Studies on the Role of NGALR in Triple-Negative Breast Cancer

A thesis submitted for the degree of

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To

INDIAN INSTITUTE OF TECHNOLOGY, GUWAHATI

By

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Dedicated to

MY PARENTS

To the two who gave me life and the one who made me DREAM…

DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING
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DECLARATION

I hereby declare that the contents of the research work described in this thesis titled **"Studies on the Role of NGALR in Triple-Negative Breast Cancer"**, is a presentation of my original research work carried out in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, under the supervision of Prof. Ajaikumar B. Kunnumakkara.

Sincere efforts have been made to duly acknowledge the contributions from others for their ideas, technical help, references or any other help which may be involved in the completion of this thesis work.

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CERTIFICATE

This is to certify that the work described in the thesis titled **"Studies on the Role of NGALR in Triple-Negative Breast Cancer"**, submitted by Krishan Kumar Thakur (Roll no: 156106011) to Indian Institute of Technology Guwahati, India, for the award of the degree of Doctor of Philosophy is an authentic record of the research work carried out under my supervision in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, India.

This thesis or any part thereof has not been submitted elsewhere for award of any other degree or diploma.

24 May, 2021

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Krishan Kumar Thakur

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Abstract

According to GLOBOCAN 2020, breast cancer is the most common cancer worldwide including both men and women. Among the various sub-types, triple-negative breast cancer (TNBC) is reported as the most aggressive breast cancer sub-type and approximately 12-27% of all breast cancer cases are TNBC. Surgery, radiation, and chemotherapy are the only suitable options for the treatment of TNBC, however, the adverse effects limit their uses. Recent studies have reported that iron is an essential component for cancer cell growth, cellular respiration, oxygen transport, heme synthesis, and nucleic acid synthesis. Various studies have reported an iron transporter, also known as neutrophil gelatinase-associated lipocalin receptor (NGALR), which is associated with numerous cancers and its dysregulation is linked with cancer cell proliferation, survival, invasion, and metastasis. However, its role has not been deciphered in TNBC to date. Our study showed that NGALR was overexpressed in breast cancer tissues and associated with poor overall survival. Further, we observed significant overexpression among various sub-types of breast cancer including TNBC. We have also reported that NGALR was significantly upregulated in TNBC cell lines compared to normal cells. Moreover, TNF-α and TNF-β promoted TNBC cell proliferation, survival, epithelial-mesenchymal transition (EMT), and migration by increasing the expression of NGALR. However, knockdown of NGALR decreased the proliferation, survival, invasion, EMT, migration, and angiogenesis and induced autophagy by inhibiting Akt/mTOR and JAK/STAT pathways in TNBC cell lines. Therefore, NGALR could be used as a novel therapeutic target for the treatment of TNBC patients. However, further *in vivo* and clinical studies are required to validate these findings.

Chapter 1

Introduction and Review of Literature

1.1. Introduction

Breast cancer is the most common cancer worldwide including both males and females (Ferlay et al., 2020; Thakur et al., 2018; Schmid et al., 2020; Kumar et al., 2013; Anastasiadi et al., 2017). As per the report of Global Cancer (GLOBOCAN) 2020, 2.3 million (11.7%) new cases and 0.68 million (6.9%) deaths of breast cancer were registered in both sexes in a year globally, despite the significant advancements in research and innovation in this field (Figure 1.1). On average, one in four cancer cases and one in six deaths among women is due to breast cancer (Sung et al., 2021; Ferlay et al., 2020). Breast cancer incidences, mortality, and 5-year prevalence in both sexes are highest in Asia than in other parts of the world (Ferlay et al., 2020; Bray et al., 2018). In India, breast cancer reported 1.6 lakhs (14%) cases in both males and females, while approximately 27.7% cases in females alone in 2018 (Thakur et al., 2018; Bray et al., 2018; Dydjow-Bendek D & Zagozdzon P, 2020).

Breast cancer is considered as a heterogeneous disease, and depending on the expression of various receptors, it is categorized into different sub-types. Among the various sub-types of breast cancer, triple-negative breast cancer (TNBC) is reported as the most aggressive breast cancer sub-type (Dai et al., 2016; Joensuu et al., 2013; Abubakar et al., 2019). Pathologically, the major prognostic hallmarks of breast cancer, which includes estrogen receptor (ER) progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), are absent in TNBC sub-types (Alwan NAS & Tawfeeq FN, 2019; Dai et al., 2016; Fragomeni et al., 2018). TNBC has poor overall survival (OS) and high recurrence than any other sub-types (Reddy et al., 2018; Zhu et al., 2020). It causes approximately 12-27% of all breast cancer cases and is most prevalent among younger women (<45 years) (Thakur et al., 2018; Wahba HA & El-Hadaad HA, 2015). TNBC is reported as the most aggressive sub-types of breast cancer

as it lacks the presence of major three receptors (ER, PR, and HER2); therefore, conventional breast cancer therapies that target these hormonal receptors are not helpful in the treatment of TNBC (Bergin ART & Loi S, 2019; Yao et al., 2017; Al-Mahmood et al., 2018). Currently, surgery, radiation, and chemotherapy are the only suitable options for the treatment of TNBC. However, the adverse effects (such as chemoresistance, tumor recurrence, and radioresistance) limit their use (Doval et al., 2020; Bergin ART & Loi S, 2019; Wahba HA & El-Hadaad HA, 2015; Feng et al., 2018). Therefore, there is an urgent need to identify important biomarkers and therapeutic targets that would help in the regulation of TNBC.

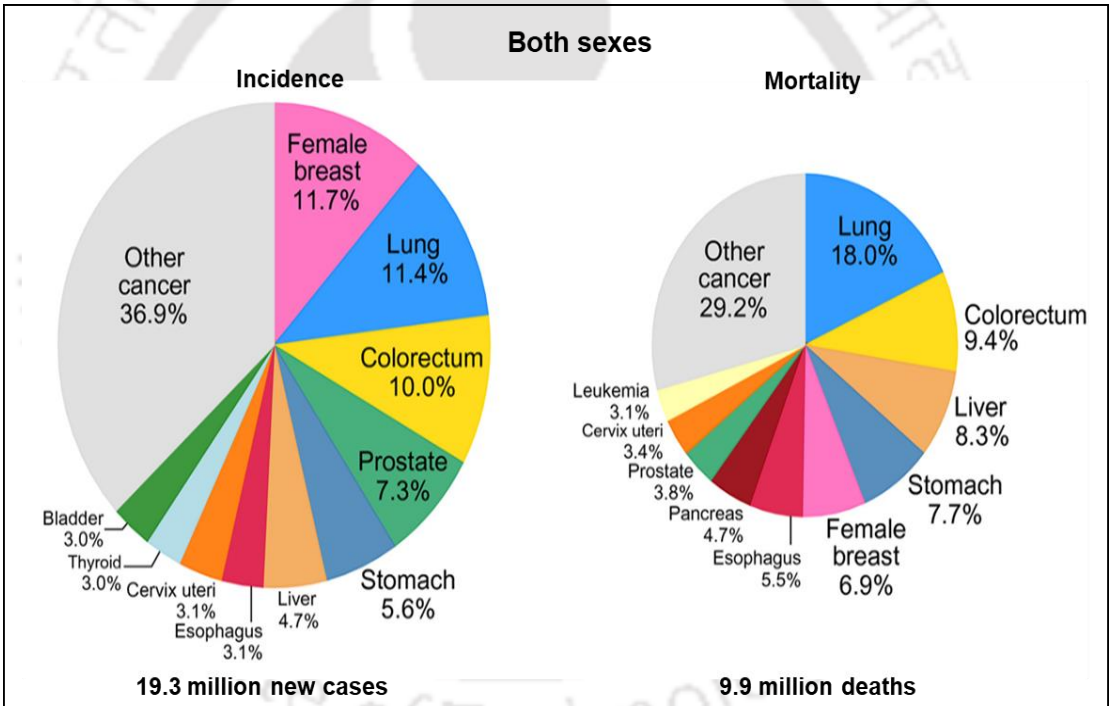

Figure 1.1. Estimated age-standardised incidence and mortality rates, both sexes, all ages, worldwide (GLOBOCAN 2020).

Recent studies have reported that dysregulation in iron homeostasis is associated with the proliferation and survival of cancer cells (Lelievre et al., 2020; Jung et al., 2019). Iron is an essential component for cancer cell growth, cellular respiration, oxygen transport, heme synthesis, and nucleic acid synthesis (Chen et al., 2019; Steegmann-Olmedillas JL, 2011; Jung et al., 2019). Therefore, regulation of iron

acquisition, storage, and export represent opportunities for novel approaches for cancer treatment. Interestingly, various studies have reported an iron transporter, also known as neutrophil gelatinase-associated lipocalin receptor (NGALR), and its association with various cancers, such as renal cell carcinoma (RCC), hepatocellular carcinoma (HCC), oesophageal squamous cell carcinoma (ESCC), glioma, and lymphocytic leukemia (Wei et al., 2020; Liu et al., 2018; Zhang et al., 2012a; Tan et al., 2014; Du et al., 2011; Liu et al., 2011; Bauvois et al., 2020). The dysregulation of NGALR is linked with the proliferation, survival, invasion, and metastasis of cancer cells (Wei et al., 2020; Santiago-Sanchez et al., 2020). However, the potential role of NGALR in the progression of TNBC is not reported to date. Moreover, the underlying molecular mechanism of NGALR in the development of TNBC needs to be unraveled. Therefore, our study focused on the potential role and association of NGALR in TNBC pathogenesis.

1.2. Sub-types of breast cancer

Breast cancer is classified into various sub-types based on numerous parameters, such as heterogenicity, presence of various genes and proteins, extrinsic and intrinsic classification. Among these various classifications, 'intrinsic' sub-types of the breast is widely accepted and is best-characterized molecular classification (Howlader et al., 2014; Anderson et al., 2014; Dai et al., 2015; Seo et al., 2020; Kunheri et al., 2020). According to intrinsic classification, breast cancer is categorized into five sub-types, which includes luminal A, luminal B, HER2-enriched, triple-negative or basal-like, and normal-like breast cancer (Yeo et al., 2017; Lehmann et al., 2011; Puppe et al., 2020; Odle TG, 2017). However, based on the presence/absence of hormone receptors (HRs), breast cancer can be categorized mainly into four sub-types (Figure 1.2). Among them, two are HR-positive sub-types (luminal A and B), and two are HR-negative sub-types

(HER2-enriched and triple-negative/basal-like) (Anderson et al., 2014; Waks AG & Winer EP, 2019; Howlader et al., 2014; Harbeck N & Gnant M, 2017).

1.2.1. Luminal A

It is the most common sub-types of breast cancer and represents approximately 50-55% of all breast cancer. This sub-type is characterized by the presence of ER and PR, while HER2 is absent. Luminal A tumors are usually associated with a lower grade and reduced expression of proliferative genes (CCNB1, CCNE1, CCND1, MKi67, and MYBL2), while higher expression of ER-associated genes (such as Bcl-2, CCND1, GATA3, and FOXA1) (O'Brien et al., 2010; Clarke et al., 2012; Harbeck et al., 2019; Al-Thoubaity FK, 2019; Bernhardt et al., 2016). Also, luminal A tumors are more common in postmenopausal women than premenopausal women (Saha et al., 2016; Ellingjord-Dale et al., 2017). When compared with other breast cancer sub-types, luminal A tumors have a better prognosis; therefore, the disease-free survival (DFS) and OS rates are high. Moreover, luminal A breast cancers are well documented to respond well to hormonal therapies. (Fragomeni et al., 2019; Hennigs et al., 2016; Tubtimhin et al., 2018; Harbeck N & Gnant M, 2017).

1.2.2. Luminal B

This sub-type is positive for ER and HER and may or may not be positive for PR. It is more aggressive and tends to have higher-grade tumors as compared to luminal A tumors. It represents approximately 10-15% of all breast cancer, with a worse prognosis than luminal A tumors (Li et al., 2016; Hashmi et al., 2018; Jaaskelainen et al., 2020; Bernhardt et al., 2016). Luminal B tumors are most commonly observed in premenopausal women than postmenopausal women. Moreover, Luminal B cancers display a higher expression of proliferative genes than ER-related genes. Hormonal

therapies and adjuvant chemotherapy are the most suitable combination for the treatment of luminal B tumors. Luminal B tumors also have high recurrence scores and relapse compared to luminal A sub-types (Dai et al., 2015; Tubtimhin et al., 2018; Al-Thoubaity FK, 2019; Fisusi et al., 2019).

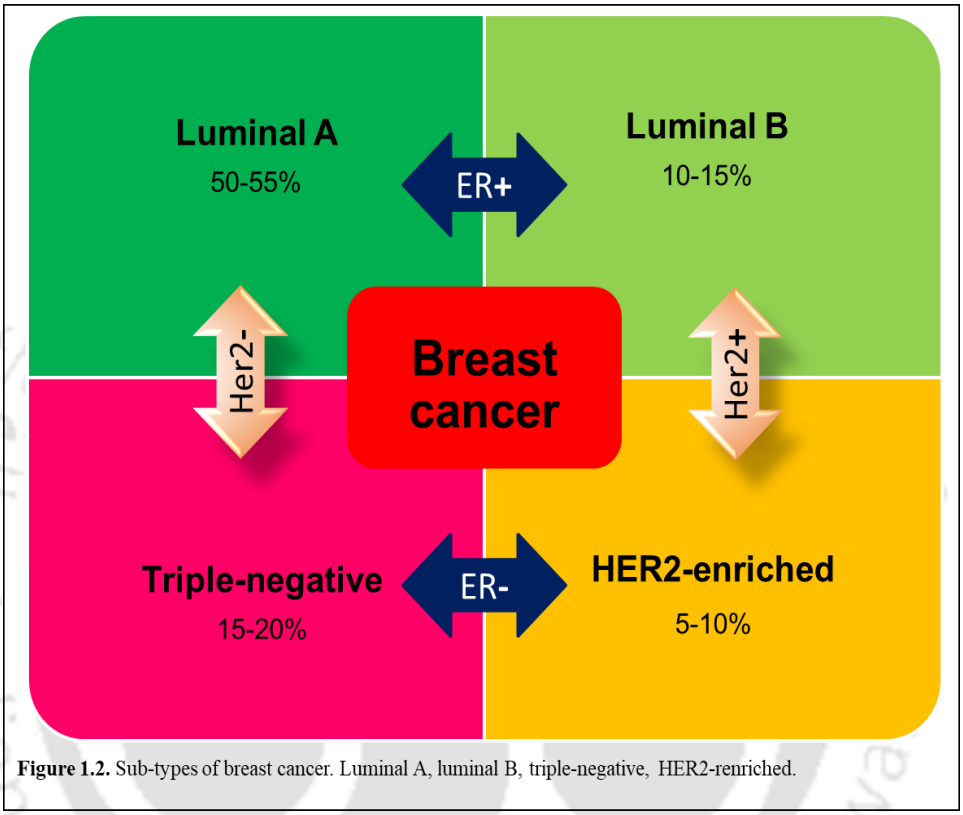

Figure 1.2. Sub-types of breast cancer. Luminal A, luminal B, triple-negative, HER2-renriched.

1.2.3. Triple-negative/basal-like

Basal-like breast cancers mimic the basal epithelial cells and normal breast-myoepithelial cells of other parts of the body. Basal-like breast cancers are having a higher expression of basal genes (such as EGFR, keratins 5, 6, 14, and 17) and proliferative genes. Approximately 60-90% of basal cancer cases are TNBC (Alluri P & Newman LA, 2014; Dai et al., 2015; Lachapelle J & Foulkes WD, 2011; Jezequel et al. 2019).

TNBCs are the most aggressive sub-types of breast cancer with higher grade (grade II-III) invasive ductal carcinomas and represent 15-20% of all breast cancer

(Thakur et al., 2018; Geyer et al., 2017). It mainly affects the women who are likely to reach menarche at an early age and younger age premenopausal women (Dai et al., 2015; Yen et al., 2012; Thakur et al., 2018) Hamy et al., 2019). TNBC is significantly linked with tumor protein P53 (TP53) somatic mutations and breast cancer susceptibility gene 1 (BRCA1) germline mutations. TNBCs have larger size tumors (>2 cm) with rapid tumor growth than the other sub-types of breast cancer. Moreover, TNBC tumors have higher metastatic properties and are more likely to metastasize to the brain, bone, and lungs (Lachapelle J & Foulkes WD, 2011; Shah et al., 2012; Geyer et al., 2017).

Table 1. Various sub-types of TNBC.

TNBC	Percentage	Functions
Base-Like 1 (BL1)	10	Cellular proliferation and response to cellular damage
Base-Like 2 (BL2)	20	Growth factor signaling with myoepithelial markers
Immunomodulatory (IM)	20	Signaling mediated by immune synapsis
Mesenchymal (M)	20	Mesenchymal epithelial transition (MET) and differentiation
Mesenchymal stem-like (MSL)	10	MET, differentiation and stem cell potential; angiogenesis and growth factor signaling
Luminal androgen receptor (LAR)	10	Hormone signaling mediated by androgen receptor
Unstable	10	Cellular proliferation and response to cellular damage

TNBC is the most heterogeneous sub-type characterized by various categories, such as clinical, morphological, genetical, proteomic, transcriptomic, epigenomic, and microenvironmental (Garrido-Castro et al., 2019; Aleskandarany et al., 2018). TNBC can be further divided into six sub-types (Table 1); basal-like (BL) 1, BL2, immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), luminal androgen receptor (LAR), and one unstable sub-type (Zhao et al., 2020; Wang et al., 2019; Jiang et al., 2019; Garmpis et al., 2020; Lehmann et al., 2011; Mayer et al., 2014).

TNBC sub-types are associated with worse prognosis, OS, and DFS compared to other breast cancer sub-types. It has also been associated with a high risk of recurrence with 3-5 years of survival (Ovcaricek et al., 2011; Fayaz et al., 2019; Zhu et al., 2020). Conventional targeted therapies are not amenable in TNBC sub-type as it is negative for ER, PR, and HER2. Therefore, surgery and chemotherapy are the only therapeutic armamentarium against this fatal disease (Wahba HA & El-Hadaad HA, 2015; Sharma P, 2018; Heimes AS & Schmidt M, 2019; Lyons TG, 2019).

1.2.4. HER2-enriched

HER2-enriched sub-type represents 5-10% of all invasive breast cancers and is negative for both ER and PR while positive for HER. It is characterized by the TP53 mutation (40-80%) and activating mutations of the p110α subunit of phosphoinositide 3-kinase (PI3K) with high expression of various genes, such as post-GPI attachment to proteins 3 (PGAP3), growth factor receptor-bound protein 7 (GRB7) and epidermal growth factor receptor (EGFR). HER2-enriched tumors are more likely to be associated with grade III tumors (Dai et al., 2015; Prat et al., 2017; Fragomeni et al., 2018; Prat et al., 2020). The association of HER2-enriched tumors with age is still unknown; however, it has a poor prognosis. HER2-enriched tumors can be treated with taxane-based neoadjuvant chemotherapy, anthracycline, and HER2 targeted therapies (such as lapatinib, trastuzumab, pertuzumab) (Figueroa-Magalhaes et al., 2014; Brandao et al., 2018; Waks AG & Winer EP, 2019).

1.3. Tumor, lymph node, and metastasis (TNM) staging of breast cancer

Breast cancer stages are determined by the tumor's characteristics, such as tumor size and the presence of HRs. Breast cancer staging helps in the determination of breast cancer prognosis, treatment options, allows more precise clinical trials, and most likely

predict the outcome of the disease. Staging can be either clinical or pathological. Clinical staging is done before the surgery and depends on the results of various tests, such as physical examinations, ultrasound, mammogram, and magnetic resonance imaging (MRI) scans. In contrast, pathological staging is dependent on the surgery after removing breast tissue or lymph node. The pathological staging provides significant information about the prognosis of patients

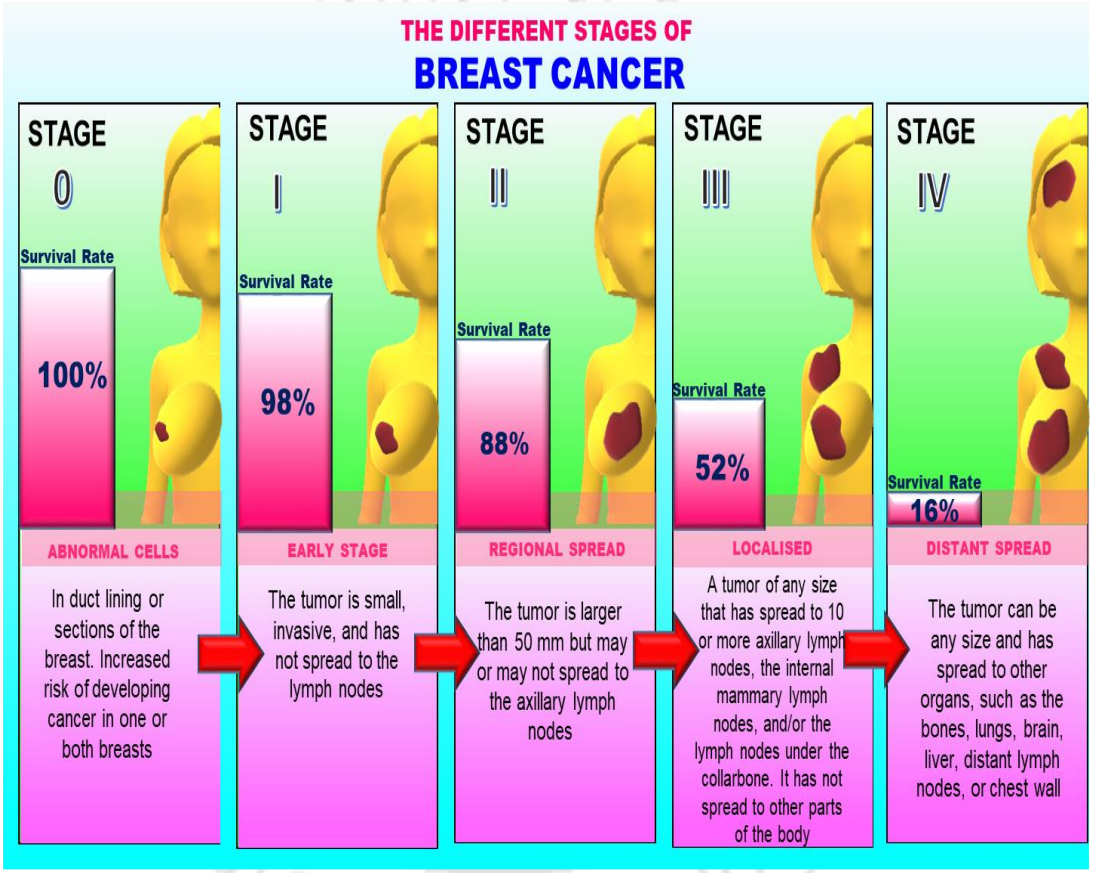

Figure 1.3. The different stages of breast cancer. Stage 0, Stage I, Stage II, Stage III, Stage IV.

The stages are more often expressed as a number on a scale of zero to four (0-IV) (Figure 1.3). Stage 0 denotes non-invasive tumors which are remained at their original location, while stage IV indicates invasive tumors, which are metastasized from the breast to the other organs of the body (Maughan et al., 2010; Giuliano et al., 2017; Li et al., 2015; Feng et al., 2019; Koh J & Kim MJ, 2019).

The breast cancer staging is described by the TNM staging system, which is supervised by the American Joint Committee on Cancer (AJCC). It is a group of experts in cancer who regularly updated and oversee how cancer can be categorized and communicated (Cserni et al., 2018; Sawaki et al., 2019; Edge et al., 2019; Hortobagyi et al., 2018).

- **T-** primary tumor site
- **N-** regional lymph node involvement
- **M-** presence or otherwise of distant metastatic spread

In 2017, a new version (eighth edition) of the TNM staging system was revised and published. According to the revised edition, the disease stage is defined and the TNM categories are assigned at the time of diagnosis. Also, the clinical stage is determined depending on physical examination and imaging studies. Moreover, the pathological stage is examined after the surgery (Koh J & Kim MJ, 2019; Sawaki et al., 2019; Giuliano et al., 2018; Chippa V & Barazi H, 2020).

1.4. Risk factors of breast cancer

Several studies have demonstrated the risk factors that lead to the progression of breast cancers (Figure 1.4), which includes gender, age, physical activity, diet, weight, smoking, alcohol consumption, excessive exposure to estrogen, frequent use of oral contraceptive, family history, race, pregnancy, breastfeeding, genetic factors stress, and anxiety. (Momenimovahed Z & Salehiniya H, 2019; Jeronimo et al., 2017; Sun et al., 2017; Feng et al., 2018; Barnard et al., 2015; Bray et al., 2018). The various risk factors which are associated with breast cancer progression are discussed below:

1.4.1. Gender

Most of the breast cancer cases (>99%) appear in females, while only very few cases (<1%) of all the breast cancers are reported in males. Therefore, being a woman itself is a risk factor for breast cancer. Breast cells are regularly growing and changing primarily due to the effect of the female hormones (such as estrogen and progesterone), which put them at significant risk of breast cancer (Sharma et al., 2010; Abdelwahab Yousef et al., 2017)

1.4.2. Age

It is one of the most common risk factors for breast cancer. Various studies have reported that women with age groups between 40 to 60 years are more likely to get breast cancer (Siegel et al., 2017; Kumar et al., 2013; Akram et al., 2017). In America (2016), approximately 99% of breast cancer-related deaths were reported in 40-60 years and 71% of deaths over 60 years old women (Siegel et al., 2017).

1.4.3. Weight

Several studies have reported the strong link between overweight and postmenopausal condition of women (Momenimovahed Z & Salehiniya H, 2019; Kim et al., 2015; Bravi et al., 2018). Adipose tissues are primary sources of estrogen production in obese and postmenopausal women. On the other hand, obesity stimulates insulin and insulin-like growth factor (IGF) levels, helping in cancer progression. Thereby, having more fatty tissue will lead to high estrogen levels that can significantly increase breast cancer risks (Kerlikowske et al., 2016; Miller et al., 2018; Rosner et al., 2017; Chen et al., 2016).

1.4.4. Diet

Diet is also one of the important risk factors for breast cancer development. Emerging studies are finding the link between diet and risk of breast cancer (Kotepui M, 2016; Holmes MD & Willett WC, 2004). Various studies suggested that a diet with high fat

increases breast cancer risk, while a diet containing fibrous protein and less fatty acid decreases breast cancer risk (Jeronimo et al., 2017; Dydjow-Bendek et al., 2019; Thakur et al., 2018). However, a controversial relationship was observed between the consumption of caffeine (tea and coffee) and the risk of breast cancer. Some studies have reported that coffee consumption decreased breast cancer risk, while other studies have pointed out the opposite effect (Momenimovahed Z & Salehiniya H, 2019; Yaghjyan et al., 2018; Boggs et al., 2010).

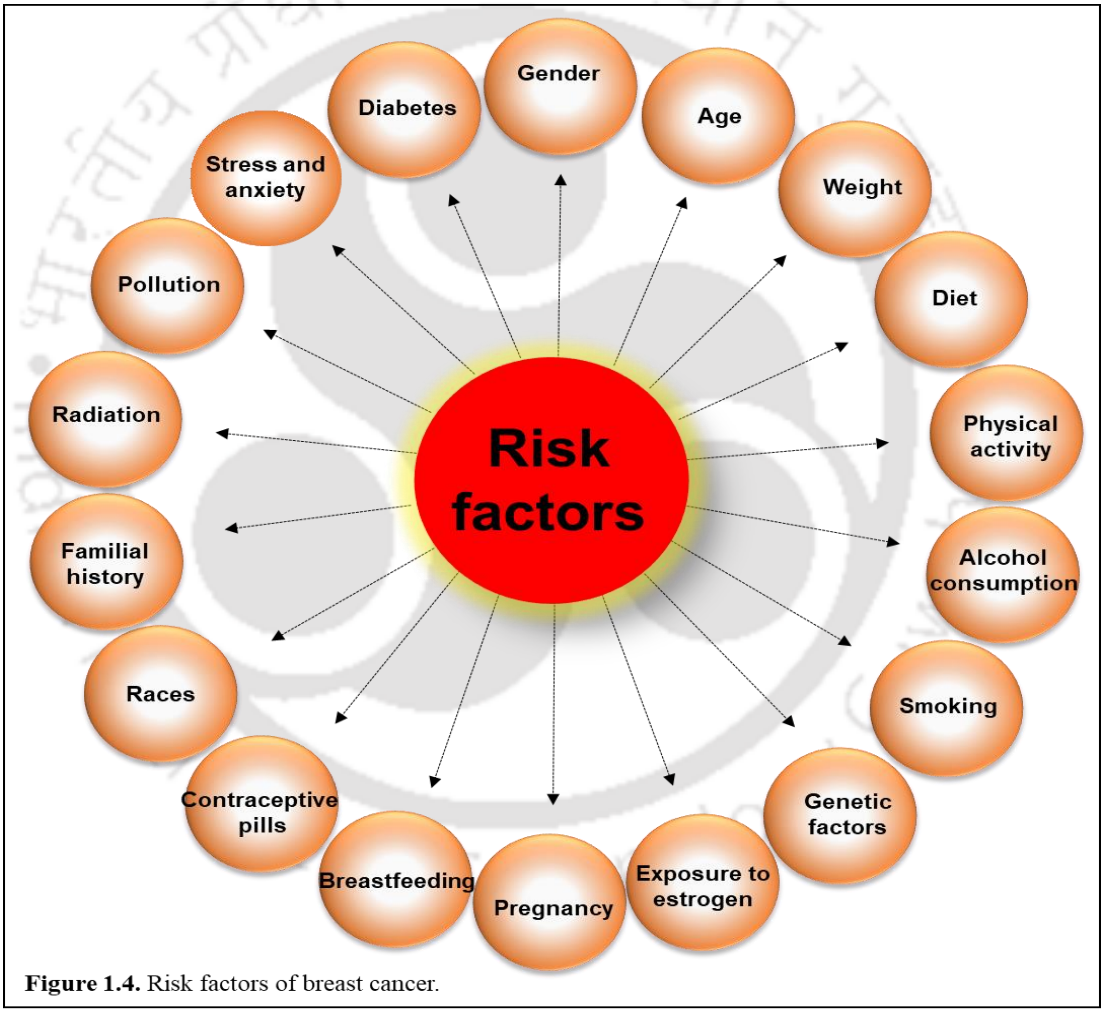

Figure 1.4. Risk factors of breast cancer.

1.4.5. Physical activity

Mounting pieces of evidence suggested that increased exercise can decrease the risk of breast cancer development (Graf C, Wessely N, 2010; Holmes et al., 2005). According to the American Cancer Society (ACS), 45-60 minutes of exercise for 4-5 days in a

week can decrease breast cancer risk. Engaging in physical activity can reduce the mortality and morbidity of breast cancer cases (Holmes et al., 2005; Holick et al., 2008; Feng et al., 2018; Abdelwahab Yousef et al., 2017).

1.4.6. Alcohol consumption and smoking

Various studies have addressed the role of alcohol as a carcinogen and its link with breast cancer progression. Alcohol has a potential role in the marinating blood estrogen level via limiting the ability of the liver, thereby can increase breast cancer risk (Miller et al., 2018; Vieira et al., 2018; Mannell A, 2017; Sun et al., 2017; Zhao et al., 2017). Smoking can cause a small increase in breast cancer risk. However, active smoking, especially in prenatal and postmenopausal women, is related to a high risk of breast cancer (Momenimovahed Z & Salehiniya H, 2019; Luo et al., 2011).

1.4.7. Genetic factors

Various mutations in genes, such as BRCA1, BRCA2, TP53, and matrix metalloproteinase (MMP-2 c-735-T), are associated with high breast cancer risk. However, mutations in the BRCA1 and BRCA2 genes are associated with approximately 40% of hereditary breast cancer cases. Moreover, a mutation in MMP-2c gene increase the breast cancer risk by 1.64-fold in younger age women (Feng et al., 2018; Yari et al., 2014; Cobain et al., 2016).

1.4.8. Exposure to estrogen

Estrogen hormone stimulates the growth of breast cells in women; thereby, long-term exposure to estrogen hormone makes them prone to breast cancer risk. Factors ~~which~~ that can stimulates the estrogen hormone level include hormone replacement therapy, alcohol, obesity, estrogens in the environment (meat and pesticides), starting menstruation cycle at an early (<12 years) age, reaching menopause at an older (>50

years) age, and (Rojas K & Stuckey A, 2016; Akram et al., 2017; Momenimovahed Z & Salehiniya H, 2019; Barnard et al., 2015).

1.4.9. Pregnancy and breastfeeding

Emerging studies have demonstrated that the breast cancer risk decrease with increasing parity (Kim et al., 2015; Ma et al., 2010). Having the first childbirth at an older stage is the most potential reproductive risk factor of breast cancer. It is also evident that every delivery decreases the breast cancer risk. Pregnancy decreases the overall number of menstrual cycles, which helps in reducing breast cancer risk. Conversely, increased abortion incidences were associated with increased breast cancer risks (Sun et al., 2017; Momenimovahed Z & Salehiniya H, 2019; Bhadoria et al., 2013).

Various studies have reported the protective and preventive effects of breastfeeding against breast cancer risk in women (Anstey et al., 2017; Holm et al., 2017). The protective effect of breastfeeding increases with increasing periods of breastfeeding. On average, the breast cancer risk could reduce by up to 50% in women having two or more children with breastfeeding for more than 13 months (Holm et al., 2017; Jeronimo et al., 2017; Jeong et al., 2017).

1.4.10. Other factors

Besides the above-mentioned factors, various other factors are also contributed to increased breast cancer risks (Figure 1.4). Some of these factors include regular consumption of oral contraceptive pills, races (black, white, Hispanic, etc.), radiation therapy, personal or familial history of breast cancer, air pollution, diabetes, duration of sleep, stress, and anxiety (Feng et al., 2018; Jeronimo et al., 2017; Momenimovahed Z & Salehiniya H, 2019; Sun et al., 2017).

1.5. Sign, symptoms, and clinical features of breast cancer

Early diagnosis is critically important for a better prognosis and to reduce the mortality of breast cancer. Whenever someone demonstrates suspected breast cancer symptoms, they must be speed up the diagnostic procedures to prevent lymph node metastasis (Santen et al., 2017; Thulesius et al., 2004; Rahman et al., 2019; O'Grady S & Morgan MP, 2018). The vital sign and symptoms of breast cancer include a lump in the breast, swelling or thickening of the breast, breast pain and tenderness, nipple discharge, changes in the nipple (dimpled, pulls inward, itches, burns, sores, redness or flaky skin), breast changes (size, texture, contour, or temperature) and weight loss (Koo et al., 2017; Santen et al., 2017; Winters et al., 2017; Thulesius et al., 2004).

TNBC usually has a higher histologic grade, stromal content, elevated mitotic count, central necrosis, lymphocytic stromal response, pushing margins of invasion, and multiple apoptotic cells. Histologically, TNBC is largely ductal; however, other unusual pathophyisology are also observed, such as metaplastic, atypical or atypical medullary or adenoid cystic carcinomas (Irvin WJ Jr & Carey LA, 2008; Livasy et al., 2006; Geyer et al., 2017).

1.6. Molecular alterations in TNBC

TNBC has an enormous complex and diverse molecular and genetic profile, which challenges the researchers to focus more on these sub-types. The lack of therapeutic markers increases the hurdles in a bid to advance disease outcomes (Sporikova et al., 2018; Ciriello et al., 2015). Molecular alterations that lead to TNBC progression are discussed below.

1.6.1. TP53

It is one of the most crucial genes associated with maintaining genomic integrity and homeostasis throughout DNA repair, cell cycle arrest, and apoptosis in TNBC

(Sporikova et al., 2018; Silwal-Pandit et al., 2017). TP53 alterations stimulate aberrant p53 expression in all breast cancer sub-types. TP53 is the most repeatedly mutated (60-80%) gene in TNBC (Pop et al., 2018; Sporikova et al., 2018). The aberrant expression of mutated p53 gene stimulated cell proliferation, recurrence, and early death in TNBC. Increased metastatic risk and worse OS were also observed with decreased p53 function in TNBC patients. Moreover, TP53 mutation was found to be a predictor of chemoresistance in TNBC (Coates et al., 2012; Powell et al., 2016; Chae et al., 2009).

1.6.2. BRCA1 and BRCA2

BRCA1 and BRCA2 genes play a critical role in TNBC cell proliferation, survival, cell cycle arrest, and stimulation and transcriptional regulation of DNA damage. Importantly, BRCA1 and BRCA2 proteins display an important role in DNA repair (double-strand break) through homologous recombination mechanism and maintain the stability of the DNA (Roy et al., 2011; Blasiak et al., 2020; Denkert et al., 2017). BRCA1 gene mutation contributed primarily (80%) in the progression of TNBC, BRCA germ-line mutation contributed approximately 15%, while the remaining cases are shared by BRCA1/2 mutation (Engel et al., 2018; Pop et al., 2018; Wengner et al., 2020). Poly (ADP-ribose) polymerase (PARP) inhibitors are significantly effective in BRCA1/2 deficient patients (Plummer R, 2011; Bartsch et al., 2010).

1.6.3. PI3K Pathway

PI3K/Akt/mammalian target of rapamycin (mTOR) pathway is one of the important mechanisms associated with cell proliferation, survival, growth, and motility. PI3K propagates signals from the various growth factors and stimulates the Akt kinase. Stimulation of Akt causes the phosphorylation of mTOR that further triggers protein synthesis and cell growth (Bordoloi et al., 2020; Nedeljkovic M & Damjanovic A,

2019; Pop et al., 2018; Ciriello et al., 2015). Imbalance in the PI3K/Akt/mTOR pathway gives the cancerous cells a significant advantage for the progression of tumor. The PI3K/Akt/mTOR signaling pathway is negatively controlled by the tumor suppressor phosphatase and tensin homolog (PTEN) (Li L & Ross AH, 2007; Nedeljkovic M & Damjanovic A, 2019; Sporikova et al., 2018; Gonzalez-Angulo et al., 2009). Importantly, due to PTEN loss, PI3K/Akt/mTOR pathway is hyperactivated and responsible for aggressive tumors, adverse clinical effects, and poor outcomes in TNBC. Moreover, chemoresistance is also associated with PTEN loss (Inanc et al., 2014; Steelman et al., 2008).

1.6.4. Tyrosine kinase receptors

EGFR is significantly overexpressed in TNBC than the other breast cancer sub-types. It is expressed in approximately 64% of cases and can be considered as one of the crucial hallmarks of TNBC. EGFR and IGF-1 receptor (IGF-1R) is present at the upstream of PI3K/Akt/mTOR pathway and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) 3 pathway (Park et al., 2014; Nedeljkovic M & Damjanovic A, 2019; Nakai et al., 2016). It is reported that increased EGFR gene and IGFs expression are associated with a worse prognosis in TNBC. However, EGFR mutations are not very common; only 11% of cases were found EGFR mutated (Teng et al., 2011; Sporikova et al., 2018; Yuan et al., 2018).

1.6.5. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway

NF-κB pathway is an important modulator of TNBC through regulating apoptosis, angiogenesis, and inflammatory response. Various studies reported that NF-κB is overexpressed in TNBC tissues compared to normal breast tissue and contributed to

TNBC progression, chemoresistance, and poor prognosis (Ossovskaya et al., 2011; Fusella et al., 2017; Nedeljkovic M & Damjanovic A, 2019). Normally, IκB binds with dimer and keep them inactive. Once the signals are received from upstream, it activated the IκB kinase (IKK) complex and phosphorylated IκB, which further releases the NF-κB dimers. This NF-κB can freely move in the nucleus and stimulates the transcription of various genes (Fan et al., 2008; Fusella et al., 2017; Kunnumakkara et al., 2020).

1.6.6. JAK/STAT pathway

JAKs are cytoplasmic proteins, which are linked with transmembrane receptors. Once the upstream ligands, such as interleukin (IL)-6 and IL-8, bind with JAKs, trans-phosphorylation occurs, which further phosphorylate STAT monomers. The activated STAT enters the nucleus and modulates the transcription of various target genes (Guanizo et al., 2018). Dysregulation of the JAK/STAT pathway has been related to cell proliferation, survival, metastasis, angiogenesis, immune suppression, and anti-apoptosis. It is reported that IL-6 and IL-8 are overexpressed in TNBC and induces JAK/STAT signaling pathways (Nedeljkovic M & Damjanovic A, 2019; Guanizo et al., 2018; Furth et al., 2014).

1.7. Treatment modalities of TNBC

Treatment of TNBC is still challenging all around the world. Surgery and radiation, along with chemotherapy, is the prime treatment available for TNBC. However, in the past years, various advancements have been made for the treatment of this devasting disease (Denkert et al., 2017; Bartsch et al., 2010; Al-Mahmood et al., 2019). The recently available therapies for the treatment of TNBC includes hormonal therapy (aromatase inhibitors), immunotherapy, gene therapy (EGFR, VEGF), targeting DNA repair pathway (PARP inhibitors, platinum salts), PI3K/Akt/mTOR pathway inhibitors,

and antibody-drug conjugates (Vagia et al., 2020; Bergin ART & Loi S, 2019; Costa et al., 2018; Sporikova et al., 2018).

1.7.1. Surgery and radiation

Surgery is one of the best suitable treatments for the treatment of metastatic breast cancer. Once the surgery is over, one can choose radiation or chemotherapy, or hormonal therapies based on the cancer conditions on physicians' advice (Matter et al., 2000; Murphy et al., 2015; Feys et al., 2015). Surgery can be used alone or combined, depending on the patients characteristics or other clinical situations. Surgery can improve OS and reduce TNBC mortality by resecting metastases (brain, bone, lung, liver). Moreover, surgery can provide asymptomatic treatment and disabled various complications. However, in the early postoperative period, surgery can increase peripheral oxidative damage to macromolecules. On the other hand, radiation can cause high resistance and relapse (7-12%) among patients with five years. Therefore, combination therapy is a preferred modality to omit these drawbacks (Al- Mahmood et al., 2019; Murphy et al., 2015; Szychta et al., 2014).

1.7.2. Chemotherapy

Chemotherapy is the widely accepted therapy for the treatment of TNBC, especially in patients with a high risk of relapse. However, conventional chemotherapies are associated with severe side effects, resistance, and poor OS in TNBC patients (Nedeljkovic M & Damjanovic A, 2019; Cazzaniga et al., 2017; Bergin ART & Loi S, 2019). Most commonly used chemotherapy includes; anthracyclines (doxorubicin, epirubicin), taxanes (docetaxel, paclitaxel), carboplatin, gemcitabine, capecitabine, and vinorelbine. Most chemotherapies are used in combinations to overcome the side

effects and resistance in TNBC (Lyons TG, 2019; Garrido-Castro et al., 2019; Al-Mahmood et al., 2018).

1.7.3. EGFR inhibitors

EGFR is the potential target for TNBC treatment, with the patients having increased expression of the EGFR gene. Various EGFR inhibitors, such as cetuximab, gefitinib, erlotinib, and vandetanib, are used for the treatment of TNBC (Costa et al., 2107; Zakaria et al., 2019; Nakai et al., 2016). The combination of cetuximab and chemotherapy/radiotherapy gives synergistic effects in TNBC treatment. Erlotinib is used for the treatment of pancreatic cancer and non-small cell lung cancer; however, it is a potent EGFR inhibitor and can also be used for TNBC treatment. Vandetanib is also used for TNBC treatment, which ~~as~~ is an antagonist of EGFR, vascular endothelial growth factor receptor (VEGF), and receptor tyrosine-kinase (De Luca et al., 2014; Al-Mahmood et al., 2018; Bao et al., 2017; Nakai et al., 2016).

1.7.4. PARP inhibitors

Emerging studies have reported the significant role of PARP inhibitors in the TNBC treatment (Bergin ART & Loi S, 2019; Lyons TG, 2019; Vagia et al., 2020). PARP inhibitors used for the treatment of TNBC include olaparib, rucaparib, veliparib, niraparib and talazoparib. Extensive clinical trials are going on for the treatment of TNBC for the use of PARP inhibitors. TNBCs with mutated BRCA1 and BRCA2 genes (faulty homologous recombination DNA-repair) are prime targets for PARP inhibitors (Bartsch et al., 2010; Plummer R, 2011; Wengner et al., 2020; Denkert et al., 2017; Blasiak et al., 2020).

1.7.5. Aromatase inhibitors

It can inhibit aromatase enzymes, which are associated with the production of estrogens from androgenic substrates. Aromatase inhibitors are of two types: type-1 inhibitors (steroidal), such as exemestane, and type-2 inhibitors (non-steroidal), such as anastrozole. Type-1 inhibitors are irreversible inhibitors of aromatase, while type-2 inhibitors bind reversibly. First-generation aromatase inhibitors are associated with various side effects; thereby, second and third generation of the aromatase inhibitors (letrozole and formestane) were used for the TNBC treatment (Chavarri-Guerra et al., 2014; Chalakur-Ramireddy NKR & Pakala SB, 2018).

1.7.6. Inhibition of PI3K/Akt/mTOR pathway

Various studies have demonstrated the active role of PI3K/Akt/mTOR pathway in TNBC progression through the loss of PTEN and activation of PI3K catalytic subunit alpha (PI3KCA) or Akt1 (Costa et al., 2018; Sporikova et al., 2018; Inanc et al., 2014; Gonzalez-Angulo et al., 2009). Thereby, therapies targeting the Akt pathway would be an effective treatment for TNBC. Various clinical trials are ongoing with Akt inhibitors (such as ipatasertib, capivasertib) and paclitaxel for the treatment of TNBC (Kim et al., 2017; Dent et al., 2018; Schmid et al., 2020).

1.7.7. Immunotherapy

Immunotherapy is a newly evolved therapy for TNBC treatment. In most cancer cases, immunity is a balance of immune cells between activating and inhibiting the destruction of tissue. STAT3 regulates the genes linked with cell proliferation and in the production of antiapoptotic and angiogenic factors. Thereby, ablating STAT3 signaling can be an effective immuno-therapeutic approach in TNBC treatment (Song et al., 2019; Qin et al., 2019). Furthermore, induction of type-I immunity microenvironment improves TNBC therapy and decrease ~~the~~ recurrences (Alistar et al., 2014).

1.7.8. Others

Apart from the above-mentioned therapies, several other treatment strategies have been applied for the treatment of TNBC. HER2 inhibitors (such as trastuzumab, pertuzumab, ado-trastuzumab, and ertumaxomab) can be used to treat patients with metastatic TNBC. Further, other therapies including VEGF inhibitors (bevacizumab), EGFR and HER2 inhibitors (lapatinib), histone deacetylase inhibitors (vorinostat), mTOR inhibitors (everolimus), MMP inhibitors (BAY 12-9566), androgen receptor inhibitors (bicalutamide and enzalutamide), IGF inhibitors, platinum salts (cisplatin and carboplatin), inhibition of plasminogen activator system, neratinib and afatinib are also used for the treatment of TNBC (Al-Mahmood et al., 2018; Vagia et al., 2020; Lyons TG, 2019; Jankowitz et al., 2013; Bergin et al., 2019; Collignon et al., 2016; O'Reilly et al., 2015).

1.8. Challenges with TNBC Therapies

The lack of targeted markers for TNBC makes its treatment more challenging. Moreover, tumor recurrence, chemoresistance, and poor prognosis make TNBC treatment critically vulnerable, especially tumors with advanced stages (Bianchini et al., 2016; Podo et al., 2010; McArthur HL, 2018).

1.8.1. Tumor recurrence

Recurrence is the return of cancer and is sometimes called relapse. TNBC can reoccur locally (local recurrence) or distantly (metastatic cancer), including bones or organs. It is an important factor driving mortality in early-stage TNBC patients. Local recurrences are more common in younger age women. Moreover, recurrent TNBCs are extremely refractory to chemotherapy; thereby, efficacious targeted therapies are warranted to met TNBC treatment (Hancock et al., 2019; Freedman et al., 2009; Goncalves et al., 2018).

However, radiation therapy can decrease the risk of local recurrences of TNBC along with adjuvant therapy. It was observed that TNBC represents a higher recurrence rate within the first three years. Moreover, there is a reduction in recurrence after five years. Thereby, TNBC does not have long post-therapy. Also, TNBC patients with early-stage tumor usually have a shorter treatment period as compared to other breast cancer sub-types (Fitzpatrick A & Tutt A, 2019; Collignon et al., 2016; Podo et al., 2010; Bianchini et al., 2016; Freedman et al., 2009; Chintalapani et al., 2019).

1.8.2. Chemoresistance

Chemoresistance is one of the biggest roadblocks for the treatment of any kind of cancer. Due to chemoresistance, 90% of chemotherapeutics drugs are failed to produce its efficacy against metastatic TNBC. Therefore, the cure for TNBC continues to escape even after the significant advancement in the chemotherapeutic approaches (O'Reilly et al., 2015; Nedeljkovic M & Damjanovic A, 2019; Wein L & Loi S, 2017; Crown et al., 2012). Tumour environmental stresses, including hypoxia, starvation, and DNA damage, have been displayed to induce such fates as senescence and autophagy. These cellular fates have been significantly associated with cancer cell progression and chemoresistance. Various mechanisms of chemoresistance associated with TNBC includes; ATP-binding cassette (ABC) transporters, mutations in DNA repair enzymes (DNA mismatch repair enzymes and topoisomerase II), overexpression of β-tubulin, alterations in genes associated with apoptosis, aldehyde dehydrogenase 1 (ALDH1) and glutathione/glutathione-S-transferase (GST), and NF-κB signaling pathways (Zhao J, 2016; Nedeljkovic M & Damjanovic A, 2019; Yuan et al., 2018; O'Reilly et al., 2015; Bao et al., 2017).

1.9. Neutrophil gelatinase-associated lipocalin receptor

NGALR, also known as lipocalin 2 receptor (LCN2R), is the organic cation transporter, which is also known as solute carrier family 22, member 17 (SLC22A17). NGALR is a specific cell-surface receptor isolated from murine FL5.12 cells, which is also known as 24p3R (Fang et al., 2007; Cui et al., 2008; Cao et al., 2012; Zhang et al., 2012a). Zhang et al. (2012) performed immunohistochemistry (IHC) in human fetal, embryonic, and normal adult tissues and demonstrated that NGALR was significantly expressed in the cerebrum and neural tube during the nervous system development period. In embryos, both NGAL and NGALR were prevalent in the gastrointestinal tract (GIT) and the lung alveolar epithelium; however, in later developmental stages, expression was almost undetectable. Moreover, the two proteins were moderately expressed in the cortex and medulla in the embryonic adrenal glands. In adults, NGALR was prevalent in the cortex and medulla (Zhang et al., 2012). In mouse kidneys, NGALR was expressed in the collecting ducts and apical membranes of distal tubules (Langelueddecke et al., 2012).

NGALR plays a crucial role in iron transport and apoptosis. Emerging studies have suggested that NGALR exerted its function through the NGAL-mediated mechanism. Moreover, co-expression of NGAL and NGALR was reported in the progression of various cancers (Du et al., 2011; Liu et al., 2011; Liu et al., 2018; Lv et al., 2010; Monisha et al., 2018). However, the underlying mechanism of NGALR and its association with cancer progression is yet to be unraveled.

1.10. Association of NGALR with iron transport and apoptosis

It has been reported that iron is an essential component for bacterial and viral cell growth. The high concentration of iron can invite bacterial and viral infections that can increase oxidative stress. Therefore, the availability and concentration of iron have to be maintained appropriately inside the body. However, bacteria can adopt a new

mechanism and generates iron chelators-siderophores. These siderophores can chelate and acquire the iron by high-affinity binding from the host. Therefore, an iron-limiting

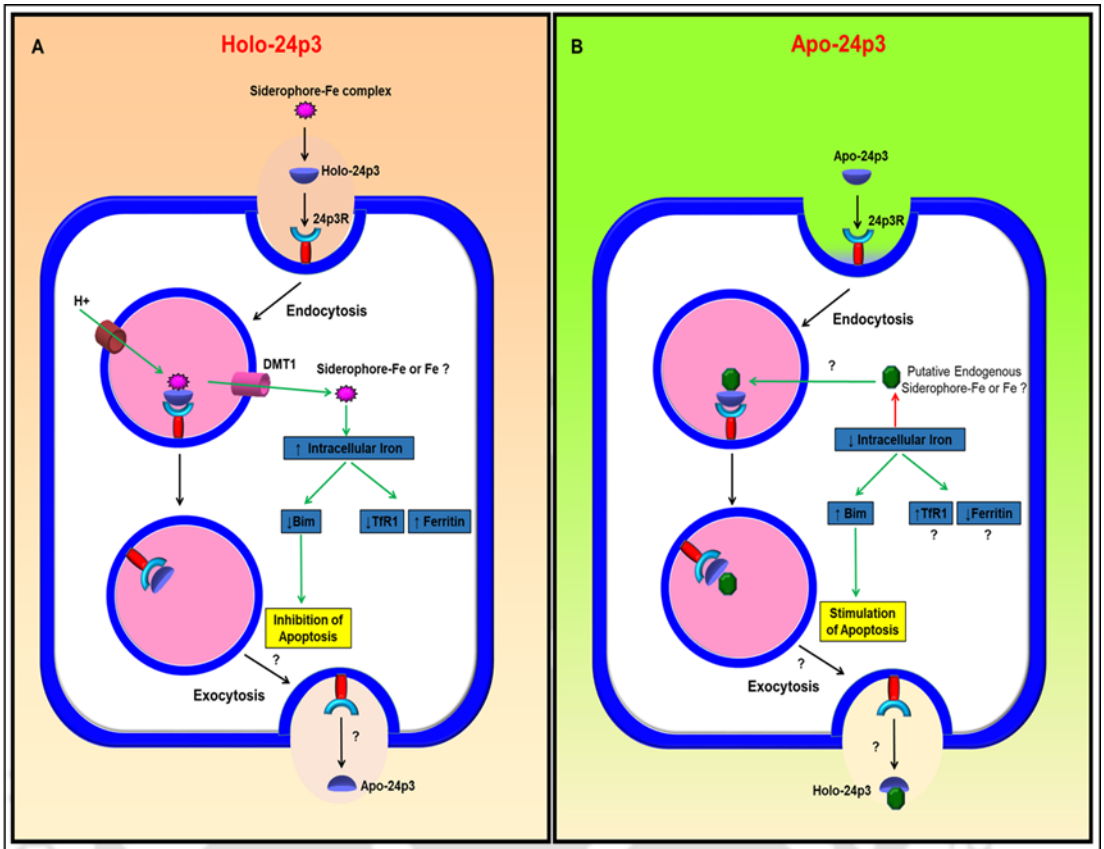

Figure 1.5. Role of NGALR in iron transport and apoptosis. (A) Internalization of Holo-24p3 (containing the iron complex of the bacterial siderophore, enterobactin and 24p3) through the 24p3 receptor leads to the uptake of iron into the cell. Elevated level of iron to the cell leads to the decrease in TfR1 and Bim (proapoptotic protein) expression and increase in ferritin levels, which suppresses apoptosis. (B) Internalization of Apo-24p3 through 24p3 receptor leads to decrease of intracellular iron. Decrease of iron from the cell results in the upregulation of the Bim, which leads to apoptosis.

strategy can counter-attack against these bacterial infections. (Flow et al., 2004; van Heijningen S, 2015).

It has emerged that NGAL can bind to bacterial siderophores with high affinity and can be a significant iron-limiting agent. NGAL is an important biomarker of kidney injury and has a role in regulating innate immunity (Goetz et al., 2002; Richardson DR, 2005). Mounting evidence suggested that iron also has a role in cell proliferation; therefore, iron chelators are among the cancer treatment targets (Richardson DR, 2005; Devireddy et al., 2001). Devireddy et al. (2005) demonstrated that NGAL and NGALR have a crucial role in the transportation of iron and regulation of apoptosis (Devireddy

et al., 2005). Bacterial cells used iron for its survival and proliferation; thereby, mammalian cells sequestered iron and secured it from being used by bacteria (van Heijningen S, 2015; Flo et al., 2004).

NGAL does not bind with bound iron (bacterial siderophore and enterobactin) while acts as an iron transporter to carry iron to the cells by modulating various genes. Remarkably, by obeying this response, it plays a vital role in regulating cell apoptosis (Figure 1.5). Iron-bound NGAL binds to NGALR, and through the endocytosis process, it increases intracellular iron concentrations and inhibits apoptosis (by decreasing the Bim protein). On the other hand, iron-free NGAL stimulates apoptosis by reducing intracellular iron and inducing the Bim protein expression (Yang et al., 2003; Richardson DR, 2005; Devireddy et al., 2005).

1.11. Association of NGALR with cancers

Recent studies demonstrated that NGALR had been associated with the progression and modulation of various cancers (Bauvois et al., 2020; Liu et al., 2018; Tan et al., 2014; Chi et al., 2020; Zhang et al., 2012a). Both *in vitro* and *in vivo* studies demonstrated that NGALR is overexpressed in ESCC and resulted in cancer progression. Further, NGALR overexpression was strongly related to poor survival and prognosis in ESCC patients (Tan et al., 2014; Du et al., 2011; Cui et al., 2008; Fang et al., 2007).

Recently, a study displayed that NGALR overexpression was involved with resistance in apoptosis in chronic lymphocytic leukemia (CLL) patients cells. NGALR was associated with apoptosis inhibition by activating the STAT3 signaling pathway (Bauvois et al., 2020). Another study reported that NGALR limited iron-transport and induced cell growth in the leptomeningeal metastases mouse model (Chi et al., 2020). Further, overexpression of NGALR was observed in endometrial carcinoma tissue samples. NGALR overexpression was significantly correlated with invasion,

metastasis, and poorer survival in endometrial carcinoma patients (Miyamoto et al., 2011).

Both NGAL and NGALR were upregulated in HCC tissues and the upregulation was significantly correlated with the poor prognosis and OS. Further, it has been associated with HCC cell proliferation and invasion (Zhang et al., 2012a). The co-expression of NGAL and NGALR were significantly upregulated in the specimens of colorectal carcinoma (CRC) patients. It was observed that NGALR was associated with high-grade tumors and invasion. Moreover, NGAL and NGALR were significantly related to poorer cellular differentiation of CRC (Lv et al., 2010). Further, Liu et al. (2011) also demonstrated that overexpression of NGAL and NGALR was significantly linked with poor prognosis and OS in human gliomas tissue samples (Liu et al., 2011). On the other hand, significant downregulation of NGAL and NGALR was observed in RCC patients (Liu et al., 2018).

1.12. Importance of the study

Increasing studies reported that breast cancer has emerged as one of the leading causes of cancer-associated deaths among women. In India, breast cancer accounts for the highest cancer-related incidences, including both males and females. In a study, we reported that TNBC has the highest incidence (27%) in India. TNBC mostly affects younger age women compared to older age women. Also, it has a poorer prognosis, high recurrences, and poor OS. The lack of hormonal receptors makes this cancer more vulnerable and challenging for the targeted treatment. Surgery in combination with radiation and chemotherapy is the only suitable treatment reliable for TNBC. Recent advancements are made to treat this devasting disease, but the mortality rates are still high for this particular sub-types of breast cancer. Therefore, TNBC required special attention to find a better target for effective treatment. Recently, NGALR has reported

an oncogenic role in the progression and development of various cancers. However, its role has not been deciphered in breast cancer, specifically in TNBC to date. Thus, unraveling the role of NGALR in TNBC would help us to identify novel therapeutic targets for the development of highly efficacious therapy for this fatal disease.

1.13. Objectives

Based on the review of literature, we have framed our objectives as follows:

1. To determine the expression of NGALR in TNBC human tissues and cell lines.
2. To determine the role of TNF-α and TNF-β in the regulation of NGALR in TNBC cell lines.
3. To determine the role of NGALR in regulating different molecular mechanisms involved in the development of TNBC.

Chapter 2

Expression of NGALR in breast cancer

2.1. Introduction

Breast cancer accounts for the highest number of cancer cases, and the number of incidences are increasing each year (Ferlay et al., 2020; Sung et al., 2021). TNBC is the most aggressive breast cancer sub-type which requires advanced treatment strategies to decrease its associated mortality. Though significant advancements have been made to treat breast cancer, the OS and prognosis are still very poor. Therefore, a detailed understanding of the pathogenesis and identification of novel targets would significantly help us to design efficacious therapy for TNBC. As discussed in the earlier chapter, TNM staging helps in the determination of TNBC prognosis, better treatment, more precise clinical trials, and the outcome of disease. Further, the TNBC sub-type is often characterized by higher grade and advanced stages tumors. Moreover, younger age women are more susceptible to TNBC than older age women (Singh et al., 2018; Agarwal G & Ramakant P, 2008; Al jarroudi et al., 2017; Thakur et al., 2018; Yao et al., 2019). It is well established that NGALR is associated with iron transport and apoptosis and the dysregulated expression of NGALR has been resulted in the progression of various cancers. However, the expression of NGALR protein has not been reported in breast cancer to date. Therefore, investigating the expression of NGALR in breast cancer, specifically in the TNBC sub-type would help us to design a novel target for TNBC treatment. Hence, the current chapter was designed to determine the expression of NGALR in breast cancer tissues and cell lines. Before determining the expression of NGALR, the OS of breast cancer patients was examined using the cancer genomics Atlas (TCGA) database.

2.2. Materials and Methods

2.2.1. TCGA analysis

The OS of breast cancer patients was analyzed from the data obtained from the TCGA data sets available online at the cBIoPortal website (http://www.cbioportal.org). The survival of breast cancer patients was evaluated through the Kaplan-Meier survival curve. To obtained the OS curve, we have followed certain steps: open the home page of the website www.cbioportal.org, clicked on "Query," selected "Breast," selected listed studies, clicked on "Query by gene," typed gene name "SLC22A17", clicked on "Submit Query" and a result window appeared. Further, clicked on "Comparison/Survival," then clicked on "survival" and downloaded the Kaplan-Meier Estimate.

2.2.2. Tissue Microarray

The expression of NGALR in breast cancer tissues and various sub-types of breast cancer was examined through IHC analysis. Tissue microarray (TMA) slide containing normal and malignant breast tissues were procured from US Biomax, USA (Cat No. BR1503e). The TMA slide had a total of 75 females breast tissues and 150 cores (duplicate cores per case) The slide contains three normal and seventy-two malignant breast patients tissue samples.

2.2.2.1. Tissue Microarray details

Name: BR1503e

Description: Breast cancer TNM, age, pathology grade, clinical stage, and with IHC results.

Cases: 75

Cores: 150

Core Diameter: 5 mm

Row number: 10

Column number: 15

Table 2.1. Breast cancer tissue array details (table adapted from https://www.biomax.us/BR1503e).

Position	No.	Age	Pathology diagnosis	TNM	Grade	Stage	Type	Molecular subtype
A1	1	46	Adjacent normal breast tissue	-	-	-	NAT	-
A2	2	46	Adjacent normal breast tissue	-	-	-	NAT	-
A3	3	43	Adjacent normal breast tissue	-	-	-	NAT	-
A4	4	43	Adjacent normal breast tissue	-	-	-	NAT	-
A5	5	43	Adjacent normal breast tissue	-	-	-	NAT	-
A6	6	43	Adjacent normal breast tissue	-	-	-	NAT	-
A7	7	19	Fibroadenoma	-	-	-	Benign	-
A8	8	19	Fibroadenoma	-	-	-	Benign	-
A9	9	19	Fibroadenoma	-	-	-	Benign	-
A10	10	19	Fibroadenoma	-	-	-	Benign	-
A11	11	54	Fibroadenoma	-	-	-	Benign	-
A12	12	54	Fibroadenoma	-	-	-	Benign	-
A13	13	49	Cystosarcoma phyllodes	T2N0M0	-	G1 IB	Malignant	Luminal A
A14	14	49	Cystosarcoma phyllodes	T2N0M0	-	G1 IB	Malignant	Luminal A
A15	15	69	Cystosarcoma phyllodes	T2bN0M0	-	G3 III	Malignant	Luminal A
B1	16	69	Cystosarcoma phyllodes	T2bN0M0	-	G3 III	Malignant	Basal like
B2	17	49	Intraductal carcinoma	TisN0M0	-	-	Malignant	Basal like
B3	18	49	Intraductal carcinoma	TisN0M0	-	-	Malignant	Basal like
B4	19	50	Intraductal carcinoma with early infiltrate	TisN0M0	-	-	Malignant	Basal like

B5	20	50	Intraductal carcinoma with early infiltrate	TisN0M0	-	-	Malignant	Her-2 type
B6	21	31	Intraductal carcinoma	TisN0M0	-	-	Malignant	Her-2 type
B7	22	31	Intraductal carcinoma	TisN0M0	-	-	Malignant	Her-2 type
B8	23	55	Intraductal carcinoma	TisN0M0	-	-	Malignant	Her-2 type
B9	24	55	Intraductal carcinoma with early infiltrate	TisN0M0	-	-	Malignant	Her-2 type
B10	25	36	Intraductal carcinoma	TisN0M0	-	-	Malignant	Luminal A
B11	26	36	Intraductal carcinoma	TisN0M0	-	-	Malignant	Luminal A
B12	27	43	Intraductal carcinoma	TisN0M0	-	-	Malignant	Basal like
B13	28	43	Intraductal carcinoma	TisN0M0	-	-	Malignant	Her-2 type
B14	29	50	Intraductal carcinoma (sparse)	TisN0M0	-	-	Malignant	Her-2 type
B15	30	50	Intraductal carcinoma	TisN0M0	-	-	Malignant	Basal like
C1	31	60	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Her-2 type
C2	32	60	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Her-2 type
C3	33	50	Invasive ductal carcinoma	T4cN0M0	2	IIIB	Malignant	Basal like
C4	34	50	Invasive ductal carcinoma	T4cN0M0	2	IIIB	Malignant	Basal like
C5	35	55	Invasive ductal carcinoma	T2N0M0	1	IIA	Malignant	Luminal A
C6	36	55	Invasive ductal carcinoma	T2N0M0	1	IIA	Malignant	Luminal A
C7	37	43	Invasive ductal carcinoma	T2N0M0	1	IIA	Malignant	Luminal A
C8	38	43	Invasive ductal carcinoma	T2N0M0	1	IIA	Malignant	Basal like
C9	39	43	Invasive ductal carcinoma	T1N0M0	1	I	Malignant	Her-2 type

C10	40	43	Invasive ductal carcinoma	T1N0M0	1	I	Malignant	Her-2 type
C11	41	54	Invasive ductal carcinoma	T2N0M0	1	IIA	Malignant	Her-2 type
C12	42	54	Invasive ductal carcinoma	T2N0M0	-	IIA	Malignant	Basal like
C13	43	44	Invasive ductal carcinoma	T2N2M0	2	IIIA	Malignant	Her-2 type
C14	44	44	Invasive ductal carcinoma	T2N2M0	2	IIIA	Malignant	Her-2 type
C15	45	28	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Luminal A
D1	46	28	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Luminal A
D2	47	60	Invasive ductal carcinoma	T2N1M0	2	IIB	Malignant	Luminal A
D3	48	60	Invasive ductal carcinoma	T2N1M0	2	IIB	Malignant	Luminal A
D4	49	71	Invasive ductal carcinoma	T4N2M0	2	IIIB	Malignant	Luminal A
D5	50	71	Invasive ductal carcinoma	T4N2M0	2	IIIB	Malignant	Luminal A
D6	51	39	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Luminal A
D7	52	39	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Luminal A
D8	53	70	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Her-2 type
D9	54	70	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Her-2 type
D10	55	64	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Basal like
D11	56	64	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Basal like
D12	57	47	Invasive ductal carcinoma	T3N0M0	2	IIB	Malignant	Luminal A
D13	58	47	Invasive ductal carcinoma	T3N0M0	2	IIB	Malignant	Luminal B
D14	59	34	Invasive ductal carcinoma	T4N0M0	2	IIIB	Malignant	Luminal B
D15	60	34	Invasive ductal carcinoma	T4N0M0	2	IIIB	Malignant	Luminal B
E1	61	51	Invasive ductal carcinoma	T2N2M0	2	IIIA	Malignant	Basal like

E2	62	51	Invasive ductal carcinoma	T2N2M0	2	IIIA	Malignant	Basal like
E3	63	64	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Her-2 type
E4	64	64	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Her-2 type
E5	65	52	Invasive ductal carcinoma	T2N2M0	2	IIIA	Malignant	Luminal A
E6	66	52	Invasive ductal carcinoma	T2N2M0	2	IIIA	Malignant	Luminal A
E7	67	45	Invasive ductal carcinoma	T1N0M0	2	I	Malignant	Basal like
E8	68	45	Invasive ductal carcinoma	T1N0M0	2	I	Malignant	Basal like
E9	69	74	Invasive ductal carcinoma	T2N1M0	2	IIB	Malignant	Luminal A
E10	70	74	Invasive ductal carcinoma	T2N1M0	2	IIB	Malignant	Luminal A
E11	71	54	Invasive ductal carcinoma	T3N1M0	2	IIIA	Malignant	Basal like
E12	72	54	Invasive ductal carcinoma	T3N1M0	2	IIIA	Malignant	Basal like
E13	73	34	Invasive ductal carcinoma	T3N0M0	2	IIB	Malignant	Basal like
E14	74	34	Invasive ductal carcinoma	T3N0M0	2	IIB	Malignant	Luminal A
E15	75	60	Invasive ductal carcinoma	T2N2M0	2	IIIA	Malignant	Her-2 type
F1	76	60	Invasive ductal carcinoma	T2N2M0	2	IIIA	Malignant	Her-2 type
F2	77	70	Invasive ductal carcinoma	T2N1M0	2	IIB	Malignant	Her-2 type
F3	78	70	Invasive ductal carcinoma	T2N1M0	2	IIB	Malignant	Luminal B
F4	79	65	Invasive ductal carcinoma	T2N1M0	2	IIB	Malignant	Luminal B
F5	80	65	Invasive ductal carcinoma	T2N1M0	2	IIB	Malignant	Her-2 type
F6	81	40	Invasive ductal carcinoma	T2N1M0	3	IIB	Malignant	Basal like
F7	82	40	Invasive ductal carcinoma	T2N1M0	2	IIB	Malignant	Luminal A
F8	83	48	Invasive ductal carcinoma	T2N2M0	2	IIIA	Malignant	Luminal B

F9	84	48	Invasive ductal carcinoma	T2N2M0	2	IIIA	Malignant	Luminal B
F10	85	38	Invasive ductal carcinoma	T2N1M0	2	IIB	Malignant	Luminal B
F11	86	38	Invasive ductal carcinoma	T2N1M0	2	IIB	Malignant	Her-2 type
F12	87	45	Invasive ductal carcinoma	T4N2M0	2	IIIB	Malignant	Luminal A
F13	88	45	Invasive ductal carcinoma	T4N2M0	2	IIIB	Malignant	Luminal A
F14	89	59	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Basal like
F15	90	59	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Basal like
G1	91	52	Invasive ductal carcinoma	T3N0M0	2	IIB	Malignant	Basal like
G2	92	52	Invasive ductal carcinoma	T3N0M0	2	IIB	Malignant	Basal like
G3	93	51	Invasive ductal carcinoma	T3N1M0	2	IIB	Malignant	Her-2 type
G4	94	51	Invasive ductal carcinoma	T3N1M0	2	IIB	Malignant	Her-2 type
G5	95	48	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Luminal B
G6	96	48	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Luminal B
G7	97	43	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Luminal A
G8	98	43	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Luminal A
G9	99	40	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Luminal A
G10	100	40	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Luminal A
G11	101	45	Invasive ductal carcinoma	T3N0M0	2	IIB	Malignant	Basal like
G12	102	45	Invasive ductal carcinoma	T3N0M0	2	IIB	Malignant	Basal like
G13	103	30	Invasive ductal carcinoma	T4N0M0	2	IIIB	Malignant	Luminal B
G14	104	30	Invasive ductal carcinoma	T4N0M0	2	IIIB	Malignant	Luminal B
G15	105	34	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Basal like

H1	106	34	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Basal like
H2	107	60	Invasive ductal carcinoma (sparse)	T2N0M0	2	IIA	Malignant	Her-2 type
H3	108	60	Invasive ductal carcinoma (sparse)	T2N0M0	2	IIA	Malignant	Her-2 type
H4	109	59	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Luminal A
H5	110	59	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Luminal B
H6	111	33	Invasive ductal carcinoma	T1N0M0	2	I	Malignant	Basal like
H7	112	33	Invasive ductal carcinoma	T1N0M0	2	I	Malignant	Basal like
H8	113	56	Invasive ductal carcinoma	T3N0M0	2	IIB	Malignant	Luminal A
H9	114	56	Invasive ductal carcinoma	T3N0M0	2	IIB	Malignant	Luminal A
H10	115	43	Invasive ductal carcinoma	T4N2M0	2	IIIB	Malignant	Her-2 type
H11	116	43	Invasive ductal carcinoma	T4N2M0	2	IIIB	Malignant	Her-2 type
H12	117	44	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Basal like
H13	118	44	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Basal like
H14	119	58	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Her-2 type
H15	120	58	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Her-2 type
I1	121	43	Invasive ductal carcinoma	T2N1M0	2	IIB	Malignant	Luminal B
I2	122	43	Invasive ductal carcinoma	T2N1M0	2	IIB	Malignant	Luminal B
I3	123	63	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Basal like
I4	124	63	Invasive ductal carcinoma	T2N0M0	3	IIA	Malignant	Basal like
I5	125	35	Invasive ductal carcinoma	T2N0M0	3	IIA	Malignant	Basal like
I6	126	35	Invasive ductal carcinoma	T2N0M0	3	IIA	Malignant	Basal like

I7	127	50	Invasive ductal carcinoma	T2N0M0	3	IIA	Malignant	Luminal B
I8	128	50	Invasive ductal carcinoma	T2N0M0	3	IIA	Malignant	Luminal B
I9	129	62	Invasive ductal carcinoma	T2N1M0	2	IIB	Malignant	Basal like
I10	130	62	Invasive ductal carcinoma	T2N1M0	2	IIB	Malignant	Basal like
I11	131	49	Invasive ductal carcinoma	T3N1M0	2	IIIA	Malignant	Basal like
I12	132	49	Invasive ductal carcinoma	T3N1M0	2	IIIA	Malignant	Basal like
I13	133	42	Invasive ductal carcinoma	T2N2M0	2	IIIA	Malignant	Luminal B
I14	134	42	Invasive ductal carcinoma	T2N2M0	2	IIIA	Malignant	Luminal B
I15	135	48	Invasive ductal carcinoma	T2N1M0	2--3	IIB	Malignant	Luminal B
J1	136	48	Invasive ductal carcinoma	T2N1M0	3	IIB	Malignant	Luminal B
J2	137	50	Invasive ductal carcinoma	T2N2M0	3	IIIA	Malignant	Basal like
J3	138	50	Invasive ductal carcinoma	T2N2M0	3	IIIA	Malignant	Basal like
J4	139	62	Invasive ductal carcinoma	T1N0M0	3	I	Malignant	Basal like
J5	140	62	Invasive ductal carcinoma	T1N0M0	2	I	Malignant	Basal like
J6	141	58	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Basal like
J7	142	58	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Basal like
J8	143	50	Invasive ductal carcinoma	T2N0M0	3	IIA	Malignant	Her-2 type
J9	144	50	Invasive ductal carcinoma	T2N0M0	3	IIA	Malignant	Her-2 type
J10	145	45	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Basal like
J11	146	45	Invasive ductal carcinoma	T2N0M0	2	IIA	Malignant	Basal like
J12	147	57	Invasive ductal carcinoma	T3N0M0	3	IIB	Malignant	Basal like
J13	148	57	Invasive ductal carcinoma	T3N0M0	3	IIB	Malignant	Basal like

J14	149	56	Invasive ductal carcinoma	T2N0M0	3	IIA	Malignant	Luminal A
J15	150	56	Invasive ductal carcinoma	T2N0M0	3	IIA	Malignant	Basal like

2.2.3. Immunohistochemistry (IHC)

The IHC analysis was performed on TMA slide using Histostain-Plus IHC kit (Cat No. 34065, Invitrogen), metal enhanced 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate kit (Cat No. 34065, Invitrogen), and anti-NGALR monoclonal antibody (Cat No. ab124506, Abcam, USA). We have followed the manufacturer's protocol which includes, deparaffinization of TMA slide by xylene, rehydration by alcohol, washings with PBS, peroxidase quenching by hydrogen peroxide in methanol, antigen retrieval by citrate buffer, blocking, primary antibody incubation, secondary antibody incubation, addition of chromogen DAB, counterstained by hematoxylin, and mounted by DPX mountant (Cat No. DC4DF64352, Merck, USA).

2.2.4. Scoring

The immunostained TMA slide was analyzed by the Nikon Eclipse YS100 microscope. The intensity and number of positive cells for NGALR-immunoreactivity were examined and given the score mentioned in Table 2.2.

Table 2.2. Scoring method for IHC.

Score (P)	**0**	**1+**	**2+**	**3+**	**4+**
Positive Cells	<10%	10-25%	25-50%	50-75%	>75%
Score (I)	**1**	**2**	**3**	Total expression score **Q= P*I**	
Intensity of Stain	weak stain	moderate stain	strong stain		

The final score was calculated by multiplying the scores of intensity and the number of positive cells (Monisha et al., 2018; Charafe-Jauffret et al., 2004).

2.2.5. Cell culture

HaCaT (immortalized human skin epithelial cells), MDA-MB-231 (TNBC), and MDA-MB-468 (TNBC) cell lines were acquired from NCCS, Pune, India. All three cell lines were maintained with Dulbecco's Modified Eagle Medium (DMEM; Gibco™, Life Technologies, USA), which contained with 1X Pen-Strep (Invitrogen, USA), and fetal bovine serum (FBS) 10% (Gibco®, USA), and incubated the cells into a carbon-dioxide (CO_2)-controlled incubator (37 °C and 5% CO_2).

2.2.6. Western blot analysis

The expression of NGALR in normal and TNBC cell lines were determined by Western blot analysis. All three (HaCaT, MDA-MB-231, and MDA-MB-468) cell lines were harvested and lysed with lysis buffer containing protease/phosphatase inhibitors (2mM EDTA, 20mM HEPES, Triton-X100 (0.1% v/v), 250mM NaCl, 2µg/ml aprotinin, 2µg/ml leupeptin hemisulfate, 1mM DTT, 1mM PMSF). The isolated protein samples were quantified by Bradford reagent (Cat No. 500-0205, Bio-Rad, USA) and BSA. 30µg of each quantified protein was mixed with 5X Laemmli Buffer (10% SDS, 250mM Tris HCl, 5% β-mercaptoethanol, 0.02% bromophenol blue, 30% glycerol) and run in an 8% SDS-polyacrylamide gel. Then gel was electrotransferred into a nitrocellulose membrane (Bio-Rad, USA). Ponceau-S stain (Cat No. ML045, Hi-Media) was used for confirmation of transfer and the membrane was blocked with non-fat milk (5%) prepared in tris-buffer saline containing 1% tween 20 (TBST). Further, the blots were incubated overnight with anti-NGALR and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies at 4°C. After incubation, the blots were washed

and incubated with horseradish peroxidase (HRP)-conjugated antibody for the next 2 hours at room temperature. The blots were then visualized in a gel doc (ChemiDoc™ XRS system, Bio-Rad, USA) using a clarity Western ECL substrate kit (Bio-Rad, USA). GAPDH was used as a loading control.

2.2.7. Statistical analysis

The statistical analysis of our results was determined through Student's t-test. Statistically significant values were denoted by *. p-value < 0.05 was denoted by *, and p-value < 0.005 represented by **. All the data are represented as mean ± standard deviation (SD).

2.3. Results and Discussion

In the current study, we have demonstrated the OS and the NGALR expression in breast cancer patient's tissues samples from the TCGA data sets and IHC analysis, respectively. IHC analysis demonstrated the expression of NGALR in various sub-types and stages of breast cancer. Further, we have determined the NGALR expression in TNBC cell lines using Western blot analysis.

2.3.1. NGALR decreases the survival of breast cancer patients

OS of breast cancer patients was analyzed using the TCGA cBioPortal database, which provides visualization, analysis of large-scale breast cancer genomics data online. In the TCGA portal, we have selected the data of 960 patients/samples. The OS of breast cancer patients was calculated using the Log-rank test (p-value= 0.733) and Kaplan-Meier analysis. We have observed that NGALR alterations decreased the OS of breast cancer patients (Figure 2.1 A). The median month survival of the breast cancer patients decreased from 129.6 months to 83.8 months. Similarly, Liu et al. (2018) has performed a TCGA analysis of NGALR on 22 clear cell RCC (ccRCC) data sets and demonstrated

that NGALR was implicated in the clinical prognosis of the disease (Liu et al., 2018). Recently, a study has reported that the overexpression of NGALR leads to the poor OS in gastric cancer patients (Wei et al., 2020). Similarly, poor OS was also reported in the patients having a higher expression of NGALR protein in endometrial cancer (Miyamoto et al., 2011).

2.3.2. Overexpression of NGALR in breast cancer tissues

Expression of NGALR was assessed in breast TMA samples by IHC analysis. The expression was compared in normal and malignant breast tissues. Our results demonstrated that NGALR protein was significantly overexpressed in breast cancer tissues than the normal breast tissues (Figure 2.1 B, C, D). Similar to our study, NGALR was also significantly upregulated in endometrial carcinoma tissues than normal tissues and associated with poor prognosis of the disease (Miyamoto et al., 2011). The elevated expression of NGALR was also observed in gastric carcinoma tissue samples than in normal tissues. The overexpression of NGALR was associated with shorter survival of gastric carcinoma patients; thus suggesting that the expression of NGALR has prognostic significance in gastric carcinoma (Wei et al., 2020). Further, the overexpression of NGALR was observed in biopsy samples of ninety-three glomerulonephritis patients through IHC analysis. In this study the expression of NGALR was significantly elevated by IL-1β (Mao et al., 2011). In addition, Cui et al. (2008) reported that hypomethylation of NGALR was associated with increased NGLR expression in ESCC tissues than normal esophageal epithelium tissues, and the overexpression plays a crucial role in the pathogenesis of the disease (Cui et al., 2008). In a similar study, NGAL and NGALR were overexpressed in ESCC tissues and related to poor prognosis and lower survival in ESCC patients (Du et al., 2011).

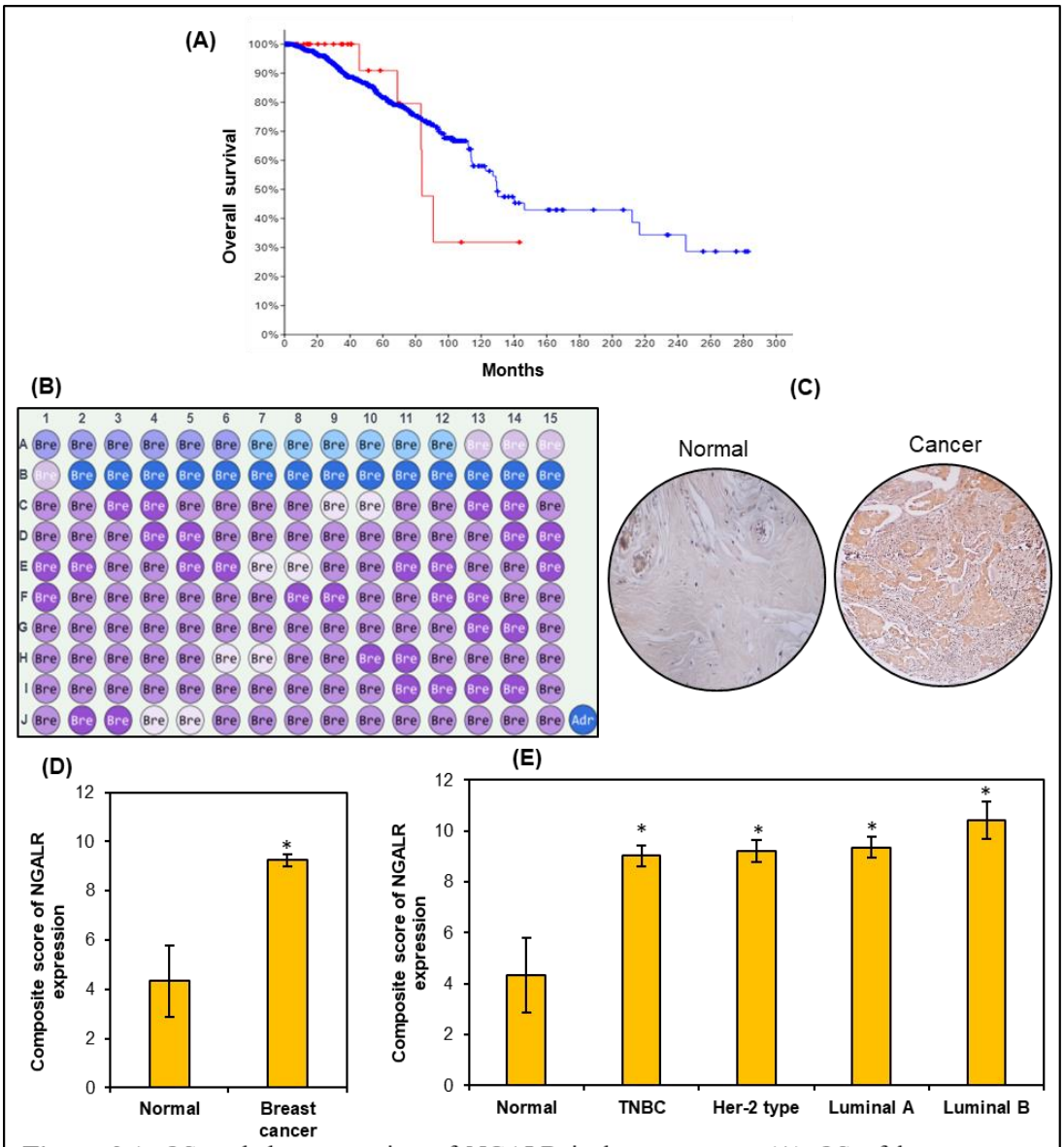

Figure 2.1. OS and the expression of NGALR in breast cancer. **(A)** OS of breast cancer patients (960) was analyzed by Kaplan-Meier Estimate. **(B)** Representative tissue microarray (TMA) slide containing 75 females breast tissues, 150 cores. Bre -Breast, Benign tumor, - Malignant tumor, - Malignant tumor (stage G1 IB), - Malignant tumor (stage G3 III), - Malignant tumor (stage I), - Malignant tumor (stage IIA), - Malignant tumor (stage IIB), - Malignant tumor (stage IIIA), - Malignant tumor (stage IIIB), - NAT. **(C)** Representative images for immunohistochemical staining of NGALR in normal and breast cancer tissues. **(D)** Graphical representation of NGALR expression in normal (3) and breast cancer (72) tissues. . **(E)** Graphical representation of NGALR expression in normal (3) tissues versus various sub-types of breast cancer, TNBC (27), Her-2 enriched (16), Luminal A (16), Luminal B (10). Data are represented as mean ± standard error (SE). * = $p<0.05$ compared to the normal tissues.

Both NGAL and NGALR were overexpressed in the HCC tissue samples and associated with TNM stage, vascular invasion and tumor recurrence. Further, the expression was positively correlated with poor prognosis and unfavorable clinicopathologic characteristics. Thus, NGALR can be a potential prognostic and therapeutic marker in HCC modulation (Zhang et al., 2012). Similarly, co-expression of NGAL and NGALR was found to be associated with poorer cellular differentiation and an invasion in the CRC. NGALR was overexpressed in the CRC tissues than normal colorectal tissues and significantly associated with a high degree of TNM stages, deeper invasion, and increased cell proliferation (Lv et al., 2010). On the other hand, a study reported that downregulation of NGALR expression was strongly related with poor prognosis in ccRCC patient samples (Liu et al., 2018). Moreover, our study found that NGALR was overexpressed in breast cancer tissues and can be served as a potential target for breast cancer therapy.

2.3.3. Expression of NGALR in different sub-types of breast cancer

As discussed earlier, NGALR was significantly overexpressed in various cancers. However, the expression was not reported among various sub-types of breast cancers. This is the first study where we have addressed the expression of NGALR in various sub-types of breast cancer. Our study demonstrated that the expression of NGALR was significantly overexpressed in various sub-types of breast cancer, including luminal A (n=16), luminal B(n=10), TNBC (n=27), and HER2-enriched (n=16) tissues compared to normal breast tissues (n=3) (Figure 2.1 E). Except for TNBC, all the three sub-types represent elevated HR and account for approximately 70-75% of all breast cancer. The elevated expression of HR can be served as a potential target for various targeted hormonal therapies. However, the absence of HR made TNBC (20-25%) sub-types more vulnerable and difficult to treat by targeted hormonal therapies (El Hachem et al.,

2019; Thakur et al., 2018). Interestingly, the lack of potential targets made us focus on TNBC sub-types to find out suitable therapeutic targets against this aggressive disease. Thereby, our next aim was to decipher the expression of NGALR in the TNBC sub-type.

2.3.4. Expression of NGALR in different age groups of TNBC patients

Emerging studies suggested that breast cancer survival is worse in younger age women (>45 years) than older women. Also, the demography of breast cancer is shifting from older to younger age women. Moreover, the TNBC sub-type was more frequently observed in younger age women than older. However, the existing cause behind this inconsistency is yet to be revealed (Lee HB & Han W, 2014; Anders et al., 2009; Aapro M & Wildiers H 2012; Yeh et al., 2017). Our study determined the expression of NGALR among various age group patients through IHC analysis in TNBC tissue samples. We observed that NGALR was significantly overexpressed in the tissue samples of 30-45 years (n= 10) and 45-60 years (n= 12) age groups (Figure 2.2 A). However, the overexpression was not significant in the age group of 60-75 years (n= 5).

Similar to our study overexpression of NGALR was observed in glioma tissues and was significantly associated with the patient age, tumor grade, lower survival, and poor prognosis (Liu et al., 2011). Lv et al. (2010) demonstrated that NGALR overexpression was highly associated with colorectal cancer patients under 57 years of age and resulted in poor survival (Lv et al., 2010). Recently, a study reported that the survival and metastasis of TNBC sub-types patients were more frequently associated with women < 40 years of age as compared to older age (> 74 years) women (Tzikas et al., 2020). A comparative study reported that women under the age of 45 years were more susceptible to TNBC (Dolle et al., 2009). The prevalence of breast cancer in the

same age groups in non-TNBC patients was less than in TNBC patients (Dolle et al., 2009). Our study also showed that the overexpression of NGALR was significantly associated with 45-60 years age group women.

2.3.5. Expression of NGALR in different process of the development of TNBC

It has been well-known that inflammation plays a critical role in the development and progression of multiple cancers, including breast cancer. Inflammation further leads to the activation of various carcinogenesis processes, such as cell proliferation, survival, invasion, metastasis, and angiogenesis (Gupta et al., 2010; Grivennikov et al., 2010; Kunnumakkara et al., 2020; Mills RC, 2017). Recent studies reported that various inflammatory mediators helped in the progression and advancement of TNBC (Liubomirski et al., 2019; Romero-Cordoba et al., 2019; Charan et al., 2020). Therefore, we have examined the expression of NGALR in various developmental stages of TNBC. We found that NGALR was significantly overexpressed in stage 1 (n=3) and stage 3 (n= 5) TNBC tissues compared to the normal tissues (n= 3) (Figure 2.2 B). However, the overexpression of NGALR was not significant in stage 2 (n= 15) TNBC tissues. In accordance with our study, NGALR expression was significantly overexpressed in stage 3 and stage 4 endometrial carcinoma tissues and implicated with higher invasion and poorer survival (Miyamoto et al., 2011). The overexpression of NGALR was significantly associated with a higher TNM stage and increased cellular differentiation in CRC tissues (Lv et al., 2010). Further, the overexpression of NGALR and its ligand were implicated in the TNM stage, poor prognosis, and tumor recurrence in HCC patient's tissues (Zhang et al., 2012). Recently, a study reported that NGALR was overexpressed in gastric cancer patients and associated with higher TNM stages (Wei et al., 2020). Our results also advocated that NGALR overexpression was linked

with higher TNBC stages and was significantly upregulated in stage 1 and stage 3 tumor tissues.

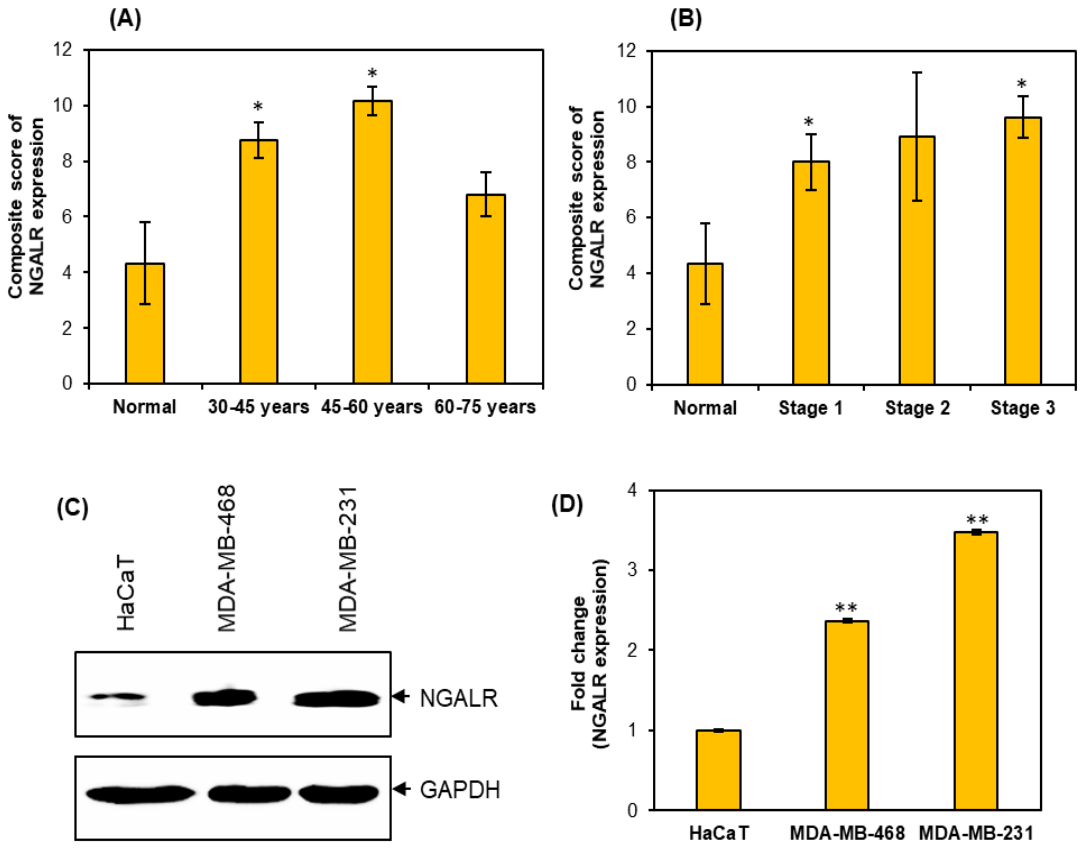

Figure 2.2. Expression of NGALR in TNBC tissues and cell lines. **(A)** Graphical representation of NGALR expression among various age groups, normal (3), 30-45 years (10), 45-60 years (12), 60-75 years (5) TNBC tissues. **(B)** Graphical representation of NGALR expression in various TNBC stages, such as stage 1 (3), stage 2 (15), stage 3 (5) tissues. **(C)** Expression of NGALR in different TNBC cell lines (MDA-MB-231, and MDA-MB-468) compared to normal cells (HaCaT) through Western blot analysis. **(D)** Quantification of NGALR expression in HaCaT, MDA-MB-231, and MDA-MB-468 cells. Data are represented as mean ± SE. ** = $p<0.005$.

2.3.6. Expression of NGALR in TNBC cell lines

The TCGA and IHC analysis have already reported the survival and overexpression of NGALR in breast cancer tissues. Further, we have revealed the overexpression of NGALR in TNBC tissue samples by IHC analysis. In addition, we have performed

Western blot analysis and determined the NGALR expression in various TNBC cell lines. The expression of NGALR was determined in HaCaT (normal) and MDA-MB-231 and MDA-MB-468 (TNBC) cell lines. Our results showed that NGALR was significantly upregulated in MDA-MB-231 and MDA-MB-468 cell lines than HaCaT cells (Figure 2.2 C, D).

Recently, Bauvois et al. (2020) revealed the expression of NGALR in CLL cells. The study suggested that NGAL and NGALR were upregulated in CLL cells compared to normal peripheral blood mononuclear cells (PBMCs) and associated with apoptotic resistance by modulating the STAT3/Mcl-1 signaling pathway (Bauvois et al., 2020). Both Western blot and real-time PCR (RT-PCR) studies suggested that NGAL and NGALR were significantly upregulated in human gliomas and linked with poor survival (Liu et al., 2011). Similarly, Mao et al. (2011) have performed Western blot and RT-PCR analysis and reported that both NGAL and NGALR proteins were upregulated in human mesangial cells (HMC). The expression was further elevated upon treatment with IL-1β by activating MAPK/ERK signaling (Mao et al., 2011). Furthermore, NGALR3 (a spliced variant of NGALR) was upregulated in oesophageal carcinoma cells (Fang et al., 2007).

2.4 Conclusion

The current chapter has demonstrated the expression of NGALR in breast cancer. The breast cancer cases with NGALR alteration have shown lower survival months compared to those without alterations. The IHC analysis revealed that NGALR has significantly overexpressed in breast cancer tissues and various sub-types of breast cancer than normal tissues. It showed that NGALR might be implicated in the poor prognosis of breast cancer. The expression of NGALR was significantly higher in younger TNBC women (30-60 years). Further, NGALR was significantly

overexpressed in stage 1 and stage 3 TNBC tumor tissues. Moreover, the expression of NGALR protein was significantly upregulated in TNBC cells compared to normal cells. Our results showed that NGALR overexpression has a role in poor survival and breast cancer progression, specifically TNBC sub-types. However, further studies are required to decipher the exact role and potential mechanism of NGALR in TNBC progression.

Chapter 3

Effect of TNF-α and TNF-β on the regulation of NGALR in TNBC cell lines

3.1. Introduction

In the preceding chapter, we have demonstrated that the overexpression of NGALR is associated with poor survival and progression of breast cancer. Further, we have shown that NGALR was significantly overexpressed in the TNBC sub-type. Various studies have reported that inflammation is significantly related with breast cancer progression (Mills RC, 2017; Agnoli et al., 2017). The OS of breast cancer patients was reduced with increasing levels of inflammatory markers, including tumor necrosis factor (TNF)-alpha (TNF-α), TNF-beta (TNF-β), and ILs (Agnoli et al., 2017; Cai et al., 2017). Recent studies have reported that dysregulation of TNFs are strongly associated with cancer progression (Niture et al., 2018; Feng et al., 2019; Hsing et al., 2019; Kunnumakkara et al., 2019). However, it is also a known fact that TNF plays a dual role in cancer progression and chemoresistance. TNF can act both as an anticancer agent and a cancer-promoting agent (Montfort et al., 2019; Bertazza L & Mocellin S, 2010). TNFs also play a dual role in breast cancer progression and chemoresistance (Cruceriu et al., 2020; Zhang et al., 2018; Pooja et al., 2011). Wolczyk et al. (2016) showed that TNF-α plays an important role in the migration and progression of breast cancer by activating the MAPK/ERK signaling (Wolczyk et al., 2016). Both *in vitro* and *in vivo* studies reported that transmembrane TNF-α induced doxorubicin resistance in breast cancer cells (Zhang et al., 2018; Mercogliano et al., 2020).

TNF-α and TNF-β are pro-inflammatory cytokines having a diverse array of roles in cell proliferation, survival, invasion, angiogenesis, and metastasis (Kunnumakkara et al., 2019; Wang X & Lin Y, 2008). Further, Pooja et al. (2010) reported that TNF-α and TNF-β polymorphisms were significantly associated with breast cancer (Pooja et al., 2011). TNF-α and TNF-β were highly expressed oral cancer blood samples than healthy individuals. Further, TNFs polymorphisms reportedly lead

to an increased risk of oral cancer (Yapijakis et al., 2009). TNF-α and IL-1β regulate the expressions of NGAL in polymorphonuclear granulocytes and human epithelial cells (Arena et al., 2010; Cowland et al., 2003). These studies advocated that TNF-α and TNF-β are implicated in cancer development and progression. However, the potential role of TNF-α and TNF-β have not been studied on the expression of NGALR in TNBC. Therefore, we have examined the effect of TNF-α and TNF-β on the progression of TNBC in MDA-MB-231 and MDA-MB-468 cells.

3.2. Materials and methods

3.2.1. Cell culture

All cell lines details, media, and other supplements used for the cell culture have already been reported in Chapter 2, Section 2.2.5. 'Cell culture'.

3.2.2. MTT assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the effect of TNF-α and TNF-β on MDA-MB-231 and MDA-MB-468 cells proliferation. MDA-MB-231 and MDA-MB-468 cells were seeded in ninety-six well plates at a density of 2000 cells/100µl and kept at a CO_2-controlled incubator overnight. Further, 0.05nM of TNF-α and TNF-β treatments were given to both the cell lines. Then, at a period of 0hr and 24hr, 10µl of MTT (5mg/ml) (Cat No. M2128, Sigma-Aldrich, USA) was added and incubated at a CO_2-controlled incubator for 2hr. After incubation, the whole supernatant was removed from each well and 100µl of dimethyl sulfoxide (DMSO) (Cat No. 1.16743.0521, Merck, Germany) was added. Then, the absorbance was recorded at 570nm in a multi-mode spectrophotometer (SpectraMax iD3, Molecular Devices, USA).

3.2.3. Colony formation assay

The clonogenicity of MDA-MB-231 and MDA-MB-468 cells were evaluated after treating with TNF-α and TNF-β through colony formation assay. Five hundred cells/well were seeded in six-well plate and stored at 37°C overnight at a CO_2-controlled incubator. Then, 0.05nM of TNF-α and TNF-β treatments were given to both the cell lines for 24hr. After 24hr incubation, the media was discarded and fresh media was added to each well. This step was repeated until the cells formed the colonies. Then, colonies were washed using 1X phosphate-buffered saline (PBS), fixed with ethanol (70%), and stained with crystal violet (0.3%) solution (Cat No: 548-6209; SRL Pvt. Ltd., India). Further, the stain was washed using 1X PBS and dried in the air.

Finally, the images of colonies were captured by Nikon-500 camera and quantified by ImageJ software. Moreover, the survival fraction of the cells was calculated by the following formula:

Plating efficiency = (No. of counted colonies /No. of cells seeded) × 100

Survival fraction = (Plating efficiency of the treated sample/Plating efficiency of the untreated sample) × 100

3.2.4. Epithelial–mesenchymal transition (EMT) assay

MDA-MB-231 and MDA-MB-468 cells were seeded on glass coverslips in a twelve-well plate at a density of $6x10^4$ cells/well. After overnight incubation, the cells were treated with 0.05nM of TNF-α and TNF-β for 24hr. Further, cells were fixed with formaldehyde (3.7%) at room temperature for 20 minutes. For the permeabilizations of cells, 0.1% Triton X-100 was used for 10 minutes and blocked in 2% BSA. Further, the cells were incubated overnight in human vimentin (Cat No. NL493, R&D systems) and Snail (Cat No. NL557, R&D systems) conjugated antibodies. Then cells were rinsed

with 1X PBST and counterstained using 0.01µg/ml 4,6-diamidino-2-phenylindole (DAPI; Hi-Media) for 5 minutes. The coverslips with cells were then appropriately dried and mounted on a glass slide using the DPX mountant. The mounted slides were visualized under an upright fluorescence microscope (Olympus, BX43, Japan). Further, the color intensity of the captured images was determined using Olympus CellSens software.

3.2.5. Wound healing assay

The wound healing assay was carried out to determine the migration potential of MDA-MB-231 and MDA-MB-468 cells. Both TNBC cells were seeded in twelve-well plates at a density of $6x10^5$ cells/well and $8x10^5$ cells/well, respectively. Once the cells formed a monolayer, the whole media was changed by serum-free DMEM media and allowed the cells to incubate for the next 8hr. The whole media was discarded, and a straight scratched was created at the middle of the well using a 10µl pipette tip. Then, scratched cells were removed by washing with 1X PBS, treated with 0.05nM of TNF-α and TNF-β, and 0hr images were captured in an inverted light microscope (Nikon Eclipse T1-SM, Japan) attached with a Nikon-500 camera. Further, the cells were kept at a CO_2 incubator, and images were captured at different time intervals (24, 48, 72, and 96 hr). The recorded images were analyzed by ImageJ software, and migration potential was calculated.

3.2.6. Cell death analysis

In order to confirm whether TNF-α and TNF-β induced cell death, we performed propidium iodide (PI) flow cytometry analysis. MDA-MB-231 and MDA-MB-468 cells were seeded into a six-well plate to give the plating density of 0.5 x 10^5/well and incubated overnight. Then, cells were treated with 0.05nM of TNF-α and TNF-β for

48hr. After incubation, the entire supernatant was collected and transferred into the new polystyrene test tubes (5x77mm). Then cells were trypsinized after washing with 1X PBS and centrifuged with supernatant media, which we have transferred earlier in polystyrene tubes. Then, the cell pellets were washed with 1X PBS and centrifuged again. Now, pellets were incubated with PI (10µg/mL) stain for 5-10 minutes. Finally, the cell counts were recorded at the flow cytometer (BD FACSCelesta™ Flow Cytometer, USA) and analyzed using FACSDiva software.

3.2.7. Western blot analysis

The NGALR expression in TNBC cell lines was determined through Western blot analysis. MDA-MB-231 and MDA-MB-468 cells were harvested (after treating with 0.05nM of TNF-α and TNF-β for 24hr) and lysed in lysis buffer. Further, we have followed the same steps as mentioned earlier in Chapter 2, Section 2.2.6. Western blot analysis.

3.2.8. Statistical analysis

The statistical analysis of our results was determined through Student's t-test. Statistically significant values were denoted by *. p-value < 0.05 was denoted by *, and p-value < 0.005 denoted by **. All the data are represented as mean ± SD.

3.3. Results and Discussion

In the current chapter, we have determined the effects of TNF-α and TNF-β in various cellular processes of TNBC progression, such as cell proliferation, survival, EMT, migration, and cell death. Further, we have evaluated the role of TNF-α and TNF-β on the NGALR expression in TNBC cell lines.

3.3.1. Effect of TNF-α and TNF-β on the proliferation and survival of TNBC cells

As discussed earlier, TNF has a dual role in cancer progression; therefore, we have determined the effect of TNF-α and TNF-β on the proliferation and survival of MDA-MB-231 and MDA-MB-468 cells through MTT assay. Our results demonstrated that both TNF-α and TNF-β have significantly increased the proliferation of MDA-MB-231 and MDA-MB-468 cells compared to untreated cells (Figure 3.1 A, B). Recently, Liu et al. (2020) also revealed that TNF-α increased breast cancer cell growth by inducing transcriptional coactivator with PDZ-binding motif (TAZ) and activating NF-κB pathway (Liu et al., 2020). Similarly, TNF-α augmented cell proliferation by inducing hepatitis B X-interacting protein (HBXIP), TNF receptor (TNFR) 1, NF-κB, and p-STAT3 in both *in vitro* and *in vivo* models of breast cancer (Cai et al., 2017). Further, lymphotoxin-alpha (also known as TNF-β) polymorphism was associated with non-Hodgkin's lymphoma (NHL) prognosis (Zhang et al., 2013). Recently, various studies demonstrated that TNF-α increased the proliferation of MDA-MB-231 cells while decreased the proliferation of MCF-7 cells (Lyu et al., 2017; Huang et al., 2018). TNF-α augmented estrogen-induced cell proliferation by activating the NF-κB pathway in T47D breast cancer cells (Rubio et al., 2006). Similarly, TNF-α induced breast cancer growth by Akt/NF-κB and p42/p44 MAPK/JNK-dependent pathways (Rivas et al., 2008). Moreover, Qiao et al. (2016) revealed that AP1- regulated the TNFα-mediated TNBC progression (Qiao et al., 2016). Our results also supported that TNF-α and TNF-β significantly increased the proliferation of MDA-MB-231 and MDA-MB-468 cells.

On the other hand, Su et al. (2012) showed that TNF-α decreased the adhesion and proliferation of breast cancer cells by regulating hypoxia-inducible factor-1alpha (HIF-1α) expression (Su et al., 2012). Similarly, an *in vitro* and *in vivo* studies reported that artemisinin possessed anticancer effects in breast cancer cells by increased TNF-α

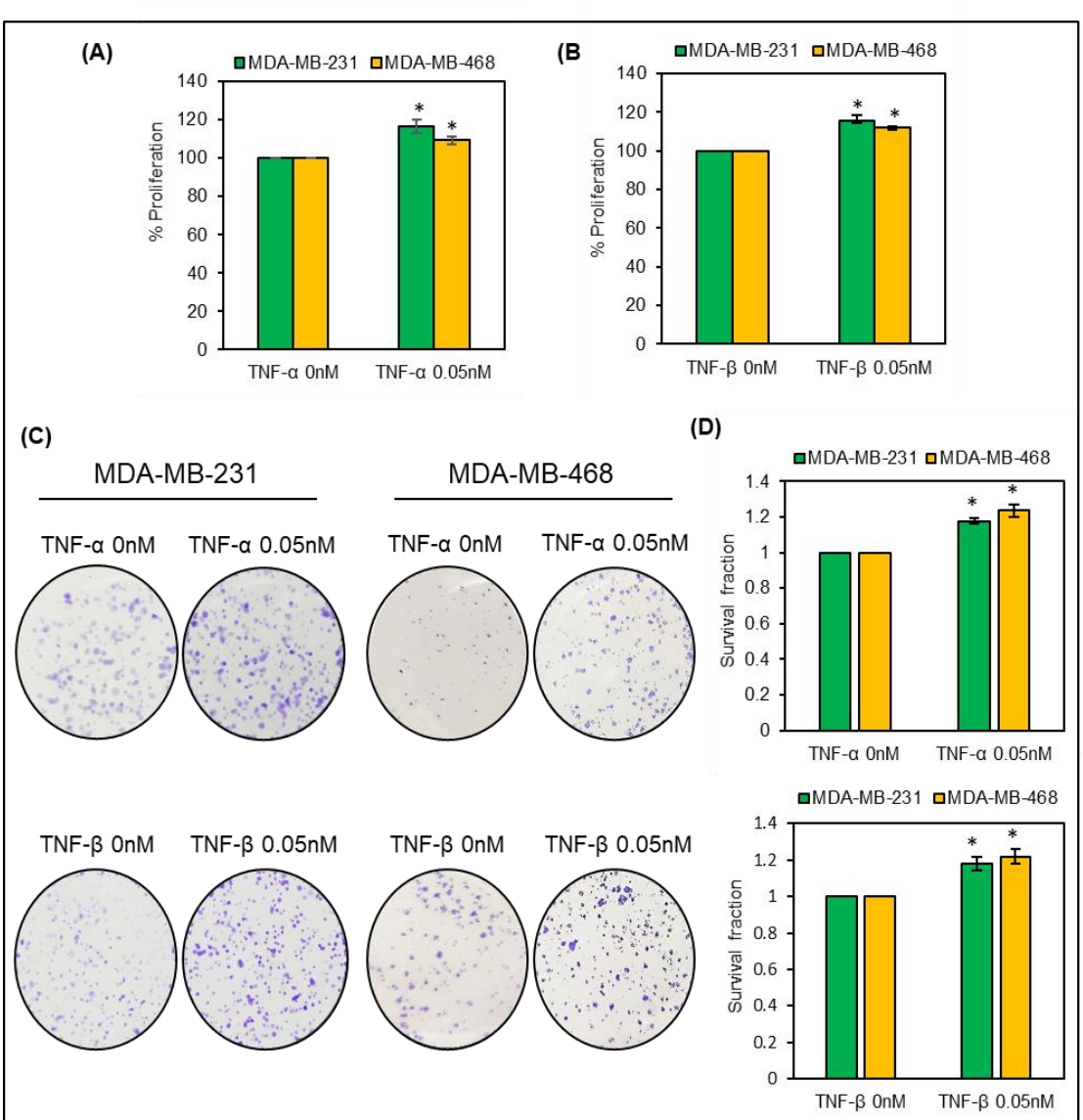

Figure 3.1. Effect of TNF-α and TNF-β on the proliferation and survival of TNBC cells. **(A-B)** Percentage proliferation of MDA-MB-231 and MDA-MB-468 cells determined by MTT assay. **(C-D)** Representative images and graphical representation of the survival fraction of MDA-MB-231 and MDA-MB-468 cells determined by colony formation assay. Data are represented as mean ± SE. * = $p<0.05$ compared to untreated cells.

and decreased transforming growth factor (TGF-β) mRNA levels (Cao et al., 2019). Recently, a study demonstrated that calcitriol has antiproliferative effects on TNBC cells through induction of TNF-α and IL-1β (Martinez-Reza et al., 2019). Further, TNF-α-sensitized breast cancer cells to docetaxel, cisplatin, and 5-fluorouracil (5-FU) in both *in vitro* and *in vivo* models (Wu et al., 201). Apart from this, a xenograft mice model of

lung cancer demonstrated that TNF-α inhibited cell proliferation by increasing the expression of HIF-1α and decreasing vasodilator-stimulated phosphoprotein (VASP) expression (Liu et al., 2019).

Further, we have determined the survival of MDA-MB-231 and MDA-MB-468 cells through clonogenic assay and showed that both TNF-α and TNF-β significantly increased the survival fraction of MDA-MB-231 and MDA-MB-468 cells (Figure 3.1 C, D). Similarly, knockdown of TNF-α decreased the cell proliferation and motility while induced apoptosis in TNBC cells (Hs578T) (Pileczki et al., 2012). Wang et al. (2015) reported that TNF-α increased the proliferation and survival of nucleus pulposus cells by modulating p38 MAPK, JNK, and NF-κB signaling pathways (Wang et al., 2015). *In vitro* and *in vivo* models of breast cancer suggested that antagonizing TNF-α suppressed breast cancer growth and metastasis (Yu et al., 2013). Furthermore, butein inhibited the proliferation and survival of TNBC cells by inhibiting TNF-α-induced CC-chemokine ligand (CCL) 2 expression (Mendonca et al., 2019). Recently, Buhrmann et al. (2019) revealed that TNF-β induced survival and invasion in colon cancer, which was reversed by the co-treatment of resveratrol and 5-FU (Buhrmann et al., 2018). Moreover, our findings suggested that both TNF-α and TNF-β are significantly associated with the proliferation and survival of TNBC cells.

3.3.2. Effect of TNF-α and TNF-β on EMT of TNBC cells

We have already determined the role of TNF-α and TNF-β on the survival and proliferation of MDA-MB-231 and MDA-MB-468 cells. It has been well established that inflammation plays a crucial role in breast cancer prognosis and recurrences. Pro-inflammatory cytokines (TNF-α and TNF-β) were found in the stromal and malignant cells in biopsy specimens of breast cancer patients with a worse prognosis. Also, TNF-

α has been associated with EMT, which is an essential step for the migration and metastasis of cancer cells (Mercogliano et al., 2020; Cole SW, 2009; Asiedu et al., 2011; Cohen et al., 2015). Therefore, we have also determined the effect of TNF-α and TNF-β on the EMT of MDA-MB-231 and MDA-MB-468 cells through EMT assay.

Our results demonstrated that both TNF-α and TNF-β significantly induced the expression of vimentin and Snail protein in MDA-MB-231 and MDA-MB-468 cells (Figure 3.2 A, B). Similarly, Asiedu et al. (2011) reported that TNF-α and TGF-β induced breast cancer stem cells (BCSCs) through EMT (Asiedu et al., 2011). TNF-α induced BCSC-like phenotype by upregulating SLUG and NF-κB/HIF-1α signaling (Storci et al., 2010). Further, TNF-α enhanced EMT by activation of AP-1 signaling and induced zinc finger E-box binding homeobox (ZEB) 2 expression in BT549 and Hs578T TNBC cells (Qiao et al., 2015). A study revealed that TNF-α induced EMT by upregulating the expression of inhibitors of IκB kinase β (IKKβ) and NF-κB and subsequently induced Twist1 protein expression in breast cancer cells (Li et al., 2012). Similarly, Dong et al. (2006) revealed that TNF-α induced EMT in MCF-7 cells by activating NF-κB and upregulating the expression of Snail protein (Dong et al., 2006). However, knockdown of the expression of Snail inhibited the NF-κB activation and subsequently suppressed invasion and migration in breast cancer cells (Wu et al., 2009). TNF-α was also reported to induced Snail-mediated EMT by activating the Akt pathway and suppressing glycogen synthase kinase-3 beta (GSK-3β) activity in CRC (Wang et al., 2013). Bigatto et al. (2015) reported that TNF-α induced MET transcription via NF-κB signaling and leading to the upregulation of Snail and downregulation of E-cadherin in various cancer cell lines (Bigatto et al., 2015). Further, TNF-α stimulated the expression of Snail in cholangiocarcinoma cells (Techasen et al.,

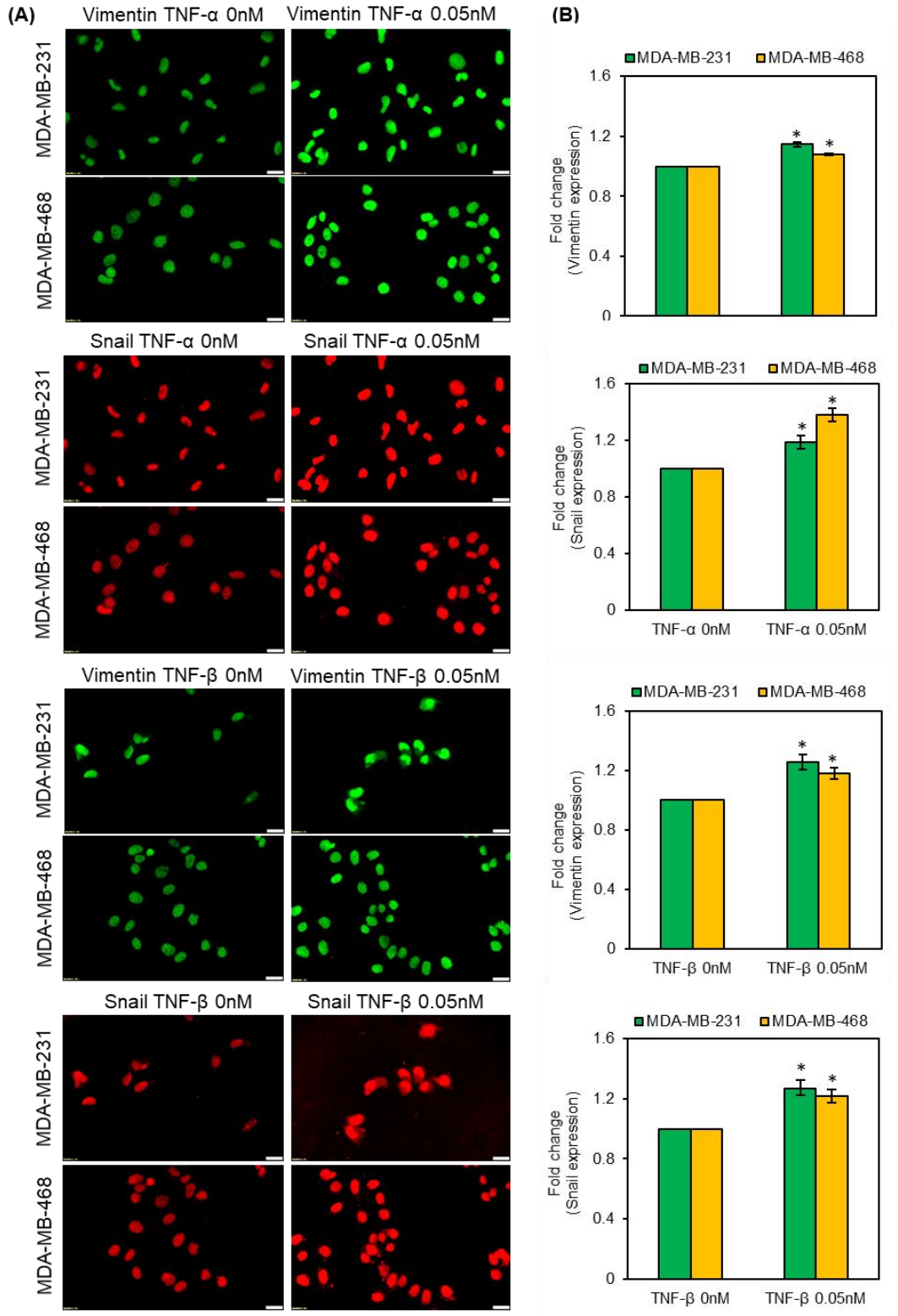

Figure 3.2. Effect of TNF-α and TNF-β on EMT of TNBC cells. **(A)** Representative images of vimentin and Snail expression in MDA-MB-231 and MDA-MB-468 cells determined by immunocytochemistry (ICC). Original magnification 40X objective. Scale bar = 20μM. **(B)** Quantification of vimentin and Snail expression in MDA-MB-231 and MDA-MB-468 cells using CellSens software. Data are represented as mean ± SE. * = $p<0.05$.

2012). Our results also showed that TNF-α and TNF-β significantly induced the expression Snail protein in MDA-MB-231 and MDA-MB-468 cells.

Further, Soria et al. (2011) demonstrated that the expression of inflammatory mediators, such as TNF-α, IL-1β, CCL2, and CCL5 were strongly associated with EMT in breast cancer (Soria et al., 2011). TNF-α induced EMT-mediated metastasis by increasing the expression of vimentin, N-cadherin, and Twist and decreasing E-cadherin expression by stimulating the NF-κB signaling pathway in hypopharyngeal cancer (Yu et al., 2014). TNF-α along with IL-6 and TGF-β induced EMT and metastasis in inflammatory breast cancer cells by increasing the expression of fibronectin 1 (FN1), vimentin, transglutaminase 2 (TGM2), and ZEB1 (Cohen et al., 2015). Similarly, both TNF-α and IL-6 stimulated EMT-mediated metastasis by upregulated the expression of vimentin and N-cadherin and downregulated the expression of E-cadherin in lung cancer (Shang et al., 2017). Further, TNF-α and TGF-β1 enhanced EMT by increasing the expression of vimentin and N-cadherin and decreasing the expression of E-cadherin via the NF-κB pathway in colon cancer (Li et al., 2018). A study recently revealed that TNF-β-induced EMT by upregulated the expression of vimentin and N-cadherin and downregulated the expression of E-cadherin via the NF-κB pathway. However, resveratrol treatment reversed the effect by inhibiting TNF-β and NF-κB activation in colon cancer cells (Buhrmann et al., 2019). Our results also showed that TNF-α and TNF-β induced the expression of vimentin protein significantly in MDA-MB-231 and MDA-MB-468 cells. Overall, both TNF-α and TNF-β induced EMT by significantly upregulating the expression of vimentin and Snail in TNBC cells.

3.3.3. Effect of TNF-α and TNF-β on the migration of TNBC cells

Our previous results have showed the role of TNF-α and TNF-β on the EMT of TNBC cells. It has been well established that EMT is strongly associated with breast cancer cell invasion and migration (Singh et al., 2019; Zhao P & Zhang Z, 2018). Various studies have revealed that inflammatory cytokines play an important role in cancer cell invasion and migration. TNF-α at high doses induced cancer cell apoptosis, while at long-time administration of low doses promoted cancer cell invasion and metastasis (Zhao P & Zhang Z, 2018; Wu et al., 2009; Singh et al., 2019; Paramanantham et al., 2020). Therefore, we have determined the effect of TNF-α and TNF-β on the migration of MDA-MB-231 and MDA-MB-468 cells by wound-healing assay. Our results demonstrated that TNF-α and TNF-β significantly induced the migration of MDA-MB-231 and MDA-MB-468 cells compared to untreated cells (Figure 3.3 A, B, C, D). The percentage wound area remaining was 60-70% after treating with TNF-α and TNF-β in both MDA-MB-231 and MDA-MB-468 cells. These results also strengthened our previous findings that TNF-α and TNF-β induced EMT in MDA-MB-231 and MDA-MB-468 cells.

Wolczyk et al. (2016) also observed similar effects and reported that TNF-α induced migration of breast cancer cells by upregulating the expression of matrix metallopeptidase (MMP) -9, fibroblast activation protein alpha (FAP-α), and CD26 (Wolczyk et al., 2016). Similarly, TNF-α augmented invasion by upregulating MMP-9 in mammary epithelial cells (Montesano et al., 2005). Recently, *in vitro* and *in vivo* studies have demonstrated that TNF-α induced invasion and metastasis by downregulating pentraxin 3 expression in gastric cancer (Cui et al., 2020). Further, TNF-α induced migration and lymph node metastasis in prostate cancer cells by upregulating CC-chemokine receptor 7 (CCR7) expression (Maolake et al., 2018).

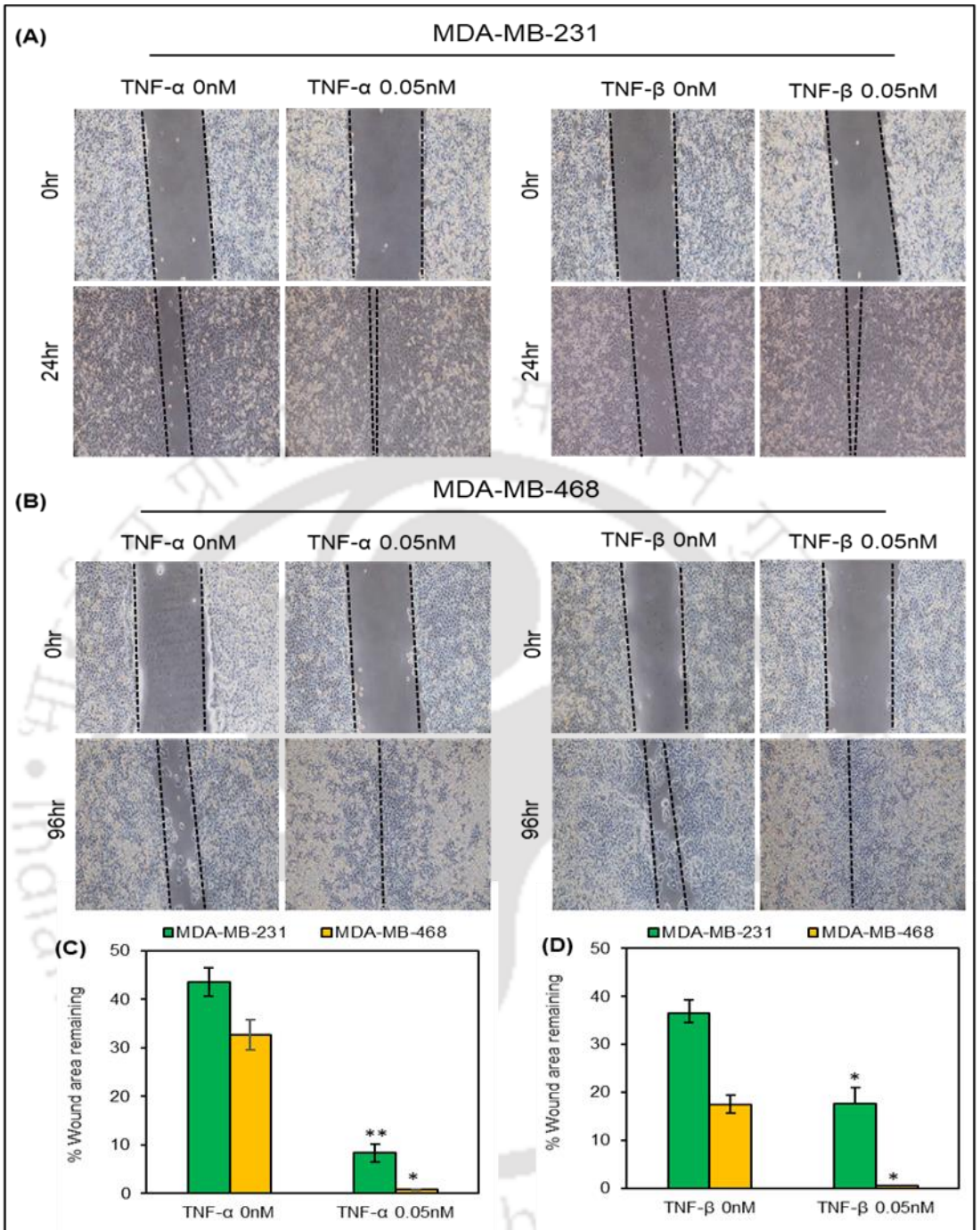

Figure 3.3. Effect of TNF-α and TNF-β on migration of TNBC cells. **(A-B)** Representative images of migration of MDA-MB-231 and MDA-MB-468 cells determined by wound healing assay. **(C-D)** Graphical representation of percentage wound area remaining of MDA-MB-231 and MDA-MB-468 cells using ImageJ software. Data are represented as mean ± SE. * = $p<0.05$ and ** = $p<0.005$ compared to untreated cells.

Recently, a study revealed that TNF-α induced cell invasion and migration in colon cancer through upregulation of tumor-associated calcium signal transduction protein-2

(TROP-2) expression (Zhao P & Zhang Z, 2018). Similarly, Kobelt et al. (2020) reported that TNF-α and interferon-gamma (IFN-γ) stimulated tumor growth and metastasis in colon cancer cells (Kobelt et al., 2020).

On the other hand, AIM (an anthocyanin isolated from Meoru) suppressed TNF-α induced EMT, invasion, and metastasis in breast cancer cells via inhibition of TNF-α associated NF-κB activation. AIM also decreased the expression of MMP-2 and MMP-9 and increased the expression of E-cadherin in breast cancer (Paramanantham et al., 2020). Similarly, NOD-like receptor family member X1 (NLRX1) modulated TNF-α-associated mitochondria-lysosomal crosstalk and decreased invasion and metastasis of breast cancer cells (Singh et al., 2019). Kanglaite, along with TNF-α, decreased EMT-mediated cell invasion and migration in colon cancer via NF-κB inhibition (Shi et al., 2017). Similarly, our results also advocated that TNF-α and TNF-β significantly induced the migration of both TNBC cells.

3.3.4. Effect of TNF-α and TNF-β on TNBC cell death

Several studies have already reported the dual role of TNF-α and TNF-β on cancer cell death (Bertazza L & Mocellin S, 2010; Wang X & Lin Y, 2008; Qiao et al., 2016). Therefore, we have examined the role of TNF-α and TNF-β on TNBC on cell death by PI- flow cytometric analysis. As PI specifically binds to dead cells nucleic acids (RNA/DNA), we have determined the percentage cell death based on the staining of PI. Our results demonstrated that TNF-α and TNF-β do not have any cytotoxic role against MDA-MB231 and MDA-MB-468 cells and decreased the percentage cell death compared to untreated cells (figure 3.4 A, B, C, D). These results further supported our previous findings that TNF-α and TNF-β increased the survival and proliferation of MDA-MB-231 and MDA-MB-468 cells. Similar to our findings, the knockdown of

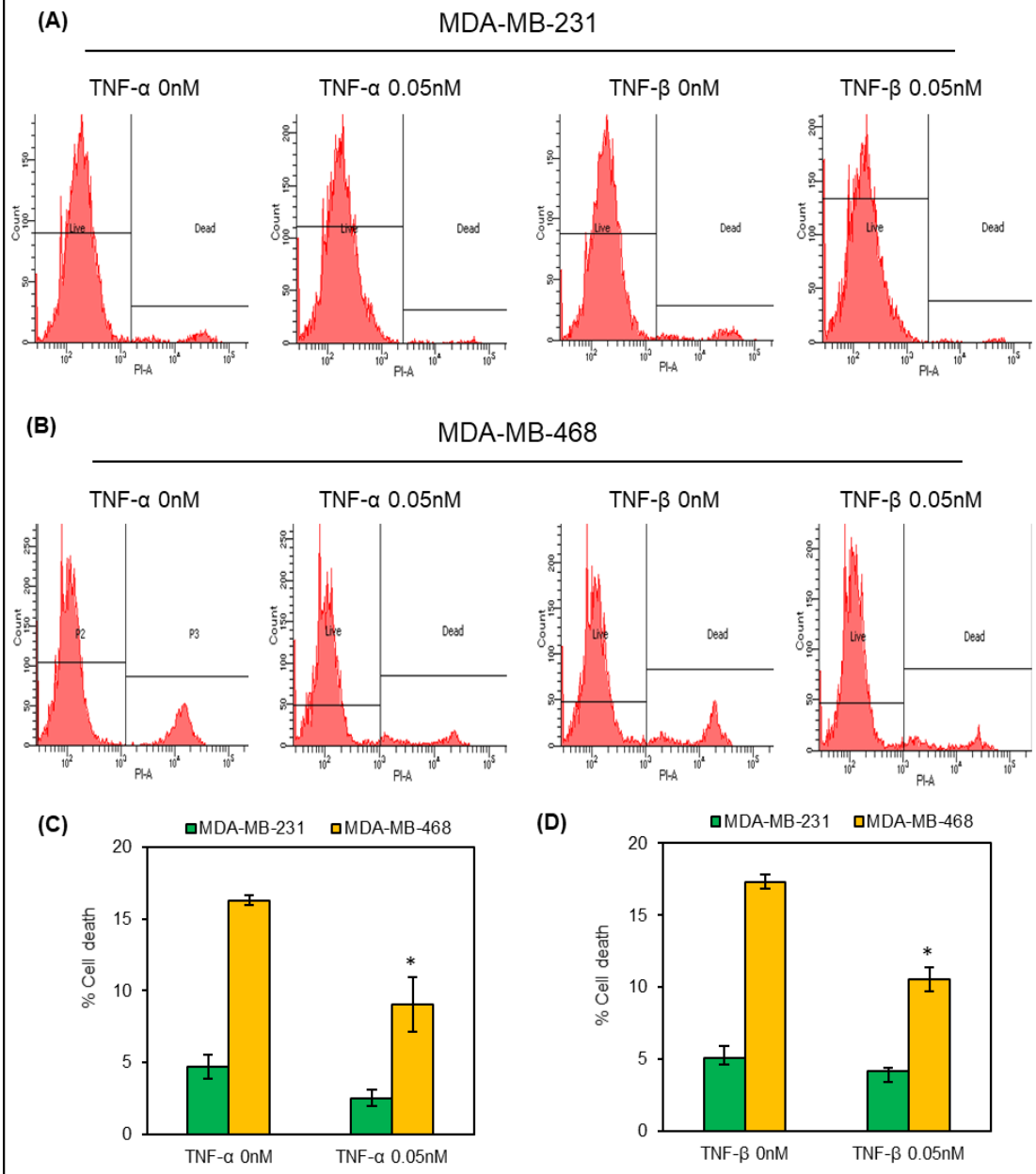

Figure 3.4. Effect of TNF-α and TNF-β on cell death of TNBC cells. **(A-B)** Representative histograms of MDA-MB-231 and MDA-MB-468 cells determined by wound healing assay. **(C-D)** Graphical representation of percentage wound area remaining of MDA-MB-231 and MDA-MB-468 cells using BD FACSDiva software. Data are represented as mean ± SE. * = $p<0.05$ compared to untreated cells.

TNF-α inhibited the proliferation and accelerated apoptosis in Hs578T cells (Pileczki et al., 2012). On the contrary, TNF-α induced apoptosis via activation of p73 and c-ABL and reactive oxygen species (ROS) production (Chau et al., 2004; Kim et al., 2010). Similarly, TNF-β increased necroptosis, apoptosis, and inflammatory signals

through TNFR 1-mediated signaling (Etemadi et al., 2013). Further, TNF-α sensitized breast cancer cells to 5-FU, docetaxel, and cisplatin and induced cytotoxicity both *in vitro* and *in vivo* (Wu et al., 2017). Recently, a study showed that TNF-α induced necrosis and apoptosis in the *Staphylococcus aureus*-infected A549 cells by increased expression of receptor-interacting protein-3 (RIP3) and cleaved caspase-1 protein (Wen et al., 2019).

3.3.5. Effect of TNF-α and TNF-β on the expression of NGALR in TNBC cells

In our earlier studies, we have demonstrated the effects of TNF-α and TNF-β on TNBC cell proliferation, survival, EMT, migration, and cell death and observed that both the cytokines were strongly associated with TNBC progression. However, the effect of TNF-α and TNF-β on the NGALR expression in TNBC still remains unclear. Therefore, we have determined the effect of TNF-α and TNF-β on the expression of NGALR in MDA-MB-231 and MDA-MB-468 cells. Our results showed that both TNF-α and TNF-β significantly upregulated NGALR expression in MDA-MB-231 and MDA-MB-468 cells compared to untreated cells (Figure 3.4 A, B, C, D). This is the first study to report the expression of NGALR after TNF-α and TNF-β treatment in TNBC cell lines. Mao et al. (2011) showed that IL-1β induced NGALR expression by modulating MAPK/ERK signaling pathway in HMC. However, in contrast to our results, TNF-α treatment has not upregulated the expression of NGALR (Mao et al., 2011). Moreover, a study has hypothesized that inhibition of TNF-α might play a critical role in the regulation of breast cancer progression (Mercogliano et al., 2020).–Chi et al. (2020) took the cerebrospinal fluid (CSF) from leptomeningeal metastasic patients and observed that both NGAL and NGALR were upregulated in cancer cells than macrophages (Chi et al., 2020). Further, in mouse collecting duct cells,

lipopolysaccharides (LPS)/toll-like receptor 4 (TLR4) activation downregulated the expression of NGALR (Probst et al., 2019).

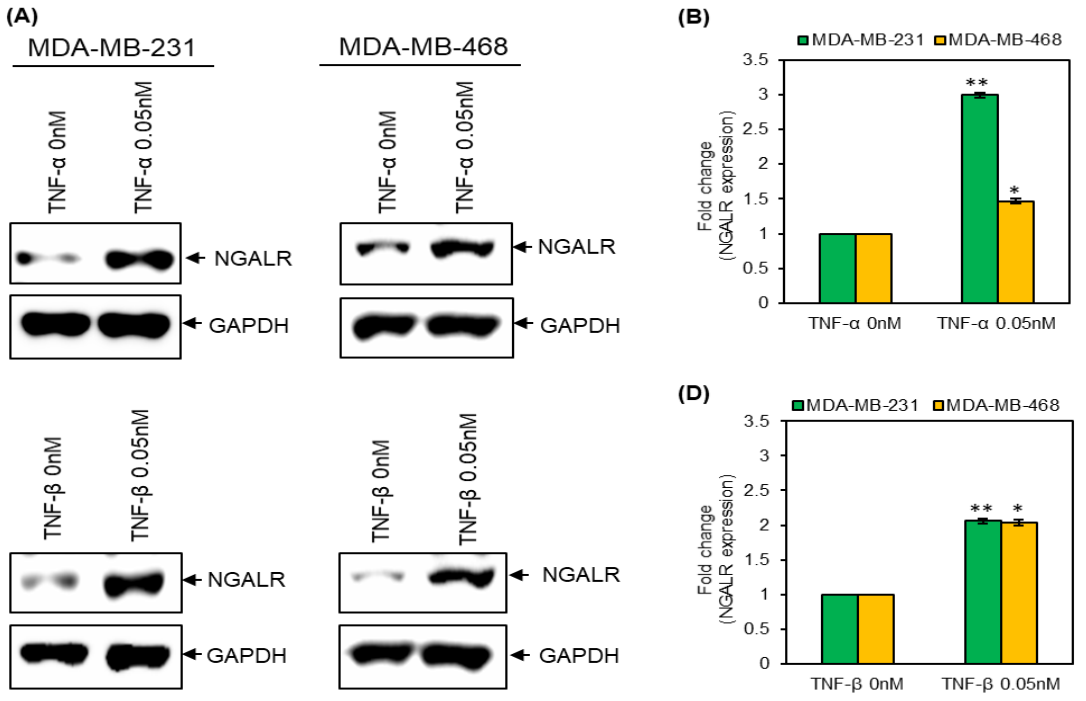

Figure 3.5. Effect of TNF-α and TNF-β on the expression of NGALR in TNBC cells. **(A)** Representative images showing the expression of NGALR in MDA-MB-231 and MDA-MB-468 cells determined by wound healing assay. **(B)** Quantification of the NGALR expression in MDA-MB-231 and MDA-MB-468 cells using Image Lab software. GAPDH was used as the loading control. Data are represented as mean ± SE. * = $p<0.05$ and ** = $p<0.005$ compared to untreated cells.

3.4. Conclusion

Taken together, the current chapter demonstrated that both TNF-α and TNF-β are strongly associated with TNBC progression. Our results revealed that TNF-α and TNF-β significantly increased cell proliferation, survival, EMT, and migration and suppressed cell death in MDA-MB-231 and MDA-MB-468 cells. Further, TNF-α and TNF-β significantly upregulated the expression of NGALR in MDA-MB-231 and MDA-MB-468 cells. These results comply with our previous chapter findings, where we have shown that overexpression of NGALR leads to TNBC progression. Therefore,

NGALR might be an important molecule in the progression and regulation of TNBC. However, these are preliminary findings, which required further mechanistic validation.

Chapter 4

Role of NGALR in the development of TNBC

4.1. Introduction

Our previous chapter showed that NGALR is overexpressed in various sub-types of breast cancer and is associated with poor survival. Further, TNF-α and TNF-β were strongly associated with TNBC progression through modulation of cell proliferation, survival, EMT, migration, and cell death. Moreover, NGALR expression was significantly upregulated upon treatment with TNF-α and TNF-β in MDA-MB-231 and MDA-MB-468 cells compared to untreated cells. These results strongly advocated that NGALR is associated with breast cancer progression and regulation, specifically TNF-α and TNF-β mediated TNBC progression. Therefore, in this chapter, we have determined the role of NGALR in the pathogenesis of TNBC. In order to achieve this goal, we have performed small interfering RNA (siRNA)-mediated knockdown of NGALR in both MDA-MB-231 and MDA-MB-468 cell lines and examined the role of NGALR in various cancer hallmarks, such as proliferation, survival, autophagy, EMT, invasion, and migration. Further, we have determined the expression of various downstream proteins involved in the pathogenesis and progression of TNBC. In addition, we have analyzed their association with TNF-α and TNF-β-mediated TNBC and deciphered the underlying mechanism of action. Notably, this is the first study to report the role of NGALR in TNBC progression.

4.2. Materials and Methods

4.2.1. Cell culture

The cell lines details, media, and other materials used for the cell culture have already been reported in Chapter 2, Section 2.2.5. ‘Cell culture’.

4.2.2. Silencing of NGALR gene

Silencing of NGALR gene was accomplished via transfection with targeted siRNA sequences in both MDA-MB-231 and MDA-MB-468 cells. The siRNA sequences were selected from the research studies of Cheng et al. (2014) and Huang et al. (2015), which were further confirmed through bioinformatics (Cheng et al., 2014; Huang et al., 2015). These siRNAs were procured from Eurofins Genomics, Germany (Table 4.1).

Briefly, MDA-MB-231 and MDA-MB-468 cells were seeded into six-well plates and once 50%-60% confluency was achieved, the siRNA transfection was initiated by following manufacturer's instructions. Both scramble (Scr) and NGALR (siNGALR) were mixed with lipofectamine RNAiMAX (Invitrogen) reagent using Opti-MEM media. The mixture was added to each well and incubated for the next 72hr in a CO_2-regulated incubator. Finally, cells were harvested for protein isolation and various other experiments were performed.

Table 4.1. siRNA target sequences.

Gene	**Sequence**
Scramble Sense (5'→3')	UUCUCCGAACGUGUCACGU
Scramble Antisense (3'→5')	AAGAGGCUUGCACAGUGCA
NGALR(A) Sense (5'→3')	CCUCAAGGAUUGGGACUAU
NGALR (A) Antisense (3'→5')	GGAGUUCCUAACCCUGAUA
NGALR(B) Sense (5'→3')	GAUUCCUCUUGGGCUUUCU
NGALR (B) Antisense (3'→5')	CUAAGGAGAACCCGAAAGA

4.2.3. MTT assay

The effect of NGALR knockdown on the proliferation of MDA-MB-231 and MDA-MB-468 cells were determined by MTT assay. The Scr and siNGALR transfected TNBC cells were seeded in ninety-six well plates at a density of 2000 cells/100µl and stored overnight in a CO_2-regulated incubator. Then, at every indicated interval (0 and 24hr), 10µl of MTT (5mg/ml) was added to all wells and the same steps were followed as mentioned earlier in Chapter 3, Section 3.2.2. 'MTT assay'.

Additionally, the effect of TNF-α and TNF-β on the proliferation of Scr and siNGALR transfected MDA-MB-231 and MDA-MB-468 cells were examined through MTT assay. Both the transfected cells were seeded in ninety-six well plates at a density of 2000 cells/100µl. Then both the cells were treated with 0.05nM of TNF-α and TNF-β and the same protocol was followed as mentioned above.

4.2.4. Colony formation assay

The clonogenicity of Scr and siNGALR-transfected MDA-MB-231 and MDA-MB-468 cells was determined by colony formation assay. Briefly, 500 cells/well were seeded in a six-well plate and stored overnight in a CO_2-controlled incubator. Then, we have followed the same procedure as mentioned earlier in Chapter 3, Section 3.2.3. 'Colony formation assay'.

Further, the colony formation assay was performed to determine the effect of TNF-α and TNF-β on the clonogenicity of transfected TNBC cells. After seeding of transfected MDA-MB-231 and MDA-MB-468 cells, the treatment of 0.05nM of TNF-α and TNF-β were given for 24hr, and the same procedure was followed as mentioned above thereafter.

4.2.5. Cell cycle analysis

The percentage of Scr and siNGALR transfected TNBC cells at the different phases of cell division were analyzed by cell cycle analysis. The post-transfected MDA-MB-231 and MDA-MB-468 cells were seeded into the six-well plates at a density of $1x10^5$ cells/well and stored in a CO2-controlled incubator for 48hr. Then, cells were trypsinized and washed with 1X PBS. The cells were fixed by pouring 70% ice-cold ethanol dropwise through vortexing and stored overnight at -20°C. The cells were centrifuged again and washed with 1X PBS, and incubated with PI/RNase buffer for 20 minutes at room temperature (RT) in the dark. Further, the distribution of the cells in each cell cycle phase was examined through a flow cytometer (BD FACSCelesta™, USA) and quantified by FCS Express software.

4.2.6. Immunocytochemistry (ICC) analysis

The post-transfected MDA-MB-231 and MDA-MB-468 cells were seeded on glass coverslips in a twelve-well plate to give a plating density of $6x10^4$ cells/well and allowed to reach 60-70% confluency. The cells were fixed with formaldehyde (3.7%) for 20 minutes at RT. 0.1% Triton X-100 was used to permeabilize the cells for 10 minutes, and 2% BSA was used thereafter for blocking the cells for 10 minutes. Subsequently, the cells were incubated overnight with 5µg/mL anti-LC3B polyclonal antibody (Ref. L10382, Life technologies) at 4°C. Further, cells were washed with 1X PBST and incubated with Alexa Fluor 594-conjugated antibody (Invitrogen, USA) at RT for 45 minutes. The cells were again washed with 1X PBST and counterstained with 0.01µg/ml DAPI for 5 minutes. The coverslips with cells were then appropriately dried and mounted on a glass slide using DPX mountant. The mounted slides were then observed under an upright fluorescence microscope and images were captured. Further, the fluorescent color intensity was determined using Olympus cellSens software.

We have followed the same procedure for the EMT assay as we performed in Chapter 3, Section 3.2.4. 'EMT assay'.

4.2.7. Invasion assay

The invasiveness of Scr and siNGALR transfected MDA-MB-231 and MDA-MB-468 cells were determined by Boyden's invasion chamber assay. Briefly, after successful transfection with these siRNAs, both MDA-MB-231 and MDA-MB-468 cells were incubated with serum-free media for 18hr. Then $5x10^4$ cells/500µl/well were seeded into the upper chamber of the Transwell migration chamber, which is pre-coated with Matrigel (Cat No. 354480, USA) using serum-free DMEM media. Further, the lower chamber of Transwell was filled with 750µl of DMEM medium (10% FBS) as a chemo-attractant and the cells were incubated for 48hr in a CO_2 incubator. The whole media was discarded, and the chamber was washed the 1X PBS and fixed with 70% ethanol. The migration chamber was again washed and incubated with 0.01 % (w/v) crystal violet. The cells that remained (non-migrated) on the upper surface of the membrane were scraped-off using cotton swabs. However, the stained cells were observed under an inverted light microscope (Nikon Eclipse TS100, Japan), and respective images were captured using a Nikon 500 camera (which was attached at the top of the microscope). Further, the Transwell were incubated with 1% SDS for 1hr, and the absorbance was recorded at 590nm in a multi-mode microplate reader (SpectraMax iD3, Molecular Devices, USA).

4.2.8. Wound healing assay

The wound healing assay was used to determine the migration of Scr and siNGALR transfected TNBC cells. The siRNA transfected MDA-MB-231 and MDA-MB-468 cells were seeded at a density of $6x10^5$ cells/well and $8x10^5$ cells/well in a twelve well

plates, respectively. Further, we have followed the same protocol as discussed earlier in Chapter 3, Section 3.2.5. 'Wound healing assay'.

In addition, the effect of TNF-α and TNF-β on the migration of Scr and siNGALR transfected MDA-MB-231 and MDA-MB-468 cells was also examined through wound healing assay. The transfected cells were seeded in twelve well plates at a density of $6x10^5$ cells/well and $8x10^5$ cells/well respectively. Then both the cells were treated with 0.05nM of TNF-α and TNF-β and the same protocol was followed as mentioned above.

4.2.9. Western blot analysis

The expression of siNGALR in TNBC cell lines was examined by Western blot analysis to confirm the successful knockdown. Further, we have determined the expression of various proteins in post-transfected cells by the same assay. Briefly, post-transfected MDA-MB-231 and MDA-MB-468 cells were lysed with lysis buffer. Then, the same steps were followed as discussed previously in Chapter 2, Section 2.2.6. Western blot analysis. Moreover, the blocking was done in 5% BSA for the phospho (p) antibodies (Table 4.2). Further, this assay was also used to determine the effect of TNF-α and TNF-β on Scr and siNGALR transfected TNBC cells. For this purpose, the post-transfected MDA-MB-231 and MDA-MB-468 cells were treated with 0.05nM of TNF-α and TNF-β for 24hr and then lysed with lysis buffer. Thereafter, we followed the same procedure as mentioned above.

Table 4.2. Details of the various antibodies used for Western blot.

Name of Antibody	**Cat No, procured**	**Dilutions used**
Anti-SLC22A17 (NGALR)	ab124506 abcam®, USA	1:7000

Anti-GAPDH	2118S; Cell Signaling Technology, USA	1:2000
Anti-Phospho-Akt (Ser473)	4060S; Cell Signaling Technology, USA	1: 4000
Anti- Akt1	2938S; Cell Signaling Technology, USA	1: 2000
Anti-Phospho-mTOR (Ser2448)	5536T; Cell Signaling Technology, USA	1: 2000
Anti-mTOR	2983T; Cell Signaling Technology, USA	1: 2000
Anti-Phospho-STAT3 (Ser727)	9134T; Cell Signaling Technology, USA	1: 2000
Anti-STAT3	9139T; Cell Signaling Technology, USA	1: 2000
Anti-Phospho-JAK-2 (Tyr1007/1008) (C80C3)	3776S; Cell Signaling Technology, USA	1: 1000
Anti-JAK-2	3230S; Cell Signaling Technology, USA	1: 1000
Anti-p21	10-7526; ABGENEX Pvt. Ltd., India	1:1000
Anti-Cyclin D1	15071; Cell Signaling Technology, USA	1: 2000
Anti-LC3B	2775S; Cell Signaling Technology, USA	1: 1000
Anti-Bcl-2	15071; Cell Signaling Technology, USA	1: 1000
Anti-COX-2	12282P; Cell Signaling Technology, USA	1: 2000
Anti-Survivin	2808BC; Cell Signaling Technology, USA	1: 2000
Anti-CXCR4	ab124824; abcam®, Cambridge, USA	1: 2000
Anti-VEGF-A	ab46154; abcam®, Cambridge, USA	1: 2000
Anti-Twist1	46702S; Cell Signaling Technology, USA	1: 2000
Anti-N-cadherin	13116S; Cell Signaling Technology, USA	1: 1000
Anti-E-cadherin	3195S; Cell Signaling Technology, USA	1: 2000
Anti-TNF-α	3707S; Cell Signaling Technology, USA	1: 2000
Anti-TNF-β	ab100844; abcam®, Cambridge, USA	1: 2000

Anti-rabbit secondary antibody	ab97080; abcam®, Cambridge, USA	1: 6000
Anti-mouse secondary antibody	ab97040; abcam®, Cambridge, USA	1: 6000

4.2.10. Statistical analysis

The statistical analysis of our results was determined through Student's t-test. Statistically significant values were denoted by *. p-value < 0.05 was denoted by *, and p-value < 0.005 denoted by **. All the data are represented as mean ± SD.

4.3. Results and Discussion

In the present chapter, we have demonstrated the role of NGALR in TNBC progression and the modulation of various hallmarks of cancer. First, we have confirmed the knockdown of NGALR gene in both MDA-MB-231 and MDA-MB-468 cells. Subsequently, we have evaluated the effect of siNGALR on the proliferation, survival, autophagy, EMT, invasion, and migration of MDA-MB-231 and MDA-MB-468 cells. Then, we have identified the various downstream molecular targets of NGALR. Moreover, the effects of TNF-α and TNF-β on the proliferation, survival, and migration of transfected TNBC cells were also studied.

4.3.1. Silencing of NGALR in TNBC cells

siRNA can be used as a potential tool to determine the single gene function *in vitro* and *in vivo*, thereby it plays a crucial role in designing targeted therapies (Dana et al., 2017). In order to establish the role of NGALR in TNBC progression, siRNA silencing of NGALR was carried out and confirmed by Western blot analysis. Our results showed that siRNA knockdown significantly reduced the expression of NGALR in both MDA-MB-231 and MDA-MB-468 cells than Scr control (Figure 4.1 A, B). After successful knockdown of NGALR, we have determined the role of siNGALR in the regulation of

various hallmarks of cancer, such as proliferation, survival, autophagy, EMT, invasion, migration, and angiogenesis.

4.3.2. Silencing of NGALR decreased proliferation and survival of TNBC cells

Cancer cells are strongly characterized by increased proliferation and survival and reduced apoptosis through the regulation of various signaling pathways and molecules (Bordoloi et al., 2019; Fouad YA & Aanei C, 2017). Therefore, we have evaluated the effect of NGALR knockdown on the proliferation and survival of human TNBC cells through MTT and colony formation assay. Our results demonstrated that silencing of NGALR significantly decreased the proliferation and survival of both MDA-MB-231 and MDA-MB-468 cells compared to Scr control (Figure 4.1 C). Moreover, we have also determined the expression of various proteins which are associated with the proliferation and survival in TNBC cells (Figure 4.1 D, E). We observed that silencing of NGALR downregulated the expression of survivin, COX-2, and Bcl-2 in MDA-MB-231 and MDA-MB-468 cells than Scr control (Figure 4.1 F). Similarly, various studies have reported that COX-2 overexpression was associated with poor OS and increased death of TNBC patients (Mosalpuria et al., 2014; Abdel-Fatah et al., 2013). Further, COX-2 overexpression was significantly associated with increased Bcl-2 protein expression, genomic instability, and doxorubicin resistance in breast cancer cells (Singh et al., 2008). However, siRNA knockdown of COX-2 decreased the stemness of TNBC cells (Tain et al., 2017). Recently, another study demonstrated that silencing of COX-2 gene markedly suppressed the proliferation and growth of breast cancer cells by downregulating the expression of survivin and Bcl-2 (Han et al., 2014). Similarly, siRNA-mediated knockdown of the survivin gene suppressed growth and induced apoptosis of breast cancer cells by Bid cleavage and caspase-3 activation

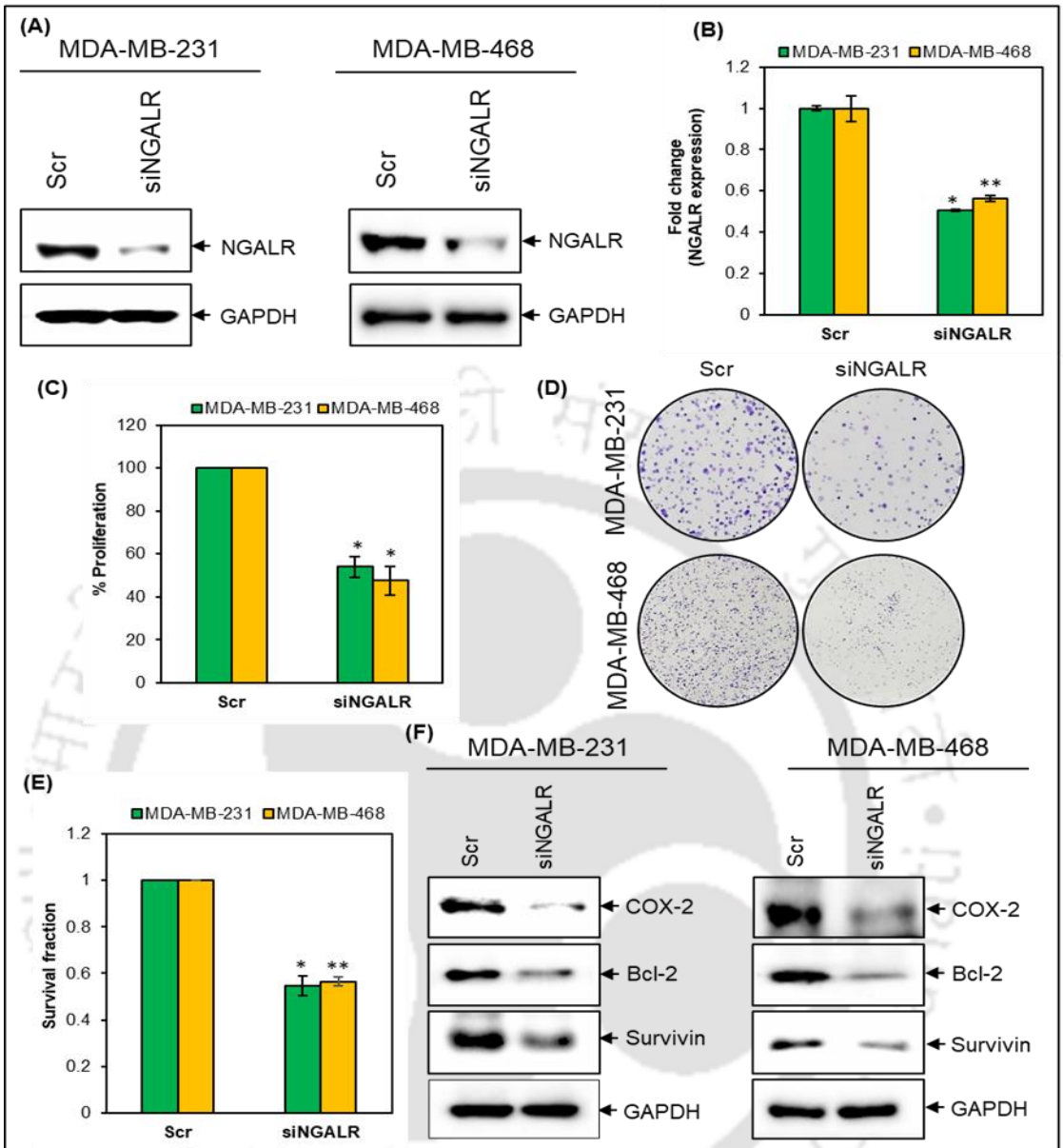

Figure 4.1. Effect of siNGALR knockdown on the expression of NGALR and proliferation and survival of TNBC cells. **(A-B)** Representative images and of the NGALR expression in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells determined by Western blot analysis. **(C)** Percentage proliferation of siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells determined by MTT assay. **(D-E)** Representative images and graphical representation of the survival fraction of siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells determined by colony formation assay **(F)** Representative images of the expression of various proteins associated with proliferation and survival of siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells determined by Western blot analysis. Data are represented as mean ± SE. * = $p<0.05$ and ** = $p<0.005$ compared to Scr control.

(Croci et al., 2008). Moreover, siRNA knockdown of Bcl-2 sensitized TNBC cells to doxorubicin (Inao et al., 2018). Notably, our results also showed that silencing of

NGALR significantly suppressed proliferation and survival of MDA-MB-231 and MDA-MB-468 cells by downregulating the expression of COX-2, survivin and Bcl-2.

4.3.3. Silencing of NGALR induced S phase arrest of TNBC cells

We have already shown that silencing of NGALR decreased TNBC cell proliferation and survival in our previous study. To further confirm these findings, we have performed cell cycle analysis in transfected MDA-MB-231 and MDA-MB-468 cells through flow cytometric analysis. Our results demonstrated that silencing of NGALR induced the S phase cell cycle arrest in both MDA-MB-231 and MDA-MB-468 cells compared to Scr control (Figure 4.2 A, B). The S phase of the cell cycle is a synthetic phase where DNA replication and synthesis occur. Moreover, cell cycle arrest at any phase corresponds to the increased number of cells compared to the normal control group (Zhang et al., 2000; Tinnemans et al., 1995). Our results showed an increased percentage cell count in the S phase compared to Scr control in MDA-MB-231 and MDA-MB-468 cells. In order to confirm S phase arrest, we have performed Western blot analysis of the proteins associated with the regulation of cell cycle and observed that the expression of cyclin A2 was downregulated while the p21 expression was upregulated in MDA-MB-231 and MDA-MB-468 cells than Scr control (Figure 4.2 C). These findings strongly supported our earlier results that siRNA-mediated knockdown of NGALR suppressed proliferation and survival in MDA-MB-231 and MDA-MB-468 cells. In line with our findings, an *in vivo* study reported that cyclin A is a prognostic factor in early-stage breast cancer (Michalides et al., 2002). Further, cyclin A2, cyclin-dependent kinase 1 (CDK1), and cyclin B1 were overexpressed and associated with worse OS of TNBC patients (Lu et al., 2020). Moreover, deletion of cyclin A2 decreased tumor formation and tumorigenesis in oncogene-transformed mouse

embryonic fibroblasts (Gopinathan et al., 2014). An inverse relationship was observed between p21 and HER-2, which was associated with poor OS (Winters et al., 2003). On

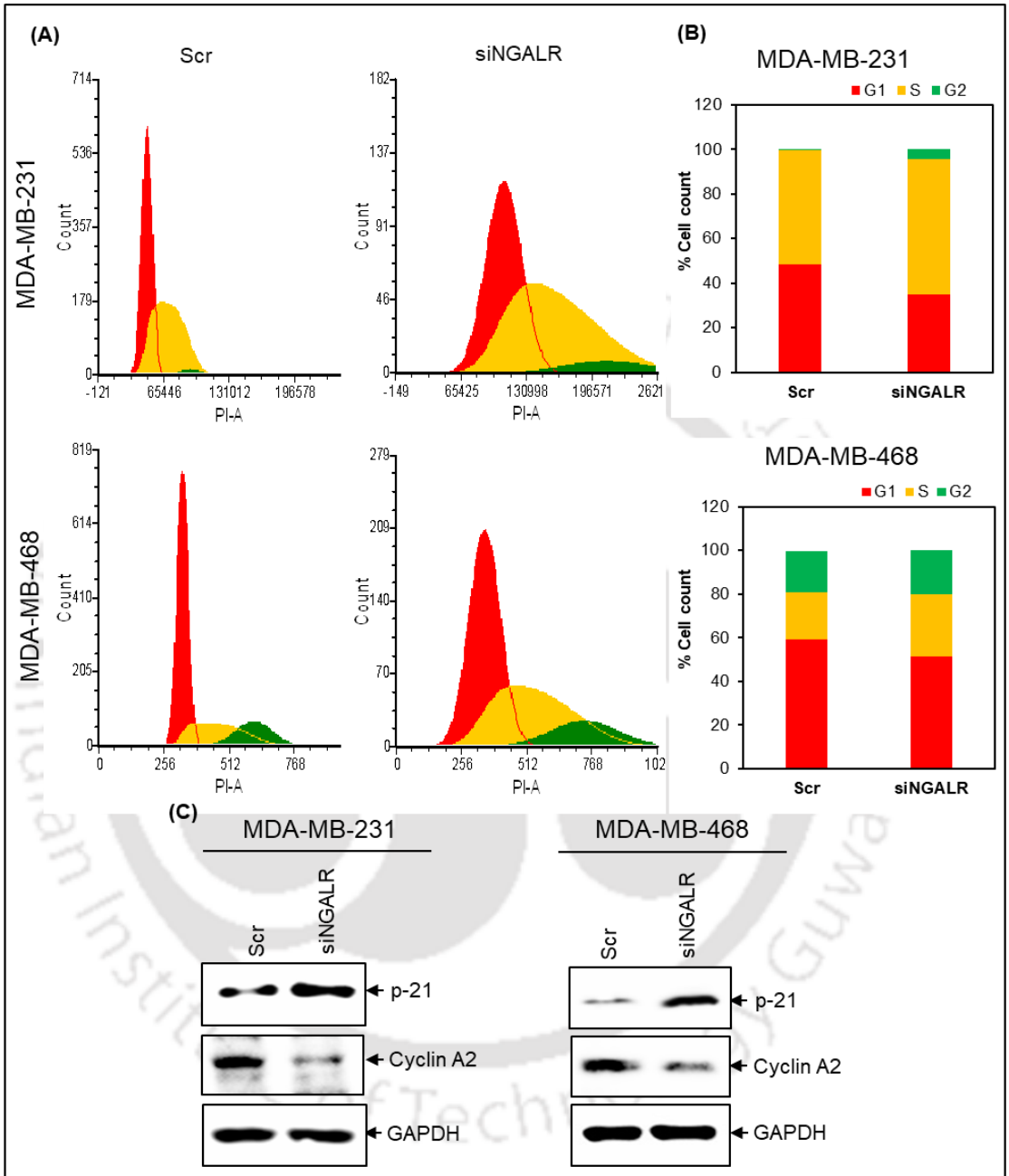

Figure 4.2. Effect of siNGALR knockdown on the cell cycle regulation of TNBC cells. **(A-B)** Cell cycle distribution and graphical representation of the percentage of siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells determined by flow cytometry and quantified by FCS Express software. **(C)** Representative images of the expression of various proteins associated with cell cycle regulation in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells determined by Western blot analysis.

the contrary, a xenograft mouse model of breast cancer recently demonstrated that the silencing of p21 suppressed primary tumor formation (Dai et al., 2017). In our study, we have shown that silencing of NGALR induced the S phase cell cycle arrest by modulating the expression of cyclin A2 and p21 in TNBC cells. Further, these results strongly advocated that silencing of NGALR decreased TNBC cell proliferation and survival by inducing the S phase arrest.

4.3.4. Silencing of NGALR induced autophagy in TNBC cells

Autophagy is an important catabolic (lysosomal degradation) process, which is crucial for survival and maintenance of homeostasis in cellular stress responses in cancer progression (Chen et al., 2013; Han et al., 2018; Ulasov et al., 2019). Various studies have demonstrated that microtubule-associated protein chain 3B (LC3B) is an important marker of autophagy and plays a dual role in cancer development and progression (Yun CW & Lee SH, 2018; Satyavarapu et al., 2018; Berardi et al., 2011; Bristol et al., 2012; Cocco et al., 2020). Therefore, we have evaluated the expression of LC3B-mediated autophagy after siNGALR knockdown in TNBC cells through ICC analysis using LC3B-antibody. Our ICC results showed that siRNA-mediated knockdown of the NGALR gene increased LC3B expression significantly in both MDA-MB-231 and MDA-MB-468 cells than Scr control (Figure 4.3 A, B). These findings were further confirmed through Western blot analysis and it was found that the silencing of NGALR upregulated the expression of LC3B protein in MDA-MB-231 and MDA-MB-468 cells compared to Scr control (Figure 4.3 C). In accordance with our findings, Chang et al. (2016) also reported that decreased expression of LC3B protein is associated with a poor prognosis of TNBC (Chang et al., 2016). Moreover, silencing of the Bcl-2 gene decreased proliferation and survival and increased

autophagic cell death by inducing LC3B expression in breast cancer cells (Akar et al., 2008).

On the contrary, various studies demonstrated that elevated expression of LC3B is involved in poor prognosis and the worst outcome of breast cancer (Zhao et al., 2013; Chen et al., 2013; Bortnik et al., 2020). Inhibition of autophagy in LC3B-overexpressed TNBC cells improved chemotherapy efficiency (Lefort et al., 2014). Recently, Li et al. (2020a) also revealed that autophagy suppression through the depletion of LC3B by inhibiting tenascin-C degradation increased T cell-mediated cytotoxicity in TNBC cells

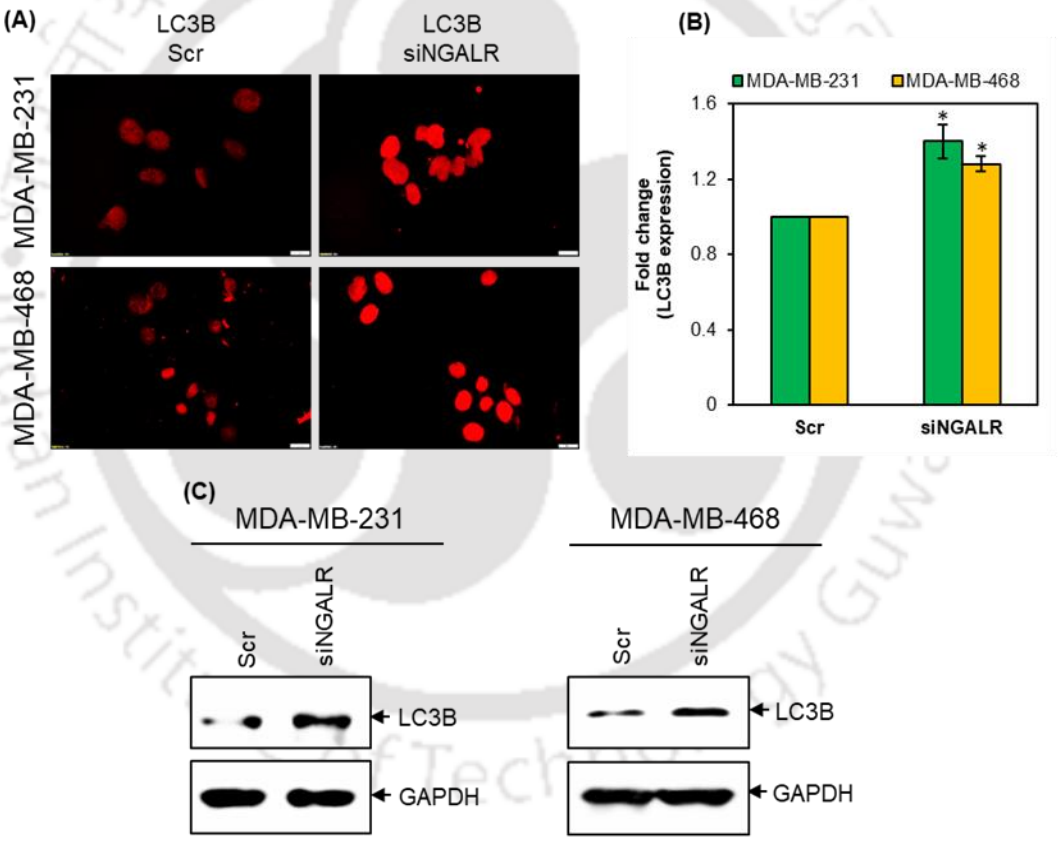

Figure 4.3. Effect of siNGALR knockdown on autophagy in TNBC cells. **(A-B)** Representative images and quantification of LC3B expression in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells determined by ICC and CellSens software respectively. Original magnification 40X objective. Scale bar = 20µM. **(C)** Expression of LC3B in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells determined by Western blot analysis. Data are represented as mean ± SE. * = $p<0.05$ compared to Scr control.

(Li et al., 2020a). Further, autophagy delayed apoptotic death following DNA damage in breast cancer cells (Abedin et al., 2007). Moreover, our findings showed that silencing of NGALR induced autophagy by increasing the expression of LC3B protein in MDA-MB-231 and MDA-MB-468 cells.

4.3.5. Silencing of NGALR suppressed EMT in TNBC cells

EMT is one of the important phenomena of large-scale cell movement during cell growth (Leggett et al., 2021; Yang et al., 2020). Cancer cells induced metastasis and tumorigenesis by recruiting various transcription factors and proteins required for EMT, such as Snail, E-cadherin, N-cadherin, vimentin, Twist, Slug, COX-2, ZEB, and FOXA-2 (Kar et al., 2019; Matysiak et al., 2017; Karamanou et al., 2020). Therefore, we have determined the expression of EMT proteins (vimentin and Snail) through ICC analysis in MDA-MB-231 and MDA-MB-468 cells after the silencing of NGALR. Our ICC results demonstrated that both vimentin and Snail proteins were significantly downregulated in siNGALR transfected MDA-MB-231 and MDA-MB-468 cells than Scr control (Figure 4.4 A, B, C, D). Various studies reported that upregulated expression of vimentin and Snail are associated with poorer OS and recurrence-free survival (RFS) in TNBC patients (Karihtala et al., 2013; Yamashita et al., 2013; Jiralerspong et al., 2010; Rashed et al., 2021). Further, elevated Snail expression was related with increased EMT, and induced cell migration and suppressed cell adhesion in breast cancer cells (Smith et al., 2014). However, silencing of the Snail suppressed motility in MDA-MB-231, and MDA-MB-468 cells, whereas overexpression of Snail increased migratory propensity (Lundgren et al., 2009). On the contrary, the expression of Snail was significantly decreased in breast cancer patients with a poor outcome, primarily those patients who had node-positive breast tumors. However, the expression

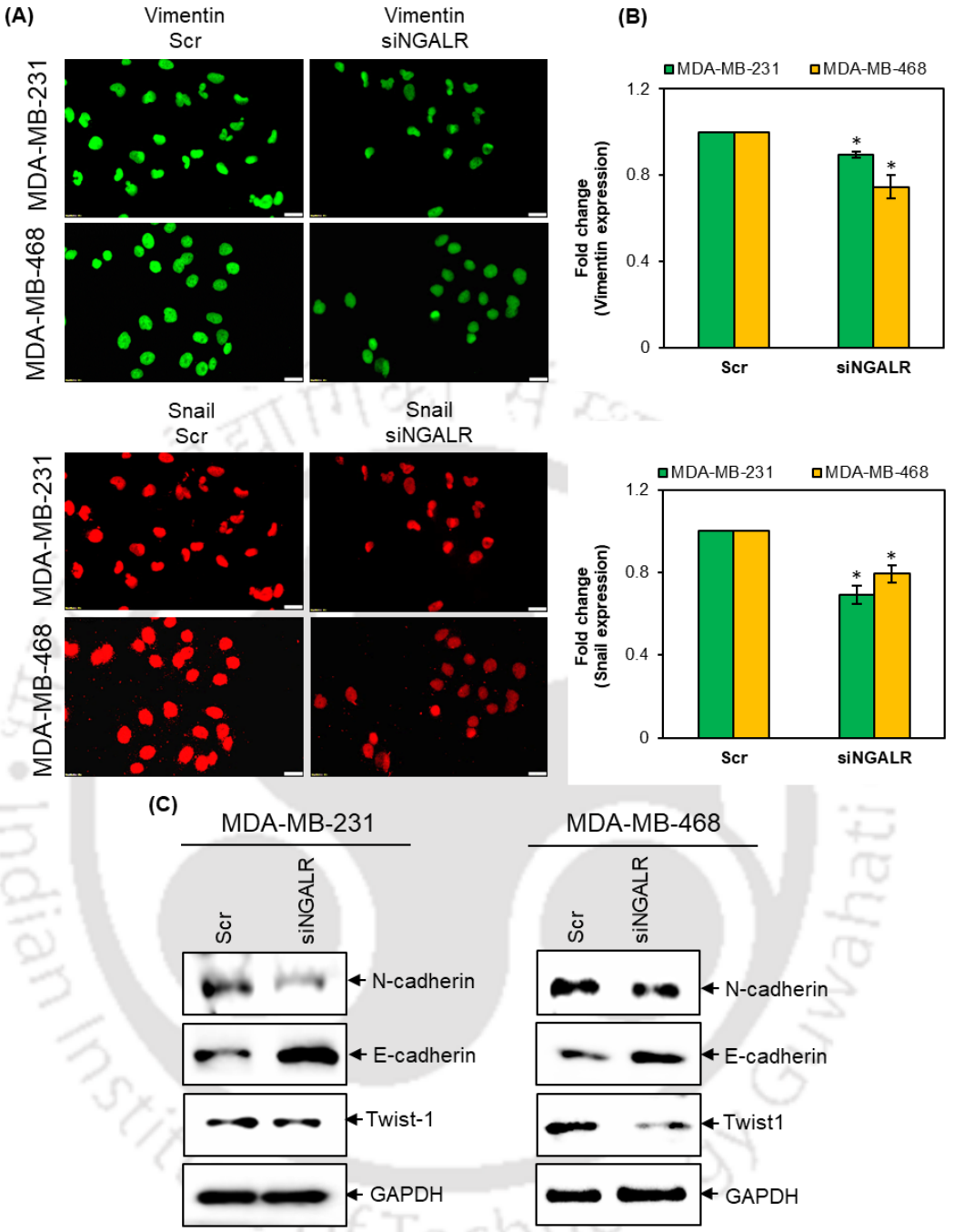

Figure 4.4. Effect of siNGALR knockdown on EMT of TNBC cells. **(A)** Representative images of vimentin and Snail expression in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells determined by ICC. Original magnification 40X objective. Scale bar = 20µM. **(B)** Graphical representation of quantification of vimentin and Snail in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells using CellSens software. **(C)** Expression of various EMT proteins in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells determined by Western blot analysis. Data are represented as mean ± SE. * = $p<0.05$ compared Scr control.

of Twist and Slug were elevated (Martin et al., 2004).

Further, we have performed Western blot analysis to confirm the role of EMT in siNGALR knockdown TNBC cells. Our results showed that silencing of NGALR downregulated N-cadherin and Twist1 and upregulated E-cadherin expression in MDA-MB-231 and MDA-MB-468 cells than Scr control (Figure 4.4 E). Similar to our study, increased expression of vimentin, Twist, and Slug and decreased E-cadherin expression was associated with poor OS of TNBC patients (Zhou et al., 2018; Shen et al., 2016). Finlay et al. (2014) showed that silencing of Twist1 suppressed invasion and migration in breast cancer cells (Finlay et al., 2014). Recently, a study reported that silencing of Snail-1 induced the S phase arrested and significantly decreased proliferation and migration and induced apoptosis in MDA-MB-468 cells (Aletaha et al., 2017). In the current chapter, we have demonstrated that the knockdown of NGALR significantly suppressed EMT by downregulation of vimentin, Snail, Twist1, and N-cadherin and upregulation of E-cadherin expression in MDA-MB-231 and MDA-MB-468 cells. It is well known that EMT is an essential step for the invasion and migration of cancer cells (Son H & Moon A, 2010). Therefore, next we have determined the role of siNGALR knockdown on invasion and migration in TNBC cells.

4.3.6. Silencing of NGALR attenuated invasion, migration, and angiogenesis in TNBC cells

Our initial findings strongly suggested that knockdown of NGALR decreased proliferation, survival, and EMT, and induced autophagy in TNBC cells. It is well established that invasion and migration are important hallmarks of cancer progression (Son H & Moon A, 2010). Therefore, we have determined the association of NGALR with invasion and migration of TNBC cells. Our matrigel invasion assay showed that

knockdown of NGALR attenuated invasion in MDA-MB-231 and MDA-MB-468 cells compared to Scr control (Figure 4.5 A, B). Similarly, silencing of NGALR significantly suppressed the migration of MDA-MB-231 and MDA-MB-468 cells than Scr control. Moreover, we found that the percentage wound area remaining was more in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells compared to Scr control (Figure 4.5 C, D). In support of these findings, Smith et al. (2014) demonstrated that the EMT marker Snail significantly promoted invasion and migration in breast cancer cells (Smith et al., 2014). Moreover, *in vitro* and *in vivo* studies suggested that the knockdown of Snail suppressed invasion and migration and attenuated chemoresistance and cancer stem-like properties in mouse breast cancer (Ma et al., 2017).

Further, we have determined CXC chemokine receptor 4 (CXCR4) protein expression which is significantly associated with the invasion and migration of cancer cells. After successful knockdown of siNGALR, the CXCR4 protein expression was determined through Western blot analysis in TNBC cells. We observed that knockdown of NGALR suppressed the expression of CXCR4 protein in both MDA-MB-231 and MDA-MB-468 cells compared to Scr control (Figure 4.5 E). In accordance with our results, siRNA-mediated silencing of CXCR4 blocked metastasis in MDA-MB-231 cells (Liang et al., 2004). Similarly, inhibiting the expression of CXCR4 with anti-CXCR4 antibody attenuated the migration and vascular permeability of MDA-MB-231 cells (Lee et al., 2004). Further, an *in vitro* study demonstrated that the knockdown of CXCR4 by RNAi significantly inhibited breast cancer cell migration (Chen et al., 2003). Li et al. (2004) showed that CXCR4 was significantly overexpressed in breast cancer patient's tissues and resulted in poor OS (Li et al., 2004). Another study reported that CXCR4 and CXCR7 stimulated metastasis in breast cancer (Muller et al., 2001). These studies further support our findings that silencing of NGALR attenuated the

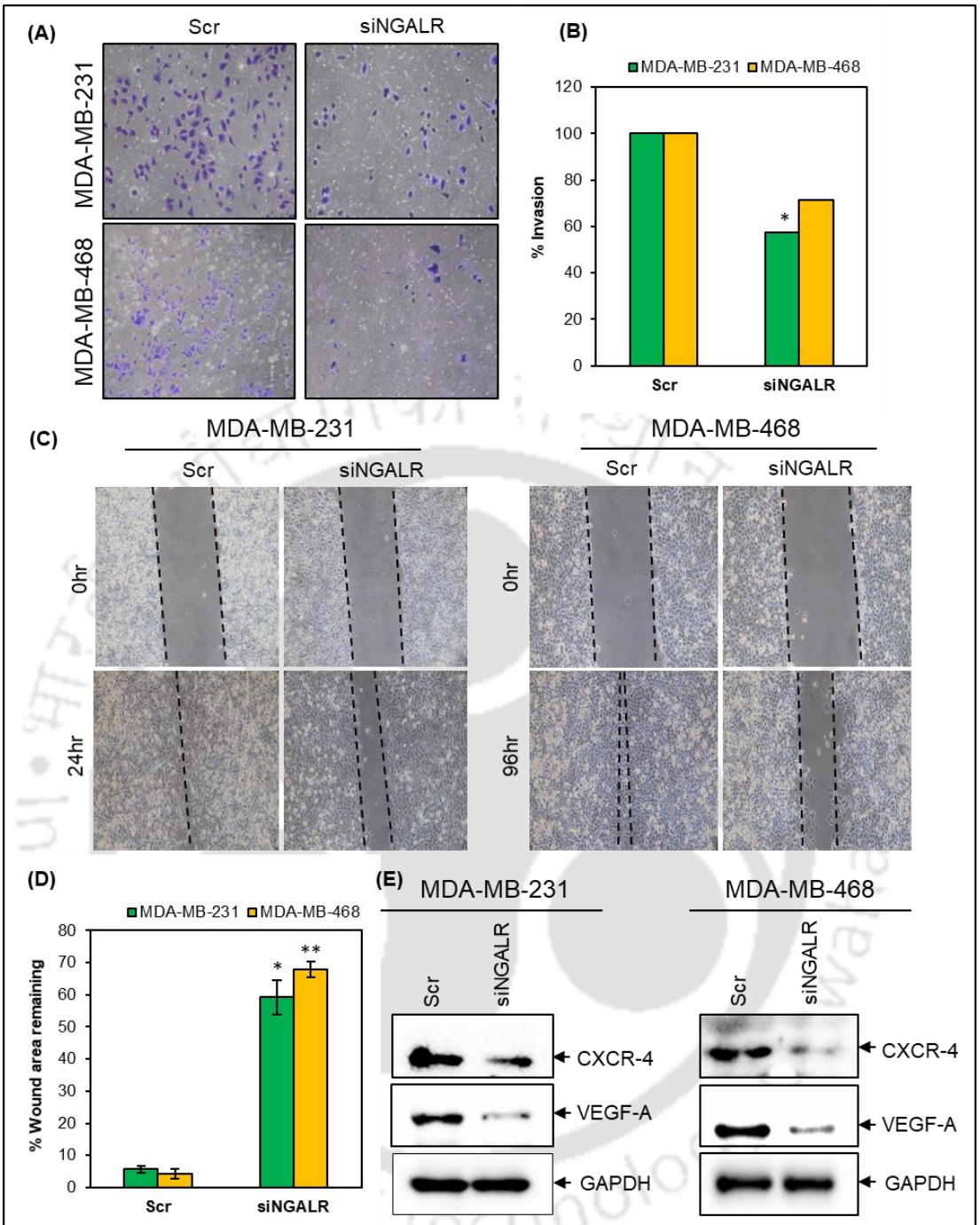

Figure 4.5. Effect of siNGALR knockdown on invasion, migration and angiogenesis of TNBC cells. **(A-B)** Representative images and graphical representation of percentage invasion of MDA-MB-231 and MDA-MB-468 cells determined by invasion assay. **(C-D)** Representative images and graphical representation of percentage wound area remaining of MDA-MB-231 and MDA-MB-468 cells determined by wound healing assay. **(E)** Expression of various proteins associated with invasion, migration and angiogenesis in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells determined by Western blot analysis. Data are represented as mean ± SE. * = $p<0.05$ and * = $p<0.05$ compared to Scr control.

invasion and migration of MDA-MB231 and MDA-MB-468 cells through the downregulation of CXCR4 expression.

Angiogenesis is one of the most critical processes required for cancer progression and is significantly associated with cell proliferation, survival, and migration. VEGF-A is a specific promoter of angiogenesis that induces vascular permeability (Goel HL & Mercurio AM, 2013; Roberts et al., 2013; Rapisarda A & Melillo G, 2012). Moreover, numerous studies have shown that increased expression of VEGF-A is associated with poor OS of TNBC patients (Ribatti et al., 2016; Marme et al., 2015; Dent SF, 2009). Our results showed that silencing of NGALR downregulated the expression of VEGF-A protein in MDA-MB-231 and MDA-MB-468 cells than Scr control (Figure 4.5 E). In line with our findings, increased expression of VEGF-A was associated with shorter RFS of TNBC patients (Linderholm et al., 2009). Su et al. (2016) reported that elevated VEGF-A expression was implicated with poor DFS and distant metastasis-free survival in the TNBC patients sample (Su et al., 2016). *In vitro* and *in vivo* models of TNBC demonstrated that oridonin inhibited VEGF-A-mediated angiogenesis and EMT by reducing the expression of HIF-1α, VEGF-A, vimentin, Snail, and N-cadherin and increasing the level of E-cadherin (Li et al., 2018). These findings strongly suggested that knockdown of NGALR decreased the angiogenesis of TNBC cells by downregulating the expression of VEGF-A.

4.3.7. Silencing of NGALR inhibited the Akt/mTOR and JAK/STAT pathways in TNBC cells

Our previous findings suggested that NGALR is significantly associated with TNBC progression. We have already demonstrated the role of NGALR in TNBC cell proliferation, survival, autophagy, EMT, invasion, migration, and angiogenesis.

However, the underlying molecular mechanism and signaling pathway through which NGALR imparted its action needs to be deciphered. It is well established that the Akt/mTOR pathway is strongly associated with proliferation, survival, migration, metabolism, apoptosis, and angiogenesis of TNBC cells (Massihnia et al., 2016; Costa et al., 2018; Khan et al., 2019; Hernandez-Aya et al., 2011; Cai et al., 2020). Therefore, we have determined the association of NGALR with the Akt/mTOR pathway in TNBC cells through Western blot analysis. We observed that silencing of NGALR decreased the expression of p-Akt (Ser473) and p-mTOR (Ser2448) in both MDA-MB-231 and MDA-MB-468 cells compared to Scr control. However, the expression of unphosphorylated Akt and mTOR were unchanged than Scr control in both TNBC cell lines. (Figure 4.6 A). Similar to our results, the PARP inhibitor was found to suppress the Akt/mTOR pathway in TNBC cells and enhanced the anticancer efficacy (De et al., 2014). Further, inhibition of the Akt/mTOR pathway repressed cell proliferation and attenuated intrinsic resistance to dasatinib in various TNBC cell lines (Haga et al., 2020). Recently a study reported that inhibiting the activation of STAT3 and Akt/mTOR signaling suppressed proliferation and survival and increased apoptosis in MDA-MB-231 and MDA-MB-468 cells, whereas reduced tumor weights in a xenograft mouse model of MDA-MB-231 cells (Qu et al., 2020). Similarly, berberine decreased the proliferation, invasion, and migration of breast cancer cells by attenuated the expression of PI3K, p-Akt, mTOR, and COX-2 and increased expression of p53 (Liu et al., 2020). Recently, a study reported that p-mTOR was significantly elevated in non-TNBC patients than in TNBC cases; however, unphosphorylated mTOR was not high (Ito et al., 2019).

Various studies have demonstrated that JAK/STAT pathway also plays a crucial role in the development and progression of TNBC (Qin et al., 2019; Song et al., 2020;

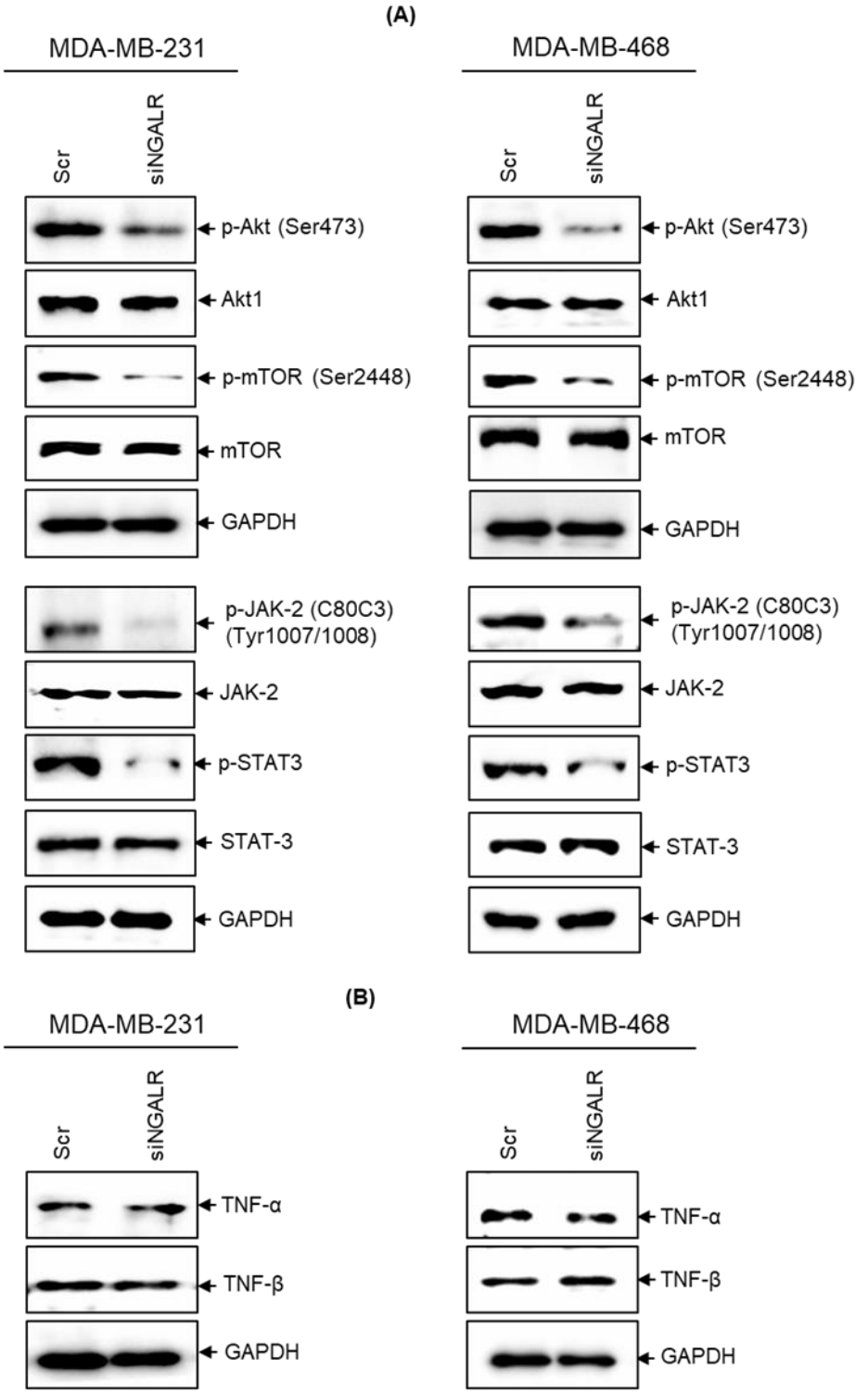

Figure 4.6. Effect of siNGALR knockdown on Akt/mTOR and JAK/STAT pathways in TNBC cells determined by Western blot analysis. **(A)** Expression of various proteins associated with Akt/mTOR and JAK/STAT pathways in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells compared to Scr control. **(B)** Expression of TNF-α and TNF-β in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells

Irey et al., 2019). Thus, we have determined the expression of various proteins associated with the JAK/STAT pathway after the knockdown of NGALR in TNBC cell lines. Our results demonstrated that knockdown of NGALR attenuated the expression of p-JAK-2 (Tyr1007/1008) (C80C3) and p-STAT3 (Ser727); however, the expression of JAK-2 and STAT3 were not changed compared to Scr control in MDA-MB-231 and MDA-MB-468 cells (Figure 4.6 A). Numerous studies have reported that the JAK/STAT pathway is widely associated with tumor development by regulating tumor cell proliferation, survival, EMT, metastasis, angiogenesis, inflammation, apoptosis, and chemoresistance (Qin et al., 2019; Jin W, 2020; Owen et al., 2019; Ma et al., 2020). In support of our findings, both *in vitro* and *in vivo* models of TNBC demonstrated that JAK-2 amplification was associated with increased stemness, metastasis, chemoresistance, and tumorigenesis (Balko et al., 2016). Similarly, increased expression of the JAK-2 and p-STAT3 resulted in TNBC progression; however, knockdown of JAK-2 decreased cell growth (Chen et al., 2018). On the other hand, Irey et al. (2019) reported that inhibition of the JAK/STAT pathway promoted TNBC progression by inducing the expression of protumorigenic inflammatory factors, such as COX-2 (Irey et al., 2019). Therefore, inhibition of the JAK/STAT pathway by targeting NGALR might prove to be an effective therapeutic approach for the management of TNBC.

In our previous chapter, we have already reported that TNF-α and TNF-β induced the expression of NGLAR and were significantly associated with increased proliferation, survival, EMT, and migration and decreased cell death of TNBC cells. To further confirm these findings, we have determined the expression of TNF-α and TNF-β in siNGALR knockdown TNBC cells through Western blot analysis. However, we have not observed any significant difference in the expression of TNF-α and TNF-

β in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells compared to Scr control (Figure 4.6 B). These results strongly advocated that TNF-α and TNF-β might

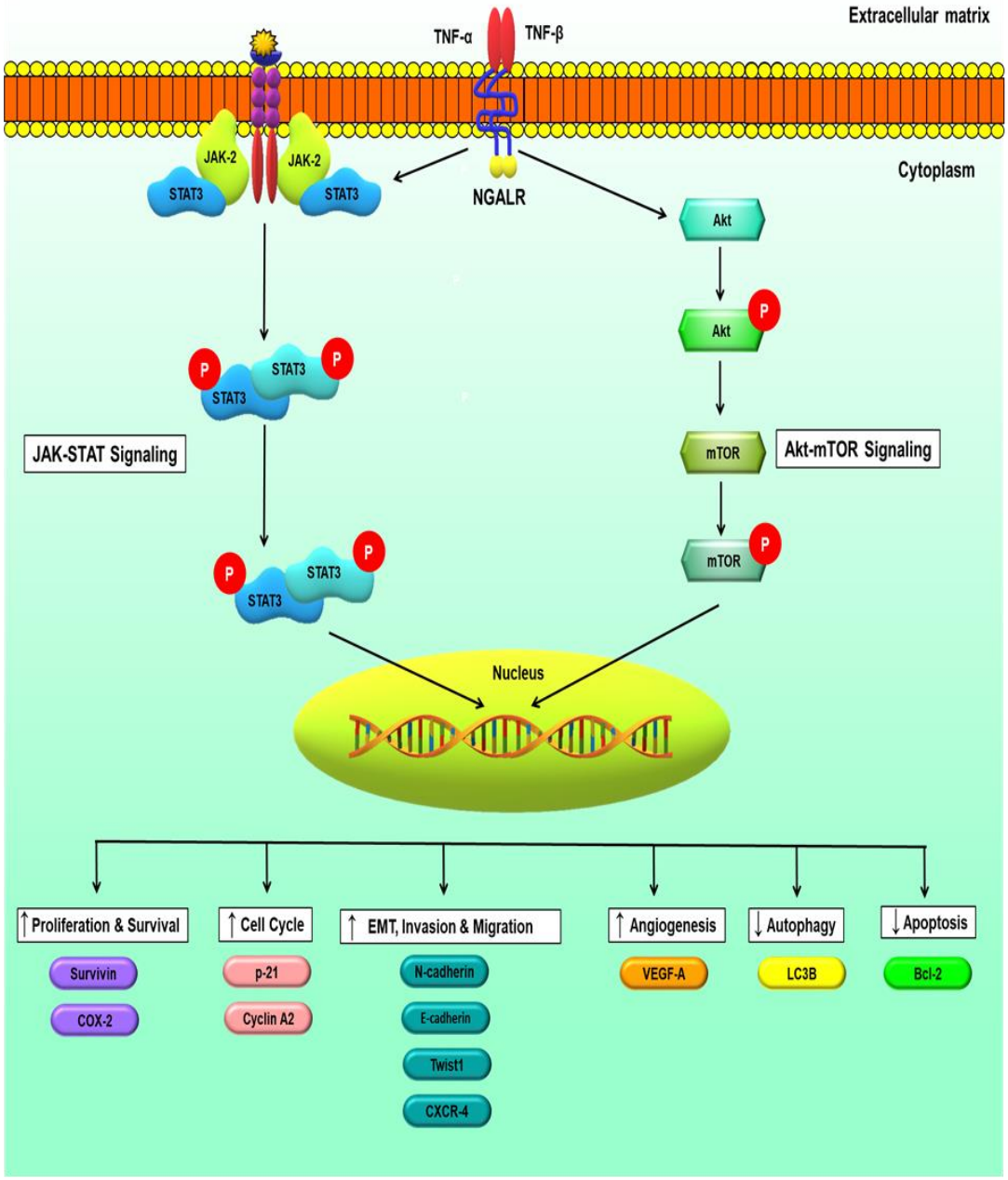

Figure 4.7. Possible mechanism of action of NGALR in TNBC. NGALR induces proliferation, survival, EMT, invasion, migration, and angiogenesis and suppresses autophagy by regulating Akt/mTOR and JAK/STAT pathways in TNBC cells.

be present upstream of NGALR and associated with NGALR-mediated tumorigenesis in MDA-MB-231 and MDA-MB-468 cells. Overall, our findings suggested that

NGALR is strongly associated with increased proliferation, survival, EMT, invasion, migration, and angiogenesis and decreased autophagy in TNBC cells by activating the AKT/mTOR and JAK/STAT pathways and regulating the expression of various downstream proteins (Figure 4.7).

4.3.8. Role of TNF-α and TNF-β on siNGALR knockdown TNBC cells

In the preceding chapters, we have already demonstrated that NGALR was significantly overexpressed in breast cancer tissues and cell lines and might be associated with poor OS. Further, NGALR expression was induced by pro-inflammatory cytokines (TNF-α and TNF-β) and was found to stimulate various tumorigenesis processes in TNBC. The silencing of NGALR significantly inhibited proliferation, survival, EMT, invasion, migration, angiogenesis, and induced autophagy by modulating the Akt/mTOR and JAK/STAT pathways in both MDA-MB-231 and MDA-MB-468 cells. These findings suggested that NGALR could serve as a promising therapeutic prognostic target for TNBC treatment. Further, we have reported that TNF-α and TNF-β are present upstream of NGALR which is strongly associated with TNBC progression and regulation. Moreover, in the previous chapter, we have shown that TNF-α and TNF-β increased NGALR-mediated TNBC tumorigenesis by regulating various hallmarks of cancer. However, the exact role of NGALR in TNF-α and TNF-β-mediated TNBC tumorigenesis remains to be elucidated. Therefore, the siNGALR knockdown TNBC cells were treated with TNF-α and TNF-β and their effect were examined on cell proliferation, survival, and migration. Further, we also determined the expression of various proteins involved in TNF-α and TNF-β-mediated TNBC progression.

Our previous chapter has shown that treatment of TNF-α and TNF-β significantly increased the expression of NGALR in TNBC cell lines. To further

confirm these findings, we have treated the siNGALR knockdown cells with TNF-α and TNF-β and determined the NGALR expression through Western blot analysis. Our results demonstrated that after treating with TNF-α and TNF-β, the expression of NGALR was increased in Scr control, while the expression was not increased in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells (Figure 4.8). These findings suggested that both TNF-α and TNF-β exerted their tumorigenic role in TNBC by regulating the expression of NGALR.

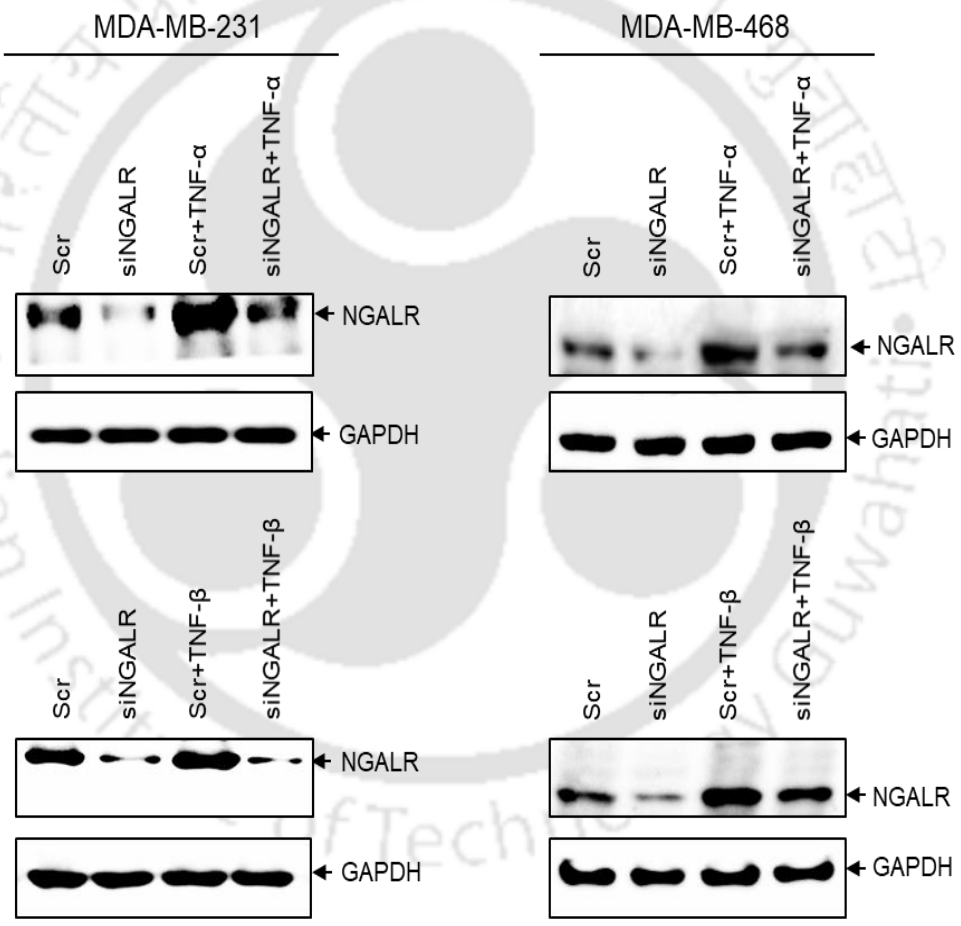

Figure 4.8. Effect of TNF-α and TNF-β on the expression of NGALR in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells determined by Western blot analysis.

4.3.8.1. Effect of TNF-α and TNF-β on the proliferation and survival of siNGALR knockdown TNBC cells

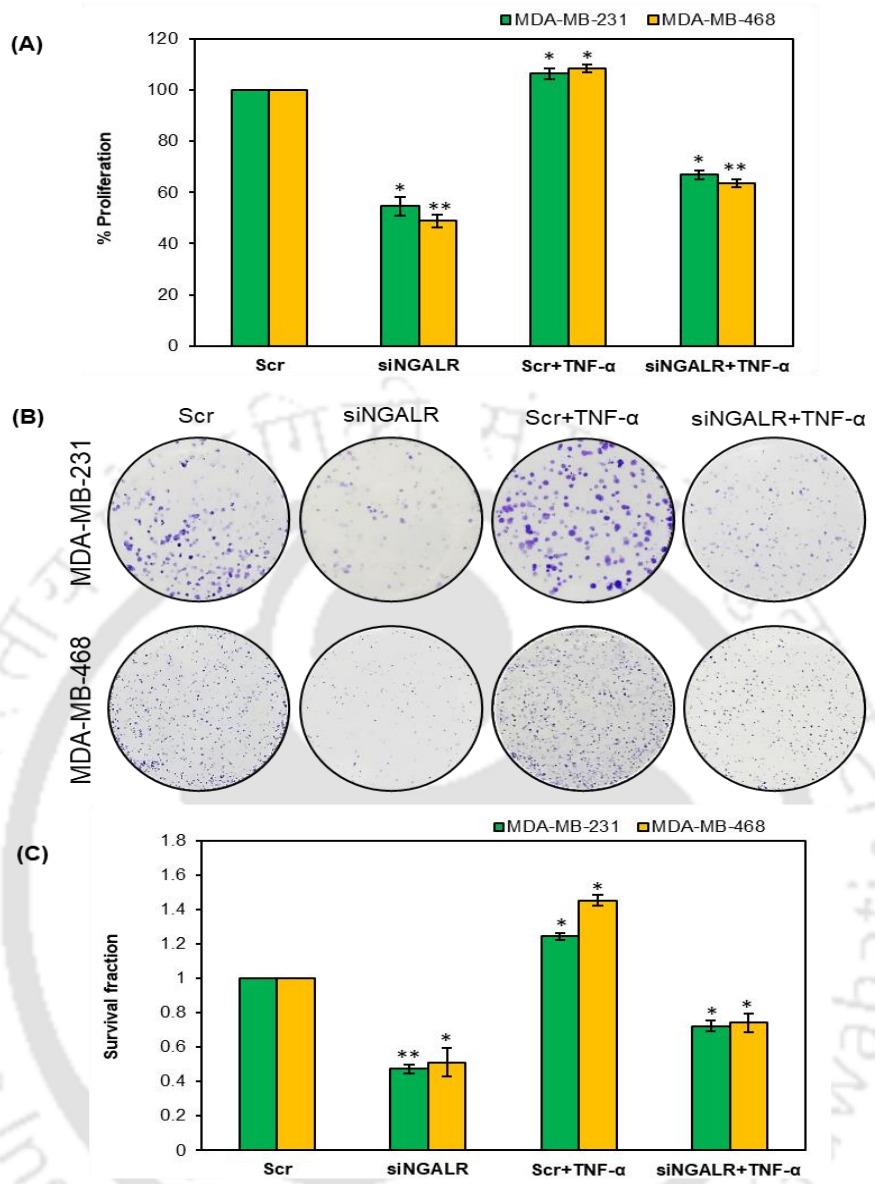

Figure 4.9. Effect of TNF-α on the proliferation and survival of siNGALR knockdown TNBC cells. **(A)** Graphical representation of the percentage proliferation of TNF-α treated siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells determined by MTT assay. **(B-C)** Representative images and graphical representation of survival fraction of TNF-α treated siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells determined by colony formation assay. Data are represented as mean ± SE. * = $p<0.05$ and ** = $p<0.005$ compared to Scr control.

Our preceding chapter has already reported that both TNF-α and TNF-β increased the proliferation and survival in MDA-MB-231 and MDA-MB-468 cells by upregulating NGALR expression. To further strengthen these findings, the siNGALR knockdown TNBC cells were treated with TNF-α and TNF-β and the percentage proliferation and

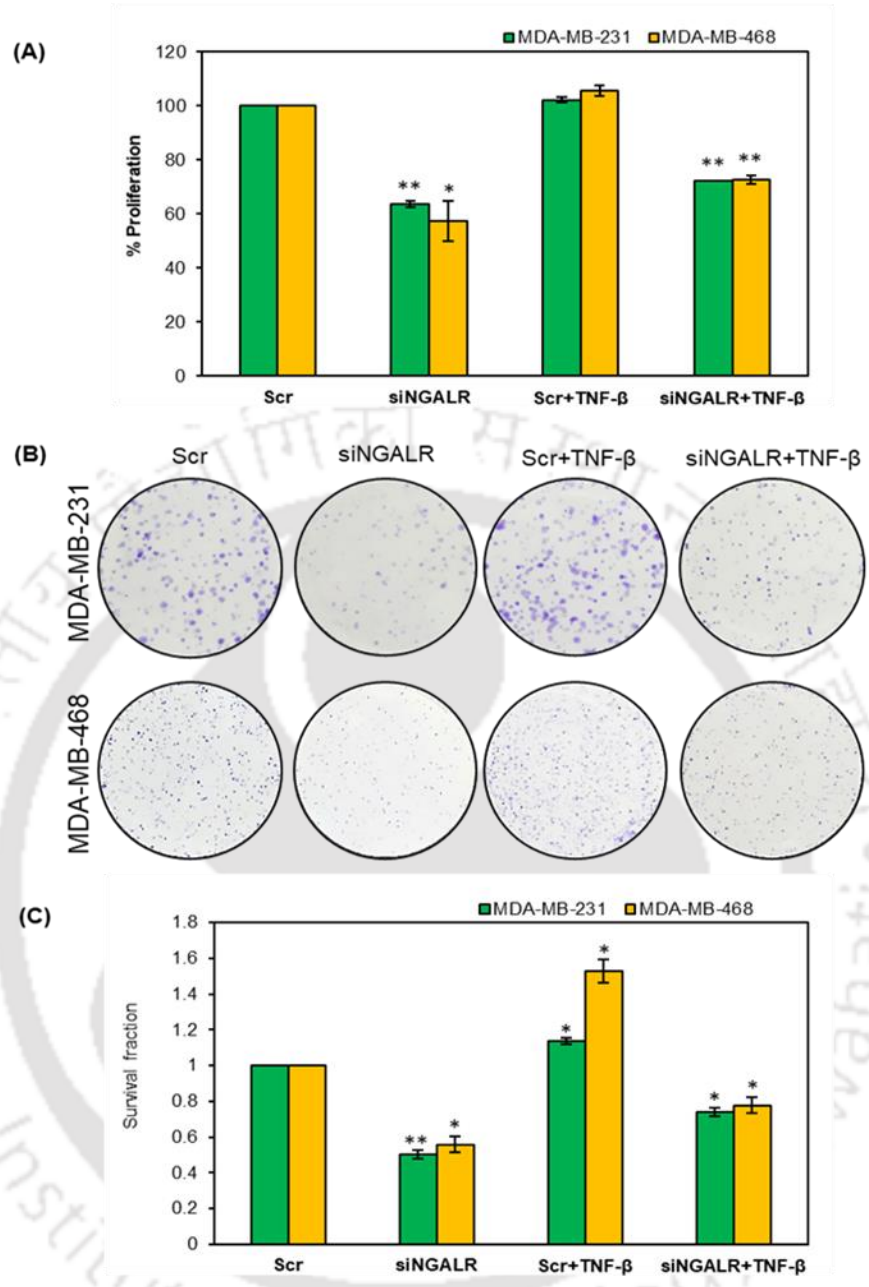

Figure 4.10. Effect of TNF-β on the proliferation and survival of siNGALR knockdown TNBC cells. **(A)** Graphical representation of the percentage proliferation of TNF-β treated siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells determined by MTT assay. **(B-C)** Representative images and graphical representation of survival fraction of TNF-β treated siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells determined by colony formation assay. Data are represented as mean ± SE. * = $p<0.05$ and ** = $p<0.005$ compared to Scr control.

survival fraction were determined through MTT and colony formation assays respectively. Our results showed that after treating with TNF-α and TNF-β, the

percentage proliferation and survival fraction of the Scr control cells were increased significantly. In contrast, the siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells showed reduced proliferation and survival compared to Scr control treated with TNF-α and TNF-β (Figure 4.9 A, B, C and Figure 4.10 A, B, C). These results strongly support our previous findings that TNF-α and TNF-β increased TNBC cell proliferation and survival by regulating NGALR expression. Various studies demonstrated that TNF-α induced growth and stemness of MDA-MB-468 cells (Liu et al., 2020; Cai et al., 2017). Further, TNF-α enhanced the proliferation and survival of breast cancer cells by upregulating the expression of TNFR2 (Garcia-Tunon et al., 2006).

Further, we have determined the effect of TNF-α and TNF-β in the expression of various proteins implicated in proliferation and survival, such as survivin, COX-2, Bcl-2, and cyclin D1 in siNGALR knockdown TNBC cells through Western blot analysis. After treating with TNF-α and TNF-β, the expression of survivin, Bcl-2, COX-2, and cyclin D1 were reduced in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells than Scr control treated with TNF-α and TNF-β (Figure 4.13, 4.14). These findings suggested that TNF-α and TNF-β increased the proliferation and survival of TNBC cells by NGALR-mediated regulation of survivin, Bcl-2, COX-2, and cyclin D1 expression.

4.3.8.2. Effect of TNF-α and TNF-β on the migration of siNGALR knockdown TNBC cells

In our previous chapter, we have already reported that TNF-α and TNF-β treatment increased the migration of TNBC cells. Moreover, it is well known that administration of TNF-α at a low dose for long-term induced cancer cell invasion and metastasis (Zhao P & Zhang Z, 2018; Wang X & Lin Y, 2008). Thus, it is important to decipher the effect

of TNF-α and TNF-β on the migration of siNGALR knockdown TNBC cells. Therefore, we have performed a wound-healing assay to determine the migration

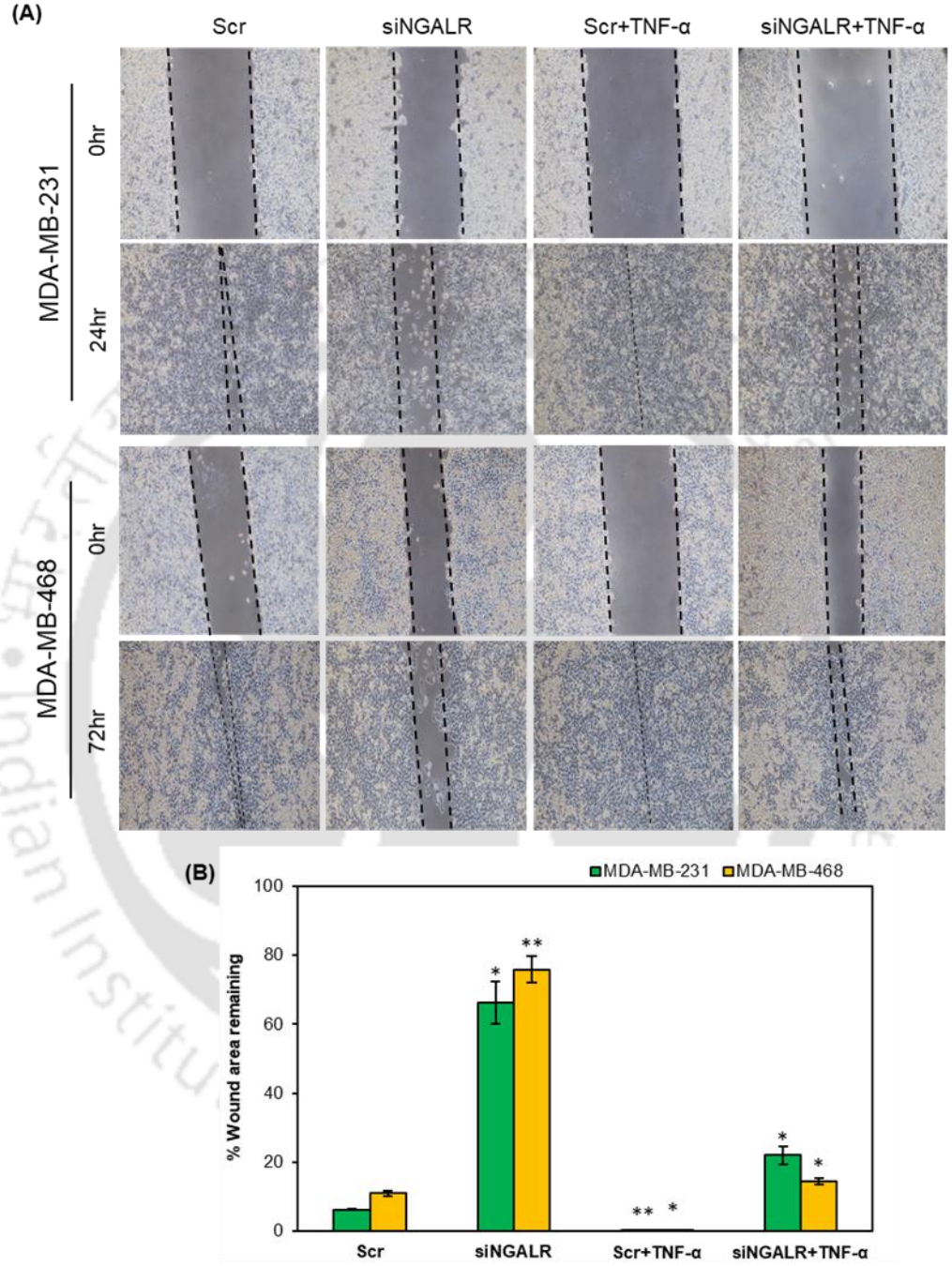

Figure 4.11. Effect of TNF-α on the migration of siNGALR knockdown TNBC cells. **(A-B)** Representative images and graphical representation of percentage wound area remaining of TNF-α treated siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells determined by wound healing assay. Data are represented as mean ± SE. * = $p<0.05$ and ** = $p<0.005$ compared to Scr control.

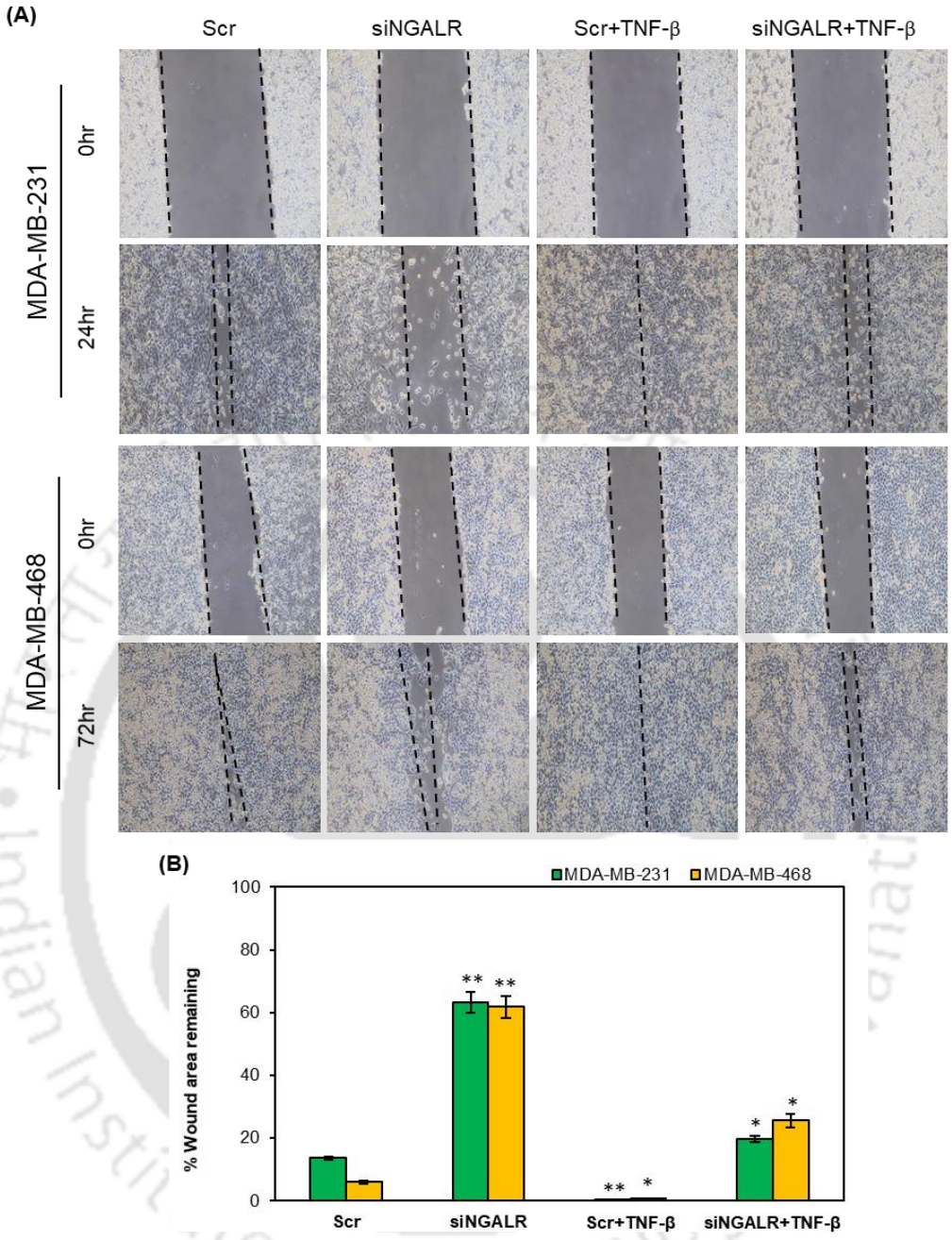

Figure 4.12. Effect of TNF-β on the migration of siNGALR knockdown TNBC cells. **(A-B)** Representative images and graphical representation of percentage wound area remaining of TNF-β treated siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells determined by wound healing assay. Data are represented as mean ± SE. * = $p<0.05$ and ** = $p<0.005$ compared to Scr control.

potential of siNGALR knockdown TNBC cells after treating them with TNF-α and TNF-β. Our results showed that the migration of TNF-α and TNF-β treated Scr cells increased significantly, while such increase was not observed in the siNGALR

knockdown MDA-MB-231 and MDA-MB-468 cells treated with TNF-α and TNF-β (Figure 4.11 A, B and Figure 4.12 A, B). The percentage of wound area remaining was more in siNGALR knockdown TNBC cells compared to the respective Scr control. Notably, TNF-α-induced migration of MDA-MB-231 by upregulating the expression of MMP-9 and MAPK/ERK signaling activation (Wolczyk et al., 2016). Similarly, TNF-α promoted invasion and migration in breast cancer cells by inducing the

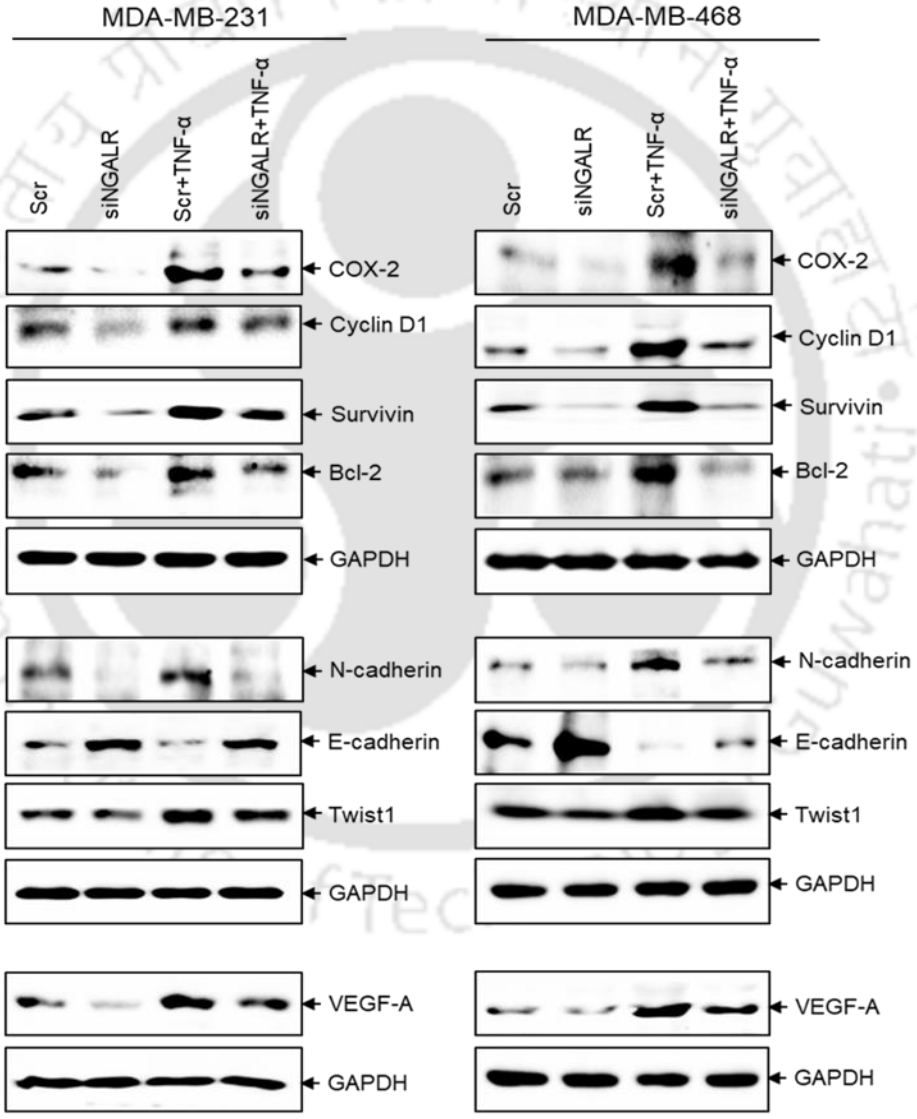

Figure 4.13. Effect of TNF-α on the expression of various proteins associated with the proliferation, survival, EMT, migration, and angiogenesis of siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells compared to the respective Scr control determined by Western blot analysis. GAPDH was used as the loading control.

expression of MMP-9 (Montesano et al., 2005). Further, it has been well established that EMT is an essential phenomenon for the invasion and migration of cancer cells, (Wang Y & Zhou BP, 2011; Campbell K, 2018; Son H & Moon A, 2010). Our previous studies have already revealed that TNF-α and TNF-β increased

the EMT and migration of TNBC cells. Therefore, we have deciphered the effect of TNF-α and TNF-β on the expression of various EMT proteins in siNGALR

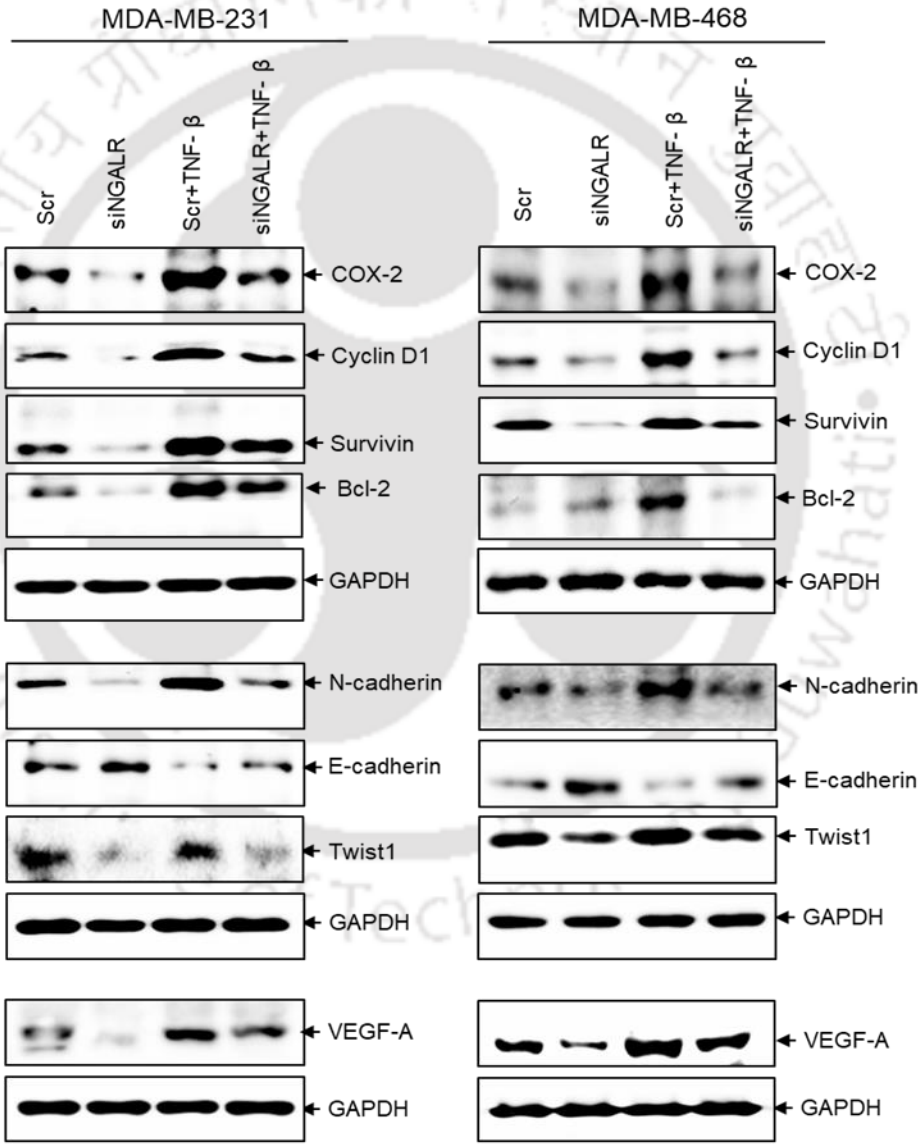

Figure 4.14. Effect of TNF-β on the expression of various proteins associated with the proliferation, survival, EMT, migration, and angiogenesis of siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells compared to the respective Scr control determined by Western blot analysis. GAPDH was used as the loading control.

knockdown TNBC cells through Western blot analysis. Our results revealed that after treating with TNF-α and TNF-β, the expression N-cadherin and Twist1 were upregulated, and E-cadherin was downregulated in Scr control; however, the expression N-cadherin and Twist1 were downregulated, and E-cadherin was upregulated in siNGALR knockdown MDA-MB-231 and MDA-MB-468 (Figure 4.13, 4.14). These findings strongly advocated that TNF-α and TNF-β increased NGALR-mediated EMT and migration of MDA-MB-231 and MDA-MB-468 cells by regulating the expression of N-cadherin, Twist1, and E- cadherin. In support of our study, Li et al. (2012) reported that TNF-α induced EMT and migration by upregulating the expression of Twist1 protein in breast cancer cells; moreover, opposite results were observed after silencing the expression of Twist1 (Li et al., 2012). Further, our results demonstrated that VEGF-A expression was upregulated in Scr control and downregulated in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells treated with TNF-α and TNF-β (Figure 4.13, 4.14).

4.3.8.3. Effect of TNF-α and TNF-β on the regulation of Akt/mTOR and JAK/STAT pathways in siNGALR knockdown TNBC cells

Our previous chapter has already reported that both TNF-α and TNF-β significantly induced NGLAR-mediated proliferation, survival, EMT, and migration and decreased cell death in MDA-MB-231 and MDA-MB-468 cells. In the current chapter, we demonstrated that TNF-α and TNF-β are present upstream of NGALR and exerted their tumorigenic role by regulating Akt/mTOR and JAK/STAT pathways in TNBC cell lines. Further, treatment of TNF-α and TNF-β upregulated the expression of NGALR in Scr control, while downregulated in siNGALR knockdown TNBC cells. However, the mechanistic pathway through which TNF-α and TNF-β exerted their tumorigenic

functions needs to be revealed. Therefore, the siNGALR knockdown TNBC cells were treated with TNF-α and TNF-β and the expression of various proteins associated with the Akt/mTOR and JAK/STAT pathways were determined through Western blot analysis. We observed that after treating with TNF-α and TNF-β, the expression of p-Akt, p-mTOR, p-JAK-2, and p-STAT3 were upregulated in Scr control, while downregulated in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells

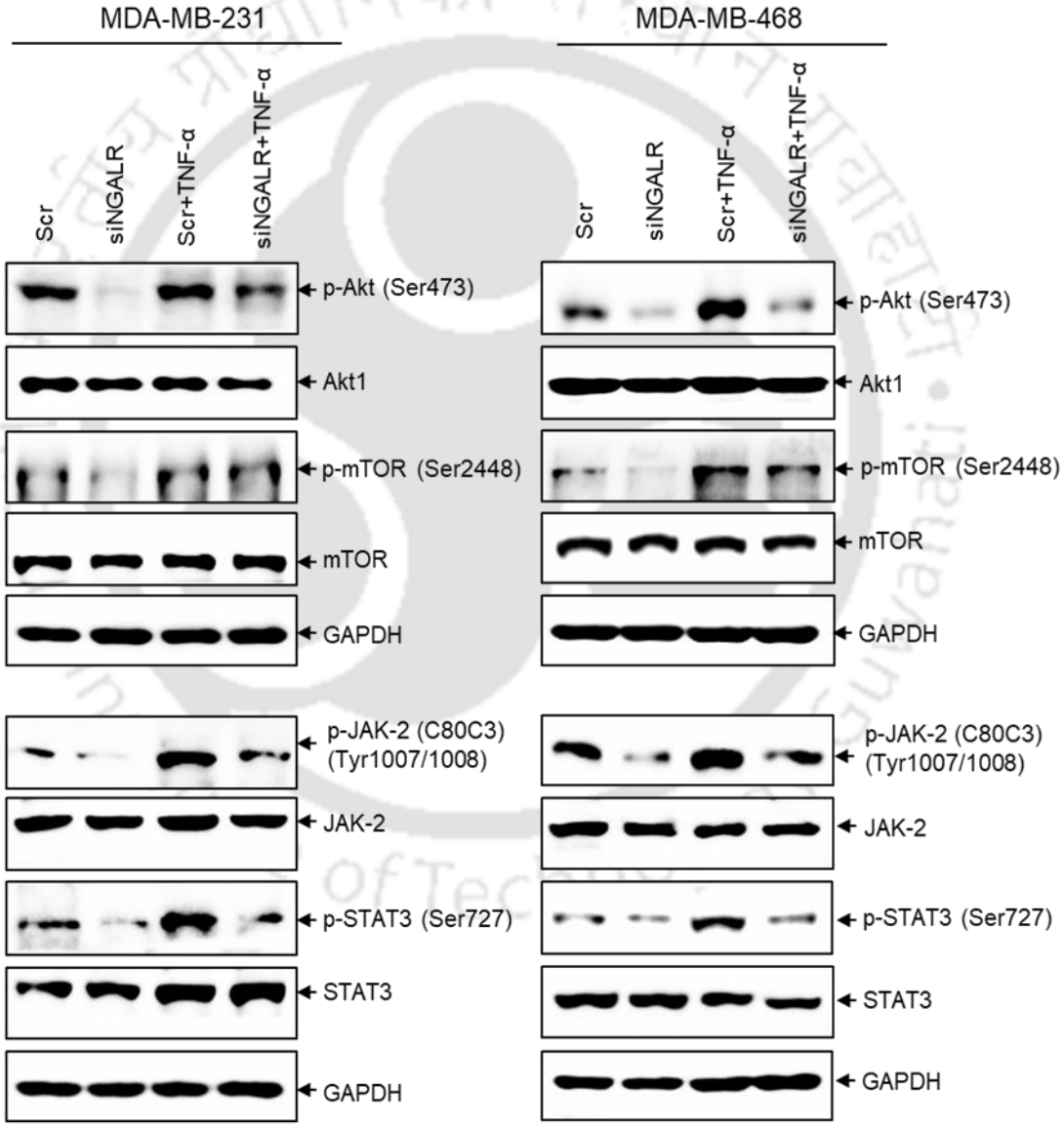

Figure 4.15. Effect of TNF-α on the regulation of Akt/mTOR and JAK/STAT pathways in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells compared to the respective Scr control determined by Western blot analysis. GAPDH was used as the loading control.

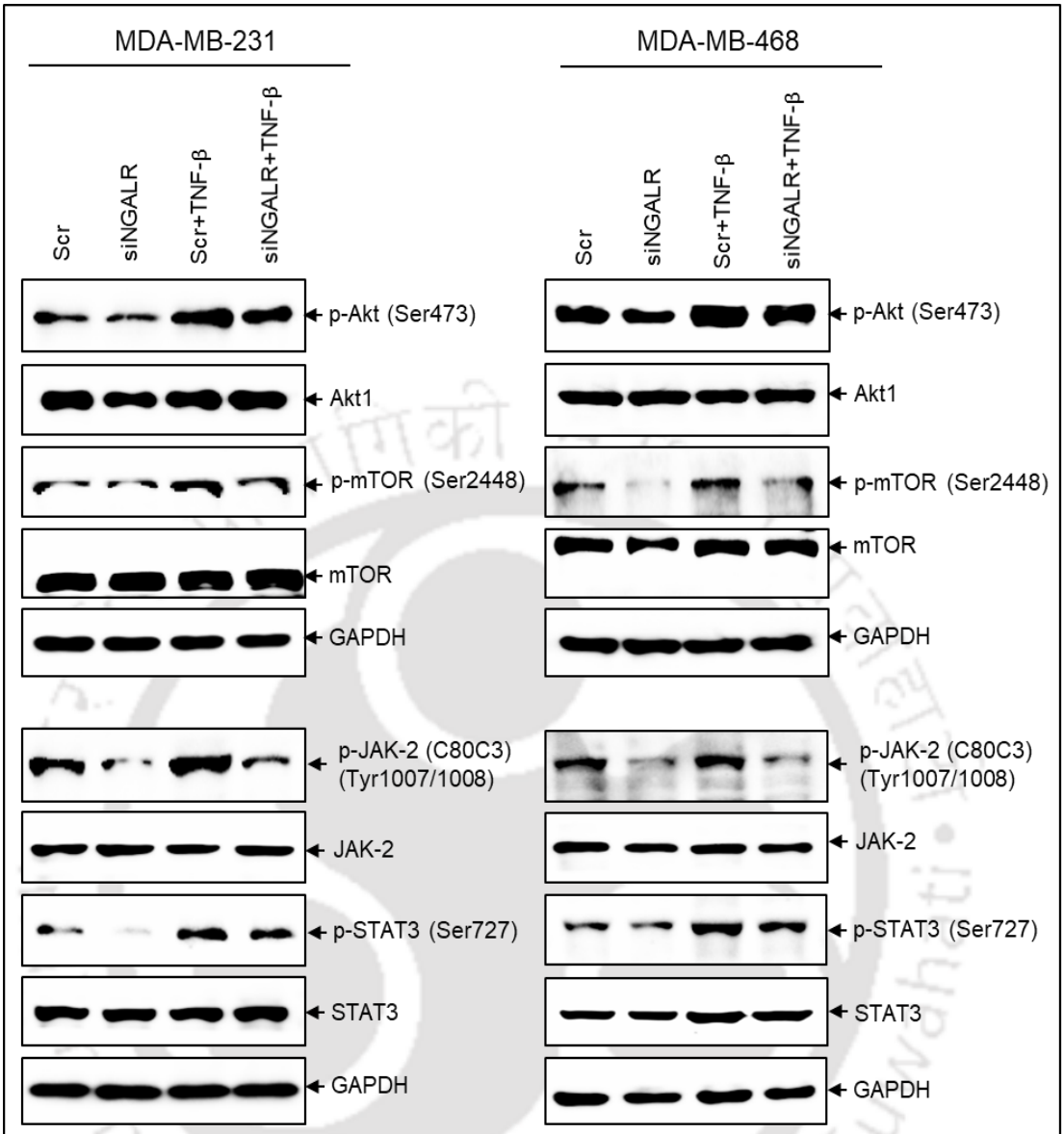

Figure 4.16. Effect of TNF-β on the regulation of Akt/mTOR and JAK/STAT pathways in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells compared to the respective Scr control determined by Western blot analysis. GAPDH was used as the loading control.

(Figure 4.15 and Figure 4.16). Moreover, the expression of Akt, mTOR, JAK-2, and STAT3 were unchanged after TNF-α and TNF-β treatments in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells compared to TNF-α and TNF-β treated Scr control (Figure 4.15 and Figure 4.16). These findings suggested that TNF-α and TNF-β are present upstream of NGALR and are associated with NGALR-mediated TNBC

cell proliferation, survival, EMT, and migration by regulating the Akt/mTOR and JAK/STAT pathways.

4.4. Conclusion

The current chapter demonstrated that NGALR is significantly associated with TNBC progression by regulating the various hallmarks of cancer. Silencing of NGALR attenuated the proliferation, survival, EMT, invasion, migration, and angiogenesis and induced autophagy by regulating the expression of various proteins, such as COX-2, survivin, Bcl-2, p21, cyclin D1, cyclin A2, vimentin, Snail, Twist1, N-cadherin, E-cadherin, CXCR4, VEGF-A, and LC3B in TNBC cells lines. Our mechanistic findings suggested that NGALR drives its tumorigenic role by activating the Akt/mTOR and JAK/STAT pathway. Further, the expression of TNF-α and TNF-β were unaltered in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells, which revealed that both TNF-α and TNF-β are present upstream of NGALR. Subsequently, the expression of NGALR was not increased in siNGALR knockdown TNBC cells after treating with TNF-α and TNF-β. Moreover, TNF-α and TNF-β treatment is significantly associated with NGALR-mediated proliferation, survival, EMT, and migration of siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells by regulating the Akt/mTOR and JAK/STAT pathways. These findings strongly advocated that NGALR is significantly associated with TNBC progression and can serve as a plausible therapeutic target for TNBC management. However, these results need to be confirmed through *in vivo* and clinical studies.

Chapter 5

Discussion and Conclusion

5.1. Discussion and Conclusion

The incidences of breast cancer cases are increasing year by year and it becomes the most common cancer including both sexes which accounts for approximately 12-15% of all cancer cases (Ferlay et al., 2021; Sung et al., 2020). On average, one in four cancer cases and one in six cancer deaths among women occur due to breast cancer (Sung et al., 2020; Bray et al., 2018; Anastasiadi et al., 2017). The Asian countries reports the highest incidences, mortalities, and five-year prevalences of breast cancer (Thakur et al., 2018; Bray et al., 2018; Dydjow-Bendek D & Zagozdzon P, 2020). Numerous risk factors are involved in breast cancer development and progression, which we have already discussed in earlier chapters. Moreover, breast cancer is a heterogeneous disease and categorized into different sub-types based on the expression of various receptors. Among the various sub-types, TNBC is the most aggressive sub-type of breast cancer and accounts for approximately 12-27% of all breast cancer cases, which is most prevalent among younger women (Dai et al., 2016; 2013; Thakur et al., 2018). Pathologically, the major prognostic breast cancer markers, which include ER, PR, and HER2, are not present in TNBC sub-types (Alwan NAS & Tawfeeq FN, 2019; Dai et al., 2016; Fragomeni et al., 2018). The lack of expression of these receptors makes conventional chemotherapies ineffective for the treatment of TNBC. Further, tumor recurrence and chemoresistance resulted in poor OS and worse prognosis in TNBC (Bergin ART & Loi S, 2019; Yao et al., 2017; Al-Mahmood et al., 2018). Despite the advanced treatment approaches, the TNBC incidence and mortality rates are still high. Therefore, there is an urgent need to identify novel biomarkers and therapeutic targets that would help in the regulation of TNBC.

Various studies have reported a strong association between iron and cancer cell proliferation and survival (Chen et al., 2019; Jung et al., 2019). Interestingly, NGALR

is associated with iron transport and apoptosis. Further, dysregulated expression of NGALR leads to the development of various cancers, such as RCC, HCC, ESCC, glioma, endometrial carcinoma, and lymphocytic leukemia (Liu et al., 2018; Zhang et al., 2012a; Tan et al., 2014; Du et al., 2011; Liu et al., 2011; Bauvois et al., 2020). However, the potential role of NGALR in the tumorigenesis of TNBC has not been reported yet. Recently, Chi et al. (2020) reported that NGALR regulated iron transport and induced cell growth in the leptomeningeal metastasies mouse model (Chi et al., 2020). Further, a study demonstrated that NGALR overexpression was related to apoptosis resistance in CLL cells (Bauvois et al., 2020). Various studies reported that overexpression of NGALR was related to poor survival in ESCC patients (Tan et al., 2014; Du et al., 2011; Cui et al., 2008; Fang et al., 2007). Moreover, NGALR overexpression was significantly linked to invasion, metastasis, and poor survival in endometrial carcinoma patients (Miyamoto et al., 2011). These studies strongly advocated that NGALR is implicated in cancer development and progression. Therefore, we speculated that unraveling the role of NGALR in TNBC would help us to find a novel therapeutic target for the development of highly efficacious therapy for TNBC. As per our knowledge, this is the first study to elucidate the role of NGALR in TNBC. First, we observed the OS of breast cancer patients from the TCGA database. Subsequently, the expression of NGALR was determined in various sub-types of breast cancer tissues through IHC analysis. Further, the expression of NGALR in TNBC cell lines was examined through Western blot analysis. Moreover, we have evaluated the role of pro-inflammatory cytokines (such as TNF-α and TNF-β) in the progression of NGALR-mediated TNBC. Finally, we have deciphered the role of NGALR in TNBC and TNF-induced TNBC.

The TCGA analysis of breast cancer samples showed that the alteration in NGALR decreased the OS of breast cancer patients. Similarly, the TCGA analysis on 22 ccRCC data sets demonstrated that NGALR was implicated in the clinical prognosis of the disease (Liu et al., 2018). Further, we showed that NGALR was significantly overexpressed in breast cancer tissues than normal tissues. Recently, a study has also reported that dysregulation of NGALR was associated with poor OS in gastric cancer patients (Wei et al., 2020). Similarly, NGALR overexpression resulted in poor OS in endometrial carcinoma patients (Miyamoto et al., 2011). NGALR and its ligand NGAL were upregulated in HCC tissues and associated with poor OS and prognosis (Zhang et al., 2012a). Subsequently, our IHC results demonstrated that NGALR was significantly overexpressed among various sub-types of breast cancer, including TNBC. Various studies have already reported that TNBC is frequently observed in younger women than older age women; however, the existing cause behind this is yet to be revealed (Lee HB & Han W, 2014; Anders et al., 2009; Aapro M & Wildiers H 2012; Yeh et al., 2017). In our study, we revealed that the expression of NGALR was significantly associated with the TNBC women of 30-45 and 45-60 years age groups. Lv et al. (2010) demonstrated that NGALR was highly associated with <57 years of age in colorectal cancer patients and resulted in poor survival (Lv et al., 2010). Recently, a study also reported that the survival and metastasis of TNBC sub-types were more frequently associated with women <40 years of age compared to >74 years of age (Tzikas et al., 2020). Moreover, overexpression of NGALR was observed in glioma tissues and significantly associated with patient age, tumor grade, lower survival, and poor prognosis (Liu et al., 2011). Further, our study has demonstrated that NGALR was strongly associated with various developmental stages of TNBC and significantly overexpressed in stage 1 and stage 3 TNBC tissues. Similar to our findings, the

expression of NGALR was significantly upregulated in stage 3 and stage 4 in endometrial carcinoma tissues and related with higher invasion and poorer survival (Miyamoto et al., 2011). However, NGALR was downregulated and associated with the poor prognosis in ccRCC patient samples (Liu et al., 2018). Further, we have found that the expression of NGALR protein was significantly upregulated in both MDA-MB-231 and MDA-MB-468 TNBC cell lines. This is the first study to reveal the expression of NGALR in various TNBC cell lines. Recently, a study revealed the upregulated expression of NGALR in CLL cells than normal PBMCs and associated with apoptotic resistance (Bauvois et al., 2020). Further, upregulation of NGALR was observed in oesophageal carcinoma cells (Fang et al., 2007). These findings suggested that, like most cancers, NGALR might be associated with the progression of TNBC tumorigenesis.

Various studies have reported that inflammation is significantly associated with breast cancer progression (Mills RC, 2017; Agnoli et al., 2017). The OS of breast cancer patients was reported to reduce with increasing levels of pro-inflammatory cytokines, including TNF-α and TNF-β (Agnoli et al., 2017; Cai et al., 2017). TNF-α and TNF-β are having a diverse array of functions, such as cell proliferation, survival, invasion, angiogenesis, and metastasis (Kunnumakkara et al., 2019; Wang X & Lin Y, 2008). Pooja et al. (2011) revealed that TNF-α and TNF-β polymorphisms were significantly associated with breast cancer progression (Pooja et al., 2011). Further, TNF-α and TNF-β were highly expressed in oral cancer patients compared to healthy individuals, and the polymorphisms in TNF-α and TNF-β lead to an increased risk of oral cancer (Yapijakis et al., 2009). Moreover, it is also reported that these pro-inflammatory cytokines play a dual role in breast cancer progression and chemoresistance (Cruceriu et al., 2020; Zhang et al., 2018; Pooja et al., 2011). Therefore, we have determined the

role of TNF-α and TNF-β in the progression of TNBC tumorigenesis. Our results have demonstrated that both TNF-α and TNF-β significantly upregulated the expression of NGALR in TNBC cell lines. It is well established that TNF-α is strongly associated with various hallmarks of cancer, such as proliferation, survival, EMT, invasion, migration, and angiogenesis (Wu Y & Zhou BP, 2010; Yu et al., 2013; Wang X & Lin Y, 2008). Our results also reported that TNF-α and TNF-β significantly increased the proliferation, survival, EMT, and migration of MDA-MB-231 and MDA-MB-468 cells. Further, we have observed that TNF-α and TNF-β treatment decreased TNBC cell death compared to untreated cells. Similar to our findings, Cai et al. (2017) reported that TNF-α promoted proliferation and survival of breast cancer *in vitro* and *in vivo* (Cai et al., 2017). Similarly, *in vitro* and *in vivo* models of breast cancer reported that inhibition of TNF-α attenuated breast cancer growth and metastasis (Yu et al., 2013). EMT is an essential step for the migration and metastasis of cancer cells, and it has been well established that TNF-α is significantly involved in the EMT of cancer cells (Tsai JH & Yang J, 2013; Mercogliano et al., 2020; Asiedu et al., 2011; Cohen et al., 2015; Wang Y & Zhou BP, 2011). Our results also revealed that TNF-α and TNF-β treatment significantly increased EMT by increasing the expression of vimentin and Snail protein in MDA-MB-231 and MDA-MB-468 cells. Asiedu et al. (2011) observed similar results and reported that TNF-α and TGF-β increased BCSCs through induction of EMT (Asiedu et al., 2011). Moreover, knockdown of Snail inhibited the NF-κB activation and aatenuated invasion and migration in breast cancer cells (Wu et al., 2009). Further, we have determined the effect of TNF-α and TNF-β on migration of TNBC cells and observed that both TNF-α and TNF-β significantly induced the migration of MDA-MB-231 and MDA-MB-468 cells. Wolczyk et al. (2016) have revealed that TNF-α promoted invasion and migration of MDA-MB-231 cells by stimulating the

MAPK/ERK pathway activation. On the other hand, inhibition of TNF-α attenuated EMT, invasion, and metastasis by decreasing MMP-2 and MMP-9 and increasing E-cadherin expression in breast cancer cells (Paramanantham et al., 2020; Singh et al., 2019). Overall, our findings suggested that both TNF-α and TNF-β significantly increased TNBC cell proliferation, survival, EMT, and migration and suppressed cell death by inducing the expression of NGALR. Similarly, Pileczki et al. (2012) have shown that knockdown of TNF-α induced apoptosis and suppressed cell proliferation ~~of~~ in TNBC cells (Pileczki et al., 2012). Contrastingly, other studies have showed that TNF-α increased apoptosis via activation of p73 and c-ABL and ROS production (Chau et al., 2004; Kim et al., 2010). Similarly, TNF-β increased necroptosis, apoptosis, and inflammatory signals by TNFR1-mediated signaling (Etemadi et al., 2013).

In order to further determine the role of NGALR in the progression of TNBC, we suppressed the NGALR expression through siRNA-mediated knockdown. Our results have shown that the expression of NGALR was significantly downregulated in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells, and it leads to the decrease in the proliferation and survival of TNBC cells by inducing the S phase cycle arrest. Moreover, silencing of NGALR was associated with suppression of EMT, invasion, migration, and angiogenesis, and acceleration of autophagy in TNBC cells. Our study has also investigated the expression of various downstream proteins which are associated with the previously mentioned hallmarks of cancer in siNGALR knockdown TNBC cells. We have observed that the expression of survivin, COX-2, Bcl-2, cyclin A2, vimentin, Snail, N-cadherin, Twist1, CXCR4, and VEGF-A were downregulated, whereas p21, E-cadherin, and LC3B expression were upregulated in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells. Various studies have reported that COX-2 overexpression resulted in poor OS and increased mortality in

TNBC patients (Mosalpuria et al., 2014; Abdel-Fatah et al., 2013). However, the siRNA knockdown of COX-2 significantly suppressed cell proliferation and growth by downregulating the expression of survivin and Bcl-2 (Han et al., 2014). Moreover, silencing of the Bcl-2 gene decreased proliferation and survival and increased autophagic cell death by inducing LC3B expression in breast cancer cells (Akar et al., 2008). On the contrary, various studies reported that overexpression of LC3B was involved in poor prognosis and the worst outcome ~~of~~ in breast cancer patients (Zhao et al., 2013; Chen et al., 2013; Bortnik et al., 2020). Several studies have also reported that upregulation of vimentin and Snail are related to RFS and OS in TNBC patients (Karihtala et al., 2013; Yamashita et al., 2013; Jiralerspong et al., 2010; Rashed et al., 2021). Conversely, increased expression of LC3B was associated with poor prognosis and the worst outcome of breast cancer (Chen et al., 2013; Bortnik et al., 2020). Cancer cells induced metastasis and tumorigenesis by recruiting various transcription factors and proteins required for EMT, such as E-cadherin, N-cadherin, Snail, vimentin, Twist1, and COX-2 (Kar et al., 2019; Matysiak et al., 2017; Karamanou et al., 2020). Various studies revealed that increased expression of vimentin, Twist, and Slug and decreased expression of E-cadherin were associated with poor OS of TNBC patients (Zhou et al., 2018; Shen et al., 2016). Aletaha et al. (2017) showed that silencing of Snail-1 induced the S phase cell cycle arrest and significantly decreased proliferation and migration, and induced apoptosis in MDA-MB-468 cells (Aletaha et al., 2017). Further, knockdown of Snail suppressed invasion and migration and attenuated chemoresistance in mouse breast cancer models (Ma et al., 2017). Li et al. (2004) showed that CXCR4 was overexpressed in breast cancer patient's tissues and resulted in poor OS (Li et al., 2004). However, downregulating the expression of CXCR4 suppressed the invasion and migration of MDA-MB-231 cells (Liang et al., 2004; Lee

et al., 2004; Kim et al., 2020). *In vitro* and *in vivo* models of TNBC demonstrated that oridonin inhibited VEGF-A-mediated angiogenesis and EMT by reducing the expression of HIF-1α, VEGF-A, vimentin, Snail, and N-cadherin and increased E-cadherin (Li et al., 2018). Further, various studies demonstrated that increased expression of VEGF-A was associated with poor RFS, DFS, and distant metastasis-free survival in the TNBC patients (Linderholm et al., 2009; Su et al., 2016). Overall, our findings suggested that NGALR increased the proliferation, survival, EMT, invasion, migration, and angiogenesis, while suppressed autophagy by regulating the expression of various tumorigenesis-associated proteins in TNBC cells.

It is well established that the Akt/mTOR and JAK/STAT pathways are strongly associated with the proliferation, survival, migration, metabolism, apoptosis, and angiogenesis of TNBC cells (Massihnia et al., 2016; Costa et al., 2018; Khan et al., 2019; Cai et al., 2020; Qin et al., 2019; Song et al., 2020). Our study has also reported that knockdown of NGALR suppressed the expression of p-Akt, p-mTOR, p-JAK-2, and p-STAT3 in MDA-MB-231 and MDA-MB-468 cells. Haga et al. (2020) revealed that inhibition of the Akt/mTOR pathway decreased cell proliferation and attenuated intrinsic resistance to dasatinib in various TNBC cell lines (Haga et al., 2020). Similarly, berberine decreased the proliferation, invasion, and migration of breast cancer cells by attenuating the expression of PI3K, p-Akt, mTOR, and COX-2 and increasing the expression of p53 (Liu et al., 2020). Further, JAK-2 amplification was associated with increased stemness, metastasis, chemoresistance, and tumorigenesis, and knockdown of JAK-2 decreased cell growth (Balko et al., 2016; Chen et al., 2018). On the other hand, inhibition of the JAK/STAT pathway promoted TNBC progression by inducing the expression of protumorigenic inflammatory factors, such as COX-2

(Irey et al., 2019). However, our findings suggested that NGALR induced TNBC tumorigenesis by stimulating the Akt/mTOR and JAK/STAT pathways.

We have already reported that TNF-α and TNF-β increased TNBC cell proliferation, survival, EMT, and migration and decreased cell death by inducing the expression of NGALR. However, the expression of TNF-α and TNF-β were unchanged in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells compared to Scr control. These findings strongly advocated that TNF-α and TNF-β might be present upstream of NGALR and associated with NGALR-mediated tumorigenesis in TNBC cells. Further, we have reported the effect of TNF-α and TNF-β on the NGALR-mediated tumorigenesis in siNGALR knockdown TNBC cells. Our results showed that after treating with TNF-α and TNF-β, the expression of NGALR was increased in the Scr control, while the expression of NGALR has not increased in siNGALR knockdown TNBC cells. These results strongly suggested that both TNF-α and TNF-β exerted their tumorigenic role in TNBC by regulating the expression of NGALR. Further, we have already shown that TNF-α and TNF-β treatment increased the proliferation, survival, EMT, and migration in TNBC cells. Similarly, treatment with TNF-α and TNF-β enhanced the proliferation, survival, EMT, and migration of Scr control; however, the enhancement was not observed in siNGALR knockdown TNBC cells. Moreover, upon treatment with TNF-α and TNF-β, the expression of survivin, COX-2, Bcl-2, cyclin D1, N-cadherin, Twist1, and VEGF-A were upregulated, while the expression of E-cadherin was downregulated in Scr control, however he opposite was observed in siNGALR knockdown TNBC cells. These findings strongly advocated that TNF-α and TNF-β increased proliferation, survival, EMT, migration, and angiogenesis by regulating the expression of NGALR in TNBC cell lines. Our previous studies have also demonstrated that NGALR exerted its tumorigenic role by inducing the Akt/mTOR

and JAK/STAT pathways. However, after treating with TNF-α and TNF-β, the expression of p-Akt, p-mTOR, p-JAK-2, and p-STAT3 were not upregulated in siNGALR knockdown MDA-MB-231 and MDA-MB-468 cells than Scr control. These findings suggested that TNF-α and TNF-β are upstream of NGALR and associated with NGALR-mediated TNBC cell proliferation, survival, EMT, migration, and angiogenesis by regulating the Akt/mTOR and JAK/STAT pathways.

Overall, this is the first study to decipher the role of NGALR in the progression of TNBC. NGALR is significantly overexpressed in breast cancer tissues and associated with poor OS. The overexpression was significantly associated with younger age and advanced stages in TNBC tissue samples. Further, upregulated expression of NGALR was associated with increased proliferation, survival, EMT, invasion, migration, and angiogenesis and decreased autophagy via regulation of the Akt/mTOR and JAK/STAT pathways in TNBC cells. Moreover, NGALR was significantly associated with TNF-α and TNF-β-induced TNBC tumorigenesis.

5.2. Limitations and future prospective of the study

The current study depicted a strong association between NGALR and TNBC progression, specifically through TNF-α and TNF-β-induced tumorigenesis. Notably, certain limitations were observed, which required further attention. Breast cancer accounts for the highest number of cancer cases in India and the TMA slide which we used for the expression of NGALR lacks tissue samples from the Indian population. Further, the TMA slide does not include male tissue samples, and it has been reported that breast cancer can affect male individuals too. Moreover, the slide contains limited number of healthy and TNBC samples. Hence, it would be better to perform the expression analysis of NGALR with a greater number of tissue samples with diverse racial and ethnic differences. TMA samples are also devoid of various other details,

such as diet, OS, DFS, treatment modalities, tumor recurrence, and patients history. Our study used TMA tissue samples and TNBC cell lines; therefore, our findings need to be further validated through *in vivo* studies. Our study further reported that TNF-α and TNF-β induced the expression of NGALR in TNBC; therefore, the role of more upstream mediators need to be deciphered in the progression of TNBC. Further, we have performed partial knockdown of the NGALR gene; it will also be useful to see the effects after complete knockout of the NGALR. Moreover, investigation of chemoresistance and tumor recurrence are warranted as these are the major obstacles in the path of effective treatment for TNBC. Notably, our study provides a unique platform to design targeted therapies against NGALR and determine their efficacy in the effective treatment of TNBC.

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Abbreviations

°	Degree
%	Percentage
µg:	Microgram
µl:	Microlitre
µM:	Micromolar
g:	Gram
hr:	Hour
ml:	Millilitre
mm:	Millimettre
nm:	Nanometer
nM:	Nanomolar
SD:	Standard deviation
SE:	Standard error
w/v:	Weight per volume
5-FU:	5-fluorouracil
ABC:	ATP-binding cassette
ACS:	American Cancer Society
AJCC:	American Joint Committee on Cancer
ALDH1:	Aldehyde dehydrogenase 1
BcL2:	B-cell lymphoma 2
BCSCs:	Breast cancer stem cells
Bid:	BH3 interacting-domain death agonist
BL:	Basal-like
BRCA:	Breast cancer susceptibility gene
BSA:	Bovine serum albumin
CCL:	CC-chemokine ligand
CCNB1:	Cyclin B1
CCNE1:	Cyclin E1
CCR7:	CC-chemokine receptor 7
ccRCC:	clear cell RCC
CD26:	Cluster of differentiation 26
CDK1:	Cyclin-dependent kinase 1
CLL:	Chronic lymphocytic leukemia
CO_2:	Carbon-dioxide
CRC:	Colorectal carcinoma
CSF:	Cerebrospinal fluid
CXCR4:	CXC chemokine receptor 4
DAB:	Diaminobenzidine tetrahydrochloride
DAPI:	4,6-diamidino-2-phenylindole
DFS:	Disease-free survival
DMEM:	Dulbecco's Modified Eagle Medium

DMSO:	Dimethyl sulfoxide
EDTA:	Ethylenediamine tetraacetic acid
EGFR:	Epidermal growth factor receptor
EMT:	Epithelial–mesenchymal transition
ER:	Estrogen receptor
ESCC:	Oesophageal squamous cell carcinoma
FAP-α:	Fibroblast activation protein alpha
FBS:	Fetal bovine serum
FN1:	Fibronectin 1
FOXA1:	Forkhead box protein A1
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
GATA3:	GATA binding protein 3
GIT:	Gastrointestinal tract
GRB7:	Growth factor receptor-bound protein 7
GSK-3β:	Glycogen synthase kinase-3 beta
GST:	Glutathione/glutathione-S-transferase
HBXIP:	Hepatitis B X-interacting protein
HCC:	Hepatocellular carcinoma
HCl:	Hydrochloric acid
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2:	Human epidermal growth factor receptor 2
HIF-1α:	Hypoxia-inducible factor-1alpha
HMC:	Human mesangial cells
HR:	Hormone receptor
HRP:	Horseradish peroxidase
ICC:	Immunocytochemistry
IFN:	Interferon
IGF:	Insulin-like growth factor
IGF-1R:	IGF-1 receptor
IHC:	Immunohistochemistry
IKK:	IκB kinase
IL:	Interleukin
IM:	Immunomodulatory
JAK:	Janus kinase domain
JNK:	c-Jun N-terminal kinase
LAR:	Luminal androgen receptor
LC3B:	Microtubule-associated protein chain 3B
LCN2R:	Lipocalin 2 receptor
LPS:	Lipopolysaccharides
M:	Mesenchymal
MACC1:	Metastasis-associated in colon cancer 1
MAPK:	Mitogen-activated protein kinase
Mcl-1:	Myeloid cell leukemia-1

MET:	Mesenchymal epithelial transition
MKi-67:	Marker of Proliferation Ki-67
MMP2:	Matrix metalloproteinase2
MRI:	Magnetic resonance imaging
MSL:	Mesenchymal stem-like
mTOR:	Mammalian target of rapamycin
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MYBL2:	MYB Proto-Oncogene Like 2
NaCl:	Sodium chloride
NCCS:	National Centre for Cell Science
NF-κB:	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGAL:	Neutrophil gelatinase-associated lipocalin
NGALR:	Neutrophil gelatinase-associated lipocalin receptor
NHL:	non-Hodgkin's lymphoma
NLRX1:	NOD-like receptor family member X1
OS:	Overall survival
p:	Phospho
PARP:	Poly (ADP-ribose) polymerase
PBMCs:	Peripheral blood mononuclear cells
PBS:	Phosphate Buffered Saline
Pen-Strep:	Penicillin-Streptomycin
PGAP3:	Post-GPI attachment to proteins 3
PI:	Propidium iodide
PI3K:	Phosphoinositide 3-kinase
PI3KCA:	PI3K catalytic subunit alpha
PMSF:	Phenylmethylsulfonyl fluoride
PR:	Progesterone receptor
PTEN:	Phosphatase and tensin homolog
RCC:	Renal cell carcinoma
RFS:	Recurrence-free survival
RIP3:	Receptor-interacting protein-3
ROS:	Reactive oxygen species
RT:	Room temperature
RT-PCR:	Real-time polymerase chain reaction
Scr:	Scramble
SDS:	Sodium dodecyl sulfate
siRNA:	small interfering RNA
SLC22A17:	Solute carrier family 22, member 17
STAT:	Signal transducer and activator of transcription
TAZ:	Transcriptional coactivator with PDZ-binding motif
TBST:	Tris-buffer saline containing 1% tween 20
TCGA:	The Cancer Genomics Atlas
TGF-β:	Transforming growth factor-beta

TGM2:	Transglutaminase 2
TLR4:	Toll-like receptor 4
TMA:	Tissue microarray
TNBC:	Triple-negative breast cancer
TNF:	Tumor necrosis factor
TNFR1:	TNF receptor 1
TNM:	Tumor, lymph node, and metastasis
TP53:	Tumor protein P53
TROP-2:	Tumor-associated calcium signal transduction protein-2
VASP:	Vasodilator-stimulated phosphoprotein
VEGF:	Vascular endothelial growth factor
ZEB:	Zinc finger E-box binding homeobox

List of Tables

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List of Publications

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Abstracts Presented in Conferences

1. Krishan K. Thakur, Bethsebie Sailo, Sajin K. Fransis, Manglam S. Nair and Ajaikumar B. Kunnumakkara. "Labdane-type diterpene as an anticancer drug in lung cancer" at 8th International Translational Cancer Research Conference: "Role of Inflammation and Immune System for Cancer Prevention and Treatment" organized by Institute of Science, Banaras Hindu University, Varanasi, India, held on February 13-16, 2020.
2. Krishan K. Thakur, Sajin K. Fransis, Manglam S. Nair and Ajaikumar B. Kunnumakkara. "Anticancer effects of labdane-type diterpene on lung cancer cells" at 4th International Conference on Nutraceuticals and Chronic Diseases (INCD)-2019 organized by IIT Guwahati and Society for Nutraceuticals and chronic diseases at IIT Guwahati, India, held on September 23-25, 2019.
3. Krishan K. Thakur, and Ajaikumar B. Kunnumakkara. "Overexpression of NGALR in breast cancer and its clinical prognosis" at Indo-Japan Symposium on "Recent Advances in Biomedical Research" RABR-2019 jointly organized by IIT Guwahati and National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan, held on March 26-27, 2019.
4. Krishan K. Thakur, and Ajaikumar B. Kunnumakkara. "Overexpression of NGALR in breast cancer and its clinical prognosis" at Research Conclave 2019 organized by IIT Guwahati, Assam, India, held on March 14-17, 2019.
5. Krishan K. Thakur, Sajin K. Fransis, Manglam S. Nair and Ajaikumar B. Kunnumakkara. Gave oral presentation on the topic "Anticancer Effects of Coronarin-D on Lung Cancer Cells" at International workshop on "Introduction to basic and advanced biomedical approaches for enhancing QOL in ageing societies" under the Sakura Exchange Program in Science (JST) at AIST, Tsukuba, Japan, held on October 14-21, 2018.
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Conferences, Workshops and Trainings Attended

1. Participated in Two Days International Virtual Conference on "Frontiers in Health Sciences" organized by the Department of Zoology and Zoological Society (ZSCC) of Cotton University, Guwahati, Assam, India on August 7-8, 2020.
2. Participated at 8th International Translational Cancer Research Conference: "Role of Inflammation and Immune System for Cancer Prevention and Treatment" at Banaras Hindu University (BHU), Varanasi, India on February 13-16, 2020.
3. Participated in DHR-ICMR sponsored workshop on "Diagnostic in Oncology using low Cytometry and Real-Time Polymerase Chain Reaction (RT-PCR)" at North-East Cancer Hospital and Research Institute, Guwahati on December 6-10, 2019.
4. Participated at 4th International Conference on Nutraceuticals and Chronic Diseases (INCD)-2019, organized by IIT Guwahati and Society for Nutraceuticals and chronic diseases at Institute of Technology (IIT)-Guwahati, India on September 23-25, 2019.
5. Participated at Indo-Japan Symposium on "Recent Advances in Biomedical Research" RABR-2019 jointly organized by IIT Guwahati and AIST, Tsukuba, Japan on March, 26-27, 2019.
6. Participated at Research Conclave 2019 at IIT Guwahati, Assam, India on March 14-17, 2019.
7. Participated in National conference on "New trends in multi-modal molecular imaging applications for animal studies in drug discovery" at NIPER Guwahati, Assam, India on November 20-21, 2019.
8. Participated at International workshop on "Introduction to basic and advanced biomedical approaches for enhancing QOL in ageing societies" at AIST-Tsukuba, Japan on October 14-21, 2018.
9. Attended 7 days DAICENTER - SHIMADZU Analytics Workshop jointly organized by DAICENTER, Japan, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan and Shimadzu, India, held from September 17-21, 2018 at Shimadzu Analytical, Mumbai, India.

10. Participated at 3rd INCD-2018 organized by Sri Rama Himalaya University and Society for Nutraceuticals and chronic diseases in Rishikesh, Uttarakhand, India on September 14-16, 2018.
11. Participated at National Conference on "Ethno-medicine and Traditional Health Practices in Northeast Region of India", at NIPER Guwahati, India on Aug 25th, 2018.
12. Participated at "International conference on Trends in Biochemical and Biomedical Research", BHU, Varanasi, India on February 11-13, 2018.
13. Attended Research Training Workshop on "Understanding Human Disease and Improving Human Health Using Genomics-Driven Approaches" Co-organized by National Institute of Biomedical Genomics (NIBMG), Kalyani & Mizoram University, Mizoram, India on November 19-24, 2017.
14. Participated at 2nd INCD-2017 organized by IIT Guwahati and Society for Nutraceuticals and chronic diseases at Goa, India on September 01-03, 2017.
15. Participated at Research Conclave 2017, at IIT Guwahati, Assam, India on March 16-19, 2017.
16. Participated in 1st INCD-2016, organised by IIT Guwahati, Assam, India on September 09-11, 2016.
17. Participated at Research Conclave 2016, organized by IIT Guwahati, Assam, India on March 17-20, 2016.

Award

- Received 'Best Poster Award' for the poster entitled "Overexpression of NGALR in breast cancer and its clinical prognosis" at Indo-Japan Symposium on "Recent Advances in Cancer Research" RACR-2019 jointly organized by IIT Guwahati and AIST, Tsukuba, Japan, March, 26-27, 2019 at IIT Guwahati.

Co-curricular Activities

- Member, organizing committee of the Indo-Japan Symposium on "Recent Advances in Cancer Research" RACR-2019 jointly organized by IIT Guwahati and AIST, Tsukuba, Japan, March, 26-27, 2019 at IIT Guwahati.

- Member, local committee of the "2nd International conference on Nutraceuticals and Chronic Diseases 2017 (INCD-2017), Goa, India, 2017.

- Member, organizing committee of the Indo-Japan symposium "Hope from herbs: Research- based care and cure potentials" jointly organized by IIT Guwahati and AIST Japan at IIT Guwahati, 2017.

- Member, local committee of the "1st International conference on Nutraceuticals and Chronic Diseases 2016 (INCD-2016), Kerala, India, 2016.