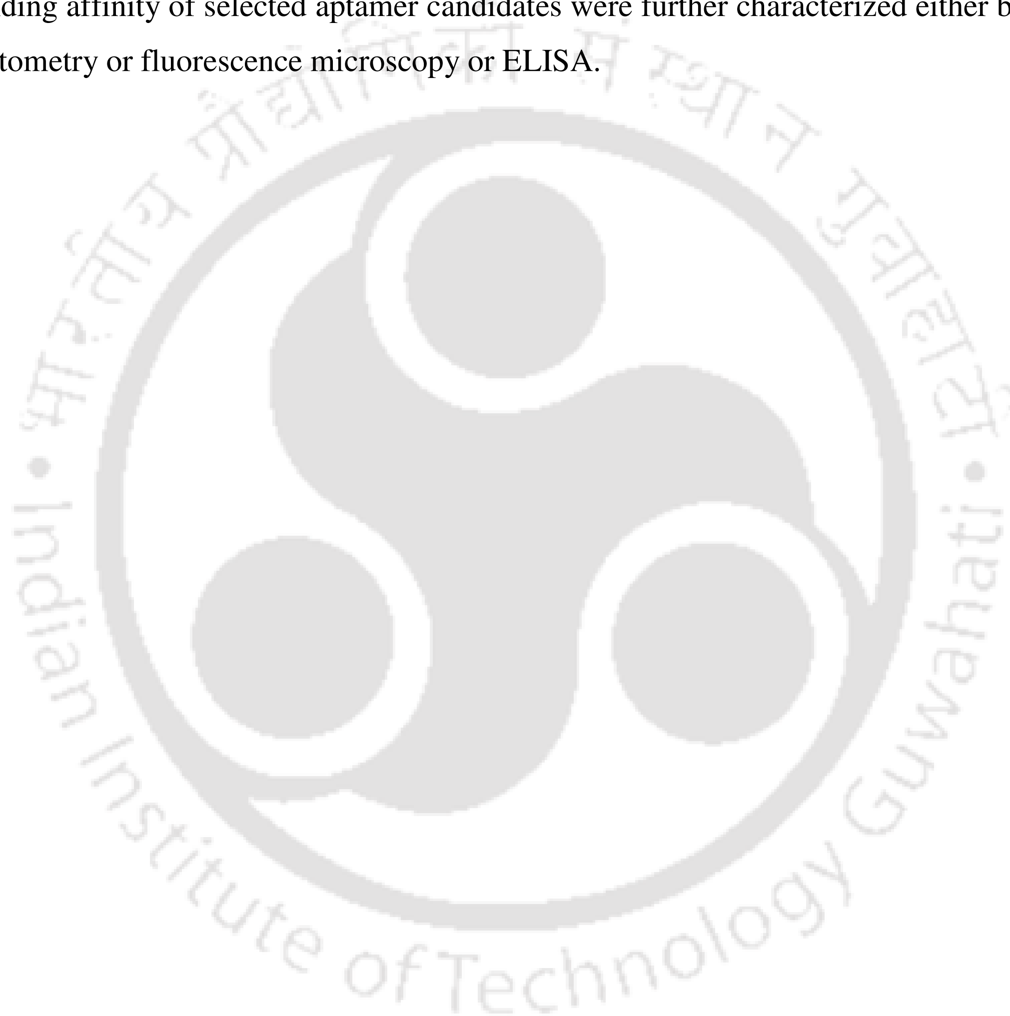
1990, this strategy has been used to identify aptamers against a wide variety of targets from small molecules to peptides, proteins, nucleic acid-based structures.

In the present study protein - SELEX methodology was employed to identify a panel of DNA aptamers against recombinant human Extra Cellular Domain (ECD) of EGFR. The selected aptamers were grouped into six families based on their sequence homology. One aptamer from each group were selected to evaluate their binding affinity and selectivity. The binding affinity of selected aptamer candidates were further characterized either by flow cytometry or fluorescence microscopy or ELISA.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3.2 Materials and Methods

3.2.1 Aptamer library & Primers for SELEX

A degenerate oligodeoxynucleotide library (**Fig. 3.1**) containing a central random region of 40 nucleotides and flanked by invariant primer annealing sites:
5′- ATACCAGTCTATTCAATT–N40-AGATAGTATGTGCAATCA-3′ was purchased from IDT. Two sets of primers were synthesized:
1st set constitute unmodified *viz* APT_F1 (Forward primer): 5′-ATACCAGTCTATTCAATT-3′ and APT_R1 (Reverse primer): 5′-TGATTGCACATACTATCT-3′.
2nd set includes 5′ modified primers i.e. FAM labelled Apt_F1 and Biotin labelled Apt_R1.
The unmodified forward primer (Apt_F1) and Biotinylated reverse primer were used for PCR amplification during SELEX rounds. At the end of 14th round of selection, unmodified Apt_F1 and Apt_R1 were used for amplification of enriched aptamer library. FAM labelled Apt_F1 were used for the labelling of aptamer library.

Figure 3.1: Molecular architecture of Aptamer Library.

3.2.2 Optimization of PCR amplification of aptamer library

The conditions for PCR amplification of aptamer library were optimized as mentioned in **Table 3.1**. The number of cycles of amplifications were optimized. Finally, PCR amplification was carried out in a final reaction volume of 50µl containing Mg^{2+} ions (2.5mM), dNTPs (0.2mM); Primers (1.5µM), 1.0 µl of Taq DNA polymerase (1µl of 1Unit/µl) , 1.0 µl of DMSO (2%) and 1 µl of Aptamer Library DNA in a thermal cycler

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(Takara). The amplicons were run on a 2 % (w/v) agarose gel in the presence of NEB low molecular weight ladder.

Table 3.1: Conditions for PCR thermal cycles for amplification of Aptamer Library.

Steps		Time
I. Denaturation at 94°C		4 min
II. 14 cycles of	i) Denaturation at 94°C	30 sec
	ii) Annealing at 45°C	30 sec
	iii) Extension at 72°C	45 sec
III. Final extension at 72°C		5min

3.2.3 *In vitro* selection of aptamers against Extra Cellular Domain of EGFR by SELEX

The 76 mer long ssDNA library was used as an initial pool. Selection steps were performed at room temperature RT, in ABB (Aptamer Binding Buffer: 50mM Sodium Phosphate buffer, 150 mM NaCl, 1mM $MgCl_2$, 5mMKCl, pH: 7.2). A 500 pmol of initial DNA library was used for the first round of selection. Every time prior to use, DNA library was heated at 95°C, cooled at 4°C and placed at RT for 10 min each. Initially, Negative SELEX was performed by incubating DNA library with NC (Nitro cellulose) membrane in order to eliminate candidates capable of nonspecific binding to NC membrane. The unbound DNA were collected and used in successive SELEX rounds. Simultaneously, for protein SELEX rounds EGFR ECD protein were immobilized on NC membrane by incubating recombinant EGFR ECD protein with NC membrane at RT for 1hour with gentle shaking. The protein immobilised membrane were washed extensively and then incubated with renatured aptamer library for 1hr. After incubation, the membrane was washed thrice with ABB to remove weakly and unbound aptamers. The EGFR specific aptamers were collected by heat elution method. DNase-free water (200 µl) was added to the membrane and the candidate aptamers bound to the target were eluted by heating at 95°C for 2 min. The eluted ssDNA pool was amplified by PCR using Apt_F1 and Biotinylated Apt_R1 primers (Takara PCR thermal cycler) as described in

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Section 3.2.2. The sense ssDNA strands were separated from the biotinylated antisense ssDNA according to the protocol mentioned in section 3.2.4. Separated ssDNA aptamers served as a starting pool for next SELEX rounds. The aptamer pools were subjected to negative SELEX after 8th, 11th and 13th round to filter out sequences that may bind to NC membrane. To enrich the aptamers with high affinity and specificity, the stringency of selection was enhanced gradually by extending the washing time with increasing volume of washing buffer and increasing number of washes after 5th round. Furthermore, the size of NC membrane, amount of protein immobilized and incubation time were gradually reduced. The detailed selection parameters were listed in **Table 3.3**. The enrichment of aptamer sequences during SELEX rounds were monitored by 2% agarose gel electrophoresis. The entire selection process was repeated for 14 rounds according to the extent of enrichment.

3.2.4 Single strand separation of PCR amplified aptamer pool

After every PCR amplification, the double stranded PCR product were separated into single stranded DNA sequence based on alkaline denaturation and streptavidin magnetic beads (New England Biolabs). A 50 µL suspension of the streptavidin magnetic beads were taken in a 1.5 µl eppendorf tube and was washed thrice with 500 µl of Streptavidin Binding Buffer (0.5 M NaCl, 20 mM Tris-HCl (pH 7.5) 1 mM EDTA). The PCR amplified dsDNA were then added to the beads and the mixture was allowed to incubate at room temperature for 1 hour under gentle shaking. The beads were then washed thrice with 500 µl of Streptavidin Binding Buffer. The beads were re-suspended in 100 µl of water and the non-biotinylated DNA strand was separated from the immobilized complementary strand by addition of 0.1 M NaOH to the beads. The supernatant was collected in a fresh eppendorf tube and neutralized by the addition of 1 M NaH_2PO_4. This supernatant containing single stranded DNA served as an enriched ssDNA pool for the next SELEX rounds.

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3.2.5 Cloning and Sequencing of EGFR specific enriched Aptamer pool

3.2.5.1 Cloning of enriched aptamer pool

At the end of fourteen round of SELEX, eluted enriched DNA aptamer pool were PCR amplified using unmodified APT_F1 and APT_ R1 primers as mentioned in section 3.2.2. The PCR amplified enriched aptamer pool were ligated with pTZ57R/T vector using InsTA cloning kit (Thermo Scientific) following the components of ligation reaction as mentioned in **Table 3.2**. The ligation reactions were setup in 1.5 ml microcentrifuge tubes and incubated at 4°C overnight to get maximum number of transformants. The reactions were setup at an insert: vector molar ratio of 3:1, where the amount of insert required in a reaction was calculated using the following formula:

$$\frac{\text{Amount of vector (ng)} \times \text{Size of insert(kb)}}{\text{Size of vector (kb)}} \times \text{Insert : Vector molar ratio} = \text{Amount of insert (ng)}$$

$$\frac{55 \text{ (ng)} \times 76 \text{ (bp)}}{2886 \text{ (bp)}} \times \frac{3}{1} = 4.3 \text{ ng}$$

Table 3.2: Components for ligation reaction setup for TA cloning of EGFR specific enriched aptamer pool.

Reaction components	**volume (µl)**
10X Ligation Buffer	1.0
pTZ57R/T Vector (50ng)	1.0 (55ng)
PCR product	5.0 (4.3 ng)
T4 DNA Ligase (3 units/µl)	1
Nuclease-free water	1.0
Total reaction volume	10.0

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After 16 hours of incubation, ligated construct was transformed into competent *E. coli* (DH5α) cells following the protocol mentioned in section 2.2.3.3.

3.2.5.2 Screening of positive TA clones carrying Aptamer candidates

The positive clones were identified by blue white screening method as described in section 2.2.3.4. About 84 white colonies were picked from LB plate and grown overnight in 5 ml LB medium supplemented with Ampicillin (100 µg/ml). The plasmid DNA from this 5 ml culture was isolated by NID method following the protocol mentioned in Section 2.2.3.5. 1 µl plasmid DNA isolated from individual colonies were subjected to PCR amplification using Apt_F1 and Apt_R1 primers as described in section 3.2.2. The PCR amplicons were checked on 2% agarose gel.

3.2.5.3 Sequencing of selected positive clones carrying aptamer candidates

The plasmid of TA clones which were found positive in PCR amplifications were isolated using Sigma plasmid isolation miniprep kit according to the manufacturer's instructions. The isolated plasmid DNA were then sent for sequencing to Scigenome Pvt. Ltd, India. The sequencing was performed using M13 Forward and Reverse primers.

3.2.6 Sequence Analysis and Secondary Structure Prediction of selected aptamers

Altogether, 20 individual clones were sequenced based on Sanger's method (Scigenome Pvt. Ltd, India). The sequencing results were subjected to multiple sequence alignment analysis with the Clustal Omega to identify highly conserved motifs in the enriched DNA pool. Secondary structures of the selected aptamers were predicted by the Zuker algorithm (Zuker, 2003) using Mfold (version 3.2) with conditions set up at 150 mM NaCl, 1mM $MgCl_2$ and 25°C. The abundant aptamer sequences or aptamer with unique complex secondary structure representing each homologous family *viz* EGFR_A1, EGFR_A7, EGFR_A15, EGFR_A13 and EGFR_A16 were synthesized with either 6 FAM (6-fluorescein amidite) or Biotin labelling at 5' end depending on downstream experiments (ILS, India). Initially the binding of candidate aptamers EGFR_A1, EGFR_A7 and EGFR_A15 were assessed by flow cytometry and fluorescence microscopy. The binding affinity of other two candidates EGFR_A13 and EGFR_A16 were assessed by ELISA.

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3.2.7 Cells and Cell culture conditions

A431 (Human squamous carcinoma), MDA MB 231 (Human triple negative breast carcinoma), A549 (Human lung carcinoma), U87-MG (Human glioblastoma), HeLa (Human cervical adenocarcinoma) and Jurkat (T-cell lymphoma) cell-lines were obtained from National Centre for Cell Science, Pune. All the cell lines were cultured in Dulbecco′s Modified Eagle′s Medium (DMEM, Sigma Aldrich) with high glucose (4.5 g/L). Media were supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific) and 100 IU/mL penicillin-streptomycin. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in air jacketed Eppendorf CO_2 incubator.

3.2.8 Binding Assay of selected aptamers with six EGFR expressing cancer cell lines by Flow Cytometry

The specificity and binding affinity of aptamer candidates were evaluated by flow cytometry. Cells were grown to mid-log phase and detached using an enzyme-free EDTA based cell dissociation solution (Phosphate Buffer Saline with 1mM EDTA). Cells were then resuspended at a concentration of 1X 10^6 cells/mL in ABB. DNA aptamers were heated at 95°C, cooled at 4°C and placed at RT for 10 min each to permit the formation of secondary structures, which are necessary for binding to the target cell surface protein. The folded aptamers- EGFR_A1, EGFR_A7 and EGFR_A15 were added to the cells of each cell line at a final concentration of 200 nM in 500µl of ABB. After forty five mins of incubation, cells were washed twice with ABB. The pellet was then resuspended in 1ml of PBS (Phosphate Buffer Saline) buffer and analysed on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) by counting 10000 events. The data were analysed by Cellquest Pro software (Becton Dickinson). All binding assays were done in duplicate and repeated thrice.

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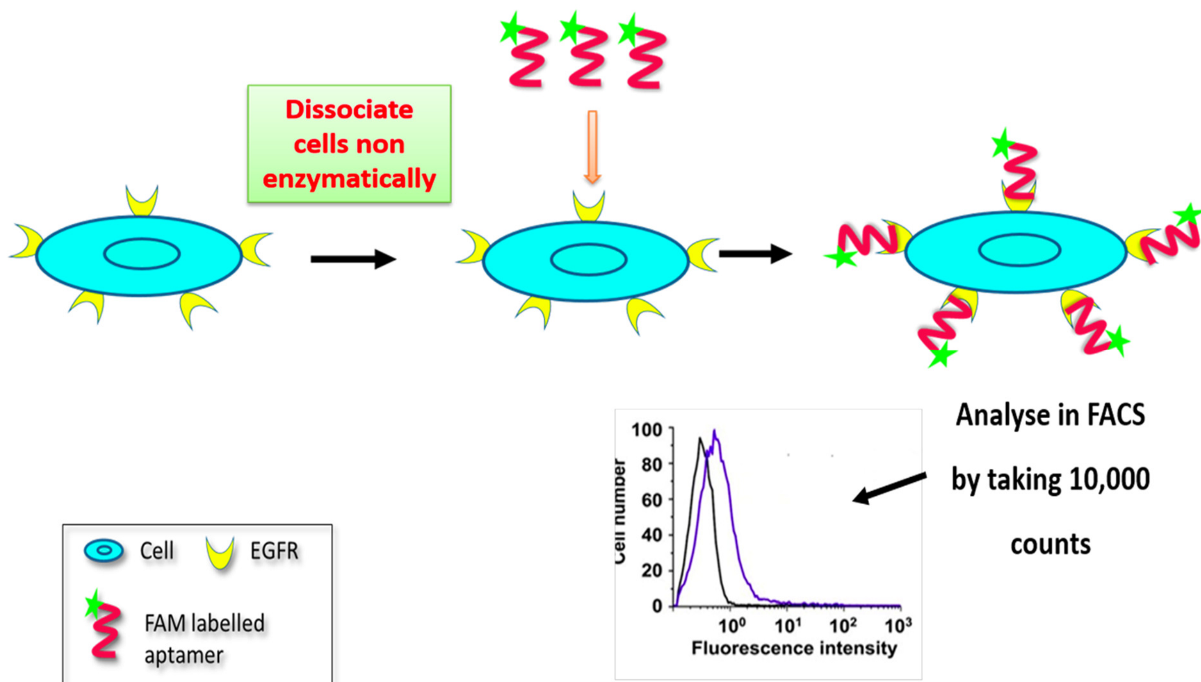

Figure 3.2: Schematic of aptamer binding assay by Flow cytometry.

3.2.9 Verification of cell surface binding of the selected aptamers on target cancer cell lines by flow cytometry

To verify the binding of aptamers to the surface of target cells, binding assays were performed after enzymatic treatment. Cells were first washed twice with PBS (500µl), to remove FBS (Fetal Bovine Serum) of complete media, which might quench the function of trypsin and then incubated with Trypsin (Sigma Aldrich, 500µL, 0.05%) EDTA (0.53mM) in HBSS at 37°C for 5 min. After incubation, the effect of trypsin was neutralized by adding DMEM complete media. The cells were pelleted down and washed with PBS (500µl) once again. Then the cells ($1X10^6$) were resuspended in ABB and used for the aptamer binding assay by flow cytometry as described in section 3.2.8.

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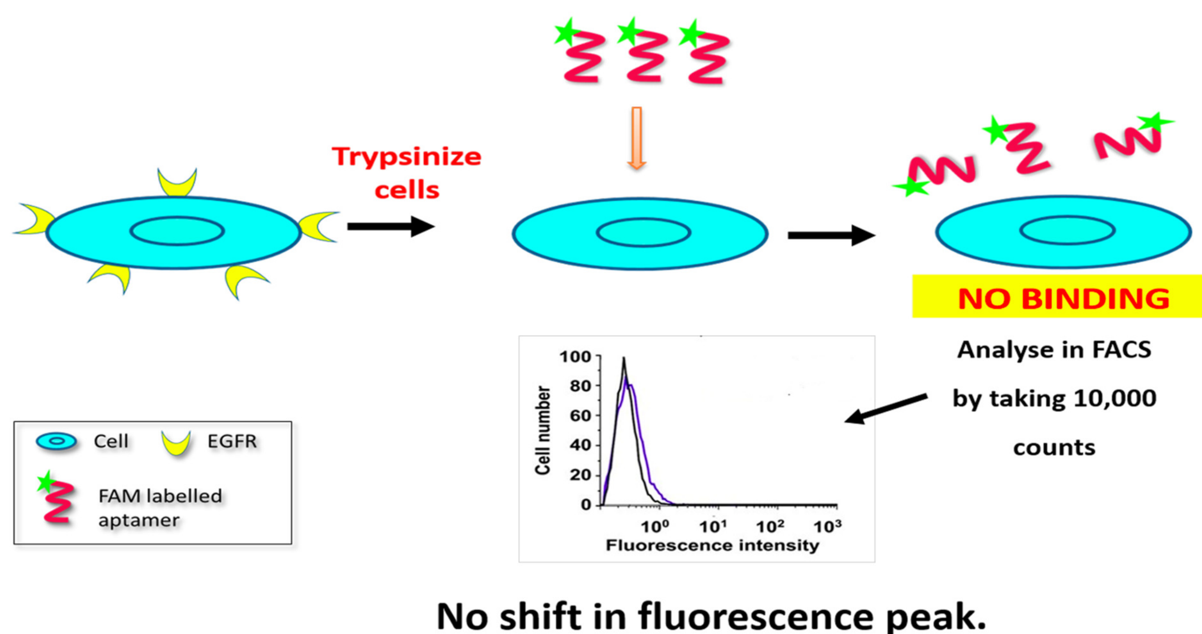

Figure 3.3: An overview of FACS analysis of aptamer binding assay of trypsin treated cells.

3.2.10 Fluorescence imaging of EGFR expressing cancer cells bound to the selected aptamers

To evaluate the potential of selected aptamer candidates as imaging probe, aptamer based immunocytochemistry of A431 and MDA MB 231 cell lines were performed. Cells were passaged when grown to 80% confluency with 1X trypsin, and cultured in 60 mm culture dish containing glass slides under standard culture conditions. After 24 hours of incubation, cells were fixed using 4% formaldehyde for 10 minutes at room temperature. The slides were washed with cold 1X PBS and blocked using 5% bovine serum albumin (BSA) in PBS for 1hr at RT. The cells were washed with PBS and incubated with the individual FAM labelled aptamers EGFR_A1, EGFR_A7 and EGFR_A15 and initial aptamer pool in dark for 45 minutes at RT. FAM-labelling of the non-enriched aptamer library was carried out by PCR amplification with FAM labelled forward primer and biotinylated reverse primer. Strand separation was performed on streptavidin magnetic beads as discussed in section 3.2.4. After incubation with aptamers, cells were washed extensively and counterstained with DAPI for 2 min and observed in fluorescence

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microscope with filters for FAM (excitation λ 450–490, emission λ 515–565) and DAPI (excitation λ 358 , emission λ 461).

3.2.11 Determination of dissociation constant of selected aptamer candidates

The dissociation constant of selected aptamers (EGFR_A15, EGFR_A13 & EGFR_A16) were determined by indirect Enzyme linked Immunosorbent Aptamer Assay (i-ELAA). i-ELAA was performed on 96 well Maxisorp flat-bottomed polystyrene immuno plates (Nunc, Denmark). The microplate were incubated with 500ng of EGFR ECD protein overnight at 4° C. After immobilization plates were washed extensively with PBST buffer (PBS-0.05% Tween 20). Blocking of plates were done by 5% BSA. A volume of 100 µL of denatured individual biotinylated aptamers was added to each well in varying concentrations ranging from 0nM-1 µM. Plates were incubated for 60 min at RT. After washing, the plates were incubated with HRP conjugated streptavidin (New Englands Biolabs). The microplates were again washed four times with PBST and then the substrate TMB (Sigma) was added for 10 min at RT. The reaction was stopped by adding 2M HCl and read at 450 nm. A calibration curve was obtained using aptamers with concentrations in the range of 0–1000 nM. A saturation curve was obtained based on these data, and the equation $Y = BmaxX/ (K_d + X)$ was used to calculate the K_d according to GraphPad Prism 5.0. Y represented the mean value of OD_{450} nm, Bmax was the maximal value of OD_{450} nm and X was the concentration of the aptamer.

3.2.12 Electrophoretic Mobility Shift Assay (EMSA) of selected aptamer with recombinant EGFR ECD protein

The binding of best aptamer candidate i.e. EGFR_A15 was also studied by EMSA. The fixed amount of FAM-labelled EGFR_A15 (0.25µM) aptamer was mixed with increasing concentration of purified recombinant EGFR ECD protein (0-20µM) in ABB and incubated for 1 hr at 25°C. The samples were then resolved on 9% nondenaturing polyacrylamide gels with low ionic strength (0.5X TBE) running buffer at 70 V. To stabilize the formation of Aptamer-Protein complex, 1mM $MgCl_2$ and 5mM KCl was added to the running buffer. The gel was washed with distilled water and then visualized under UV illumination in a gel documentation system (BioRad XR Gel documentation system). The band intensity was analysed by BioRad Image Lab 4.1 software.

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3.3 Results

3.3.1 Design of Aptamer library

The design of aptamer library plays a very crucial role in the success of any aptamer selection process. One of the primary rule involved in designing aptamer library is its stability. It should not be too stable and it can be easily denatured at 95°C for PCR amplification. The initial aptamer library 5′- ATACCAGTCTATTCAATT–N40-AGATAGTATGTGCAATCA- used here in the study had a mean melting temperature of 69.1°C which ensured that it can be denatured during PCR amplification. Further, the secondary structure analysis of aptamer library revealed that the random regions were mainly involved in the 3-D diversity of the library (**Fig. 3.4**).

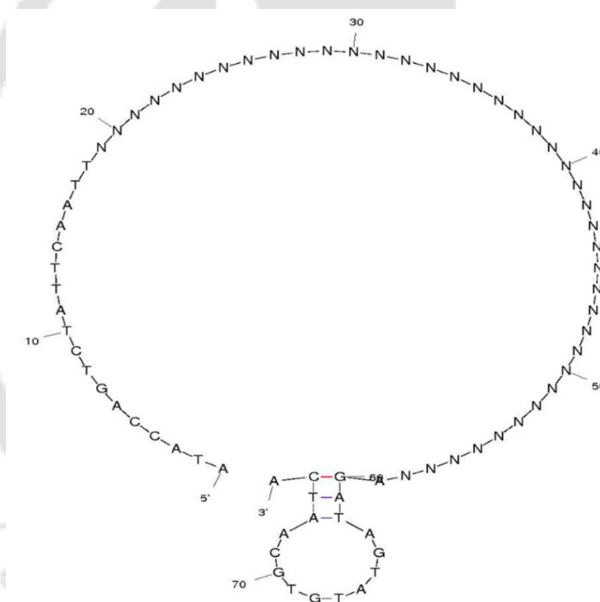

Figure 3.4: Potential secondary structure of aptamer library as predicted by Mfold software.

3.3.2 Optimization of PCR amplification of aptamer library

Initially the obtained aptamer library was PCR amplified for 30 rounds. On running on 2% agarose gel, in spite of distinct band of expected 76 bases, a smear was obtained (**Fig. 3.5 A**). The cycles of amplification were then reduced to 14. Further to prevent the

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formation of concatameric artefacts, 2% DMSO was added to PCR mix. A distinct band of 76 bases was obtained on 2% agarose gel (**Fig. 3.5 B**)

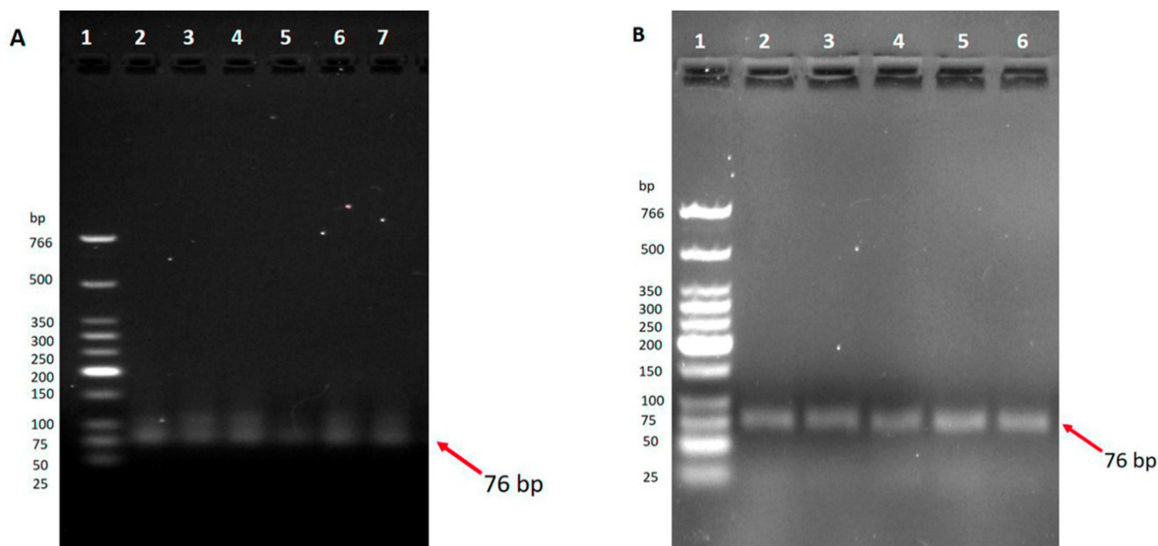

Figure 3.5: Optimization of PCR amplification of Aptamer Library.

3.3.3 *In vitro* selection of aptamers against Extra Cellular Domain of EGFR by SELEX

An ssDNA library with a random sequence of 40 nucleotide flanked by 18 bases was used as precursor pool for the selection of aptamers for EGFR. For the first round of selection, 500 pmol of ssDNA was used directly without PCR amplification. Before each round of selection, the nucleic acids were denatured and renatured to allow proper folding of the DNA into stable tertiary structures. At the end of every rounds, the eluted DNA were amplified by PCR. The reverse primer was synthesized with a Biotin group at 5′ end for ssDNA generation from dsDNA PCR product. After every PCR amplification of selected sequences, the double stranded product were converted into single stranded DNA by streptavidin-coated magnetic beads and alkaline denaturation (0.1 M NaOH).

The standard protein SELEX strategy was employed to select EGFR ECD specific aptamers. The aptamer library was subjected to sequential binding and elution with the recombinant proteins immobilized on NC membrane. The amount of protein immobilized were determined by Bradford protein assay by analysing the concentration

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of protein before and after immobilization (**Table 3.3**). To prevent the selection of nonspecific aptamers that binds to the NC membrane, a pre-selection i.e. Negative SELEX was performed before 1st, 9th, 12th and 14th rounds of SELEX. The Negative selection was carried out by incubation of the ssDNA pool with the NC membrane in the absence of target. The unbound ssDNA fraction was collected for the following SELEX rounds. The amount of protein used and selection parameters employed in each round of SELEX process is crucial for the successful screening of aptamers. After 5th round, the stringency of selection parameters were increased by reducing the amount of EGFR ECD protein, size of the NC membrane and increasing washing steps for selection of aptamers with high specificity and affinity as indicated in **Table 3.3**

Table 3.3: Selection parameters during SELEX rounds.

SELEX Round	Amount of Protein immobilized (nmol)	Reaction condition
1st	6.5	Size of NC membrane: 0.8cm^2 Incubation time: 1 hr Washing: 3 times, 2 min.
2nd	6.0	Size of NC membrane: 0.8cm^2 Incubation time: 1hr Washing: 3 times, 2 min.
3rd	5.5	
4th	2.5	Size of NC membrane: 0.8 cm^2 Incubation time: 1hr min Washing: 3 times, 2 min
5th	2.5	
6th	1.0	Size of NC membrane: 0.5 cm^2 Incubation time: 45 min Washing: 5 times, 5 min
7th	0.9	
8th	0.5	
9th &10th	0.25	Size of NC membrane: 0.4 cm^2 Incubation time: 30 min Washing: 5 times, 5 min
11th	0.25	Size of NC membrane: 0.3 cm^2

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		Incubation time: 20 min Washing: 5 times, 5 min
12th	0.015	Size of NC membrane: 0.3 cm^2 Incubation time: 15 min Washing: 5 times, 10 min
13th	0.015	
14th	0.005	Size of NC membrane: 0.3 cm^2 Incubation time: 5 min Washing: 5 times, 10 min

3.3.4 Monitoring enrichment of aptamer candidates during SELEX rounds

The enrichment of aptamer pool through SELEX rounds were monitored by 2% agarose gel electrophoresis (**Fig. 3.6**). On contrast with first selection round, by the end of 8^{th} round approx 25-30 fold increase in enrichment was attained. However after 8^{th} round, a sudden 13.4 fold reduction in enriched pool was observed. The reason could be the introduction of negative SELEX after 8^{th} round and reduction of incubation time to 30 mins which might led to the elimination of non-specific or weak binders. With the increase in SELEX rounds a steady increase in the intensity of bands were observed, indicating that the DNA sequences with better binding to EGFR ECD were being enriched. A plateau in the enriched pool was achieved by the end of 13^{th} SELEX rounds, representing a significant enrichment in the aptamer pool. Yet, an additional SELEX round was performed in highly stringent condition to generate a panel of aptamers binding to EGFR ECD with very high affinity.

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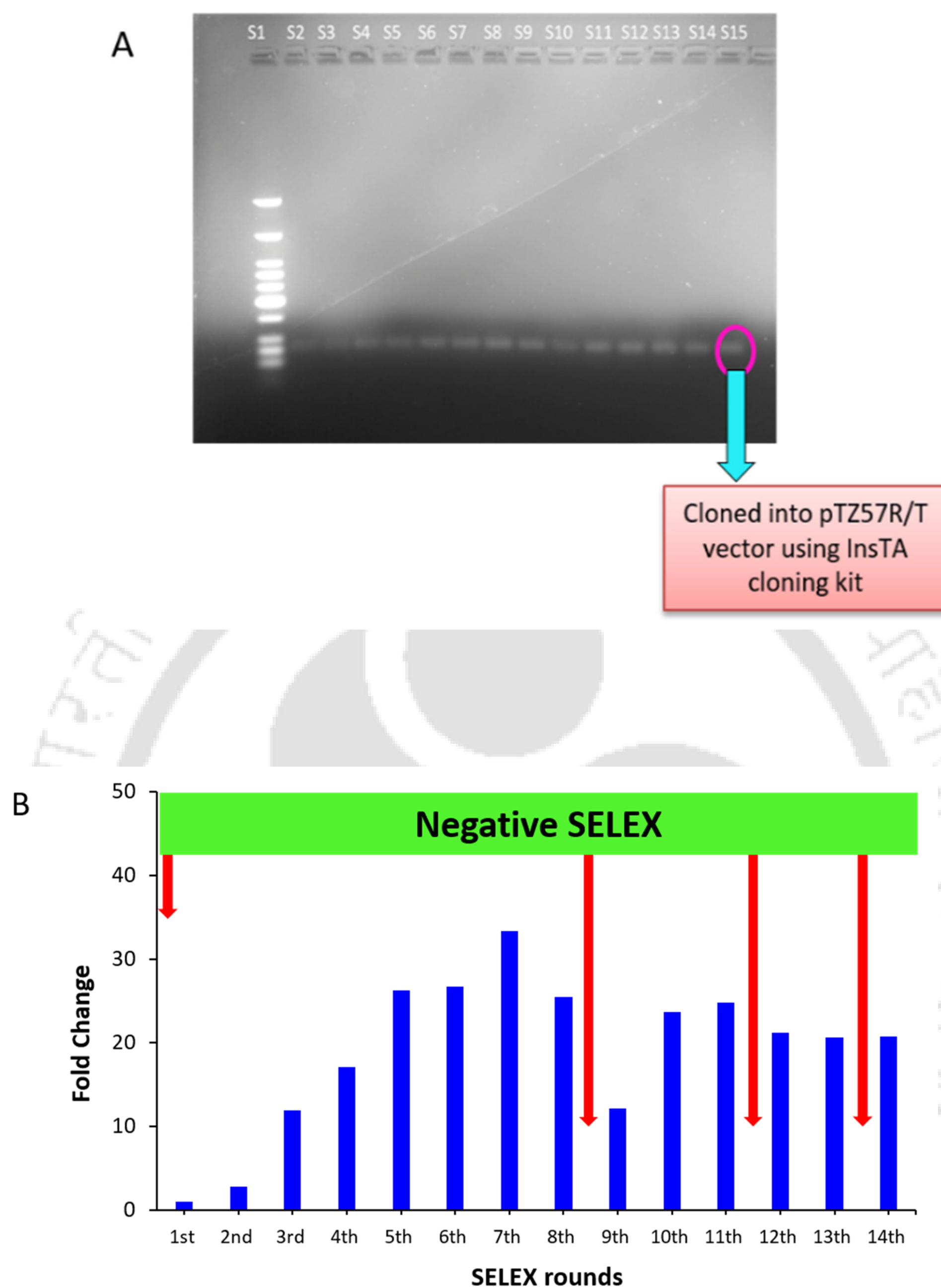

Figure 3.6: Monitoring Enrichment of aptamer candidates during SELEX rounds. (A) 2% Agarose gel depicting the enrichment of aptamer pool in different SELEX rounds. Lane S1: NEB Low Molecular weight Ladder, Lane S2-S15: SELEX rounds 1 to 14. (B) Graph depicting fold change in the enrichment of EGFR specific aptamer of each cycle.

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3.3.5 Cloning and Sequencing of EGFR specific enriched aptamer pool

The enriched aptamer candidates eluted after 14 iterative cycles of SELEX were amplified by PCR using unmodified Apt_F1 and Apt_R1. The PCR amplicons were then cloned into pTZ57R/T vector using InsTA cloning kit (Thermo Scientific). About 84 colonies were screened for the presence of selected candidate aptamers by colony PCR. Twenty positive clones containing candidate aptamers were sent for sequencing to Scigenome Pvt. Ltd, India.

3.3.6 Sequence Analysis of selected aptamer candidates

Sequence analysis of twenty clones provided sixteen independent aptamer sequences. The sequences were mainly grouped into five families based on the homology of DNA sequences of individual clones (**Fig. 3.7**). The sequence analysis exhibited higher frequency of EGFR_A1, EGFR_A7, EGFR_A15 and EGFR_A13 accounting 10% of all identified sequences. The remaining sixteen aptamers were orphan sequences. A closer inspection of the sequences of individual aptamers revealed short regions of sequence similarity. Consensus repeats like TTTTTT, GGGGG, TGTG, and CCCC were found in 25%, 20%, 50% and 35% of sequences respectively.

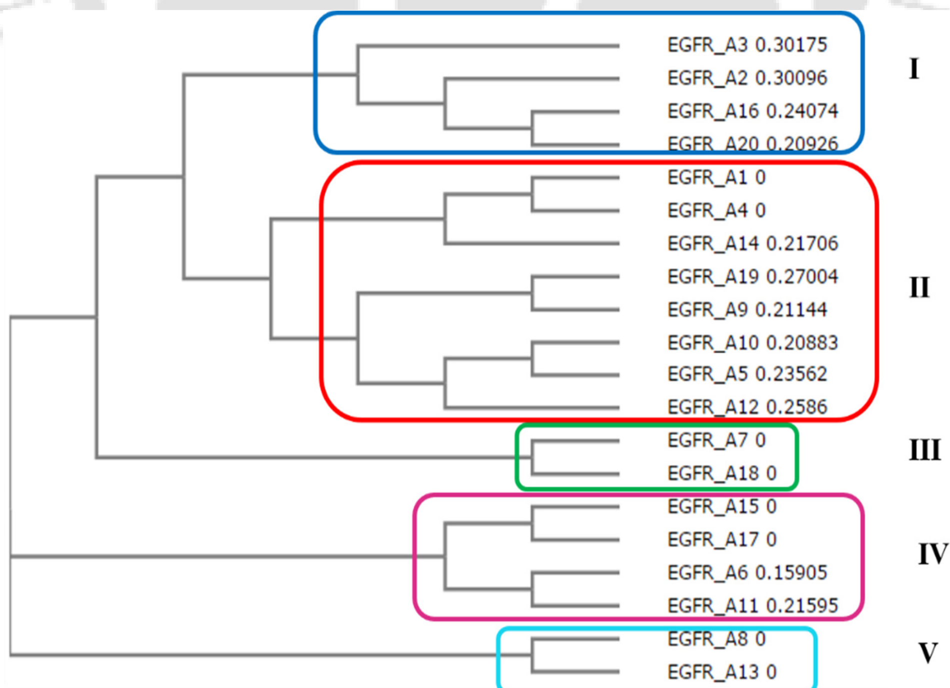

Figure 3.7: Similarity in the sequences of selected aptamer candidates.

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Table 3.4: Sequences of the aptamers candidates (EGFR_A1, EGFR_A7, EGFR_A13, EGFR_A15 and EGFR_A16) selected for characterization. Alphabet in lower case corresponds to the primer binding region.

Family	Name	Sequence	ΔG (Kcal/mol)	Abundance
I	EGFR_A13	ataccagtctattcaattGGGGGCGAAAGATCCAGTCCGTTTTTTTAGTTTTTACTGTagatagtatgtgcaatca	-3.72	10%
II	EGFR_A1	ataccagtctattcaattACCGGTTCGAGCATTTTTTTGGACTCCTTGATAGTATGTGagatagtatgtgcaatca	-2.53	10%
III	EGFR_A15	ataccagtctattcaattGGGGGGACGCGTACTTGTGTTATAGTATTACCTTTCCGGTAgatagtatgtgcaatca	-5.28	10%
IV	EGFR_A7	ataccagtctattcaattAGGTGTGTCAGAGCGGGTGTCCGAGGGAGATAGGGTCCTTagatagtatgtgcaatca	-6.28	10%
V	EGFR_A16	ataccagtctattcaattACCGCCTATATCCCACCCGATCAAACACTGTGCCCCCCGGagatagtatgtgcaatca	-2.22	5%

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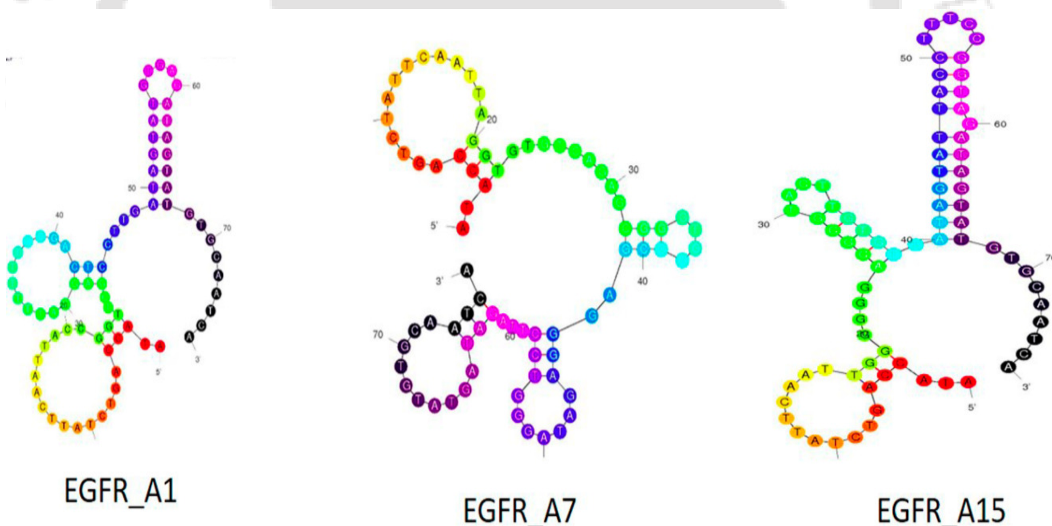
3.3.7 Secondary Structure Prediction of aptamer candidates

Secondary structures of the selected aptamers were predicted using Mfold software. Each sequence displayed different free energy values (dG) that reflects the stability of folded structures. The three-dimensional conformation of the aptamers were predominated by stem-loop or hairpin loop like structures. According to the proposed secondary structures, all the selected aptamers were found to contain some common secondary structural elements including a central bulky loop with a protruding either one loop (EGFR_A10, EGFR_A14, and EGFR_A20), two loop (EGFR_A2, EGFR_A5, EGFR_A9, EGFR_A11, EGFR_A12 and EGFR_A19), three loop (EGFR_A1, EGFR_A6 and EGFR_ A15) or four loop (EGFR_A3, EGFR_A7 and EGFR_A13) like structure. Surprisingly, only EGFR_A16 folds into different pattern and no common secondary structure elements could be assigned to this variant.



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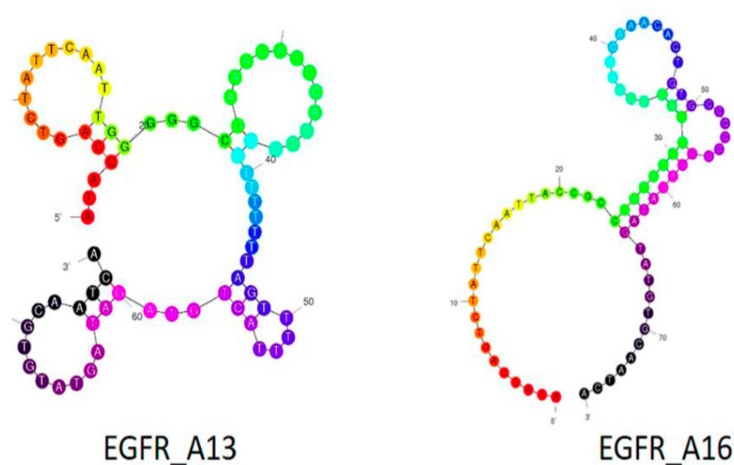

Figure 3.8: Potential secondary structure of aptamer candidates selected for study (EGFR_A1, EGFR_A7, EGFR_A15, EGFR_A13 and EGFR_A16) as predicted by Mfold software.

After sequence analysis, homologous analysis and structure prediction, five aptamer sequences representing each homologous family viz EGFR_A1, EGFR_A7, EGFR_A15, EGFR_A13, EGFR_A16 were synthesized with either FAM or Biotin labelling at 5' end to study their binding interaction with EGFR. The binding abilities of the selected sequences to target were evaluated either by flow cytometry or Enzyme Linked Aptamer assay (ELAA). Initially, the affinity of three aptamers viz: EGFR_A1, EGFR_A7 and EGGR_A15 representing family II, III and IV were studied by FACS and ICC. Later on the binding of remaining two sequences EGFR_A13 & EGFR_A16 alongwith EGFR_A15 were studied by Enzyme linked aptamer assay.

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3.3.8 Binding Assay of selected aptamers with six EGFR expressing cancer cell lines by Flow Cytometry

To investigate the selectivity and affinity of aptamer candidates, the binding assays of the selected sequences were evaluated by flow cytometry. Firstly, the most abundant three aptamer candidates EGFR_A1, EGFR_A7 and EGFR_A15 were synthesized with FAM labelling at 5′ end. The untreated cell was used as background fluorescence signal. A shift in the peak of the histogram to a higher fluorescence intensity was an indication of fluorescence intensity of the cell as a result of the binding of FAM labelled DNA sequence to the target on cell surface (**Fig. 3.9**). Six cancerous cell lines expressing different levels of EGFR protein were selected for binding studies. The cell lines used in the study are A431 (human squamous carcinoma), A549 (human lung carcinoma), MDA MB 231 (human triple negative breast cancer), U87-MG (Human glioblastoma), HeLa (Human cervical adenocarcinoma) and Jurkat Cell line (T cell lymphoma). All three aptamers were found to bind with A431 with very high affinity as compared to other cancer cell lines due to its abnormally high expression of EGFR. As expected, none of the aptamers binds to Jurkat cell line which do not expresses EGFR at all. As shown in **Fig. 3.9 B**, the observed mean fluorescence intensity of EGFR_A15 bound to A431, A549, U87 MG, MDA MB231 and HeLa cell line as compared to untreated cells was 68.16, 53.26, 45.12, 13.19, and 9.37 respectively. Whereas the observed mean fluorescence intensity of EGFR_A7 and EGFR_A1 bound to A431, A549, U87 MG, MDA MB231 and HeLa cell line compared to untreated cell was 6.59, 6.72, 6.13, 5.52, 6.15 and 5.69, 4.05,1.48, 1.42, 0.69 respectively. It was evident that among the three aptamer candidate, EGFR_A15 was binding to all EGFR expressing cancerous cell lines with very high affinity.

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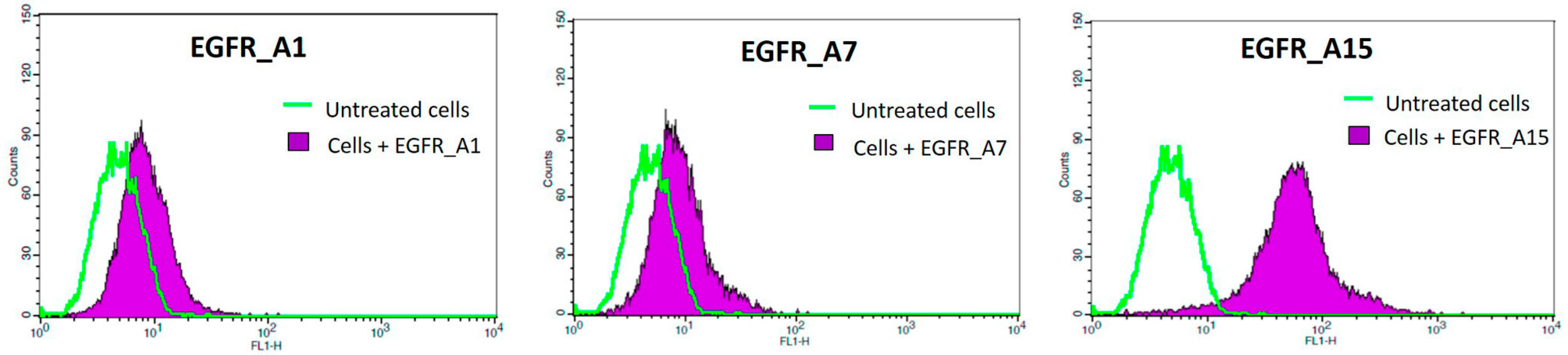
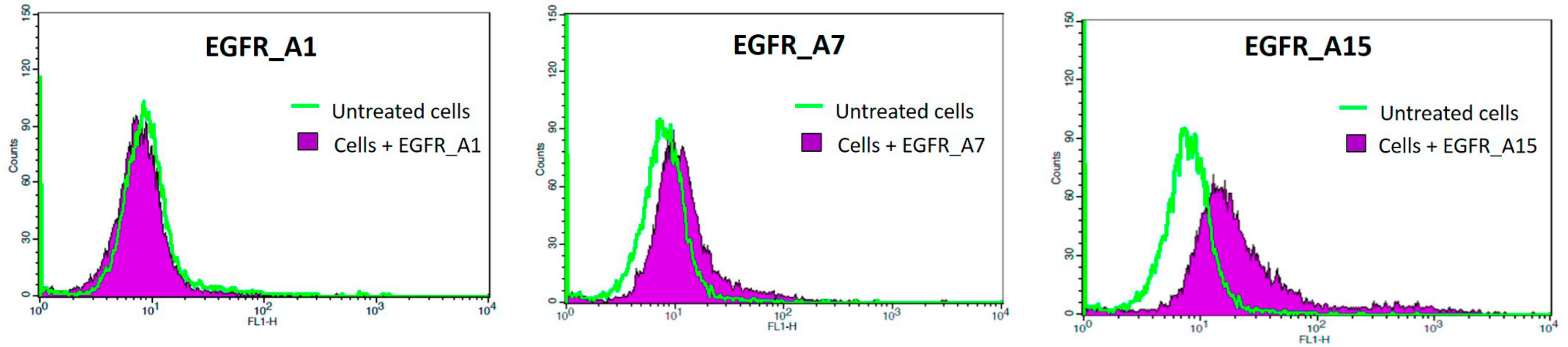
A.

Histogram Overlay Analysis of A431 Cell Line



Histogram Overlay Analysis of A549 Cell Line



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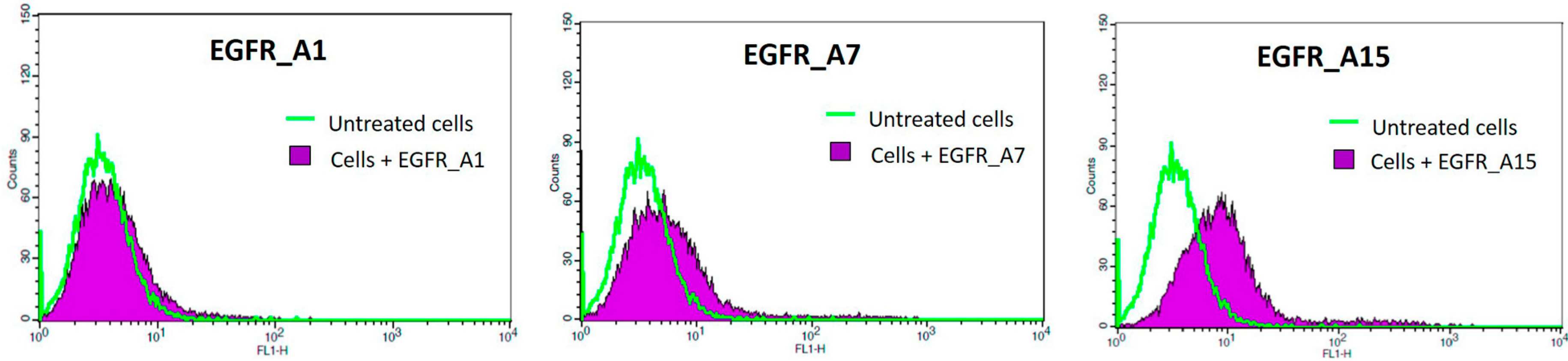
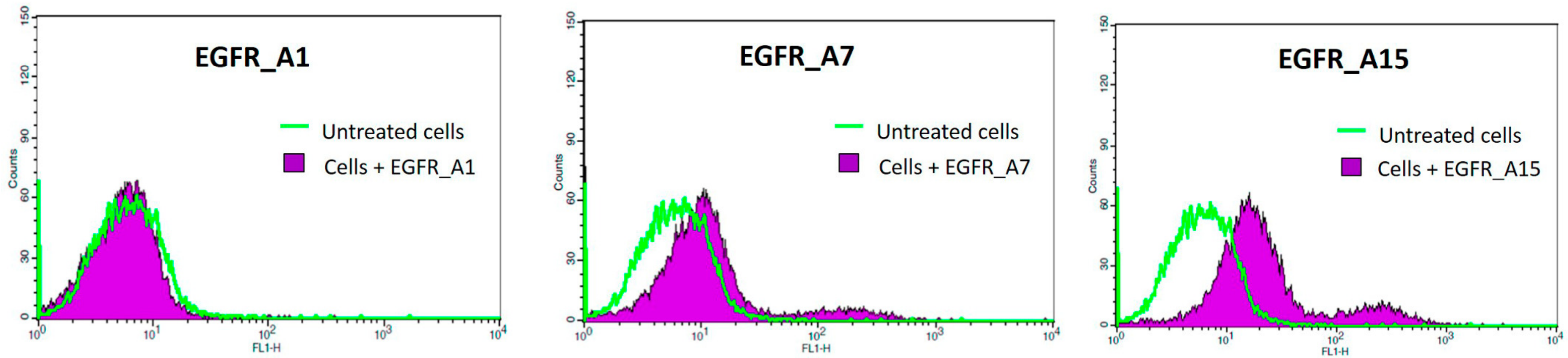
Histogram Overlay Analysis of MDA MB 231 Cell Line



Histogram Overlay Analysis of U87-MG Cell Line



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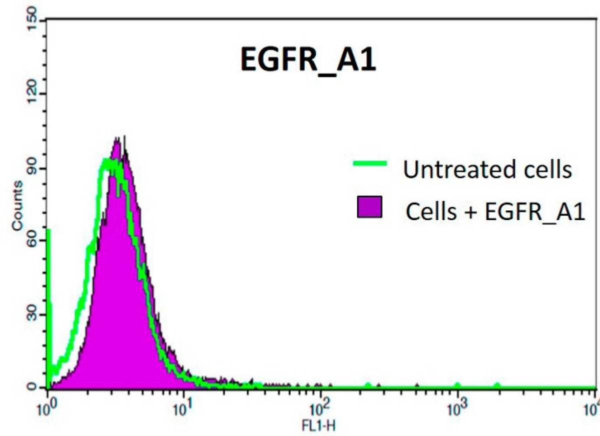
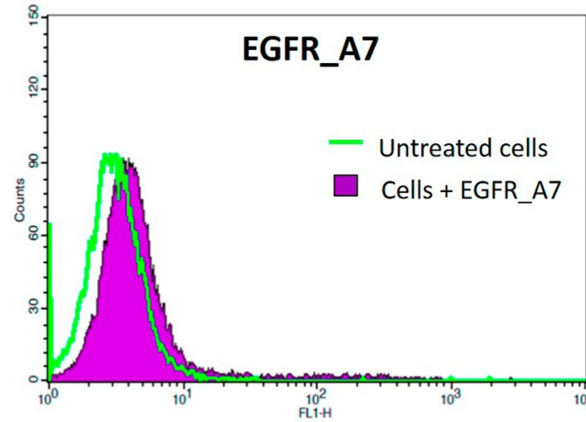
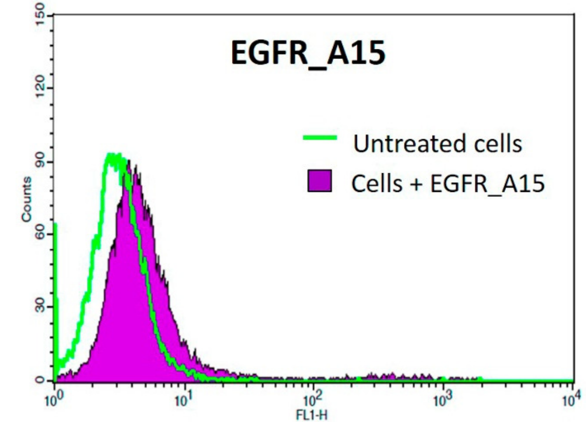
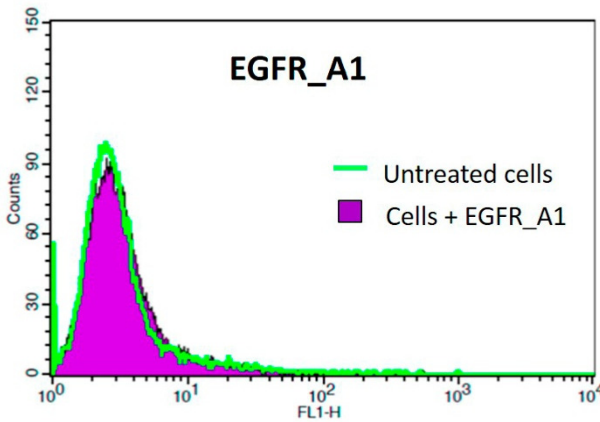
Histogram Overlay Analysis of HeLa Cell Line

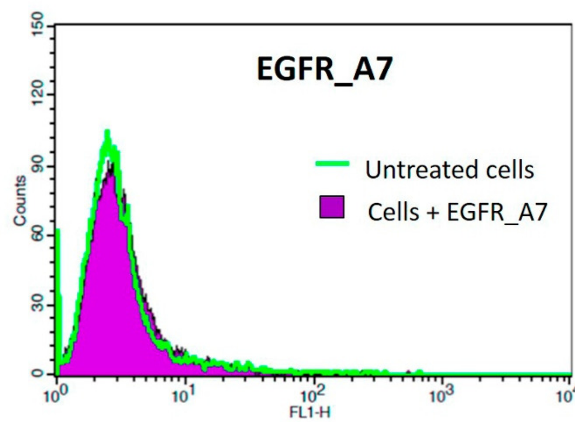


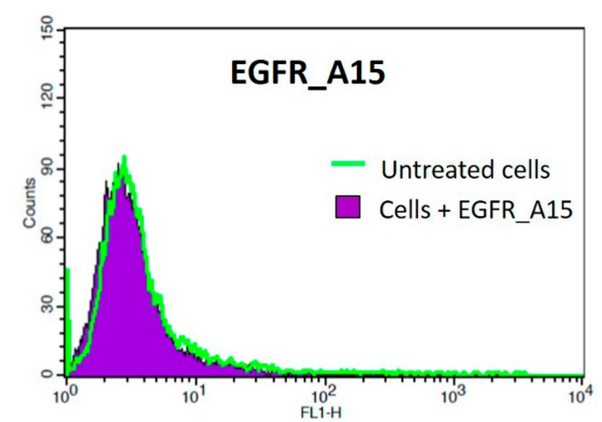




Histogram Overlay Analysis of Jurkat Cell Line







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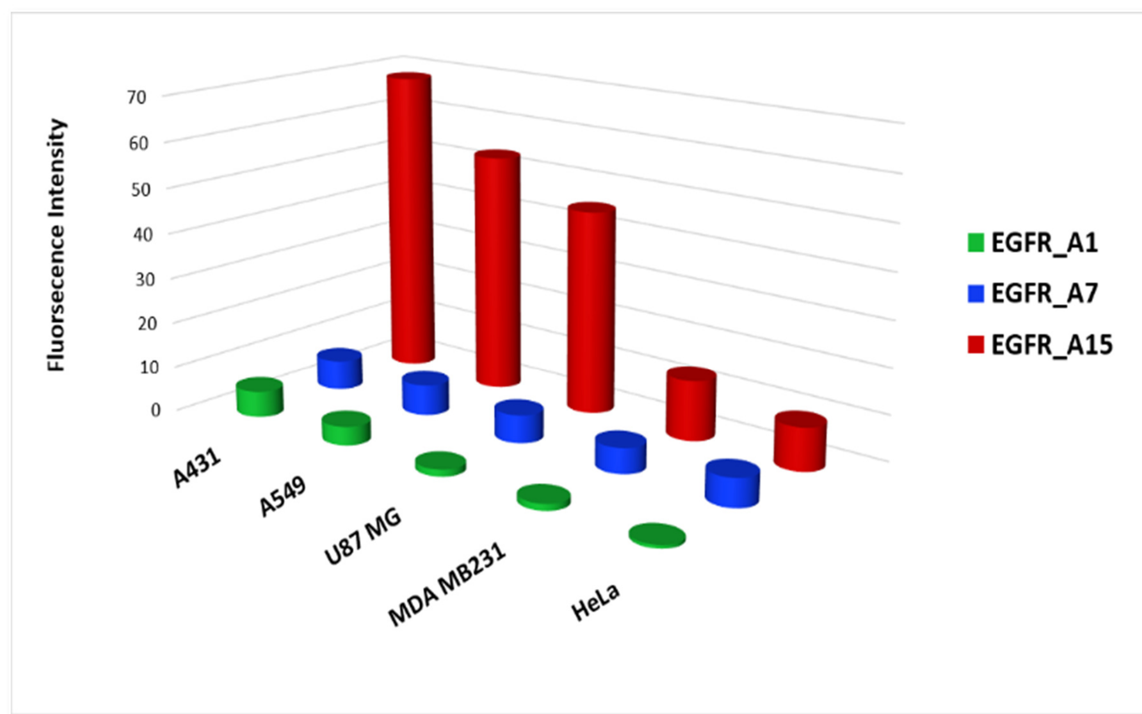

Figure 3.9: (A) Flow cytometry binding analysis of EGFR_A1, EGFR_A7 and EGFR_A15 aptamers with various EGFR expressing human cell lines: A431, A549, MDA MB 231, U87 MG, HeLa and Jurkat cell line.

(B) Pictorial representation of the binding affinity of EGFR_A1, EGFR_A7 & EGFR_A15 aptamer with EGFR expressing cell lines. The fluorescence intensity of untreated cell was set as background fluorescence signal. The fluorescence signal of the individual aptamers was then subtracted from background fluorescence and used in pictorial representation as shown.

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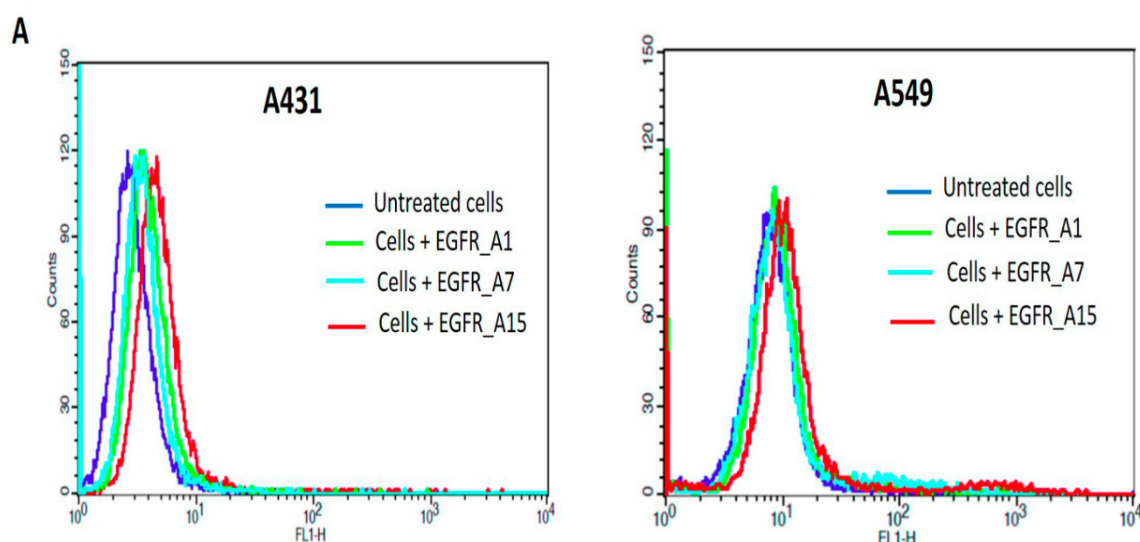
3.3.9 Verification of cell surface binding of the selected aptamer on target cell by flow cytometry.

EGFRs are receptor proteins which are expressed on the cell membrane. Thus, it was obvious for us to assume that the selected EGFR specific aptamers will bind to the cell surface. In order to determine the possible binding site of aptamers on the target cells, fluorescence binding assay were conducted after treating cells with trypsin. In this assay cells were treated with trypsin for 5 min at 37°C. The cells were quickly washed twice by centrifugation and then incubated with the FAM labelled aptamers. The fluorescence signal of untreated cells were used as background signal. **Fig. 3.10** shows the effect of trypsin on aptamer binding. It was observed that the fluorescence signals of aptamer treated cells were reduced to the background. This indicates that the binding entities of these aptamers were susceptible to trypsin cleavage, therefore the binding of aptamers with the EGFR expressing cell lines were lost. However, in case of A431 cell lines, the fluorescence signal of EGFR_A15 was reduced only marginally. This can be attributed to the abnormally high expression of EGFR on this cell line. Further, the incubation time of trypsin treatment of A431 cell lines were increased to 10 -15 mins and analysed by flow cytometry as described above. The histogram profile of trypsin treated A431 cells were compared with the cells dissociated non-enzymatically. The fluorescence intensity of EGFR_A15 were almost completely abolished by 15 min of trypsin digestion (**Fig. 3.11**) Thus the, above results clearly suggests that the binding target of aptamers were cells surface protein i.e. EGFR.



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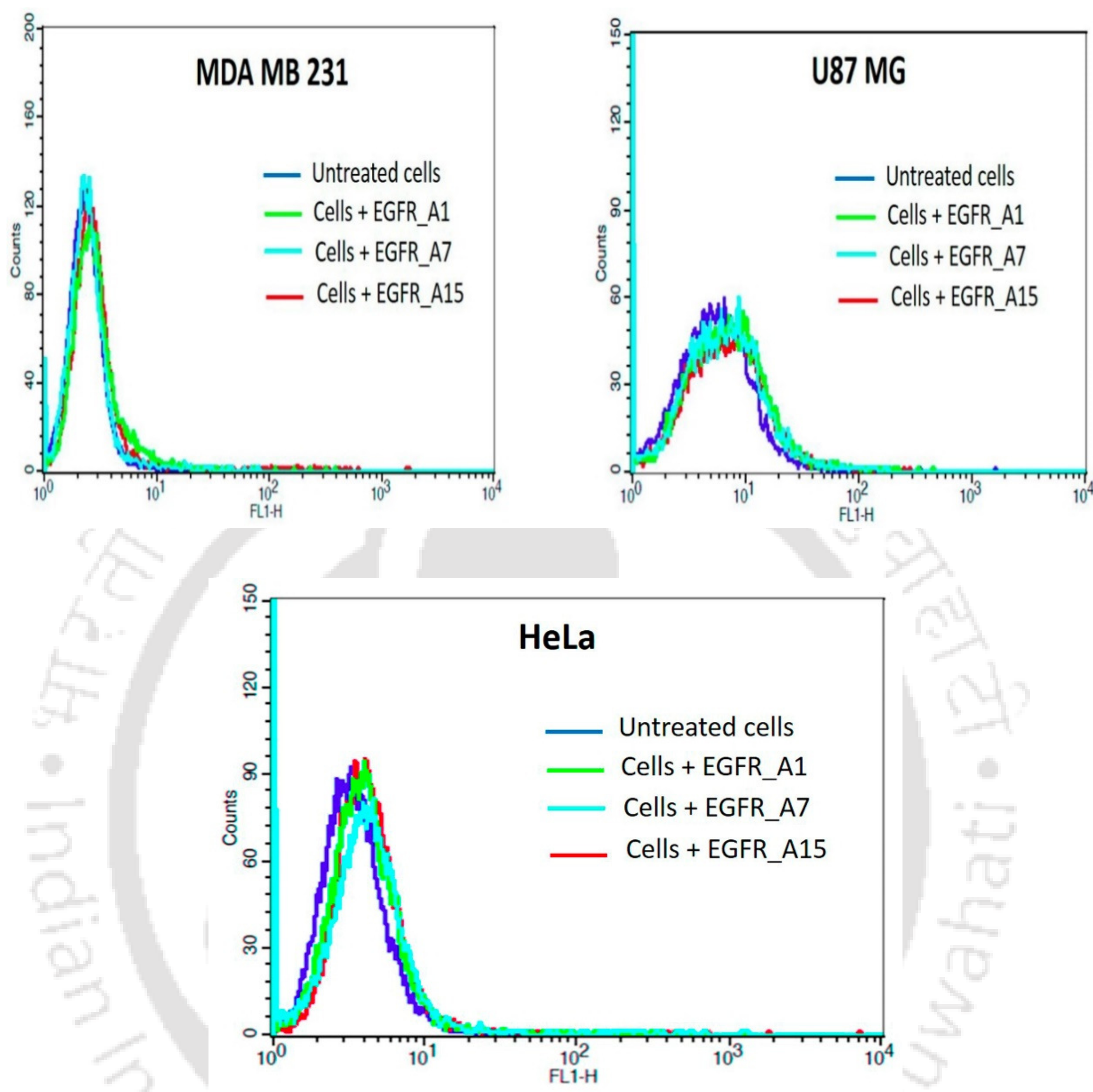

Figure 3.10: The cell surface binding of selected aptamers EGFR_A1, EGFR_A7 and EGFR_A15 to trypsin treated A431, A549, MDA MB 231, U87MG and HeLa cells. [Aptamer]=200nM.

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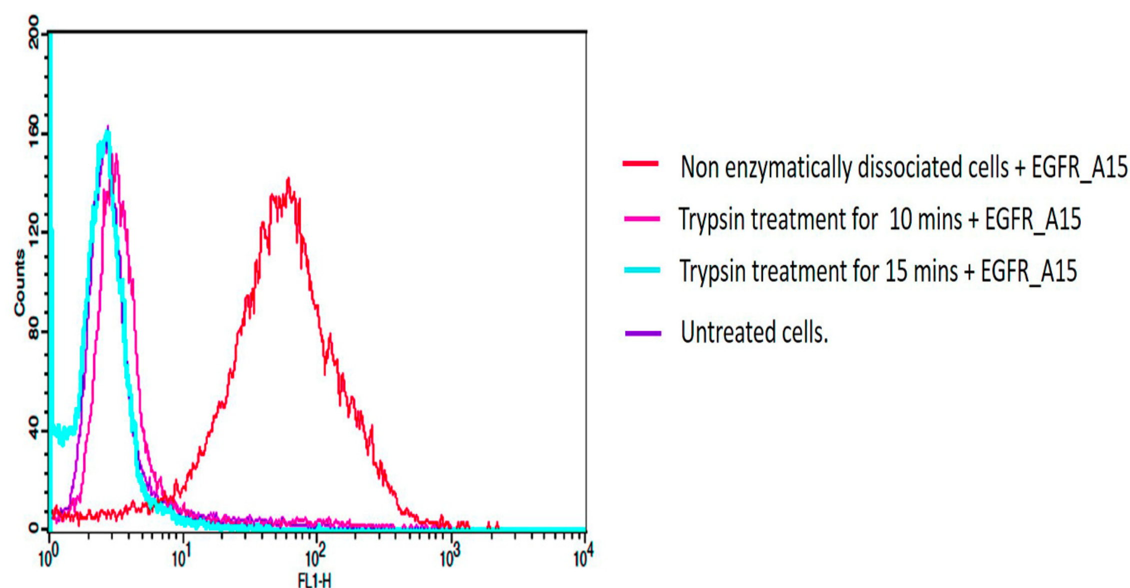

Figure 3.11: Binding of EGFR_A15 aptamer to trypsin treated A431 cell lines. The binding of EGFR_A15 was completely abolished when incubation time of trypsin treatment was increased to 15 mins.

3.3.10 Fluorescence imaging of the selected aptamers bound to the cancer cell line

The selective cell recognition property of aptamers was further established by fluorescence microscopy using FAM labelled EGFR_A1, EGFR_A7 and EGFR_A15 with A431 and MDA MB 231 cell lines. It was one step method of imaging in which cells were fixed on a tissue slide and treated with FAM labelled aptamers and random sequences in dark for 1 hour and then analysed on a fluorescence microscope. As depicted in **Fig. 3.12** bright fluorescence was observed on the periphery of cells after incubation with EGFR_A15. It was evident from the microscopic images that the aptamer EGFR_A15 was localized at the cell surface/membrane. However, the fluorescence of EGFR_A1 and EGFR_A7 appeared to be localized within the nuclei of cells. No fluorescence was observed in the Random sequence (FAM labelled initial aptamer pool) treated cells.

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The initial flow cytometry data correlated with fluorescence microscopy imaging. The aptamer which had highest signal intensity (EGFR_A15) by flow cytometry also produced brighter fluorescence signal in fluorescence microscopy. Among the three selected aptamers viz EGFR-A1, EGFR_A7 and EGFR_A15, EGFR_A15 had the strongest binding and thus it was chosen for the further studies. We were curious to study the binding affinity of remaining two homologous family. Thus two more aptamers i.e. EGFR_A16 and EGFR_A13 were selected from group I and V respectively. All three aptamers viz EGFR_A15, EGFR_A13 and EGFR_A16 were synthesized with Biotin modification at 5' end to suit our further research.

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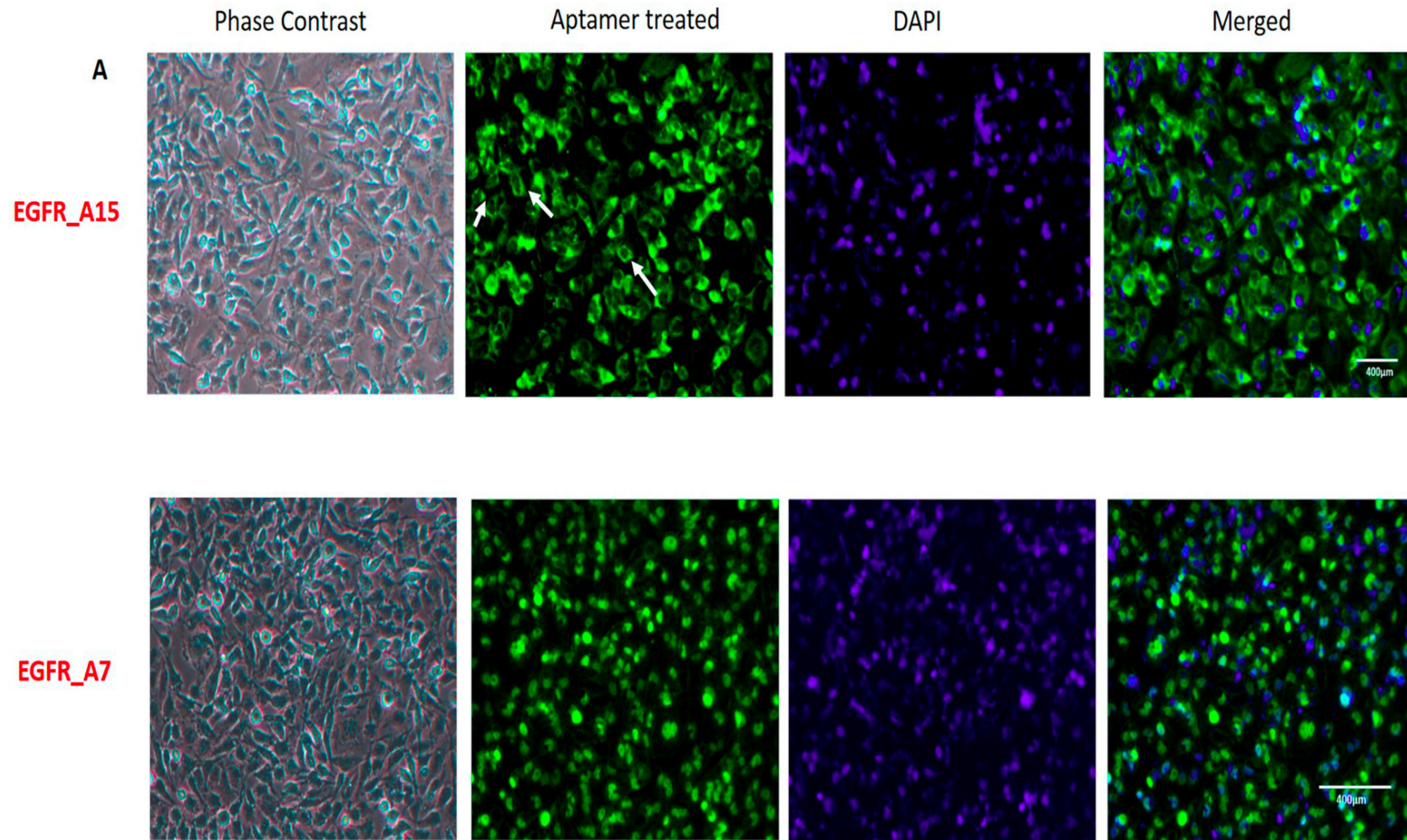

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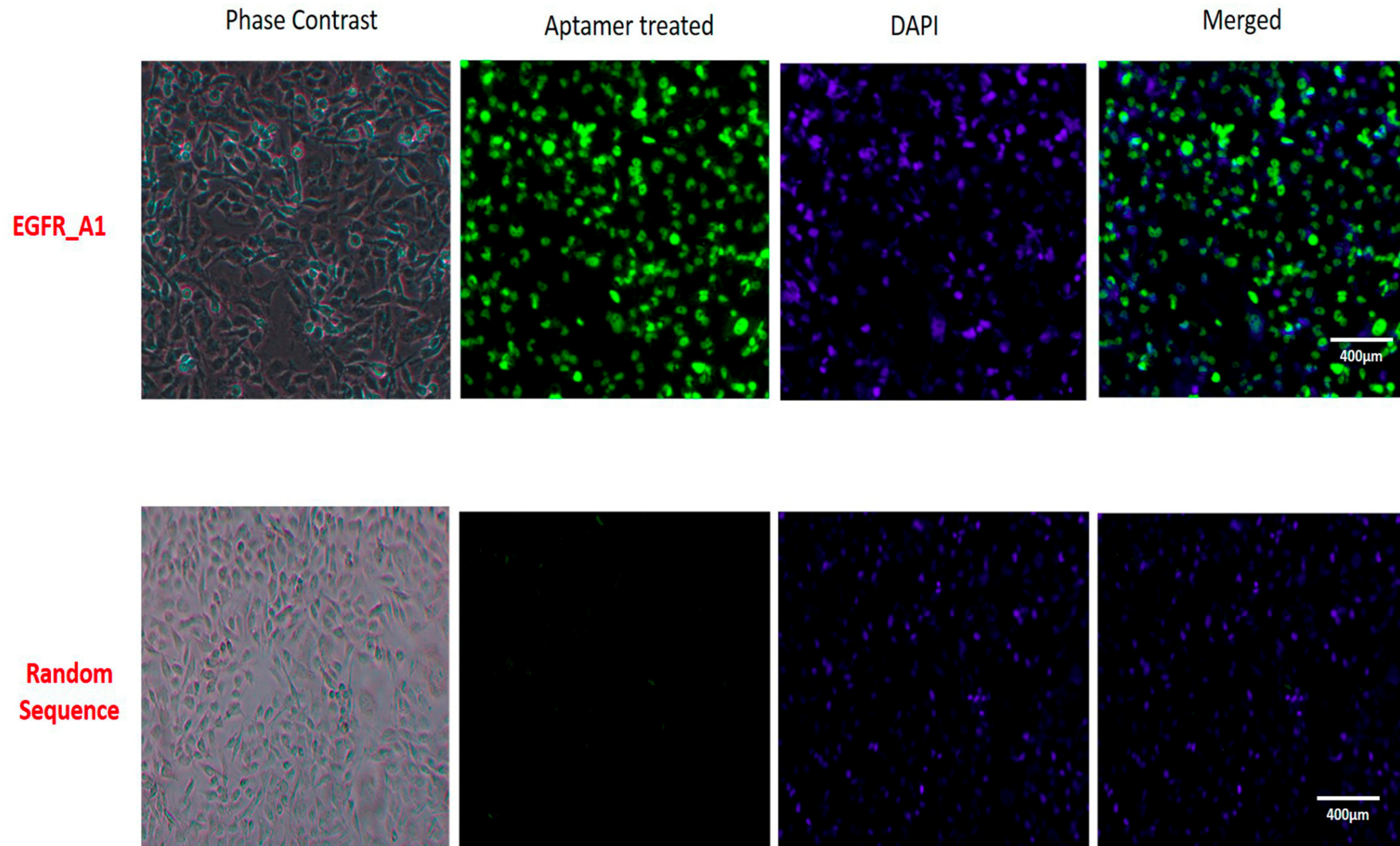

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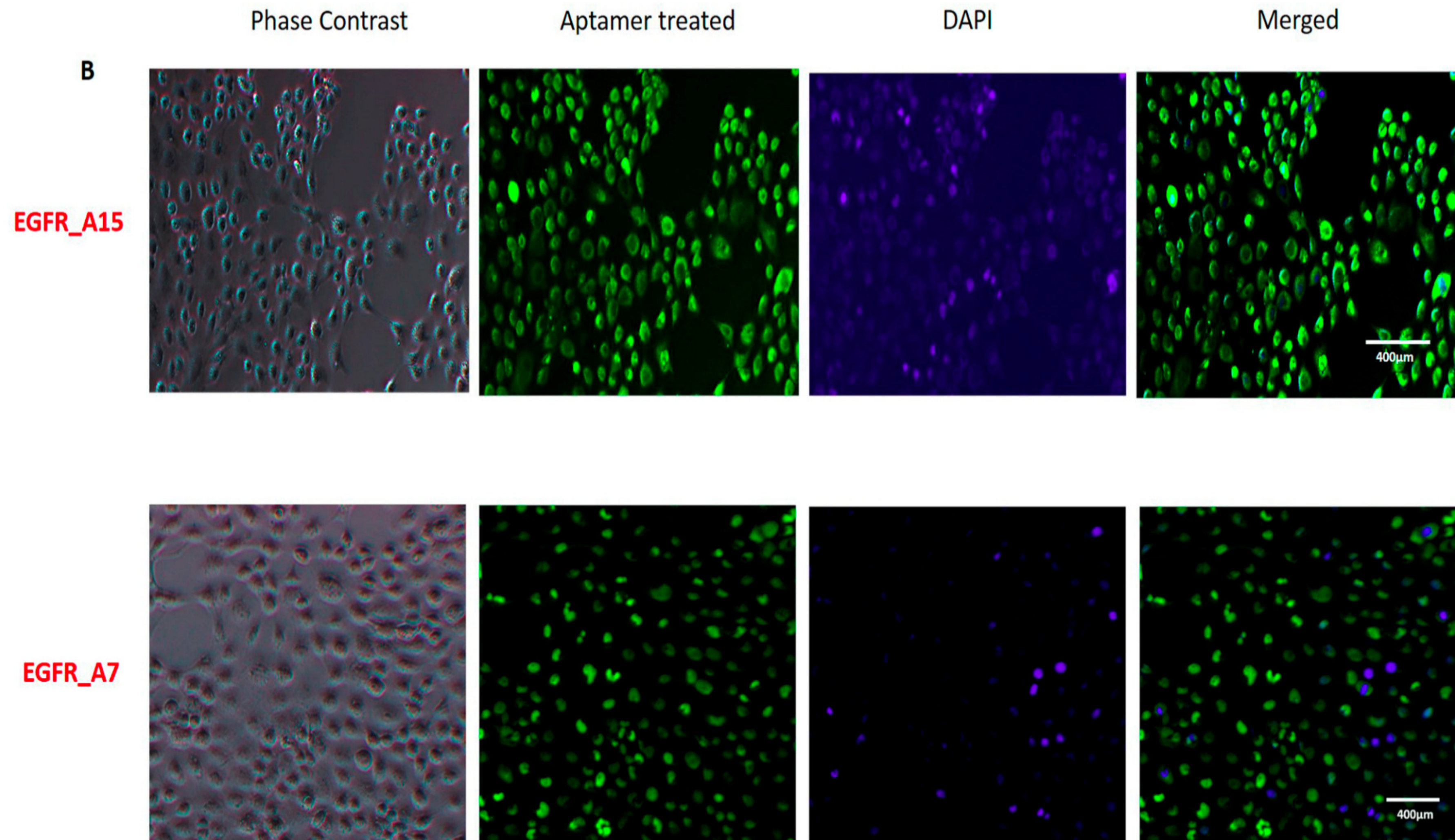

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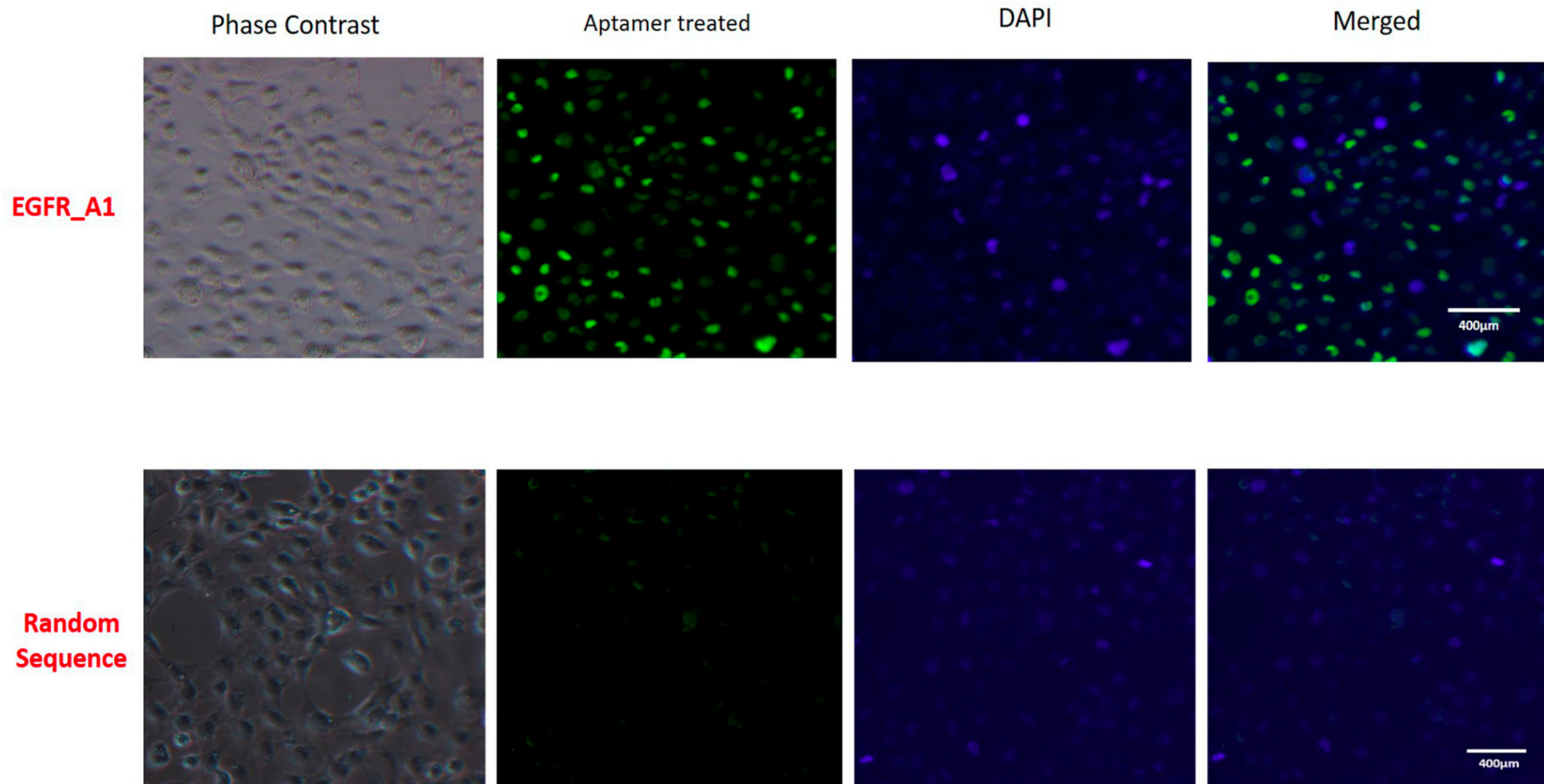

Figure 3.12: The fluorescence imaging of aptamer EGFR_A15, EGFR_A7, EGFR_A1 and Random Sequence (FAM labelled initial non enriched aptamer pool) bound to (A) MDA MB231 cells. (B) A431 cells.

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3.3.11 Determination of Dissociation constant of the selected aptamers

Initially Isothermal calorimetry (ITC) was tried to determine the dissociation constant of selected aptamers. The calorimetry experiments was performed at 25°C using a MicroCal VP-ITC (Micro Cal Inc., Northampton, MA, USA). Recombinant EGFR ECD protein and aptamers were prepared in aptamer binding buffer (ABB). The 300µL of 1.5µM EGFR ECD protein was placed in the sample cell holder. Typically, 30 serial injections (1.5µL per one injection) of 10 µM aptamer candidate at spacing of 120 sec were made with continuous stirring of the solution (at 210 rpm) in the sample cell. Each injection generated a heat-burst curve (µcal/sec) versus time (min). But no convincing results were obtained by this study

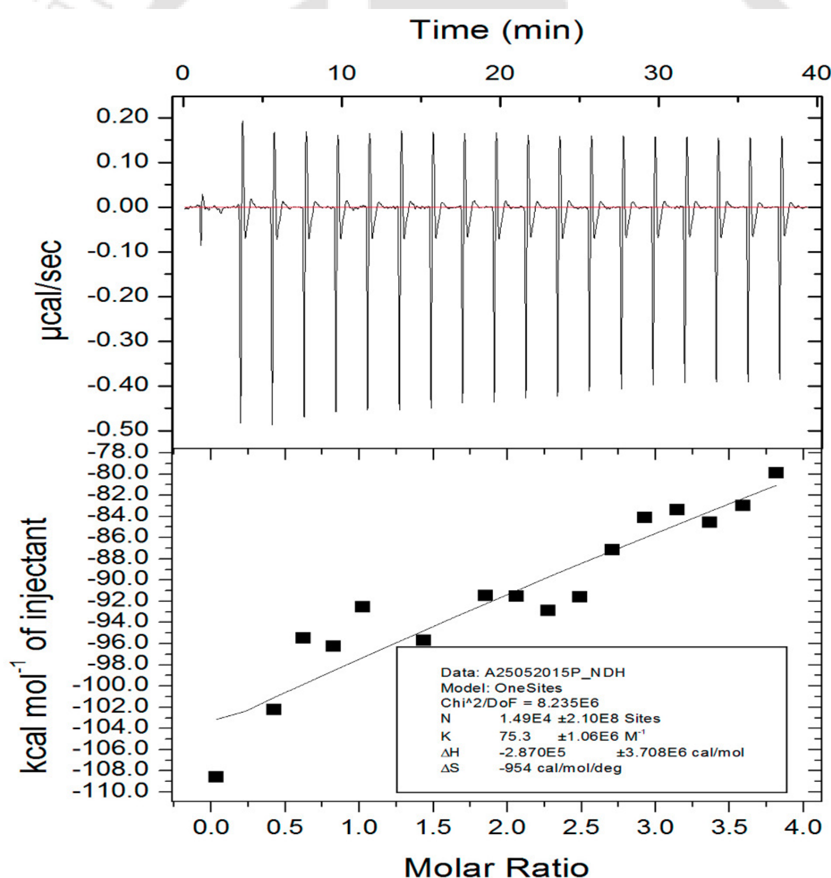

Figure 3.13: Binding study of EGFR_A15 with recombinant EGFR ECD protein by ITC. Isotherms were fitted to a one-site independent binding model.

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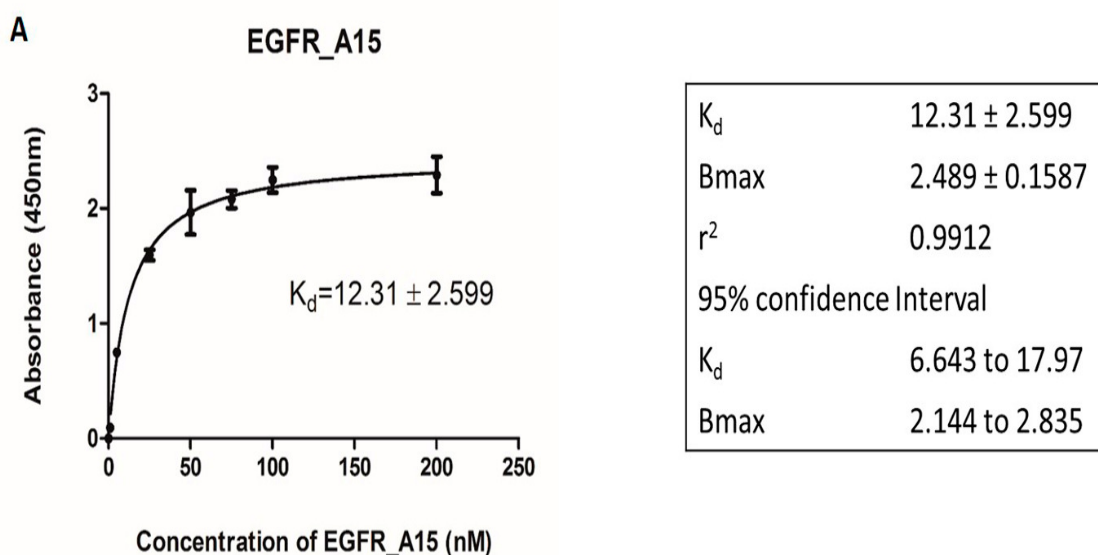
Thus an alternative method reported by Li *et al.*with slight modifications was employed to calculate the dissociation constant of selected aptamers *viz* EGFR_A15, EGFR_A13 and EGFR_A16 (Fu *et al*., 2014; Li *et al*., 2011). Binding assays were conducted by incubating variable concentration of biotinylated EGFR_A15, EGFR_A13 and EGFR_A16 aptamers with a constant concentration of EGFR ECD protein (500ng) coated on a microwell plate. Streptavidin HRP was added and the reaction was colorimetrically detected using TMB substrate. The binding datas were studied by non-linear regression analysis using GraphPad Prism 5.0. The dissociation constant of two aptamers EGFR_A15 and EGFR_A13 were in nanomolar range. Aptamer EGFR_A16 do not show any binding with the target. The K_d values of EGFR_A15 and EGFR_A13 were 12.31 ± 2.59 nM and 15.59 ± 6.02nM respectively (**Fig.3.14**). From the above experiment it was evident that EGFR_A15 is a strong binder as compared to all other selected aptamer candidates.



K_d	12.31 ± 2.599
Bmax	2.489 ± 0.1587
r^2	0.9912
95% confidence Interval	
K_d	6.643 to 17.97
Bmax	2.144 to 2.835

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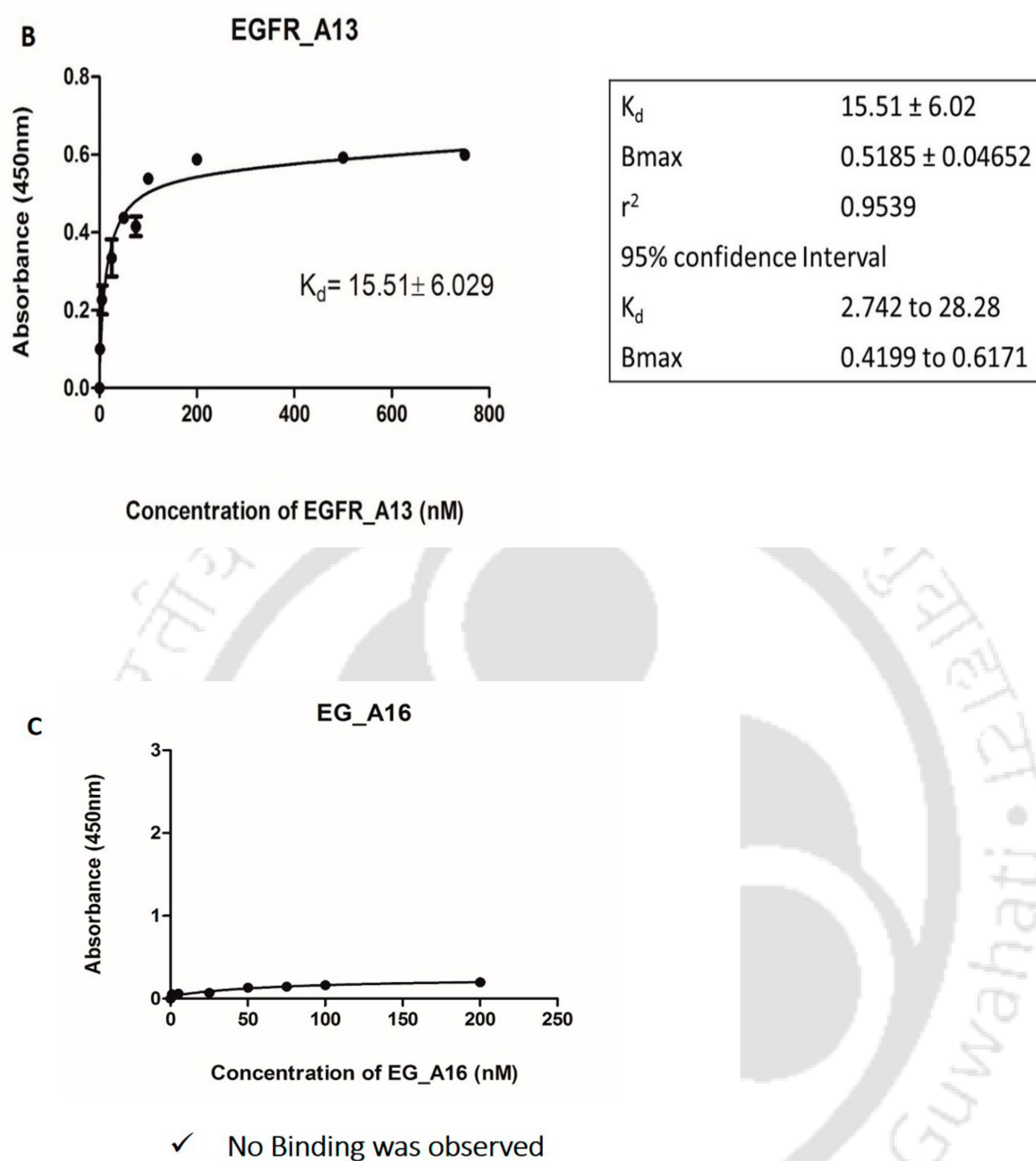

K_d	15.51 ± 6.02
Bmax	0.5185 ± 0.04652
r^2	0.9539
95% confidence Interval	
K_d	2.742 to 28.28
Bmax	0.4199 to 0.6171

Figure 3.14: Dose-dependent i-ELAA to determine the dissociation constant (K_D) of aptamers (A) EGFR_A15, (B) EGFR_A13 and (C) EGFR_A16. The fit curves were drawn by the GraphPad Prism 5.0 using various concentrations of aptamers as the binding ligands. Aptamer EGFR_A16 do not show any binding with the EGFR ECD protein.

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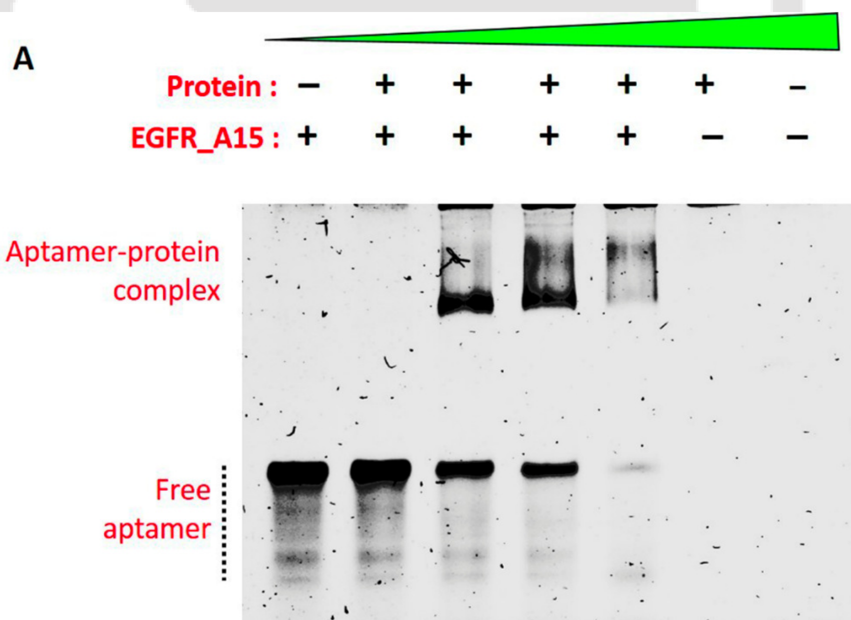
3.3.12 Electrophoretic Mobility Shift Assay (EMSA) of selected aptamer with recombinant EGFR ECD protein

Based on all the earlier studies, EGFR_A15 was found to have the best binding affinity as compared to other selected aptamers (EGFR_A1, EGFR_A7, EGFR_A13 and EGFR_A16). Further binding affinity of EGFR_A15 with recombinant EGFR ECD protein was also evaluated by EMSA study. A fixed concentration of EGFR_A15 was incubated with the increasing concentration of EGFR ECD protein and then the aptamer-protein complex and free aptamers were resolved in nondenaturing PAGE. The slower electrophoretic mobility of aptamer-protein complex as compared to free/non bound aptamer, confirmed the formation of Aptamer-ECD complex (**Fig. 3.15**). A considerable decrease in free/non bound aptamer concentration (upto 93.6%) was observed with the increasing concentration of protein. At the highest concentration of protein, 20µM EGFR ECD, a very faint band of free aptamer was visible.



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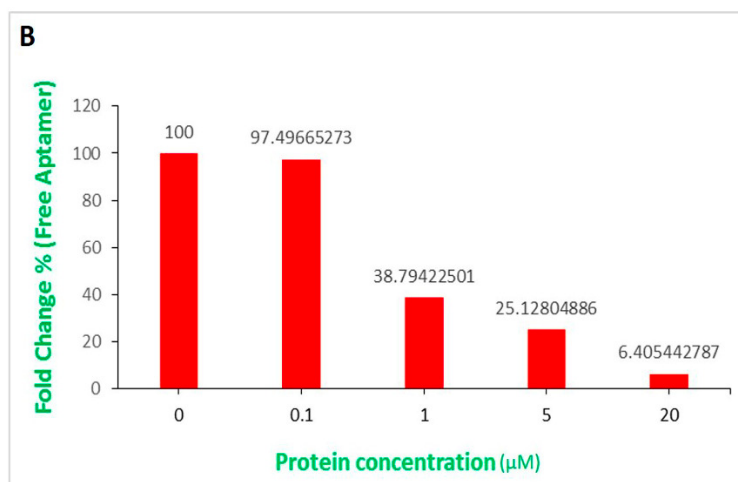

Figure 3.15: (A) Electrophoretic mobility shift assay (EMSA) of the EGFR_A15 with purified EGFR ECD protein. FAM labeled EGFR_A15 aptamer was incubated with purified proteins and resolved on non-denaturing 9% PAGE gel. The relative migration of aptamer-protein complexes and the non-bound Aptamer (Free EGFR_A15) was labelled. The dotted line on the left indicates the positions of the unbound aptamer.

(B) Graph depicting fold change in the concentration of free aptamer. The band intensity of free aptamer was measured by BioRad Image Lab 4.0 software and plotted against the concentration of protein used in EMSA assay.

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3.4 Discussions

Cancer cells typically display molecular signatures on their surface which have been exploited more recently as targets for aptamers to chaperone therapeutic cargos into cells (Orava *et al*., 2010). EGFR is one of such target which are overexpressed in most of the cancer and other diseases. Currently the diagnostic and treatment strategy for EGFR related cancer mainly rely on anti EGFR antibodies (Cetuximab, panitumumab) or small molecules which are tyrosine kinase inhibitors (gefitinib, erlotinib and lapatinib). In the present study, conventional protein SELEX strategy was employed for the selection of EGFR specific aptamers. The extra cellular domain of EGFR protein was expressed and purified from bacterial system by affinity chromatography as described in chapter 2. The recombinant EGFR ECD protein immobilized on NC membrane was used as a matrix for the selection of EGFR specific aptamers.

Success of an aptamer selection is highly dependent on the design of library. In any SELEX experiment, the *in vitro* selection conditions and aptamer library should be optimized according to the target protein properties and the potential application of selected aptamers. One of the important aspects for oligonucleotide library design is the length of the central random region as this length has a role in determining the initial abundance and likely complexity of selected structure (Knight *et al*., 2004; Knight and Yarus, 2003). The complexity of the selected structures such as hairpins, bulges within helices, pseudoknots, and G-quartets etc are associated with the affinity of aptamers to its target. Longer random region gives the library a greater structure complexity, which is particularly important for targets which are not known to be associated with or to bind to nucleic acids. Therefore, a longer random sequence pool may provide better opportunities for the identification of aptamers (Marshall and Ellington, 2000). However, it was shown in several SELEX experiments that the efficiency of aptamer selection decreased, when the random region was longer than 70 nucleotides (Legiewicz *et al*., 2005). Thus, in this study, an ssDNA library containing a 40 nucleotide long central random region was used for the in vitro selection of aptamers against EGFR.

Another important factor governing the SELEX procedure were the use of either DNA or RNA libraries. The previous studies revealed that there were no difference between

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RNA and DNA aptamers in terms of affinity and specificity to its target (Gold *et al.*, 1995). However, DNA aptamers have certain advantages over RNA aptamers. The SELEX procedure for DNA aptamers are much faster and easier than that for RNA aptamers. Unfortunately, RNA aptamers are prone to nuclease degradation, which limits its application in clinical research unless expensive modification, for example, with 2′-F-pyrimidine, is used. DNA aptamers exhibit much higher stability in a broad range of conditions including biological fluids, which makes them more suitable for clinical applications (Y. L. Song *et al.*, 2013). Moreover DNA aptamers are cheaper. Therefore, an ssDNA library was chosen instead of a RNA library in this work. Many researchers have reported the use of DNA aptamers in their study (Ara *et al.*, 2012; Boiziau *et al.*, 1999; Bruno *et al.*, 2012; Eissa *et al.*, 2013; Sefah *et al.*, 2010; Shangguan *et al.*, 2006; Y. Song *et al.*, 2013; Xiong *et al.*, 2013; Zhang *et al.*, 2014; Zhang, 2011).

Initially for the first round of selection 500 pmol aptamer library was used. For the selection of EGFR specific aptamers, the library were incubated with the immobilised EGFR ECD and then the bounded aptamers were eluted. The eluted ssDNA were PCR amplified and separated into single stranded form for next rounds of selection. In between, Negative SELEX was introduced to remove any non-specific binders. After fifth SELEX round, stringency was increased to select only high affinity aptamers. The enrichment during selection was monitored by agarose gel electrophoresis and by the end of 14 round, an overall 20.8 fold enrichment was achieved. Finally after 18 iterative cycles of SELEX including 14 rounds of protein SELEX and 4 rounds of Negative SELEX, a panel of DNA aptamers were selected.

The variety of structural elements of DNA allows it to bind to its targets and accomplish its many functions. The potential secondary structure of selected aptamer candidates were predicted using Mfold software. All the aptamers were found to share a common stem loop or hairpin like structure as predicted by Mfold. The alignment of the selected aptamers do not exhibit any conserved motifs but a short consensus repeats like TTTTTT, GGGGG, TGTG and CCCC were observed. Based on the sequence similarities, aptamers were grouped into five homologous families. The abundant aptamer sequences or aptamer with unique complex secondary structure representing each homologous family

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viz EGFR_A1, EGFR_A7, EGFR_A15, EGFR_A13 and EGFR_A16 were synthesized with either FAM or Biotin labelling at 5' end for further characterization.

The binding of aptamer to its target strictly relies on conformation complementary for target binding. The ECD protein of EGFR used for SELEX was purified from *E. coli* which is a prokaryotic system. The major drawback of this system is inefficient post-translational modification of recombinant proteins. Here in this study recombinant protein was used for the selection of aptamers. For clinical application, the selected aptamer should bind to native conformation of protein. Therefore binding studies of selected aptamers EGFR_A1, EGFR_A7 and EGFR_A15 were performed with various EGFR expressing eukaryotic cell lines by flow cytometry. Further to investigate the selectivity and affinity of aptamer candidates, cell lines expressing variable levels of EGFR and mutants of EGFR were chosen for flow cytometric studies. The untreated cell was used as background fluorescence signal. A shift in the peak of the histogram to a higher fluorescent intensity was an indication of fluorescence intensity of the cell as a result of the binding of labelled aptamer probe to the target cell (**Fig 3.9**). All three aptamers were found to bind with A431 with very high affinity as compared to other cell lines due to abnormally high expression of EGFR in this cell line. As expected, none of the aptamers binds to Jurkat cell line which do not expresses EGFR at all. It was interesting to note that the selected aptamers were also binding to U87 MG cell lines, which is a human glioblastoma cell line. The Glioblastoma multiforme (GBM) cells overexpresses epidermal growth factor receptor variant III (EGFRvIII), which has an in frame deletion of 801 bp. EGFRvIII, the most common form of mutant were commonly found in breast and lung cancers, and especially in most aggressive grade IV glioblastoma multiforme. These mutants were linked to unresponsiveness of radiation and chemotherapeutic drugs in treating brain tumor. Thus, significant shifting of peak in flow cytometry histogram of selected aptamers with U87MG cell line proposed that the aptamers were binding to deletion mutant of EGFRvIII alongwith full length EGFR as well.

Preliminary flow cytometry analysis also revealed that all the sequences which were populated throughout the SELEX or got enriched during selection were binding to our target cell line but with varying binding affinity. Some of the sequences exhibit strong

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binding while some sequences are weak binder. As it can be seen from **Fig. 3.9** in almost all the cell lines the shifting of fluorescence intensity peak of EGFR_A15 in histogram was most prominent as compared to EGFR_A1 and EGFR_A7. This implies that EGFR_A15 had the strongest binding while EGFR_A1 and EGFR_A7 had weak binding. Moreover EGFR_A15 could also recognise the varying EGFR expression level in different cell lines.

EGFRs are receptor proteins which are expressed on the cell membrane. It was obvious to assume that the selected aptamers against EGFR ECD protein will bind to cell surface protein. In order to determine the possible binding site of the candidate aptamers on the target cell, fluorescence binding assay were conducted by treating cells with proteinase. To digest the surface proteins of the cells they were incubated with trypsin and then the binding experiments were repeated using aptamers with digested cells. The fluorescence signal of untreated cells were used as background signal. **Fig. 3.10** shows that the aptamers significantly lose their binding with cells after trypsin treatment. The above results suggests that the binding target of aptamers are cells surface protein i.e. EGFR. Interestingly binding of EGFR_A15 with A431 cell line was only marginally lost. This can be attributed to abnormally high expression of EGFR on A431 cell lines. However on increasing the incubation time of trypsin, its binding was completely abolished.

Furthermore, to test the potential application of selected aptamers as imaging probe, binding of aptamers with MDA MB 231 and A431 cell lines were studied through fluorescence microscopy. Intense bright fluorescence of FAM labelled EGFR_A15 was observed at the cell surface of MDA MB 231 and A431 cell lines. Aptamers EGFR_A1 and EGFR_A7 were found to localize within the nuclei. In literature there are many reports supporting the internalization of aptamers through endocytosis after binding and its further transportation to nuclei (Tan *et al.*, 2013). However, the mechanism by which these aptamers localize to the nuclei remains unclear. Such cell internalizing aptamer serves as a delivery vehicle for chemotherapeutic drugs. Meanwhile, the flow cytometric analysis of trypsin treated cells and microscopy images of EGFR_A1 and EGFR_A7 bound cell lines indicates the possibility of transportation of these aptamers to the nuclei after binding. In future, these aptamers could be used as carriers to deliver chemical

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drugs or siRNAs to the target cells (Bagalkot *et al*., 2006; Chu *et al*., 2006). However further detailed experimentation are required.

It was exciting to find that the initial flow cytometry data correlates with fluorescence microscopy imaging. The aptamer which had highest signal intensity (EGFR_A15) by flow cytometry also produced brighter fluorescence signal in fluorescence microscopy. Among the three selected aptamers *viz* EGFR-A1, EGFR_A7 and EGFR_A15, the aptamer with highest binding i.e. EGFR_A15 was chosen for the further studies. Furthermore, to study the binding affinity of remaining two homologous family, two more aptamers EGFR_A16 and EGFR_A13 belonging to group I and V respectively were selected. All three aptamers *viz* EGFR_A15, EGFR_A13 and EGFR_A16 were synthesized with Biotin modification at 5′ end to suit our further research.

Many methods such as isothermal calorimetry, surface plasmon resonance, fluorescence anisotropy/polarization, circular dichroism, UV−vis absorption and affinity capillary are reported in literature for the determination of dissociation constant of aptamer protein interaction (Jing and Bowser, 2011; Lin *et al*., 2011). These methods require sophisticated and expensive instrument and are quite time consuming (Jing and Bowser, 2011). Initially Isothermal calorimetry was employed to determine the dissociation constant of selected aptamers with EGFR protein. For this methodology, both the protein and ligand should be present in same buffer. The buffer composition of purified EGFR ECD protein was 50mM Sodium Phosphate buffer, 400 mM NaCl, pH: 7.2 while the buffer composition of aptamers (ABB) was 50mM Sodium Phosphate buffer, 150 mM NaCl, 1mM $MgCl_2$, 5mMKCl, pH: 7.2. On dialysis of recombinant EGFR ECD protein to ABB, they do not remain stable and start precipitating. Due to this no convincing result was obtained. Thus, a very simple, cheap and convenient method reported by Li et al.with slight modifications were employed to calculate the dissociation constant of selected aptamers *viz* EGFR_A15, EGFR_A13 and EGFR_A16 (Fu *et al*., 2014; Li *et al*., 2011). The EGFR ECD protein immobilized microtiter plates were treated with varying concentration of selected aptamers. The aptamers were found binding to its target with very strong affinity in nanomolar range. This is in good agreement with K_d values obtained for aptamers against other targets. Such examples include the DNA aptamers to thrombin with K_d=25–200 nmol/l (Bock *et al*., 1992), RNA aptamer to PSMA with K_d=2

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nmol/l (Lupold *et al*., 2002), RNA aptamer to tenascin-C with K_d=5 nmol/l (Hicke *et al*., 2001) and RNA aptamer to *Trypanosoma cruzi* cell surface receptor with K_d=172 nmol/l (Ulrich *et al*., 2002).

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3.5 Conclusions

In conclusion we report herein, the successful isolation of a panel of DNA aptamers binding selectively to EGFR protein after 18 rounds of SELEX. This methodology reported here provides the possibility of rapidly selecting individual aptamers for any recombinant protein. The secondary structure and sequence similarity of aptamers were analysed. For further studies most abundant aptamers or aptamer with unique complex secondary structure (EGFR_A1, EGFR_A7, EGFR_A13, EGFR_A15 and EGFR_A16) belonging to different homologous families were synthesized with either FAM or Biotin modifications. Initially the binding affinity of the aptamer candidates -EGFR_A1, EGFR_A7 & EGFR_A15 were characterized by flow cytometry. The selected aptamers were found capable of binding to EGFR expressing cancerous cells and can recognise full length EGFR as well as deletion mutant of EGFRvIII. The aptamer with best binding ability EGFR_A15 can detect varying EGFR expression level in cancer cell lines. Further the clinical potential of aptamers (EGFR_A1, EGFR_A7 & EGFR_A15) as a bioimaging probe was evaluated by fluorescence microscopy. Aptamer EGFR_A15 displayed a distinct binding on their target cell surface. However aptamers EGFR_A1 & EGFR_A7 were found to be localized inside the cell. The K_d values of the developed aptamers (EGFR_A15, EGFR_A13 & EGFR_A16) were determined by ELAA, which was found to be in the lower nanomolar range. Among the selected aptamers, EGFR_A15 was found to have the best recognition and selectivity. Aptamer EGFR_A16 was found to be a very weak binder. Aptamer EGFR_A15 holds a great potential as a molecular probe for the detection of any human malignancies or diseases in which EGFR are overexpressed.

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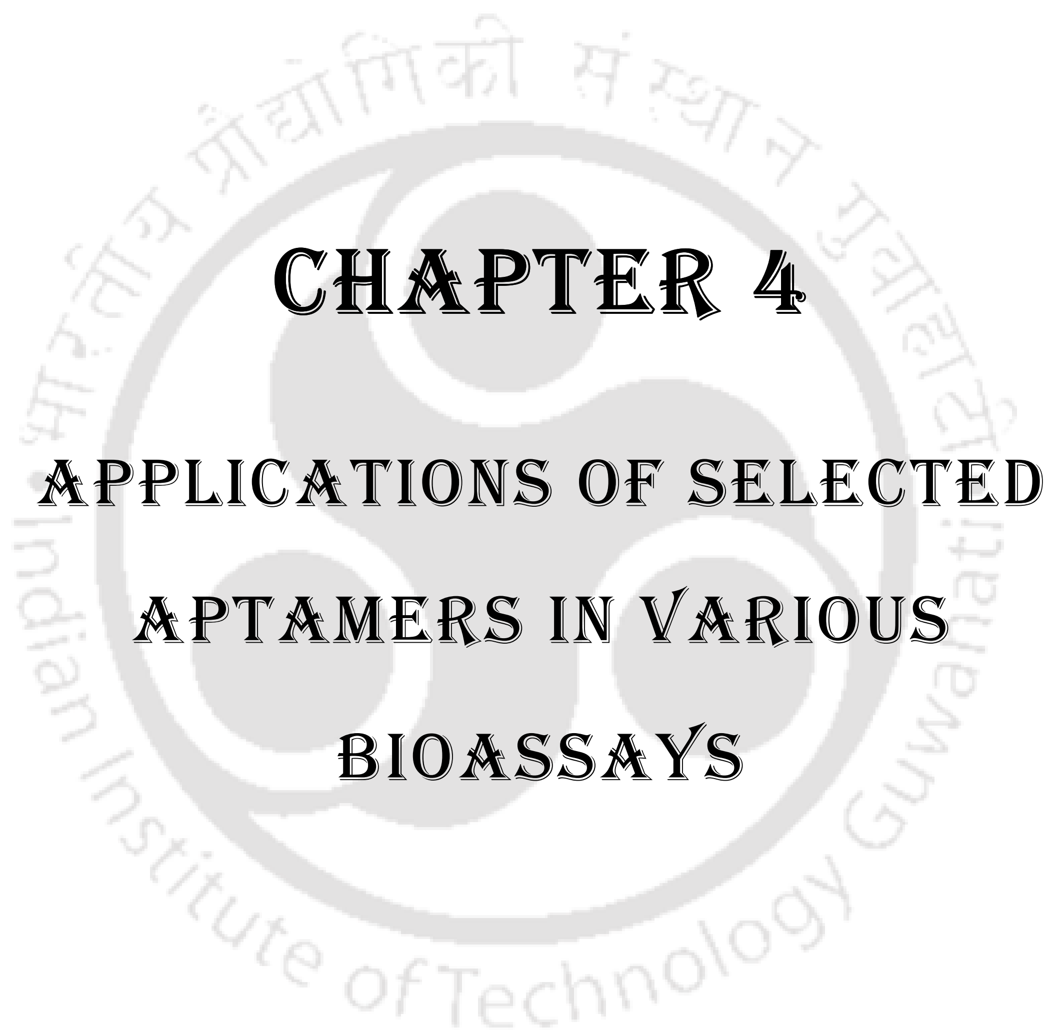

CHAPTER 4

APPLICATIONS OF SELECTED APTAMERS IN VARIOUS BIOASSAYS

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4.1 Introduction

In biomedical research, the antibodies are considered as a workhorse reagent. They are the most popular and widely used class of molecules providing molecular recognition, which plays an essential role in basic research as well as in clinical practice. They have made substantial contributions to the advancement of diagnostic assays, and novel mAb has become indispensable in most diagnostic tests that are used routinely in clinics today. Due to its high sensitivity and specificity for particular epitopes, it became an ideal reagent for research and clinical applications. Since the advent of aptamers, many examples demonstrated that the aptamers can be a substitute for antibodies in diagnostic, therapeutic and clinical applications. In last few years aptamers were used in almost all the biomedical field such as flow cytometry, ELISA like assays, western blots, microscopic studies, immunohistochemistry, therapeutic target, immunoprecipitations, purifications of proteins, imaging etc. The first report of aptamer application was written by Drolet in 1996, who reported the first enzyme-linked aptamer assay (ELAA), consisting of a mixed ELISA/ELAA sandwich to detect human vascular endothelial growth factor on microtiter plates (Drolet *et al*., 1996). Since then, different aptamer based ELISA like assay have been developed. Aptamers were either immobilized on the surface of microbeads or magnetic beads or nanobeads (Bruno and Richarte, 2016; Lu *et al*., 2015) or on the surface of microtiter plates. Labelled aptamers were used either as a detecting agent in direct or indirect ELISA (Jeong and Rhee Paeng, 2012) or capture agent in sandwich ELISA and, in some cases, exploited in the competition format (Baldrich *et al*., 2005; Barthelmebs *et al*., 2011; Eva Baldrich *et al*., 2004; Ferreira *et al*., 2008; Fu *et al*., 2014; Park and Paeng, 2011). Several other assays such as displacement assays (Baldrich *et al*., 2005) or mixed assay involving combination of antibodies and aptamer for the development of aptamer antibody sandwich assays (Ferreira *et al*., 2008) were also reported. Aptamers were also used in flow cytometric studies (Ara *et al*., 2012; Kim *et al*., 2014; Sefah *et al*., 2010; Y. Wang *et al*., 2014). Also aptamers have shown to be used in microscopy studies for the imaging of cancerous cell (Li *et al*., 2014; Y. Song *et al*., 2013; F. B. Wang *et al*., 2013). Some of the aptamers acts as therapeutic agents inhibiting the cell proliferations (Li *et al*., 2011, 2011) or signalling pathways (Camorani *et al*., 2014; Chen *et al*., 2003; Shum *et al*., 2011). Numerous aptamers have

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been selected against therapeutic targets and they showed great prosperity in tissue culture experiments and animal models (Title, 2015). Aptamers have also shown promising effect in inhibiting tumor cell migration and invasion (Botkjaer *et al*., 2012; Dassie *et al*., 2014; Iida *et al*., 2014; Zueva *et al*., 2011).

EGFR is often considered as one of the important biomarker in deciding the treatment regimen for the cancer patients. Overexpression or elevated levels of EGFR activity is often associated with various human cancer including breast, colon, pancreas, ovary, brain and lung cancers (berall *et al*., 2008). The patients with altered EGFR activity tend to have a more aggressive disease, associated with a poor clinical outcome (Holbro *et al*., 2003). Mostly antibodies were used for the detection of EGFR expression in cancer patients. Aptamers can be the most promising alternative to antibodies and it can be used for almost any application, which traditionally involves the use of antibodies.

In this chapter, the application of EGFR specific aptamers were studied in various biomedical field. Dot Blot assay was conducted to assess the specificity of selected aptamers. The therapeutic potential of aptamers were also evaluated by MTT assay. Further the effect of aptamers in inhibiting cell migration was also studied by *in vitro* scratch assay. Finally the lead aptamer sequence EGFR_A15 and EGFR_A13 was subsequently used to develop an indirect Enzyme Linked aptamer assay for the detection of EGFR.

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4.2 Materials and Methods

4.2.1 Dot Blot assay using aptamer candidates

A dot blot assay was performed to check the specificity and affinity of selected aptamers. A 0.5mg/ml stock solution of purified recombinant EGFR ECD was serially diluted 10 fold. A volume of 5µl of protein was spotted on 0.45 µm pore-size NC membrane and air dried. The immobilization of protein was visualized by Ponceau staining. The membrane was blocked with 5% BSA in PBST overnight at 4°C with gentle shaking. After blocking membrane was treated with 500nM bioaptamer EGFR_A15 and EGFR_A13 for 1hr at RT. After three washing steps, HRP conjugated streptavidin (1:1000 dilution from 1mg/ml stock) was added for 1hr. Color was developed by adding DAB (3, 3'-Diaminobenzidine) substrate. The biotinylated initial aptamer pool were used as a negative control. The biotinylation of non-enriched aptamer pool were performed by PCR using biotinylated Apt_F1 and unlabelled Apt_R1. The sense strand was obtained as described in section 3.2.4.

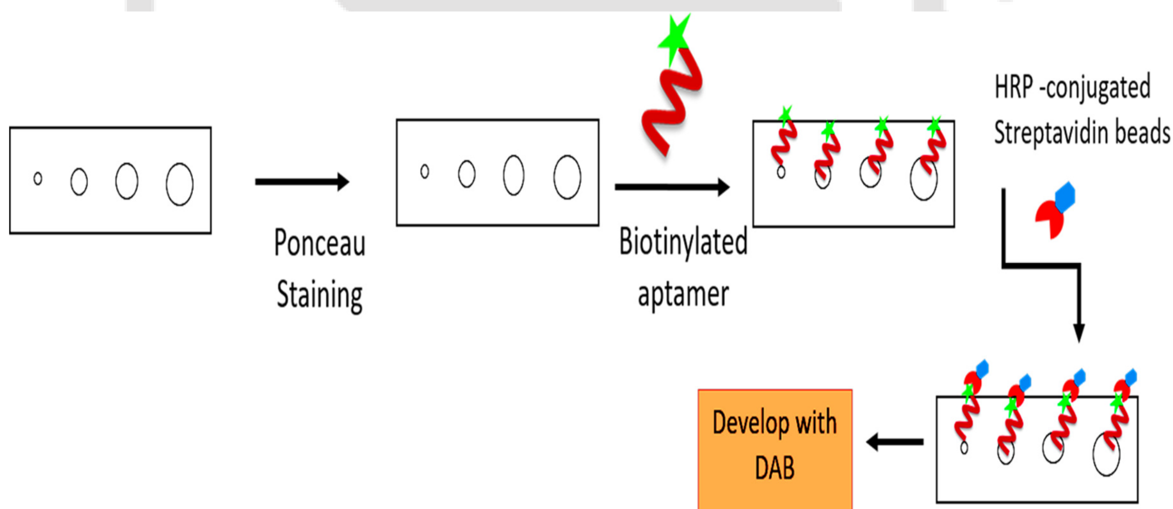

Figure 4.1: Schematic overview of Dot Blot assay.

4.2.2 *In vitro* cytotoxicity Assay of aptamer candidates against cancer cell line

Cells were counted and tested for viability before experiment. MDA MB 231 cells were seeded 24 hours prior to experiments in 96-well flat-bottom microtiter plates (Nunc) at a density of 1.0×10^4 cells/well in DMEM medium containing 10% FBS. Different concentration of selected aptamers and initial aptamer library (25nM, 50nM, 75nM,

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100nM, 200nM and 500nM) were added to the cells in DMEM incomplete medium and incubated at 37°C for 24 and 48 hours. The viability of cells were subsequently determined by adding 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) in DMEM incomplete media for 4 hours at 37°C. Formazan crystals were dissolved by adding DMSO. Absorbance was measured at λ=570 nm with background subtraction at 630 nm (Tecan Microplate reader, CA, USA). The cell viability was calculated with the following equation:

$$\text{Cell viability \%} = A_T / A_C \times 100$$

Where A_T and A_C are the absorbance of aptamer treated and untreated cells, respectively.

4.2.3 *In vitro* Scratch Assay of aptamer candidates against cancer cell lines

MDA MB 231 cells were grown to a postconfluent multilayer in 24-well plates and serum-starved for 24 hr in the presence of 0–200 nM of either the aptamer or the control (initial pool). A 200 µl pipette tip was used to make a vertical-line scratch. Migration inside the scratch was stimulated by addition of media containing 10% fetal bovine serum and cells were allowed to migrate for 8 hrs. The closing of scratch due to the migration of cells was monitored by microscopy. Cells were fixed with 4% formaldehyde and stained with crystal violet dye. Three independent experiments were performed in duplicate each.

4.2.4 Development of indirect ELAA for the detection of EGFR protein

The indirect ELAA (Enzyme Linked Aptamer Assay) was developed for the sensitive detection of Epidermal Growth factor receptor protein. The development of any enzyme linked immuno assay system involves the optimization of reagents. In other words, the working concentration of each component of the test must be assessed.

4.2.4.1 Optimization of Assay condition

The sensitivity of any assay are dependent on the interaction between biorecognition probe and analyte. Thus prior to the DNA aptamer assay, the assay parameters were investigated for the optimized method. The optimization was carried out using chessboard or checkerboard titrations (CBT). Both biotinylated DNA aptamers

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EGFR_A15 and EGFR_A13 were diluted to 1nM, 5nM, 20nM and 50nM. The stock protein solution were serially diluted.

4.2.4.2 Enzyme Linked aptamer assay for EGFR protein

The process of CBT involves the dilution of two reagents against each other to analyse the optimal concentration. The 96 well Maxisorp flat-bottomed polystyrene immuno plates (Nunc, Denmark) were used. The recombinant protein solution were diluted from column 1 to 11, while in the last column i.e. column 12 only diluent were added (**Fig. 4.2**). The microplate were then incubated overnight at 4° C with gentle shaking for the immobilization of proteins. After washing, blocking of plates were carried out by adding 200 µl of 5% BSA for 1 hour at RT. The plates were extensively washed with PBST buffer to remove unbound BSA. The diluted biotin labelled aptamers were added in different rows and incubated for 60 min at RT. After washing, the plates were incubated with HRP conjugated streptavidin (New Englands Biolabs). The microplates were again washed four times with PBST, the substrate TMB (Sigma) was added and incubated for 10 min at RT. The reaction was stopped by adding 2M HCl and read at 450 nm. A dose response curve was obtained based on these data using non-linear regression analysis in GraphPad Prism 5.0.

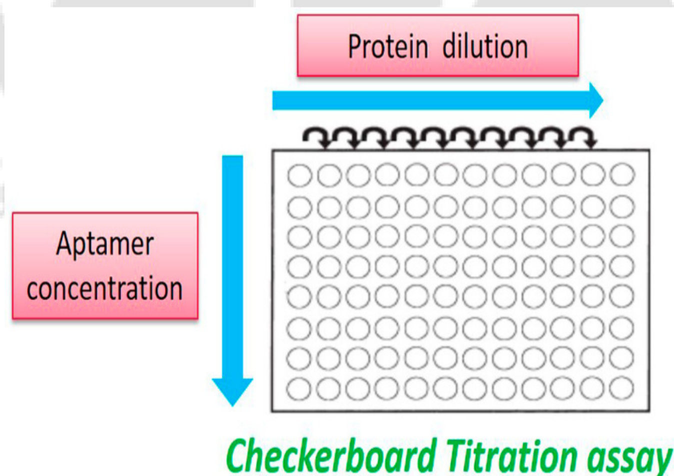

Figure 4.2: Plate layout for Checkerboard test for indirect Enzyme Linked Aptamer Assay.

The developed indirect Enzyme Linked aptamer assay using the lead aptamer sequences were also performed on complex samples of EGFR. Cell lysate of A431, MDA MB 231

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and Jurkat cell lines were coated on to the microtiter plates and incubated separately with biotin labelled 1nm of EGFR_A15, 20 nM of EGFR_A13 and anti EGFR antibody for 1hr at RT. The wells were washed with PBST buffer five times after incubation. Then Binding and detection reaction were carried out in a similar way as described above.

4.2.4.3 Establishing Indirect Competitive Enzyme Linked aptamer assay for EGFR protein

Microplates (96 wells) were coated with recombinant EGFR ECD protein as described above. The plates were washed with PBST three times and blocked with 200 µL of 5% BSA at RT for 2 h. Next, a 50-µl aliquot of excess EGFR specific monoclonal antibody at a fixed concentration (1:200 dilution in PBS) was added per well to ensure saturation of all binding sites. 50µl of 1nM Bio-EGFR_A15 was also added to the wells containing antibodies. Similarly in another assay 20nM of Bio-EGFR_A13 alongwith anti EGFR antibodies were added (**Fig. 4.3**). In last two rows only 50 µL of anti EGFR antibodies was added into the wells as a control. The plates were well mixed on a shaker and incubated at RT for 1 h. Plates were then developed as described above.

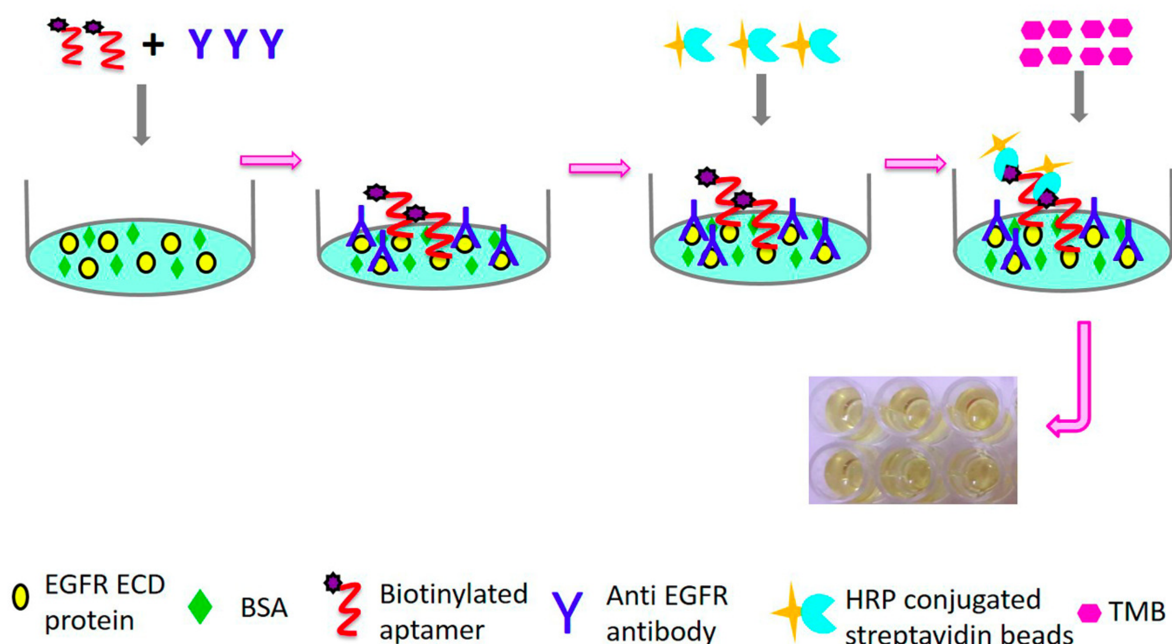

Figure 4.3: Schematic representation of indirect competitive ELAA. Anti EGFR antibody and bioaptamer are added to the blocked plate at the same time. Antibodies

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compete to inhibit the bioaptamer from binding to the coated EGFR protein, resulting in a lighter colorimetric detection. Adapted with permission from Fu *et al*., 2014.

4.2.5 Serum Recovery Experiment

4.2.5.1 Isolation of Serum from human blood

Real serum samples were collected from healthy volunteers following an established protocol and ethical guideline. In an anticoagulant free collection tube, 12 ml of blood was collected and allowed to settle for 15-30 mins to clot. The samples were then centrifuged at 2000 g for 10 mins in a refrigerated tube to remove the clot. The supernatant collected was designated as serum and stored at -80°C till further use.

4.2.5.2 Detection of EGFR in spiked serum using selected aptamers

A stock solution of recombinant EGFR ECD protein was serially diluted with serum solution. Each 50µl of EGFR ECD solution diluted in serum and PBS was added to the well. The plates were incubated overnight for the immobilization of proteins. After extensive washing and blocking with 5% BSA, plates were probed with the best recognising biotinylated aptamer sequence i.e. EGFR_A15. 1nM of EGFR_A15 aptamers and then each wells were developed following the same steps described above. Further the percent recoveries of each concentration were calculated.

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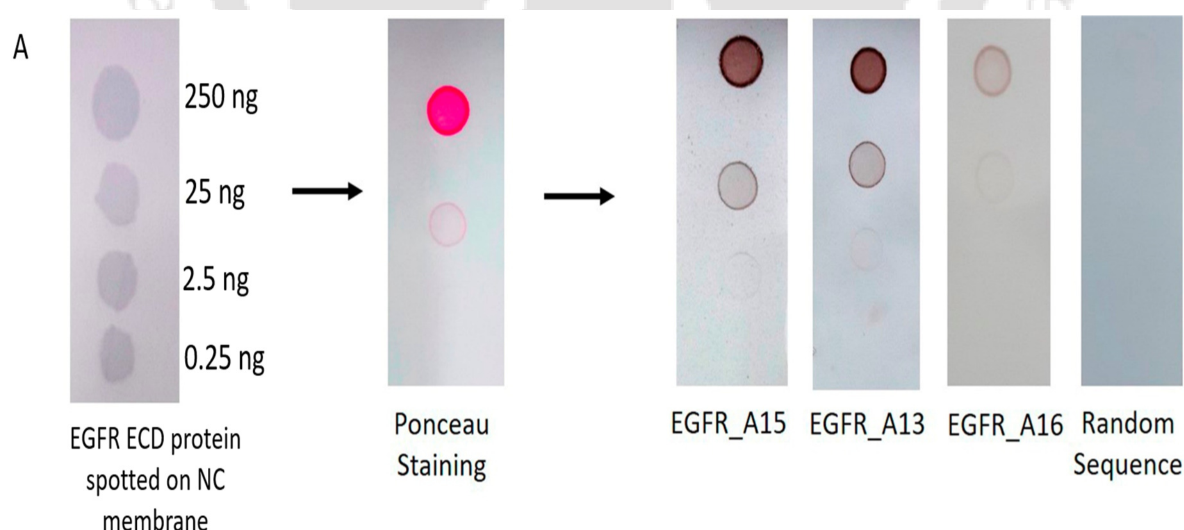
4.3 Results

4.3.1 Dot Blot assay using aptamer candidates

A dot blotting assay was performed to substantiate the specificity and affinity of aptamer and to further investigate the potential application of the selected aptamers. A series of 10 fold dilution of EGFR ECD protein in the range from 250 ng to 0.25ng were immobilized on membrane. Membranes were exposed to biotinylated EGFR_A15, EGFR_A13 aptamers alongwith control biotinylated initial aptamer library. Membrane was then probed with streptavidin HRP conjugates and developed using DAB. Both aptamers clearly displayed specific binding to EGFR ECD protein. Aptamer EGFR_A15 can detect upto 2.5ng of spotted EGFR ECD proteins while EGFR_A13 can detect upto 10 fold lower amount of spotted protein (25ng). EGFR_A16 was found detecting 250ng of protein but with very weak signal (**Fig. 4.4**). It was confirmed EGFR_A16 is a very weak binder. For further studies EGFR_A16 were not used. The biotinylated non enriched aptamer pool, used as a negative control did not show any binding with spotted proteins. The dot intensity were measured by Image J software and plotted against the corresponding protein concentration (**Fig. 4.4 B**).



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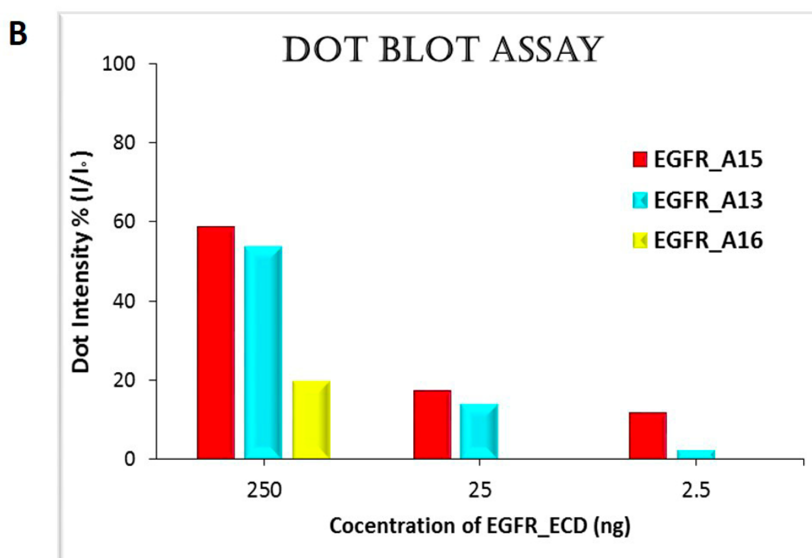

Figure 4.4: (A) Dot blot assay of selected Aptamer EGFR_A15, EGFR_A13, EGFR_A16 and Random sequence (Biotin labelled non enriched aptamer library).

(B) Histogram of dot intensity confirming the binding of biotinylated EGFR_A15, EGFR_A13 & EGFR_A16 to immobilized recombinant EGFR ECD protein.

4.3.2 *In vitro* cytotoxicity Assay of aptamer candidate against cancer cell line

In order to assess the effect of selected aptamers on the proliferation of cells expressing EGFR, *in vitro* cytotoxicity assay was conducted on MDA MB231cell lines. The cell lines were treated with varying concentration of selected aptamers and initial aptamer library ranging from 0-500nM. After 24 and 48 hrs cells were observed under microscope and analysed by MTT assay. It was found that the selected aptamers (EGFR_A1, EGFR_A7, EGFR_A15 and EGFR_A13) and initial aptamer pool have no significant effect on the proliferation of cancerous MDA MB 231 cell line (**Fig. 4.5**).

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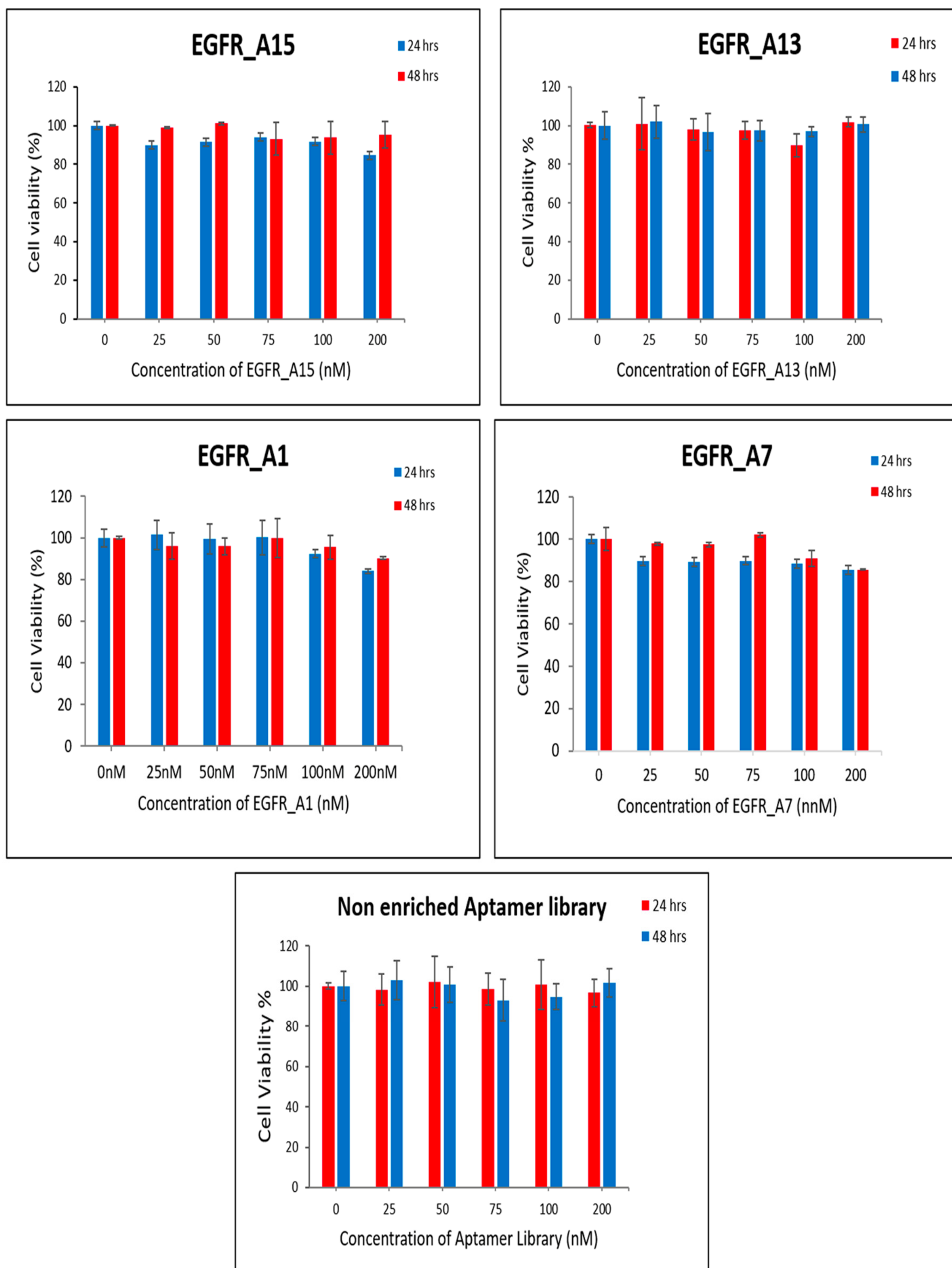

Figure 4.5: Effect of aptamers EGFR_A15, EGFR_A13, EGFR_A1, EGFR_A7 and non enriched aptamer library on the proliferation of MDA MB 231 cells. All aptamers and control aptamer pool were biocompatible do not show any significant effect on the proliferation of MDA MB 231 cancer cell line after 48 hours of treatment.

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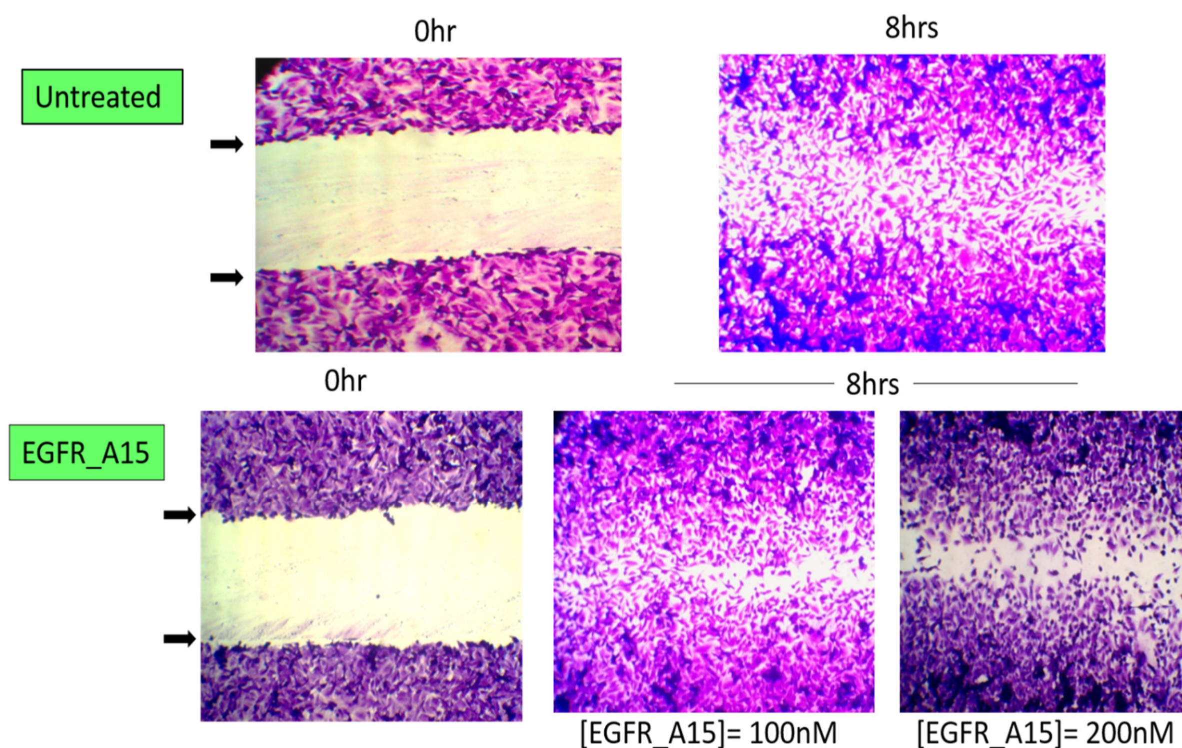
4.3.3 *In vitro* Scratch Assay of aptamer candidate against cancer cell line

The *in vitro* scratch assay was conducted to examine the effect of aptamers on the migration of cells. First the highly metastatic MDA MB 231 cells were allowed to form a monolayer. The cells were treated with aptamers: EGFR_A1, EGFR_A7, EGFR_A15, EGFR_A13 and non-enriched aptamer library in serum starved condition for 24 hours. Then, the monolayers of aptamer treated and untreated MDA MB 231 cells were scratched and the migration of cells were initiated by the addition of complete media containing 10% FBS. The migration of cells were monitored in microscope and the images were taken at 0 & 8 hours after scratching (**Fig. 4.6**). In untreated cells the scratch was closed within 8 hours due to migration of cells. All the aptamers don not exhibit similar effect. The effect of aptamers EGFR_A15, EGFR_A13 and non-enriched aptamer library treated were similar to the untreated cells. There was no delay in the migration of cells and the scratch was closed within 8 hours. However the migration of cells were significantly delayed in EGFR_A1 and EGFR_A7 treated cells, indicating that these aptamers might have some inhibitory effect on cell migration.



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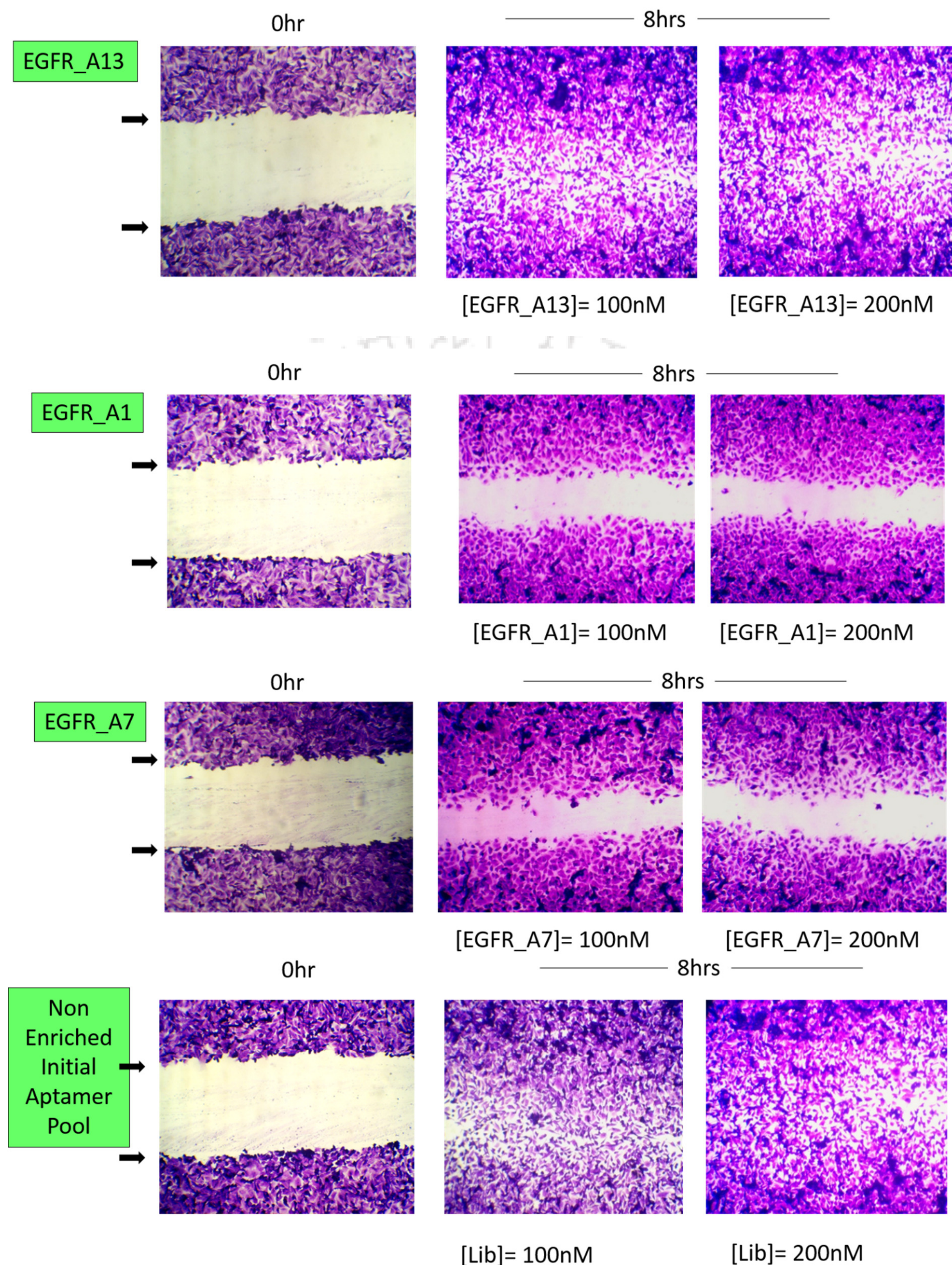

Figure 4.6: 2D locomotion of highly metastatic MDA MB 231 cells as a function of aptamer binding. Arrowheads show the size of the initial scratch created.

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4.3.4 Development of Indirect ELAA for detection of EGFR protein

In recent years, novel technologies combined with immunochemical assays have been proposed for rapid quantitative or semi quantitative analysis of biomarkers which are expressed in human malignancies and other illness. In the search of inexpensive, fast, sensitive and non-invasive methods for the detection of EGFR, an indirect ELAA was developed. The iELAA was performed using two candidate aptamers- EGFR_A15 and EGFR_A13. Both aptamers were found to have highest affinity as compared to other selected aptamers with a dissociation constant 12.31 ± 2.59 nM (EGFR_A15) and 15.59 ± 6.02nM (EGFR_A13).

The sensitivity of any immunoassay depends on both the immobilized protein concentration on the solid surface and the primary probe used for the recognition of protein. Thus, the different concentrations of aptamer (1nM, 5nM, 20nM and 50nM) and the protein were controlled to optimize the assay performance. **Fig. 4.7** shows the Dose response curve obtained by a matrix combination of both chosen reagents. The results demonstrated that the sensitivity increased when lower amounts of aptamer were used. In case of EGFR_A13, aptamer concentrations of 20nM and 50nM displayed a similar sensitivity. Meanwhile, all the concentrations of EGFR_A15 (1, 5, 20, 50 nM) exhibit almost similar response. Therefore based on conservation of reagent, concentration of 1 nM of EGFR_A15 aptamer and 20nM of EGFR_A13 was selected to give the optimum sensitivity and sufficient absorbance values for an easily detectable signal. The linear dynamic range for both aptamers was 2×10^{-6} M to 1.7×10^{-9} M. The EC50 value was found to be 2.317×10^{-7} M (1nM), 6.4×10^{-8} M (5nM), 7.558×10^{-8} M (10nM) and 8.906×10^{-8} M (20nM) for EGFR_A13. For EGFR_A15, EC50 values were 4.167×10^{-7} M (1nM), 1.67×10^{-8} M (5nM), 9.225×10^{-8} M (10nM) and 1.007×10^{-8} M (20nM).

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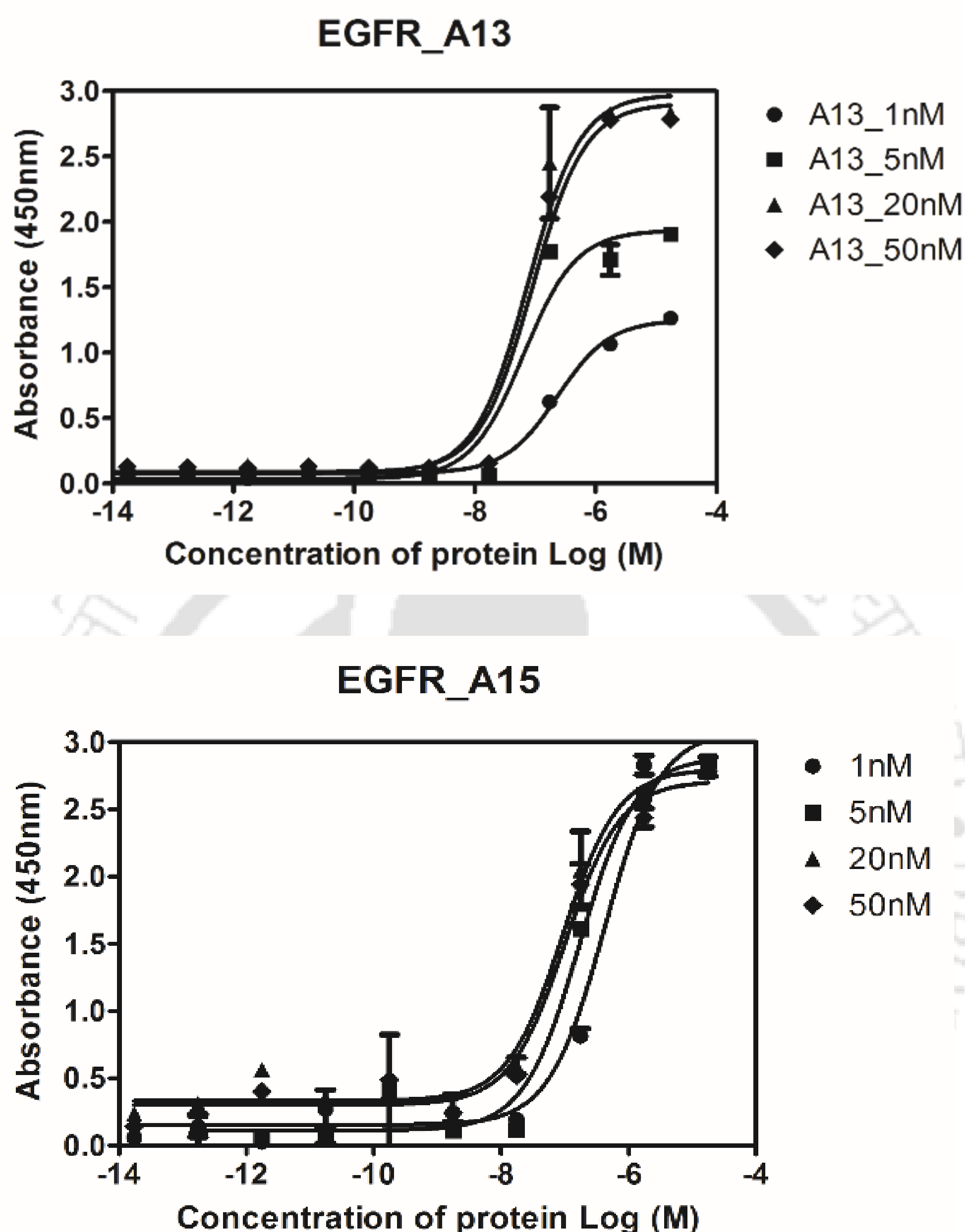

Figure 4.7: Dose response Curve for the optimization of indirect Enzyme Linked Aptamer assay condition for aptamers EGFR_A15 &EGFR_A13.

The developed indirect enzyme linked aptamer assay was validated by testing the binding of EGFR_A15 and EGFR_A13 against the cellular extracts of A431, MDA MBA 231 and Jurkat cell line (**Fig. 4.8**). Interestingly, the aptamers namely EGFR_A15 and EGFR_A13 came up with a pattern of relative binding with A431, MDA MB 231 and Jurkat cell lysate similar with the anti EGFR antibodies. A significant detectable signal was generated by both EGFR_A15 (1nM) and EGFR_A13 (20 nM) aptamers. This

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suggests that irrespective of the presence of myriad cellular components in the heterogeneous extracts of A431 and MDA MBA 231, these DNA sequences bound selectively to the EGFR present in the crude extract. Further, the negligible binding of the same DNA sequences with the cell extracts of EGFR-deficient Jurkat proves the target specificity of these sequences.

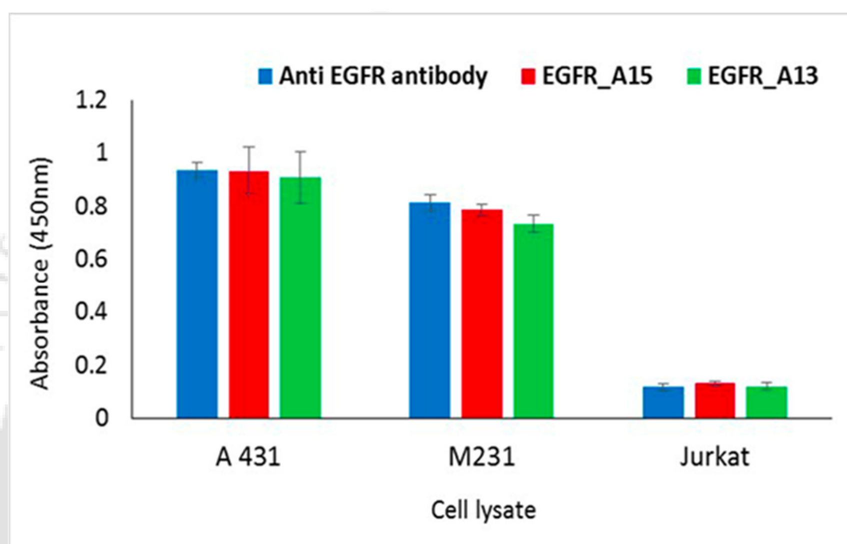

Figure 4.8: Indirect Enzyme Linked Aptamer assay performed with the optimized aptamer concentration *viz*- 1nM of EGFR_A15 and 20 nM of EGFR_A13 using cellular extract of A431, MDA MB 231 and Jurkat cell line.

4.3.4 Establishing Indirect Competitive ELAA for EGFR protein

To verify the efficacy of the aptamer in binding to the Epidermal Growth factor Receptor protein and competing with the established anti EGFR antibody for binding, a competitive ELAA was performed. The protein was adsorbed onto the microtiter plate overnight. The anti EGFR antibody was added to the plate in excess, to allow saturation and binding to all available protein. The optimized concentration of EGFR_A15 (1nM) and EGFR_A13 (20nM) were added individually. The wells were then probed with S-HRP and developed using TMB substrate. Now the antibody and aptamer will compete for binding to the EGFR. As the protein was immobilised in the wells of the plate, some of the binding sites of proteins were occupied by antibody and some sites were occupied by bioaptamer. The anti EGFR antibody will compete to inhibit the bioaptamer from

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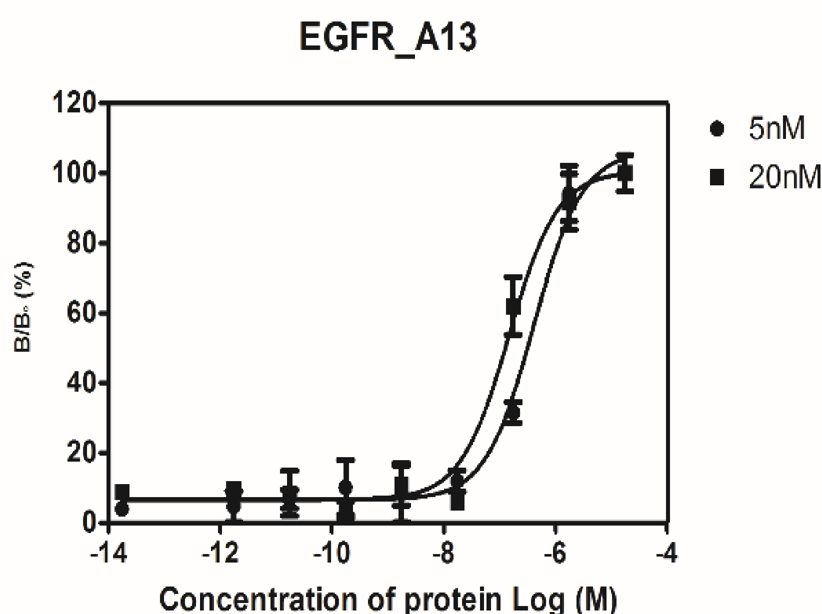
binding to its site on EGFR as depicted schematically in **Fig. 4.3.** Thus on addition of S-HRP, only the aptamers binding to the immobilised protein will interact with S-HRP leading to colour development. The antibodies that were displaced from the protein, as aptamers bound to it, were removed in the various wash steps. A well where no antibody was added was used as a positive control. The results were plotted as percentage absorbance versus aptamer concentration. Two concentrations of aptamer EGFR_A13 (5 and 20nM) and one concentration for EGFR_A15 (1nM) were investigated in the competition assays. The concentration of anti EGFR antibody causing 50% inhibition (IC50) of binding to the immobilized EGFR obtained for 20nM of EGFR_A13 aptamer was 1.3 fold higher than the IC50 value obtained for 5 nM (**Fig. 4.9**). Thus the concentration of 20 nM of EGFR_A13 aptamer was selected as the optimal concentration for the generation of detectable signal. The IC50 value for EGFR_A15 was found to be 5.428 $X10^{-7}$ M. Both the aptamers used were suitable for indirect competitive assays.



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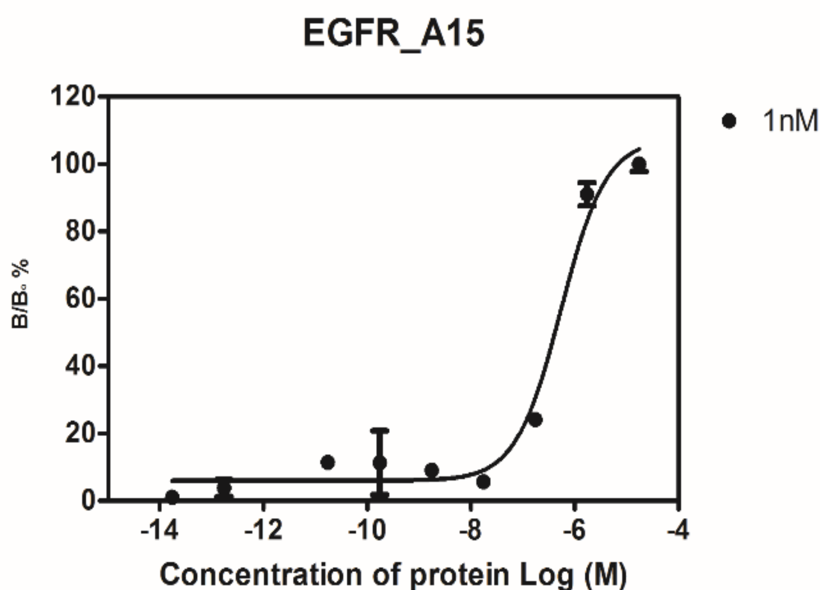

Figure 4.9: Competition curve performed with 1nM of EGFR_A15 and 5nM and 20nM of EGFR_A13.

4.3.5 Serum Recovery experiment for the detection of EGFR protein

Serum is one of the complex biological fluids that contains various compositions such as proteins (albumins, globulins). In order to examine the potential interference of the serum proteins, serum recovery experiment were performed. The serum obtained from healthy volunteer was spiked with EGFR protein. Even though the spiked serum solution was not exactly the same as the real patient samples, yet it serves as a good model. The recovery of free EGFR (0.065mg/ml and 0.0065mg/ml) spiked serum samples were 98.3% and 95.3.0%, respectively demonstrating its usability of the as a molecular probe in microtiter-based assays.

Table 4.1: Recovery of EGFR in spiked serum.

Sl.No.	Amount of EGFR added (mg/ml)	Amount of EGFR recovered (mg/ml)	Serum recovery %
1	0.065	0.0639	98.3
2	0.0065	0.0061	95.3

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4.4 Discussions

Aptamers are functional molecules, able to bind tightly and selectively to disease markers, offering great potential for applications in disease diagnosis and therapy (Ferreira *et al*., 2008). EGFR is a well-known biomarker present in various cancer and is used in immunotherapeutic and diagnostic approaches. In the previous chapter we report the selection of DNA aptamers that bind with high affinity and selectivity with recombinant EGFR protein as well as EGFR expressing cell lines. In this chapter we demonstrated the application of selected EGFR specific aptamers in various assays such as Dot Blot assay and micro-titer based assays. Also theranostic potential of selected aptamers were studied.

A dot blot assay was performed to evaluate the specificity and affinity of selected aptamers. There are reports where researchers have used dot blot assay for comparing the specificity of selected aptamers. For example Fu *et al* performed Dot blot assay for assessing the specificity of WKB-14 against the target P48 protein (Fu *et al*., 2014). This assay have many advantages such as simplicity, rapidity and sensitivity compared to other methods of detection. Moreover, due to the strong affinity between the aptamers and proteins, dots can be visible to the naked eye or quantified by a hand-held instrument which makes this assay very cheap (Wang *et al*., 2013). Also, during SELEX, NC membrane was used as a matrix for protein immobilization. Generally, such practice also leads to the enrichment of nonspecific aptamers binding to membrane alongwith the specific aptamers against the target. Although Negative SELEX was introduced before 1^{st}, 9^{th} 12^{th} and 14^{th} round to avoid the selection of nonspecific aptamers. Yet, Dot blot assay was performed to ensure the specificity of selected aptamers are only with EGFR ECD protein and to eliminate any probability of its nonspecific binding to NC membrane. From the assay it was evident that all three aptamers were binding specifically to EGFR ECD protein and do not have any affinity towards NC membrane as no background noise was observed. The aptamer EGFR_A15 and EGFR_A13 could recognize EGFR upto nanogram of EGFR ECD protein. It was also observed that EGFR_A16 have weak binding as compared to EGFR_A15 and EGFR_A13. This observation was in line with the previous studies. From all the studies it was evident that aptamer EGFR_A16 is a

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very weak binder as compared to other selected aptamers and thus it was not explored for further studies.

One obstacle in using any molecule as a vehicle for delivering chemotherapeutic drugs, siRNA etc is its effect on the cells. Generally antibodies are larger in size and have very poor tissue penetration. Moreover they activates immune response and have many side effects. DNA aptamers are generally considered to be non-immunogenic (Foy *et al*., 2007; Orava *et al*., 2013). However to confirm the cytotoxicity of our selected aptamers, MTT assay was conducted using MDA MB 231 cell lines. All the aptamer sequences were found to be biocompatible. They do not affect the morphology of cells and its proliferation even after 24 or 48 hours of treatment. Obviously, it was reasonable to assume that the selected aptamers could be used as a drug delivery vehicle.

In cancer, metastasis of tumor cell to other organ is one of the major factor causing death of patients. Metastatic forms of cancer accounts for 90% of all cancer-related deaths (Sporn, 1996). In case of TNBC, tumors relapse quickly, and commonly metastasize to visceral organs such as lung, liver and brain (Rakha and Chan, 2011). Less than 30% of women with metastatic TNBC survive 5 years, and almost all die of their disease despite adjuvant chemotherapy, which is the mainstay of treatment (Dent *et al*., 2007). The scratch assay is a convenient and economical method to study cell migration *in vitro* (Todaro *et al*., 1965). This method is based on the observation that, upon creation of a new artificial gap, so called ''scratch'', on a confluent cell monolayer, the cells on the edge of the newly created gap will move toward the opening to close the ''scratch'' until new cell–cell contacts are established again. The basic steps involve creation of a ''scratch'' on monolayer cells, capture of images at the beginning and regular intervals during cell migration to close the scratch, and comparison of the images to determine the rate of cell migration. One of the major advantages of this simple method is that it mimics to some extent migration of cells *in vivo*. There are a number of disadvantages and limitations of the *in vitro* scratch assay compared to other available methods. It takes a relatively longer time to perform than some other methods (Liang *et al*., 2007). The methods for creating the scratches vary among different labs (Andreas Vogt, 2010) and the size, shape, and spacing of the scratches can vary from assay well to assay well within a given experiment (Hulkower and Herber, 2011; Kam *et al*., 2008; Staton *et al*., 2009). Despite these

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limitations of the method, overall, *in vitro* scratch assay is still often the method of choice to analyse cell migration in a laboratory because it is easy to set up, does not require any specialized equipment and all materials required for the assay are available in any laboratory that performs cell culture (Liang *et al.*, 2007). For instance Zueva and their group performed the classical *in vitro* scratch assay to assess the effects of individual aptamer sequences on cell migration. They found out of the 17 selected sequence, only 4 aptamers (coded D3, D42, E10 and E37) were able to inhibit migration of highly metastatic HM cells at a concentration of 200 nM (Zueva *et al.*, 2011). In this study it was observed that the two of the selected aptamer sequence; EGFR_A15 and EGFR_A13 do not have any effect on cell migration. However other two sequences EGFR_A1 and EGFR_A7 significantly inhibit the cell migration. It is important to note here that these two aptamer candidates were found to be localized within the nuclei of cells in immunocytochemistry studies (Section 3.3.10). These candidates are cell internalizing aptamers and can be explored further for its anti-migratory effect. We believe that this assay is not sufficient enough to proof the effect of sequences on cell migration. The *in vitro* cell scratch assay is a very preliminary experiment and further experiments like cell invasion assay using Trans wells/ Boyden chambers and in animal models are required.

Ectodomain shedding is a common process among many structurally and functionally unrelated transmembrane proteins (Arribas *et al.*, 1996; Hayashida *et al.*, 2010; Rose-John and Heinrich, 1994). Shedding of ectodomain of EGFR protein was found in overexpressing malignant cells (Perez-Torres *et al.*, 2008). Previously, soluble isoforms of the epidermal growth factor receptor (sEGFR) was identified in the conditioned culture media (CCM) of the vulvar adenocarcinoma cell line, A431 (Zhen *et al.*, 2003) and within exosomes of the keratinocyte cell line HaCaT (Sanderson *et al.*, 2008). These soluble receptor isoforms are generated by either limited proteolytic cleavage of the transmembrane receptor or by translation of several alternative transcripts (Reiter *et al.*, 2000; Reiter and Maihle, 1996). Adamczyk *et al* reported that pancreatic cancer cell lines release both exosomal (full length, 170 kDa) and ectodomain (110 kDa) forms of EGFR (Adamczyk *et al.*, 2011). Another group also reported the release of both full length and ectodomain of EGFR in urinary bladder cell lines (Bryan *et al.*, 2015). A soluble fragment of the EGFR protein (sEGFR) can be detected in the serum or plasma of patients with breast cancer. In addition, a follow-up study by Baron, *et al.*, stated that soluble

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EGFR concentrations are useful in detecting stage I/II and stage III/IV epithelial ovarian cancer in young, premenopausal women (Baron *et al.*, 2002). Also circulating tumor cells were found to express EGFR in the breast cancer patients (Kallergi *et al.*, 2008; Kalykaki *et al.*, 2014; Payne *et al.*, 2009; Tunca *et al.*, 2012).

The optimal method for EGFR assessment is not yet clear. Limited information is available both about the biology and the potential of sEGFR as a prognostic and predictive marker and about the changes of sEGFR during the course of therapy (Muller *et al.*, 2006). Enzyme Linked Immunoassay could be used to detect the presence of soluble EGFR in the bloodstream of patients. The enzyme-linked immunosorbent assay (ELISA) technique was developed in 1971 to replace radioimmunoassay. ELISA consists of an antigen (target), an antigen capturing agent, and a detection agent that produces a signal when the antigen is present. The robustness and simplicity associated with ELISA resulted in its widespread application (Toh *et al.*, 2015). The emergence of aptamers as an alternative molecular recognition element has the potential to replace or complement the role of antibodies in ELISA, resulting in an improved ELISA-enzyme-linked aptasorbent assay (ELASA). Different variations of the term 'ELASA' include enzyme-linked aptamer assay (ELAA) (Barthelmebs *et al.*, 2011; Jeong and Rhee Paeng, 2012; Park and Paeng, 2011), enzyme-linked oligonucleotide assay (ELONA) (Balogh *et al.*, 2010; Rotherham *et al.*, 2012), aptamer-linked immobilised sorbent assay (ALISA) (Vivekananda and Kiel, 2006).

Here we demonstrated the usability of selected aptamers as a capture agent in microtiter based assay. The results aim towards the development of another detection assay for the assessing EGFR protein expression in human malignancies and illness. Here, an indirect Enzyme Linked Aptamer assay (iELAA) was developed using the best recognising aptamers- EGFR_A15 and EGFR_A13. Different parameters such as the concentrations of aptamer (1nM, 5nM, 20nM and 50nM) and the amount of protein were optimized to find out the optimum concentrations of reagent required. It was found that EGFR_A15 was more sensitive as compared to EGFR_A13 as it can be used as low in 1nm concentration for the development of detectable signal. Moreover the presence of EGFR specific antibodies also do not affect the performance of aptamers as proved by competitive iELAA. Further the developed indirect enzyme linked aptamer assay was

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validated using optimized assay conditions (1nM for EGFR_A15 and 20nM for EGFR_A13) for the cellular extracts of A431, MDA MB 231 and Jurkat cell line. The signals generated by the aptamer sequences were similar to the commercially available anti EGFR antibodies. This suggests that the performance of newly developed EGFR aptamers remains unaffected even in the presence of plethora of cellular components. In addition, serum recovery studies were performed using the best aptamer sequence EGFR_A15. The recovery of spiked EGFR protein in spiked serum was 98.3 and 95.3 % respectively. This proves that the presence of EGFR specific antibodies, myriad of cellular components in the heterogeneous cellular extracts and other contaminating proteins of serum did not hampered the performance of EGFR specific aptamers.

In literature, there are few reports of DNA and RNA aptamers selected against EGFR (Esposito *et al*., 2011; Li *et al*., 2011; D.-L. Wang *et al*., 2014). Two RNA aptamers modulating EGFR signalling pathways were reported in same year (Esposito *et al*., 2011; Li *et al*., 2011).These aptamers were able to block the EGFR activation and prevent the proliferation of tumor cells. In this study DNA aptamers were preferred over RNA aptamers as RNA aptamer suffers from limitations of instability and high cost of modification (Y. L. Song *et al*., 2013). In another work, Wang *et al* and their group successfully identified DNA aptamer named TuTu22 with dissociation constant of 56 ± 7.3 nM. Nevertheless in the present study we have reported selection of highly specific and selective DNA aptamers against EGFR and the binding affinity of the best DNA aptamer EGFR_A15 were found better than early reported DNA aptamers (12.31 ± 2.59 nM). No similarities were found in the sequences of the selected aptamers and earlier reported anti-EGFR aptamers. Moreover the selected aptamers could bind to live cells overexpressing EGFR as evident from flow cytometry and microscopic data. The application of selected aptamers in various bioassays were also explored in this study.

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4.5 Conclusions

The present chapter focuses on the exploration of application of selected aptamers in various bio assays. The selected aptamers were found to successfully detect upto nanograms of EGFR ECD protein in Dot Blot assay. Then the therapeutic potential of aptamers were explored by MTT assay. The aptamers were found to be biocompatible and didn't showed any effect on the proliferation of MDA MB 231 cells even after 48 hours of treatment. Also a preliminary experiment was carried out with selected aptamers to assess its anti-migratory effect by *in vitro* scratch assay. Out of five selected aptamer candidate, two aptamer sequences EGFR_A1 and EGFR_A7 significantly delays the migration of highly metastatic MDA MB 231 cell line. Further the lead aptamer sequences EGFR_A15 and EGFR_A13 were used to develop a micro-titer based assay for the detection of EGFR protein. To the best of our knowledge, this is the first report of indirect ELAA using EGFR specific aptamers. It was found that the lower concentration of aptamers gives better signal and the optimized working concentration of EGFR_A15 was as low as 1nM. The optimized assay condition were validated using cellular extracts of A431, MDA MB 231 and Jurkat cell lines. The aptamer also successfully detect EGFR in EGFR spiked serum sample. The developed EGFR specific aptamer were able to generate detectable signal even in the presence of EGFR specific antibodies, myriads of cellular components and contaminating proteins of serum. The data presented herein represent preliminary proof-of-principle for an indirect enzyme-linked aptamer- assay for EGFR in buffer and serum. The aptamer EGFR_A15 selected in this study can potentially be used as a recognition element for the development of any assays for the detection of EGFR protein in cancer and other diseases wherever the EGFR expression are dysregulated.

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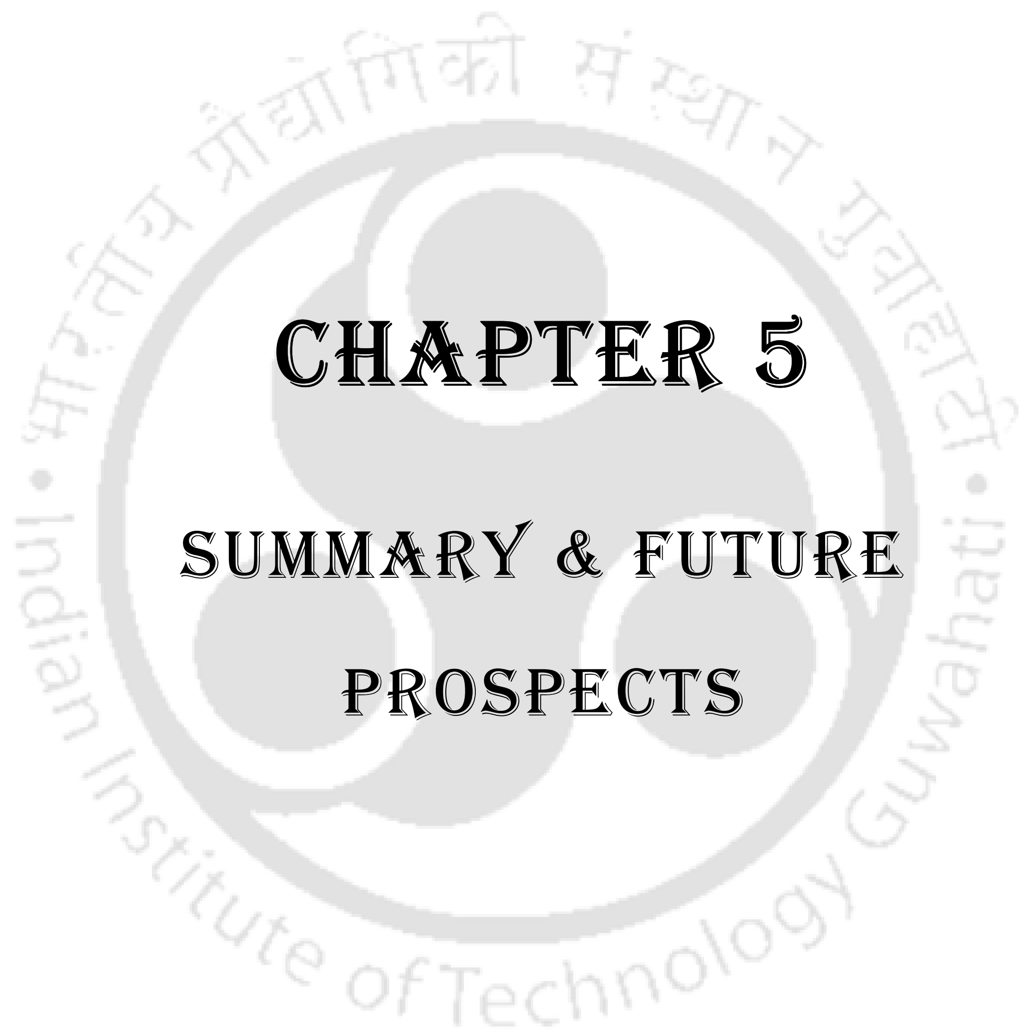

Chapter 5

Summary & Future Prospects

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Summary & Future Prospects

The Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein, which is often considered as an important biomarker for the diagnosis, detection and therapy for various human malignancies and illness. EGFR was the first receptor to be proposed as a target for cancer therapy and after two decades of intensive research, there are several anti-EGFR agents available in the clinic. EGFR has been implicated in the development of a wide range of epithelial cancers, including those of the breast, colon, head and neck, kidney, lung, pancreas, and prostate. Deregulation of EGFR often correlates with decreased disease-free and overall survival of the patients. The EGFR overexpression were also found in multiple diseases like inflammatory, cardiovascular, kidney, lungs, liver , skin, age related diseases etc (Jost et al., 2000; Komposch and Sibilia, 2016; Makki et al., 2013; Vallath et al., 2014). Aptamers binding to the EGFR with high affinity and specificity could be very useful in cancer diagnosis and therapy and also in other diseases wherever EGFR is overexpressed or dysregulated.

The present work demonstrates the cloning, expression and purification of extra cellular domain (ECD) of EGFR for aptamer selection. The primary objective of the work is to develop and characterize highly specific DNA aptamers for the detection of EGFR. The extra cellular domain of EGFR were cloned into bacterial expression vector pET 28a and expressed and affinity purified from *E. coli* by methods optimized in this study. After careful confirmation of the authenticity of the clones by restriction digestion and sequencing, the identity of the subsequently purified recombinant proteins was validated by Western blot analysis. For *in vitro* selection (SELEX) of aptamers, the recombinant ECD proteins were immobilized on NC membrane. The protein SELEX methodology was carried out from an initial pool of 10^{13}- 10^{15} ss DNA library with a 40 nucleotide long central block of randomized sequence. A total of eighteen rounds of selection were performed, of which 4 rounds were Negative SELEX and remaining 14 rounds were protein SELEX. Based on sequence homology aptamers were grouped into five homologous families. The most abundant aptamer sequences or aptamer with unique complex secondary structure, representing each homologous family *viz* EGFR_A1, EGFR_A7, EGFR_A15, EGFR_A13 and EGFR_A16 were synthesized with either FAM or Biotin labelling at 5′ end to test their interaction with EGFR. The binding abilities of

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the selected sequences to target were evaluated either by flow cytometry or Enzyme Linked Aptamer assay (ELAA).

The specificity of aptamers EGFR_A1, EGFR_A7 and EGFR_A15 were established by binding studies of selected aptamers by flow cytometry in A431, MDA MB 231, A549, U87-MG, Hela and Jurkat cell-lines. The newly developed aptamers EGFR_A1, EGFR_A7 and EGFR_A15 were found capable of binding to EGFR expressing cancerous cells. These aptamers can bind to full length EGFR as well as deletion mutant of EGFRvIII. Also aptamers were able to detect the different levels of EGFR expression in cell lines. Among the three aptamers, EGFR_A15 was a strong binders as compared to others. Next, the potential of these aptamers as imaging probe was evaluated by immunocytochemistry. The aptamers EGFR_A1 and EGFR_A7 appeared to be localized within the nuclei of cells while the aptamer EGFR_A15 was found binding to cell surface of A431 and MDA MB 231 cell lines. Thus only EGFR_A15 was chosen for further research. Two more aptamers EGFR_A13 and EGFR_A16 belonging to group V and I respectively were also chosen for further studies. The three aptamers EGFR_A15, EGFR_A13 and EGFR_A16 were synthesized with a biotin modification at 5′ end. Then the kd values of the selected aptamers were determined by iELAA. EGFR_A16 showed very weak binding with the target. The K_d values of EGFR_A15 and EGFR_A13 were 12.31 ± 2.59 nM and 15.59 ± 6.02nM respectively. Then Dot Blot assay was performed using above three aptamers. Aptamer EGFR_A15 was found to detect upto 2.5ng of spotted EGFR ECD proteins while EGFR_A13 was detecting a 10 fold lower amount of spotted protein (25ng). EGFR_A16 was found detecting 250ng of protein but with very weak signal. Therefore, it was evident that the aptamer EGFR_A16 was a very weak binder. Thus it was not used further for any application studies.

In vitro cell culture model was employed to ascertain the therapeutic potential of the selected aptamers by assessing its anti-proliferative effects by MTT assay and anti-migratory effect by scratch assay against MDA MB 231 cell lines. All the selected aptamers were found to be biocompatible in MTT assay and no effect on the proliferation of cells were observed. It could be used as drug delivery vehicle. Further in *in vitro* scratch assay, aptamer EGFR_A15, EGFR_A13 and non-enriched library demonstrated no effect on the closure of the gap. However, two aptamer candidates EGFR_A1 and

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EGFR_A7 exhibited a significant delay in the migration of cells. These two aptamers might be used as an agent for inhibition of migration of cancerous cell. However, we believe that this assay was not sufficient enough to proof the effect of sequences on cell migration. The *in vitro* cell scratch assay is a very preliminary experiment and further experiments like cell invasion assay using Trans wells/ Boyden chambers and in animal models are required.

Based on all studies, two aptamers EGFR_A15 and EGFR_A13 were found to have the best recognition ability. Further the usability of the best binding aptamers as a capture agent in microtiter based assay was demonstrated. The results aim towards the development of another detection assay for quantifying EGFR protein. The lead aptamer sequences EGFR_A15 and EGFR_A13 was subsequently used to develop an indirect Enzyme Linked aptamer assay. Different parameters such as the concentrations of aptamer (1nM, 5nM, 20nM and 50nM) and the amount protein were optimized to find out the optimum concentrations of reagent required. It was found that the lower concentration of aptamers gives better signal and the optimized working concentration of EGFR_A15 was as low as 1nM. The optimized assay condition were validated using cellular extracts of A431, MDA MB 231 and Jurkat cell lines. The aptamer also successfully detect EGFR in EGFR spiked serum sample. The developed EGFR specific aptamer were able to generate detectable signal even in the presence of EGFR specific antibodies, myriads of cellular components and contaminating proteins of serum. The data presented herein represent preliminary proof-of-principle for an indirect enzyme-linked aptamer- assay for EGFR in buffer and serum.

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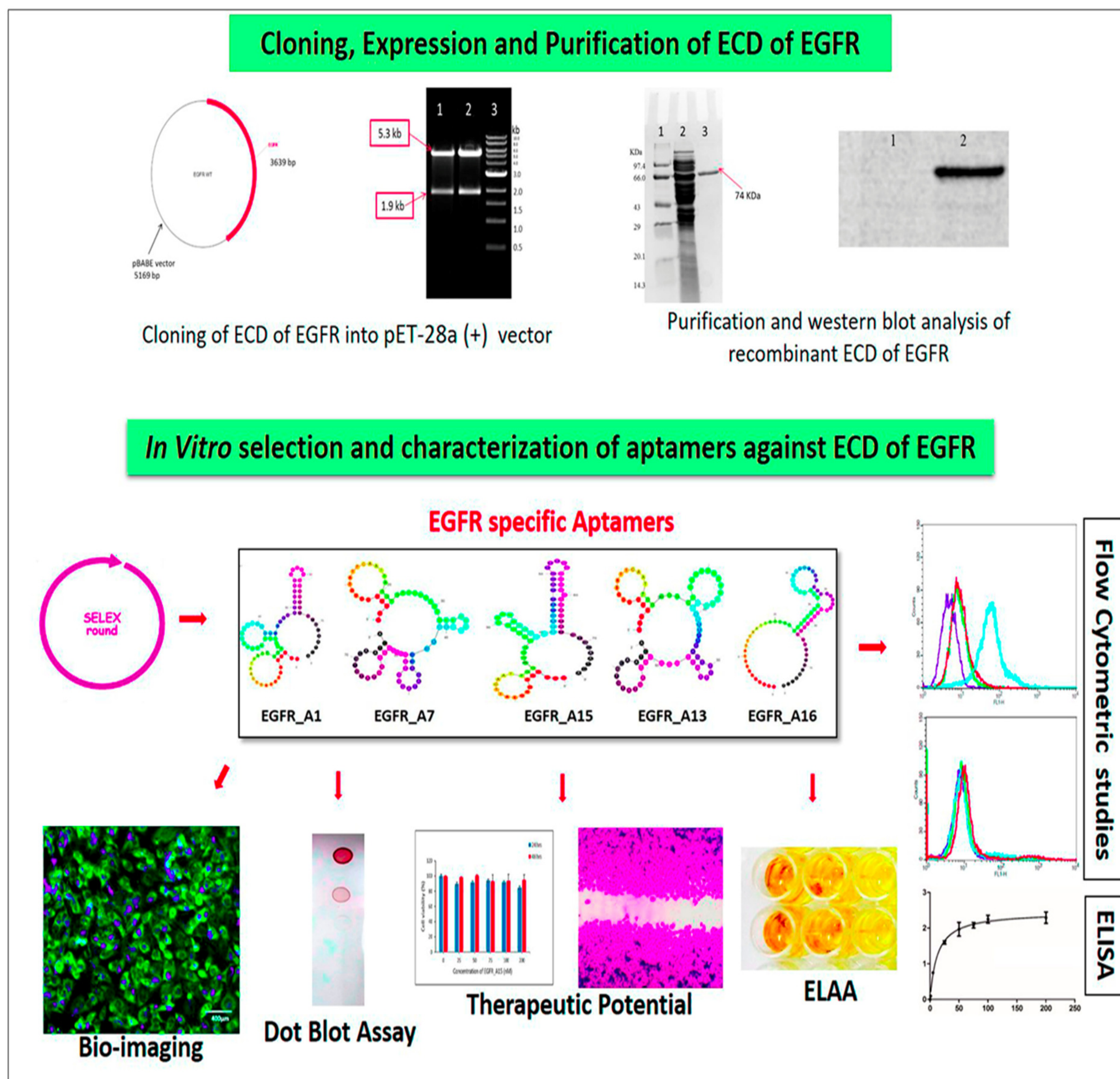

Figure 5.1: Summary of the thesis work. The figure is a schematic representation of the concluding experiments carried out in the thesis. The Extra cellular domain (ECD) of EGFR was successfully cloned, expressed, purified for aptamer selection. After 18 rounds of SELEX a panel of EGFR specific DNA aptamers were selected. Binding of selected aptamers were evaluated either by flow cytometric studies or ELISA. Further the application of aptamers as a molecular probe in different bioassays were explored.

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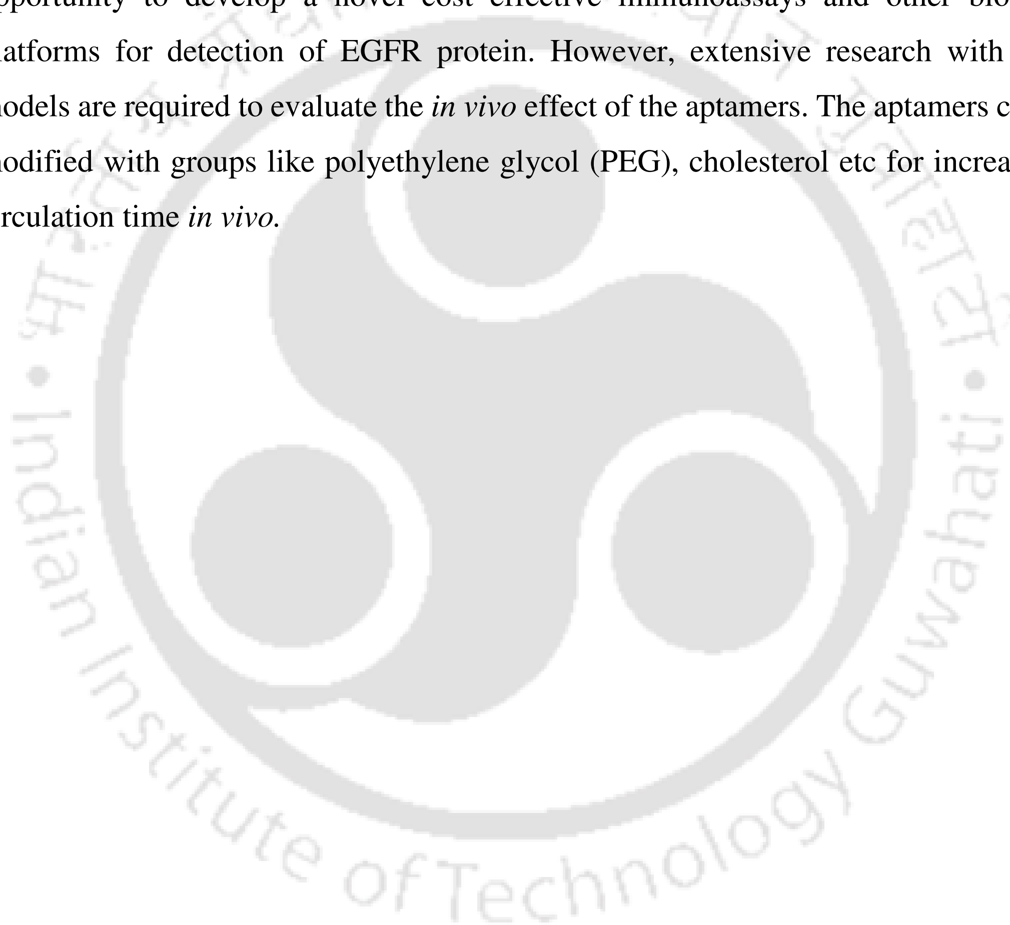
Conclusively it can be mentioned here that a panel of DNA aptamer binding specifically to EGFR protein were generated and their application in various biomedical research was assessed. Based on all the studies the aptamer EGFR_A15 was found to have the best recognition and selectivity. This aptamer holds a great potential to become a molecular imaging probe for the detection of EGFR expression in cancer and other diseases. Therefore, in future, we envisage it can be exploited for the development of a non-invasive diagnostic module for the detection of EGFR expression in cancer or any other diseases wherever EGFR expression is dysregulated. Furthermore it can also provide an opportunity to develop a novel cost effective immunoassays and other biosensing platforms for detection of EGFR protein. However, extensive research with animal models are required to evaluate the *in vivo* effect of the aptamers. The aptamers could be modified with groups like polyethylene glycol (PEG), cholesterol etc for increasing its circulation time *in vivo.*



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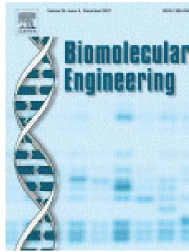

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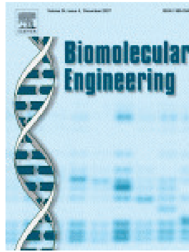

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