# STUDIES ON THE ROLE OF CYTOKINE-INDUCED PROTEINS IN BONE CANCER

**A thesis submitted for the degree of**

*Doctor of Philosophy*

**To**

**INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI**

*By*

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**April, 2021**

*Dedicated to*

*My family, friends and teachers*

*For the love, support and encouragement*

**DEPARTMENT OF BIOSCIEENCES AND BIOENGINEERING**
**INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI**
**GUWAHATI-781039**

## DECLARATION

I hereby declare that the contents of the research work described in this thesis titled **"Studies on the Role of Cytokine-Induced Proteins in Bone 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 Cytokine-Induced Proteins in Bone Cancer"**, submitted by Kishore Banik (Roll no: 146106033) 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.

April, 2021

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# ACKNOWLEDGEMENT

*I feel the opportunity to pen down an acknowledgement is an honor and a great responsibility that one can have. As I approach the finish line of this thrilling part of the scientific journey, I believe it is a responsibility to acknowledge all who helped me in this expedition of learning, development and contemplation of this beautiful scientific journey.*

*First of all, my eternal bow in admiration and gratitude to "Almighty God", whose divine blessings enabled me to complete my work on time.*

*I want to express my sincere gratitude to my supervisor, Prof. Ajaikumar B. Kunnumakkara, for his valuable suggestions, encouragement and inspiring me during every step of my work. His thirst to chase the most challenging problems in science and to encourage scientific temper can only be matched by the patience, care and independence he has bestowed upon me during my stay at Cancer Biology Laboratory (CBL), IITG. I am indebted to him for introducing me into the realm of this interesting work, training me with techniques, giving me the freedom in designing and conducting experiments.*

*I would also extend my gratitude to the members of my Doctoral committee chairperson Dr. S Senthilkumar and committee members Dr. Rajkumar P. Thummer and Prof. Chandan K. Jana for their valuable suggestions and guidance that has led to the successful completion of my research work.*

*I would like to acknowledge the Department of Biosciences & Bioengineering, IIT Guwahati, for providing the infrastructure, logistical support and facilities essential to perform my research and University Grants Commission, New Delhi, Govt. of India for the financial assistance.*

*I am extremely thankful to the present and former heads of the Department of Biosciences & Bioengineering, IIT Guwahati, Prof. Venkata V. Dasu, Prof. K. Pakshirajan and Prof. Latha Rangan for making the facilities available for carrying out research.*

*I am thankful to Dr. Nurul, Mrs. Prarthana, Mr. Niranjan, Ms. Rashmi, Mr. Chandan Kumar Nath, Mr. Dipankar Barman, Mr. Binoy, Mr. Dipankar Sharma and Mr. Pankaj Bhuyan for their administrative and technical assistance.*

*I would like to thank all the past and the present members of the CBL Dr. Monisha, Dr. Nand Kishor, Dr. Anuj, Dr. Devivasha, Dr. Padmavathi, Dr. Bethsebie, Dr. Elina, Dr. Babita, Amrita, Harsha, Krishan, Sosmitha, Parama, Aviral Kumar, Uzini, Dr. Mangala, Varsha, Nitesh, Rajesh, Jyothsna, Ramya, Aswin, Soham, Nikunj, Shabnam, Shyamananda, Raju, Amit, Heta, Nafiseh, Shailendra, Anip, Aviral, Anand, Jaiprakash, Poonam, Henamayee, Rooplekha, Bidisha, Joydeep, Shubhrojyoti, Madhuri, Gudia, Monikongkona, Semim, Pragya, Asha, Swathi, Yachna, Mangala, Olivia, Doni, Dama, Tetam, Gayathri, Srilaxmi, Srinidhi, Neha, Nabojit, Queen, Meenakshi, Anubhuti, Vandana, Ishu, Kavita, Nazia, Romi, Sharad, Elika, Mayur, Aso, Kabyashree, Anindita, Vaishali, Sandra who have given me a genial and edifying environment in the lab.*

*Special thanks to my supporting friends Abhishek, Devivasha, Harsha, Jahnu, Niten, Binita, Tulika, Kavita, Shrutika, Pooja, Rajesh, Sushil, Vijendra, Vikas, Subhrangshu, Devika, Chandana, Prashanth, Sandesh, Parag, Shivani, Nitin, Sandip, Abhijeet, Nitish, Ajinkya, Bhaskar, Gopal, Swarnima, Ayan, Snehal, Vivek, and Bhuvan for their immense support, love and care.*

*I want to express my heartfelt thanks to all my teachers of my school and colleges for inspiring me. Finally, my PhD endeavour could not have been successful without the love, support and blessings of all my family members. I owe my achievements to my mom, dad, sister, nephew,*

*brother in law and all my cousins; without their support, I would not have been able to cope up with the stress of my PhD life.*

*Kishore Banik*

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# Chapter 1

## *Introduction and Review of Literature*

# Chapter 1

## 1.1. Introduction

Primary bone cancer is an uncommon malignant tumor of the bone, originating from primitive mesenchymal cells and are responsible for 3–5% of all pediatric cancers and accounts for approximately 0.2% of all malignant neoplasms (Brown, H. K., *et al.*, 2018; Ottaviani, G., & Jaffe, N., 2009; Lam, S. W., *et al.*, 2019; Szuhai, K., *et al.*, 2012). The Surveillance, Epidemiology and End Results (SEER) –US National Cancer Institute statistics stated that the age-adjusted incidence rate rose by 0.4% per year in the last decade. The American Cancer Society-estimated that approximately 3,600 new cases and 1,720 deaths were expected in the year 2020 (Siegel, R. L., *et al*., 2020). Bone cancer has multiple subtypes, with osteosarcoma (OS), chondrosarcoma (CS), and Ewing's sarcoma (ES) being the most common. In adults, more than 40% of the primary bone cancers are CSs, which is followed by OS nearly 28%, chordomas around 10%, Ewing's tumors about 8%, and malignant fibrous histiocytoma/fibrosarcomas approximately 4%. The remainder of the cases are various rare types of bone cancers. In children and teenagers, OS accounts for 56% and Ewing's tumors, around 34% of the total cases and occur more frequently than CS. The average age of diagnosis of CSs is 51 and occurs more frequently in adults. Less than 5% of the total cases occur in patients younger than 20 years of age (Evola, F. R., *et al*., 2017; Weber, K., *et al.*, 2008; von Eisenhart-Rothe, R., *et al.*, 2011; Shweikeh, F., *et al.*, 2014; Palmerini, E., *et al.*, 2020; Ricotta, F., *et al.*, 2020; Lam, S. W., *et al.*, 2019). Chordomas are also more common in adults. Both Ewing's tumors and OS occur mostly in children and teens. Each differs in imaging appearance, biological behavior, and demographics. They are highly aggressive and entail early diagnosis, utilizing imaging and tissue biopsy. Surgical excision remains the cornerstone of curative treatment, with radiotherapy and chemotherapy frequently used in conjunction (Evola, F. R., 2017; Weber, K., *et al.*,

2008; von Eisenhart-Rothe, R., *et al.*, 2011; Shweikeh, F., *et al.*, 2014; Palmerini, E., *et al.*, 2020; Ricotta, F., *et al.*, 2020; Lam, S. W., *et al.*, 2019).

Despite the substantial improvement in overall survival of bone cancer patients accomplished in 1970s, due to the implementation of moderately effective chemotherapy, a third of all patients still die during 5 years after diagnosis mostly due to pulmonary metastasis. Additionally, the 5-year survival rate amongst children and adolescents has touched a plateau since the mid of 1980s. Moreover, the 5-year survival rate further drops to 20-30% for OS patients with metastasis (Lam, S. W., *et al.*, 2019; Maximov, V. V., & Aqeilan, R. I., 2016; Siegel, R. L., *et al.*, 2020).

Similar to other cancers, the most frequently used treatment approaches for bone cancer are surgery, chemotherapy, radiotherapy or a combination of any two or a combination of all three treatment strategies (Gaspar, N., *et al.*, 2020). Chemotherapy is used as preoperative chemotherapy or neoadjuvant and postoperative chemotherapy or adjuvant therapy evolving as a standard treatment strategy for bone cancers (Pasquali, S., & Gronchi, A., 2017; Gaspar, N., *et al.*, 2020). Some of the chemotherapeutics used either alone or in combination for the treatment of bone cancer are adriamycin (ADM), doxorubicin (DOX), cisplatin (CDDP), methotrexate (MTX) and ifosfamide (IFOS) (Wang, B., *et al.*, 2016; Bacci, G., *et al.*, 2003). Besides their low efficiency, these chemotherapeutic drugs are also found to be associated with adverse side effects. Few of them are highly toxic, affecting the patient's physiology by inducing rapid bone loss, resulting in an increased risk of fractures and osteoporosis and also decreasing the sex steroid levels, bone mineral density etc. Moreover, the use of established chemotherapeutic drugs against metastatic bone cancer are mostly associated with multiple glitches; including tumor recurrence and chemoresistance declining the overall

disease-free survival (Brown, H. K., *et al.*, 2018; Ottaviani, G., & Jaffe, N., 2009; Pantano, F., *et al.*, 2015; Picci, P., 2007; https://www.cancer.org/cancer/osteosarcoma /treating/chemotherapy.html). Hence, there is an imperative need to find novel biomarkers and targets for this disease, which would help us overcome the problems mentioned above.

The tumor necrosis factor-α-induced protein 8-like (TNFAIP8/Oxi-α/TIPE) family of proteins are a novel group of proteins identified a few years ago. The members of the TIPE family includes (TNFAIP8/TIPE), TNFAIP8 like-protein 1 (TNFAIP8L1/TIPE1), TNFAIP8 like-protein 2 (TNFAIP8L2/TIPE2), and TNFAIP8 like-protein 3 (TNFAIP8L3/TIPE3), which plays a pivotal role in regulating immune homeostasis, inflammatory responses, and cancer development (Gu, Z., *et al*., 2020). Even though the proteins of this family were first described as the modulators of tumorigenesis, inflammation, and cell death, they were also found to perform various cellular activities. TIPE family of proteins are found to be related to cancers of the bone, esophagus, brain, colon, cervix, endometrium, breast, liver, stomach, lung, and thyroid (Gu, Z., *et al*., 2020; Bordoloi, D., *et al.*, 2018; Padmavathi, G., *et al.*, 2018a). Over the last decade, several studies have demonstrated that TIPE2 protein is differentially expressed in different cells and tissues. Aberrant expression of TIPE2 protein can lead to deregulation of immune homeostasis and inflammatory responses and change the basic characteristics in cancer conditions (Bordoloi, D., *et al.*, 2018; Padmavathi, G., *et al.*, 2018a). Moreover, the importance of this protein in the etiopathogenesis of bone cancer is not yet completely understood. In consideration of the immeasurable values of TIPE2 in diagnosis, prognosis and treatment of various human diseases, unravelling the action of this protein would assist in understanding the bone tumor regulation and would serve as an early prognostic marker for this cancer. TIPE2 is primarily found in

the cytoplasm of the cell and comprises 184 amino acids. It is mainly involved in the negative regulation of cellular and innate immunity. It plays a crucial role in maintaining immune homeostasis and is vastly expressed in inflamed nervous tissue. Additionally, TIPE2 was found to be expressed in different types of cells, for instance, squamous epithelial cells in the esophagus, glandular epithelial cells in the appendix, colon, and stomach, and cervix, hepatocytes, neurons in the brain and brainstem, and transitional epithelial cells in the ureter and bladder (Bordoloi, D., *et al.*, 2018; Padmavathi, G., *et al.*, 2018a; Bordoloi, D., *et al.*, 2019).

However, it is hitherto to unravel the potential cross-talk of TIPE2 protein with diverse signaling molecules involved in bone tumorigenesis. Hence, this thesis work aims to decipher the role of TIPE2 protein in the development of bone cancer which would provide a new avenue for the identification of a potential biomarker for the early diagnosis and successful management of this disease.

### 1.2. Types of bone cancer

Bones are mainly made up of compact and hard osteoid tissues, tough and flexible cartilaginous, and fibrous tissue, as well as elements of bone marrow. Bone exerts critical functions in the body, such as support, locomotion, and protection of soft tissues, calcium and phosphate storage, and generation of bone marrow (Robling, A. G., *et al*., 2006; Datta, H. K., *et al*., 2008). Bone tumors are diverse in morphologic features and differ in their natural history from innocuous to rapidly fatal. Most of the bone cancers are classified as per the cell or tissue type they recapitulate (**Table 1.1.**). Cancer can initiate in any type of bone tissue (Franchi, A., 2012; Rajani, R., & Gibbs, C. P., 2012; https://www.cancer.org/cancer/bone-cancer/about/what-is-bone-cancer.html).

**Table 1.1.** Classification of bone cancer based on its histologic type

| HISTOLOGICAL TYPE | BENIGN | MALIGNANT |
|---|---|---|
| Hematopoietic (40%) | - | Myeloma<br>Malignant lymphoma |
| Chondrogenic (22%) | Osteochondroma<br>Chondroma<br>Chondroblastoma<br>Chondromyxoid fibroma | Chondrosarcoma<br>Dedifferentiated chondrosarcoma<br>Mesenchymal chondrosarcoma |
| Osteogenic (19%) | Osteoid osteoma<br>Osteoblastoma | Osteosarcoma |
| Fibrogenic | Fibrous cortical defect<br>*Non-ossifying fibromas*<br>fibrous histiocytoma<br>*Desmoplastic fibromas* | Fibrosarcoma |
| Unknown Origin (10%) | Giant Cell tumor<br>Unicameral cyst<br>Aneurysmal bone cyst | |
| Neuroectodermal | - | Ewing Sarcoma |
| Notochordal | Benign notochordal cell tumor | Chondroma |

### 1.2.1. Bone forming tumors

#### 1.2.1.1 Osteoma

Osteomas are benign tumors of the bone found primarily on the skull and facial bones. These tumors grow slowly and usually cause mild symptoms such as obstruction of the sinus cavity, impinge on the eye or brain, restrict the function of the oral cavity or creates cosmetic problems (Ostrofsky, M., 2019; Georgalas, C., *et al.*, 2011; Nielsen,

G. P., & Rosenberg, A. E., 2007).

#### 1.2.1.2. Osteoid osteoma and osteoblastoma

Osteoid osteoma and osteoblastoma are terms used to define benign tumor that has identical histologic features but varies in size, sites of origin and symptoms. Osteoid osteoma is usually less than 2 cm in greatest dimension and mainly occurs in the teens and early 20s. Approximately 75% of the individuals affected are less than 25 years old, and men outnumber women in the ratio 2:1. Osteoblastoma is mostly larger than 2 cm and occurs in the spine more often, and the tumor does not initiate a marked bony reaction (Dookie, A. L., & Joseph, R. M. 2020; Baumhoer, D., & Höller, S. 2020; Atesok, K. I., *et al.*, 2011; Paulin, E., *et al.*, 2019).

#### 1.2.1.3. Osteosarcoma (OS)

OS is a malignant mesenchymal tumor, where the cancerous cell produces the bone matrix. It emerges from the osteoid tissues of the bone. OS occurs most often in the upper arm and the knee. It is the most frequently occurring primary malignant tumor and accounts for nearly 20% of primary bone cancers (Selvarajah, S., *et al*., 2007; Selvarajah, S., *et al*., 2006). They occur in all age groups, but 75% of the people affected are younger than 20 years of age. This bone disease affects more men than women in the ratio of (1.6:1). The tumor typically arises in the metaphyseal region of the long bones of the extremities, and almost 50% of the tumor appears in the knee region. The majority of the OSs acquires genetic abnormalities such as ploidy change and chromosomal aberrations. OSs are characterized by a high level of genomic instability, in particular, chromosomal instability (CIN) (Selvarajah, S., *et al*., 2007; Selvarajah, S., *et al*., 2006). Frequent mutations in the retinoblastoma (*Rb*) and the *p53* genes are

observed in most OS cases (Miller, C. W., *et al*., 1996; Walkley, C. R., *et al*., 2008). Abnormalities in INK4a, which encodes p16 (a cell cycle regulator) and p14 (which regulates p53 functions), are also observed in OS. It is interesting that OS occur mainly at the site of bone growth as the frequent proliferation of the osteoblastic cells makes it more prone to acquire mutations that could lead to transformation (Hameed, M., & Mandelker, D., 2018; Maximov, V. V., & Aqeilan, R. I., 2016; Czarnecka, A. M., *et al.*, 2020; Ottaviani, G., & Jaffe, N., 2009; Evola, F. R., *et al.*, 2017).

### 1.2.2. Cartilage forming tumors

#### 1.2.2.1. Osteochondroma

Osteochondroma or exostosis is a benign cartilage capped tumor attached to the skeleton beneath with the help of a bony stalk. It is the most common benign tumor, and about 85% are solitary. Hereditary exostosis mainly occurs due to germline loss of function mutations in either the *EXT1 or EXT2* gene. These genes encode proteins that play an important role in the biosynthesis of heparin sulfates proteoglycans. The ratio of its occurrence in males and females is 3:1. It is slow-growing and becomes painful when they impinge on a nerve or if the stalk is fractured (Alabdullrahman, L. W., & Byerly, D. W., 2020; Jurik A. G., 2020; Roessner, A., *et al.*, 2020).

#### 1.2.2.2. Chondromas

Chondromas are benign tumor of hyaline cartilage that generally occur in bones of enchondral origin. It can arise within the medullary cavity (enchondromas), or on the surface of the bone (subperiosteal or juxtacortical chondromas) (Schajowicz, F., 1994; Malawer, M.M., *et al.*, 2004). It is mostly diagnosed in individuals in their 20's to 40s. Their favoured sites are the short tubular bones on the hands and feet. They are detected incidentally, and sometimes they are painful and cause pathologic fracture (Schajowicz, F., 1994; Malawer, M.M., *et al.*, 2004).

#### 1.2.2.3. Chondroblastoma

It is an uncommon benign tumor that accounts for less than 1 % of primary bone tumors. It commonly occurs in young patients in their teenage and has a male to female ratio of 2:1(Limaiem, F., *et al.*, 2020; Hmada, Y. A., 2020). Most of the chondroblastomas arise in the knee region and affects the older patients in less familiar sites such as the pelvis and ribs. They are generally painful, and due to their location mostly near a joint, they also cause effusions and confined joint mobility (Limaiem, F., *et al.*, 2020; Hmada, Y. A., 2020; De Mattos, C. B., *et al*., 2013).

#### 1.2.2.4. Chondromyxoid fibroma

It is the rarest of cartilage tumors, and for its diverse morphology, it can be easily mistaken with sarcoma. This type of tumor affects individuals in their teenage and 20's and has a male preponderance. The tumor most usually arises in the metaphysis of long tubular bones but can also involve any bone of the body (De Mattos, C. B., *et al*., 2013; Elsamanody, A., *et al.*, 2020).

#### 1.2.2.5. Chondrosarcoma (CS)

CS, which begins in the cartilaginous tissues, occurs mostly in the pelvic bones, upper leg, shoulder, and medullary canal of long bones. CSs are a heterogeneous group of tumors that can be classified into two categories based on the anatomic location 1) central, when they occur within the medullary canal 2) juxtacortical or peripheral when they appear in the cartilage cap of an exostosis (Palmerini, E., *et al.*, 2020; Limaiem, F., *et al.*, 2020; Roessner, A., *et al.*, 2020; MacDonald, I. J., *et al.*, 2019; Chow W. A., 2018). In addition to the conventional CSs that displays hyaline cartilage differentiation, there are other types of CSs such as mesenchymal, dedifferentiated, or clear cell (Kim, M. J., *et al*., 2011). The CS of the skeleton is about half frequent as OS and is the second most common malignant matrix producing tumor of the bone. Most

of the individuals get affected, usually in their 40s or older (Kim, M. J., *et al*., 2011). The tumor affects males twice as frequently as women, and nearly 15% of the CS arise from a pre-existing osteochondroma (Palmerini, E., *et al.,* 2020; Limaiem, F., *et al.,* 2020; Roessner, A., *et al.*, 2020; MacDonald, I. J., *et al.*, 2019; Chow W. A., 2018).

### 1.2.3. Fibrous tumor

#### 1.2.3.1. Fibrosarcoma

It is the collagen generating sarcomas with a fibroblastic phenotype that can occur at any age but mostly affect the older population. They have virtually equal sex distribution and frequently arise *de novo*. It presents as an enlarging painful mass that generally appears in the metaphysis region of the long bones and flat pelvic bones. Pathologic fractures are commonly observed in patients (Davis, D. D., & Kane, S. M., 2020; Folpe A. L. 2014).

### 1.2.4. Miscellaneous tumors

#### 1.2.4.1. Ewing's sarcoma

The ES Family of Tumors (ESFTs) not only occurs in bone but may also arise in soft tissues such as fibrous tissue, muscle, blood vessels fat, or other supporting tissues. ESFTs occur most commonly along the pelvis and the backbone and in the arms and legs (Jin W., 2020; Morales, E., *et al.*, 2020; Pullan, J. E., & Budh, D. P., 2020).

## 1.3. Etiology of bone cancer

The leading cause of bone cancer is not well defined; however, researchers have found various factors that enhanced the probability of developing bone tumors **(Figure 1.1)**. OS frequently occurs more in patients who have undergone high-dose external radiation therapy or treatment with certain chemotherapeutic drugs. Very few numbers of bone cancers are due to heredity. Moreover, individuals with hereditary defects of bones and

with metal implants (used as a treatment modality for fractures) are more likely to develop OS. ES is not strongly allied with any congenital childhood diseases, heredity cancer syndromes, or previous radiation exposure (Jafari, F., *et al.,* 2020; https://www.cancer.org/cancer/bone-cancer/causes-risks-prevention/risk-factors.html)

### 1.3.1. Genetic disorders

#### 1.3.1.1. Li-Fraumeni syndrome

The Li-Fraumeni syndrome, an inherited autosomal dominant disorder, makes people more susceptible to develop different types of cancer, including cancers of the breast, brain, OS, and other types of sarcoma. The majority of these cancers are mainly instigated by a mutation in the *p53* gene located on chromosome 17p13, but some are also caused by mutations in the *CHEK2* gene (Joyce, C., *et al.,* 2020; Czarnecka, A. M., *et al.,* 2020; Aedma, S. K., & Kasi, A., 2020).

#### 1.3.1.2. Rothmund-Thomson syndrome (RTS)

It is a rare autosomal recessive syndrome that can lead to bone cancer. Children suffering from this syndrome are generally short, have skeletal glitches, and rashes and are more likely to develop OS (Mojumdar, A., 2020; Mo, D., *et al.*, 2018; Lu, L., *et al.,* 2017). Abnormal changes in the gene REQL4, which belongs to the RECQ DNA helicase family, that plays an essential role in DNA metabolism are accountable for the majority of cases of RTS (Mojumdar A., 2020; Mo, D., *et al.,* 2018; Lu, L., *et al.*, 2017).

#### 1.3.1.3. Retinoblastoma (Rb)

Rb is a rare hereditary pediatric ocular tumor. The inherited form of Rb is triggered by a mutation of the *Rb1* gene. Patients with this mutation also have an augmented risk of

developing bone or soft tissue cancers. Besides, if radiation therapy is used for the treatment of Rb, the risk of OS in the bones near the eye is even higher (Lee, C., & Kim, J. K., 2020; Sun, J., *et al.*, 2020).

#### 1.3.1.4. Multiple exostoses

Multiple exostoses, also known as hereditary multiple osteochondromas (HMO), is an inherited condition characterized by the development of multiple benign osteocartilaginous masses/exostoses in the bones. These exostoses are mainly made up of cartilage. They are painful and may lead to deformed and/or fractured bones. A small percentage of the affected individuals are at risk of developing malignant sarcomas. This disorder is instigated primarily by a mutation in any of the three genes, i.e. *EXT1*, *EXT2*, or *EXT3* (Jurik, A. G., 2020).

#### 1.3.1.5. Enchondroma

Enchondroma is a common benign cartilage tumor that develops from the deregulation of chondrocyte terminal differentiation during growth plate development. Individuals having multiple tumor of this type possess a condition known as multiple enchondromatosis. These individuals have a rising risk of developing CSs (Zhang, H., & Alman, B. A. 2020; Jurik, A. G., 2020).

#### 1.3.1.6 Chordomas

Chordomas are rare, bone, axial, or extra-axial tumors derived mainly from notochordal tissues that are malignant and often recur but less commonly metastasize. Chordomas are classified into three histological types, namely, classical/conventional, chondroid, and dedifferentiated. They generally affect adults, with a minor proportion being pediatric tumors. They are chemo-resistant tumors, for which surgical resection and/or

radiotherapy are the treatments of choice (Tenny, S., & Varacallo, M., 2020; Karpathiou, G., *et al.*, 2020; Gill, C. M., *et al.*, 2020; Kremenevski, N., *et al.*, 2020).

#### 1.3.1.7. Paget's disease

It is a monostotic or polyostotic progressive skeletal disorder with a genetic predisposition. Bone areas that are affected exhibit osseous swelling, chronic pain, deformation, and fractures and are generally weaker than normal bones. It remains asymptomatic for a long duration of time which results in late diagnosis. The pathogenesis of this disease still remains unknown, but there are both genetic and environmental associations. Several viruses have been identified in the diseased bone; however, their role in the disease pathology is unclear (Klemm, P., *et al.*, 2020; Bouchette, P., & Boktor, S. W., 2020).

### 1.3.2. Previous radiation therapy

Bones that are exposed to ionizing radiation are at higher risk of developing bone cancer. The majority of radiation therapy-caused sarcomas include angiosarcoma, OS etc. Patients undergoing radiation therapy at a young age and are being treated with very high doses of radiation are at increased risk of developing bone tumors (https://www.cancer.org/cancer/bone-cancer/causes-risks-prevention/riskfactors.html; Joo, M. W., *et al.*, 2018).

### 1.3.3. Previous chemotherapy

Chemotherapy, hormonal therapy, or steroid medications used as a therapeutic strategy for the treatment of primary cancer may cause thinning of the bones, which leads to osteoporosis or joint pain and ultimately results in secondary bone cancer (Wustrack, R., *et al.*, 2020). Many chemotherapeutic drugs, including anthracyclines and alkylating

agents used for the treatment of different cancer types, may upsurge the risk of developing secondary bone tumors (https://www.cancer.org/cancer/bone-cancer/causes-risks-prevention/risk-factors.html; Wustrack, R., *et al.*, 2020).

### 1.3.4. Bone marrow transplantation

Bone marrow transplantation is used for the treatment of certain types of cancer, such as myeloma, leukemia and lymphoma, and other blood and immune system diseases that affect the bone marrow. OS has been reported in few patients who have undergone bone marrow transplantation (Ueki, H., *et al.*, 2013; Bielack, S. S., *et al.*, 2003; Asai, T., *et al.*, 2002).

### 1.3.5. Injuries

Fractures vary from other skeleton injuries, such as dislocations, though in some cases, it can be hard to differentiate. Sometimes, an individual may have more than one type of injury. However, it is assumed that fractures, tissue damages and injuries of the bone cells may lead to molecular alterations, which may cause bone cancer (https://www.cancer.org/cancer/bone-cancer/causes-risks- prevention/riskfactors.html)

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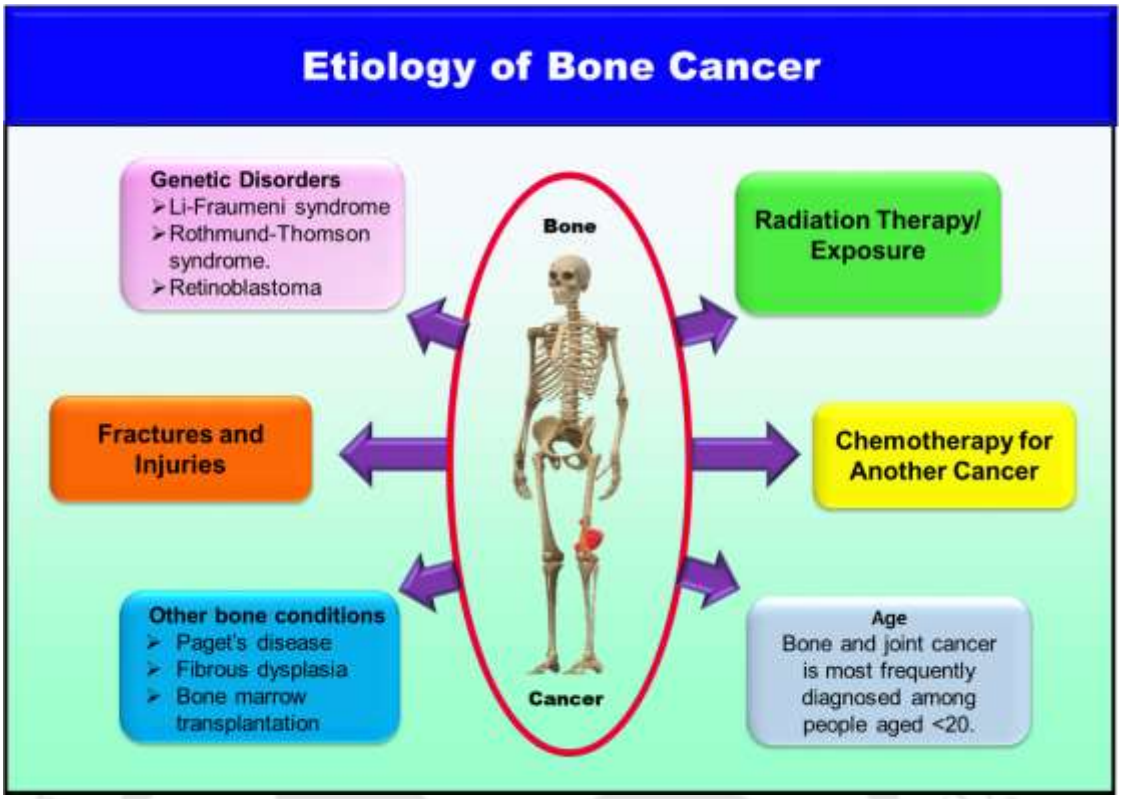


**Figure 1.1.**: The etiology of bone cancer.

## 1.4. Signs and symptoms of bone cancer

### 1.4.1. Pain

Pain is the most common symptom of patients suffering from bone tumor. At the initial stages, the pain is not constant; however, it increases with time and tumor size (https://www.cancer.org/cancer/bone-cancer/detection-diagnosis-staging/signs-html).

### 1.4.2. Swelling and stiffness

Swelling occurs in the area of pain and may appear as a lump or mass, depending on the location of the tumor. Sometimes a tumor in the neck bones may cause a lump in the back of the throat that can cause difficulty in swallowing and breathing

(https://www.cancer.org/cancer/bone-cancer/detection-diagnosis-staging/signs-symptoms.html )

### 1.4.3. Limping and fractures

Cancer in the bone deteriorates the bones and makes them more fragile and brittle, and may cause limping and fractures. Individuals with fracture in a bone which is next to or through a bone cancer pronounce sudden or severe pain in the limb (https://www.cancer.org/cancer/bone-cancer/detection-diagnosis-staging/signs-symptoms.html).

### 1.4.4. Other symptoms

Tumor of the spine can create pressure on nerves, leading to numbness and even weakness. It can also cause fatigue and weight loss. The tumor might spread to internal organs, which may cause various other symptoms, as well. For example, if cancer spreads to the lung, the patient may have trouble breathing. People with bone cancer may also have other symptoms such as fever and anemia (https://www.cancer.org/cancer/bone-cancer/detection-diagnosis-staging/signs-symptoms.html).

## 1.5. TNM staging of bone cancer

### 1.5.1. Tumor (T)

In the TNM system, the "T" plus a letter or number (0 to 4) is basically used to designate the size and location of the tumor. Furthermore, some stages are divided into subcategories that further describes the tumor in detail (**Figure 1.2.)** (Ehara, S., 2006;

Fukuma, H., *et al*., 1997). The specific tumor stage information for bone cancer is listed below in **Table 1.2**.

### 1.5.2. Node (N)

The "N" in the TNM staging system designate lymph nodes. Lymph nodes are tiny, bean-shaped organs that filter the blood and fight infections. The N factor is not so critical in bone cancers as lymph node metastases are very rare (Ehara S., 2006; Fukuma, H., *et al*., 1997). The prognosis of lymph node metastasis is utterly poor, and it is considered as distant metastasis. The inguinal and axillary nodes are mainly considered to be the regional nodes. The N factor is divided further into two grades: N0, no detectable node metastases; and N1, lymph node metastases (Ehara S., 2006; Fukuma, H., *et al*., 1997).

### 1.5.3. Metastasis (M)

In the TNM system, the "M" indicates whether cancer has undergone distant metastasis and has spread to other parts of the body (Ehara S., 2006; Fukuma, H., *et al*., 1997).

**Table 1.2.** TNM stage classification for lung cancer

| **T (primary tumor)** | Description |
|---|---|
| TX | The primary tumor cannot be evaluated |
| T0 | No primary tumor |
| T1 | Tumor ≤ 8 cm |
| T2 | Tumor > 8 cm |
| T3 | More than 1 separate tumor in the primary bone site |
| **N (regional lymph nodes)** | |
| NX | The regional lymph nodes cannot be evaluated. |
| N0 | No regional lymph node metastasis |
| N1 | The cancer has spread to the regional lymph nodes |
| **M (distant metastasis)** | |

| | |
|---|---|
| M0 | No distant metastasis |
| M1 | The cancer has metastasized to another part of the body. |
| M1a | The cancer has metastasized to a lung. |
| M1b | The cancer has metastasized to another organ |

(Table 1.2. adapted from Amin, M. B., et al., 2017; https://www.cancer.net/cancer-types/bone-cancer-sarcoma-bone/stages-and-grades).

### 1.6. Grade (G)

The G factor or the histological grade is unique to bone tumors. It is classified into four grades: G1, highly differentiated; G2, moderately differentiated; and G3 and G4, poorly differentiated. G4 is the highest grade, including ES and malignant lymphoma. Based on these factors, staging is determined as follows: IA, G1 or 2 T1N0M0; IB, G1 or 2 T2N0M0; IIA, G3 or 4 T1N0M0; IIB, G3 or 4 T2N0M0; III, not determined; IVA, G(any) T(any) N1M0; and IVB, G(any) T(any) N(any) M1 (Ehara S., 2006; Fukuma, H., *et al*., 1997) (**Table 1.3.**).

**Table 1.3.** Different grades and stages of bone cancer.

| **Grade** | |
|---|---|
| GX | The tumor grade cannot be identified. |
| G1 | The cancer cells are well differentiated. |
| G2 | The cancer cells are moderately differentiated. |
| G3 | The cancer cells are poorly differentiated. |
| **Stage** | |
| Stage IA | The tumor is low grade (G1 or GX) and 8 cm or smaller (T1). It has not spread to any lymph nodes or to other parts of the body (N0, M0). |
| Stage IB: | The tumor is low grade (G1 or GX) and larger than 8 cm (T2). It has not spread to any lymph nodes or to other parts of the body (N0, M0). |

| | |
|---|---|
| Stage IIA: | The tumor is high grade (G2 or G3) and 8 cm or smaller (T1). It has not spread to any lymph nodes or to other parts of the body (N0, M0). |
| Stage IIB: | The tumor is high grade (G2 or G3) and larger than 8 cm (T2). It has not spread to any lymph nodes or to other parts of the body (N0, M0). |
| Stage III | There are multiple high-grade (G2 or G3) tumors in the primary bone site (T3), but they have not spread to any lymph nodes or to other parts of the body (N0, M0). |
| Stage IVA | The tumor is of any size or grade and has spread to the lung(s) (any G, any T, N0, and M1a). |
| Stage IVB | The tumor is of any size or grade and has spread to the lymph nodes (any G, any T, N1, and any M), or the tumor is of any size or grade and has spread to another organ besides the lung (any G, any T, any N, and M1b). |

(Table 1.3. adapted from Amin, M. B., et al., 2017; https://www.cancer.net/cancer-types/bone-cancer-sarcoma-bone/stages-and-grades).

**1.7. Recurrent:**

OSs that have not responded to treatment or have reappeared after an initial response to treatment are regarded as recurrent. Recurrent OS appears in 30-50% of patients with initial localized disease and 80% of patients presenting with the metastatic form. Recurrent cancer might come back in the same place at the primary site, or it might reappear in a secondary site (Yu, X., *et al*., 2013; Daw, N. C., *et al*., 2015; Takeuchi, A., *et al*., 2014). The most frequent site to which OS spreads, or metastasizes, is the lungs which is also the most favorable site of recurrence. A long interval between the primary diagnosis and the appearance of recurrent tumor is attributed to a better prognosis. Besides, patients with recurrence appearing in the lungs may have a better prognosis than patients with distant metastases to other organs (Yu, X., *et al*., 2013; Daw, N. C., *et al*., 2015; Takeuchi, A., *et al*., 2014).

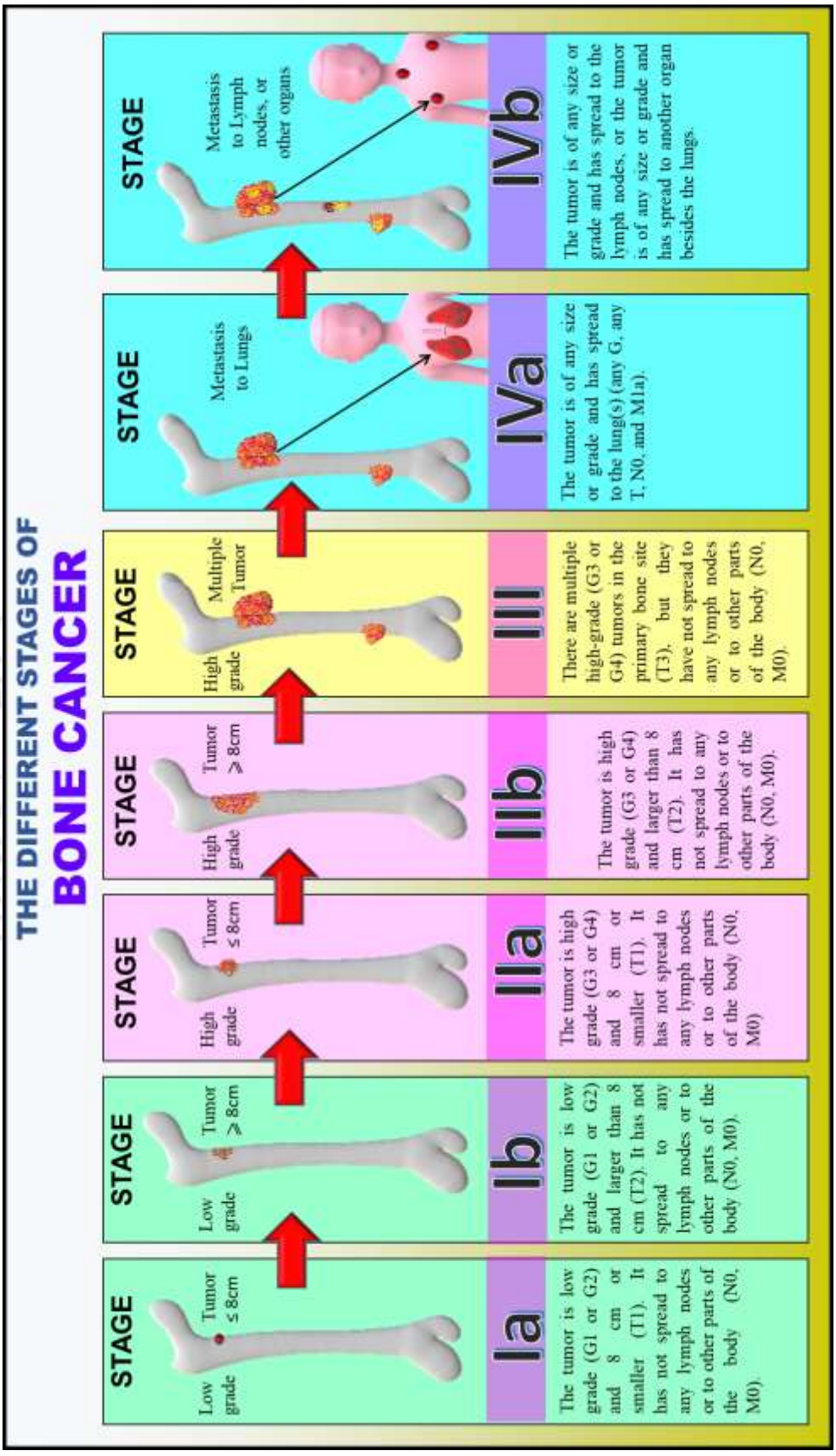


**Figure 1.2.**: The different stages of bone cancer.

### 1.8. Molecular alterations in bone cancer

The World Health Organization (WHO) has classified bone and soft tissue tumors into 58 different histologic subtypes (Fletcher, C. D., 2002). It is further classified into two groups at the molecular level, where one of the group encompasses tumors with a relatively simple karyotype with specific mutations, amplifications, or translocations. The chromosomal translocations in bone cancers represent an early event in tumorigenesis (Lam, S. W., *et al.,* 2019). Translocations cause tumorigenesis through three different molecular mechanisms, namely 1) promoter swap: A promoter of a gene which displays high expression in bone is fused to the coding sequence of the other gene, for instance, USP6, resulting in enhanced expression and thereby altered signaling; 2) creation of a chimeric gene, of which the fusion transcript performs as an aberrant transcription factor, e.g., *EWSR1-FLI1*, which triggers transcriptional deregulation; and 3) deregulation of a specific gene, resulting in altered function or inactivation of the gene, e.g., FOS. Similarly, specific hotspot mutations can change the transcriptional program when it occurs in the genes causing epigenetic changes, e.g., H3F3A/B, IDH1/2(Lam, S. W., *et al.,* 2019). Furthermore, hotspot mutations or amplification leads to aberrant signaling by activating the oncogenes (e.g., *GNAS, BRAF, MDM2*). Additionally, bone tumors harbor a complex karyotype with non-specific multiple molecular alterations. This complex genome can be attained gradually in the course of tumor progression (e.g., multistep progression model in CS) or can arise in one single cell division (e.g., chromothripsis in OS) (Lam, S. W., *et al.,* 2019).

It is a well-known fact that patients with Bloom syndrome, Werner syndrome, Rothmund–Thomson syndrome, Li–Fraumeni syndrome, and hereditary Rb have a predisposition to OS, which points to genes – *RECQL2, RECQL3, RECQL4, TP53*, and

*Rb1*, respectively. Hereditary multiple exostoses (HME) and Paget's disease are concomitant with an increased risk of OS (Ruijs, M. W., *et al.*, 2009; Ruijs, M. W., *et al.*, 2010; Marees, T., *et al.*, 2008; Hameed, M., & Mandelker, D. 2018). Subsequently, mutations in *EXT1* and *EXT2* genes are allied with HME and mutations in the SQSTM1 gene are linked with Paget's disease; therefore, these genes are also expected to contribute to OS development (Wuyts, W., *et al.*, 1998; Layfield, R., & Hocking, L. J. 2004). Noteworthy, other genes –*OPTN*, *CSF1*, *RIN3*, *PML*, *TM7SF4*, *NUP205*, and *TNFRSF11A* – have also been linked to Paget's disease and, therefore, might also be associated in osteosarcomagenesis (Maximov, V. V., & Aqeilan, R. I. 2016). The p53 signaling pathway is found to be mutated in the majority of the OS cases. This pathway is inactivated due to TP53 mutations or by amplification of MDM2. Additionally, it was also reported that COPS3 amplification is more frequently amplified in OS than MDM2, indicating that this event negatively regulates the role of p53 (Henriksen, J., *et al.*, 2003). Moreover, frequent inactivation of the *Rb1* is also reported in OS along with the genes of the p53 pathway (Indovina, P., *et al.*, 2015). Furthermore, Kovac *et al*. recognized 388 validated mutations and identified 14 genes as the main drivers that are evenly responsible for approximately 87% of OS cases. These 14 genes include *Rb1*, *BRCA2*, *TP53*, *BAP1*, *RET*, *PTEN*, *ATM*, *MUTYH*, *WRN*, *ATRX*, *FANCA*, *RECQL4*, *MDC1* and *NUMA1*. However, the roles of several genes are unknown in the context of OS, such as *FANCA*, *NUMA1*, and *MDC1* (Bousquet, M., *et al.*, 2016; Chen, X., *et al.*, 2014; Kovac, M., *et al.*, 2015; Joseph, C. G., *et al.*, 2014; Perry, J. A., *et al.*, 2014; Reimann, E., *et al.*, 2014).

In addition to the aforementioned genes, some of the other genes which were reported to be commonly altered in OS are ATRX which helps in the regulation of chromatin remodelling, nucleosome assembly, and telomere maintenance; DLG2, and the genes

of the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway, including PTEN (Chen, X., *et al.*, 2014; Zheng, C., *et al.*, 2020). The mechanistic role of cFos overexpression was also demonstrated in different *in vivo* mouse models of OS (Rüther, U., *et al.*, 1989; Wu, J. X., *et al.*, 1990; Wang, Z. Q., *et al.*, 1995). Among all the candidate OS driver genes, the role of the genes involved in the PI3K/AKT/mTOR, mitogen-activated protein kinase (MAPK), and ErbB signaling pathways have been identified as a common vulnerability for therapeutic exploration in the context of OS (Hu, K., *et al*., 2016; Rickel, K., *et al.*, 2017).

Moreover, upregulation of both – hypoxia-inducible factor 1-alpha (HIF-1α) and CXCR4 was found to be linked with metastatic bone cancer (Guan, G., *et al.*, 2015). A recent study demonstrated the association of HIF-1α overexpression and metastasis and poor survival in OS patients. In addition, it was verified that vascular endothelial growth factor (VEGF)-A acted downstream of HIF-1α and the co-expression of both the genes promoted the invasion of OS cells *in vitro* (Zhao, H., *et al*., 2015; Mizobuchi, H., *et al.*, 2008). Furthermore, it was also observed that on the silencing of c-Fos target – FGFR1 in c-Fos overexpressing murine OS cells, their metastatic potential decreased considerably (Weekes, D., *et al.*, 2016). Furthermore, PTEN, a negative regulator of PI3K/AKT, extracellular signal-related kinase (ERK), and focal adhesion kinases (FAK) signaling, could be another key player contributing to the OS metastasis (Zheng, C., *et al.*, 2020).

The miRNAs are non-coding RNAs, ranging from 16 to 27 bases, which usually suppresses gene expression at mRNAs translation levels and affect the stability of mRNAs via recruiting to miRNA-induced silencing complexes (miRISCs) (Catalanotto, C., *et al.*, 2016). The miR-20a encoded by the miR-17-92 cluster have

been found to induce metastases in OS (Huang, G., *et al.*, 2012). Osteoblast-specific knockout of the miR17-92 cluster was reported to impair bone formation in mice. Moreover, this cluster was proposed to function both as an oncogene or anti-oncogene by regulating the host immune response, depending on the type of malignancy, since different malignancies possibly express diverse sets of targets for the miR17-92 cluster (Zhou, M., *et al.*, 2014).

Researchers also proposed the involvement of PI3K/AKT, ERK, wingless-related integration site (Wnt), nuclear factor kappa-B (NF-κB), protein kinase C (PKC), hedgehog, and hippo signaling pathways that offer additional evidence for different osteosarcomagenesis mechanisms conferring to varied metastatic potential to OS (Jayarangaiah, A., & Theetha Kariyanna, P., 2020; Rickel, K., *et al.*, 2017). The conflicting roles of *Ube2d2a*, *Raf1*, and *Snap23* genes in OS primary tumor development provides the most substantial evidence of the existence of different OS genetic mechanisms conferring metastatic properties to OS (Maximov, V. V., & Aqeilan, R. I., 2016).

### 1.9. Treatment approaches for bone cancer

#### 1.9.1. Diagnosis

For the diagnosis of bone cancer, the symptoms, a physical exam, and the results of imaging tests, and blood tests suggests whether an individual has bone cancer (Geller, D. S., & Gorlick, R. 2010; Jafari, F., *et al.*, 2020). Non-invasive diagnostics methods have been extensively used over the past few decades for the diagnosis of bone cancer and comprise the use of radiography, computed tomography (CT), X-rays, positron emission tomography (PET), magnetic resonance imaging (MRI), or a combination of

these methods (Geller, D. S., & Gorlick, R. 2010; Jafari, F., *et al.*, 2020). X-rays are performed to identify the location, size, and shape of the tumor. A bone scan is a nuclear imaging test that helps diagnose and track various types of bone disease. A bone scan is performed using a small amount of radioactive material which is injected into a blood vessel, that gets accumulated in the bones and is detected by using a scanner (Jafari, F., *et al.*, 2020).

CT-scan is used for the diagnosis of bone cancer, where a series of detailed pictures of the area inside the body are taken from various angles that are generated by a computer connected to an x-ray machine (Edge, R., & Picheca, L., 2020). MRI is an important non-invasive imaging technique mainly used in radiology to produced detailed pictures of the anatomy and the physiological processes of the body. MRI scanners use strong magnetic fields, magnetic field gradients, and radio waves to create images for the diagnosis of bone cancer (Luining, W. I., *et al*., 2021; Stokkel, M. P., *et al.*, 2002; Jafari, F., *et al.*, 2020; Geller, D. S., & Gorlick, R. 2010). A PET scan is a technique that uses radioactive glucose and is administered intravenously. A scanner takes detailed pictures of areas inside the body where the glucose is consumed or used. As the cancer cells consume more glucose than normal cells, these pictures are used to locate the cancer cells in the body (Luining, W. I., *et al*., 2021; Stokkel, M. P., *et al.*, 2002; Jafari, F., *et al.*, 2020; Geller, D. S., & Gorlick, R. 2010). Bone scintigraphy or bone scan (BS) is a crucial nuclear medicine tool that is often used in combination with CT to identify metastatic disease (Luining, W. I., *et al*., 2021; Stokkel, M. P., *et al.*, 2002; Jafari, F., *et al.*, 2020; Geller, D. S., & Gorlick, R. 2010).

Numerous serum markers have been scrutinized for their effectiveness in diagnosis, progression and recurrence. Alkaline phosphatase (ALP) and lactose dehydrogenase

(LDH) are important serum biomarkers, where ALP has the most diagnostic significance in bone cancer (Limmahakhun, S., *et al.,* 2011). Blood tests are often performed to determine the alkaline phosphatase (ALP) levels in the body, which is commonly present at high levels (40% more in cancer patients) when the cells that form bone tissues are very active and are positively associated with tumor volume. SATB2 is a well-known and a sensitive marker for OS. It is non-specific and denotes osteoblastic phenotype; however, it does not indicate whether the tumor is malignant (Machado, I., *et al.*, 2016). Another very common human bone protein also used as a marker for osteoblastic phenotype is osteocalcin, which is also used as a diagnostic marker of OS (Agustina, H., *et al.,* 2018). A biopsy is a procedure where tissue or fluid sample is taken or removed from the tumor through incisional biopsy or a needle biopsy technique, to determine whether the tissues are cancerous or not (Geller, D. S., & Gorlick, R. 2010; Jafari, F., *et al.*, 2020).

### 1.9.2. Treatment modalities

Treatment modalities depend on the size, type, location, grade and stage of the bone cancer, as well as the patient's age and general health. The major treatment modalities for bone cancer include surgery, radiation therapy, chemotherapy, and cryosurgery (**Figure 1.3.**). Surgery is the main treatment option for bone cancer. In the surgery, the complete tumor is removed with negative margins (Stokkel, M. P., *et al.,* 2002; Jafari, F., *et al.*, 2020; Geller, D. S., & Gorlick, R. 2010; Tan, M. L., *et al.,* 2009). Some special surgical techniques are also used to abate the amount of healthy tissue removed along with the tumor. Drastic improvement in preoperative tumor treatment methods and surgical techniques have made it possible for bone cancer patients to avoid radical surgical procedures. Though, most of the patients who undergo limb-sparing surgery

requisite reconstructive surgery to enhance limb function (Stokkel, M. P., *et al.,* 2002; Jafari, F., *et al.,* 2020; Geller, D. S., & Gorlick, R. 2010; Tan, M. L., *et al.,* 2009).

A bone cancer patient usually receives a combination of different anticancer drugs. Commonly used chemotherapeutic drugs for bone cancer includes DOX, IFOS, cyclophosphamide, MTX, CDDP, vincristine, etc. There are several adverse side effects associated with chemotherapy, such as nausea, fatigue, vomiting, loss of appetite, risk of infection, hair loss, and diarrhea, which depends on individual to individual and the dose used for the treatment (Stokkel, M. P., *et al.,* 2002; Jafari, F., *et al.,* 2020; Geller, D. S., & Gorlick, R. 2010; Tan, M. L., *et al.,* 2009).

Radiation therapy or radiotherapy uses high-energy radiations to kill cancer cells. This treatment modality may be used in combination with surgery and is often used for the treatment of CS, which cannot be treated with chemotherapy (Stokkel, M. P., *et al.,* 2002; Jafari, F., *et al.,* 2020; Geller, D. S., & Gorlick, R. 2010; Tan, M. L., *et al.,* 2009). The most frequently used radiotherapy is called external-beam radiation therapy; the other method includes internal radiation therapy or brachytherapy (Stokkel, M. P., *et al.,* 2002; Jafari, F., *et al.,* 2020; Geller, D. S., & Gorlick, R. 2010; Tan, M. L., *et al.,* 2009). Radiation therapy is mostly used for patients where the tumor cannot be eliminated with surgery. It may also be performed before surgery to reduce the tumor size, or it may be done post-surgery to terminate any remaining cancer cells. This therapy reduces the need for extensive surgery, often preserving the leg or arm in case of bone cancer, and it may also be used to alleviate pain for people as part of remedying care. Adverse or side effects from radiotherapy may include fatigue, mild skin reactions, upset stomach, and loose bowel movements (Stokkel, M. P., *et al.,* 2002; Jafari, F., *et al.,* 2020; Geller, D. S., & Gorlick, R. 2010; Tan, M. L., *et al.,* 2009).

Cryosurgery is the use of liquid nitrogen or extreme cold to freeze and destroy the cancer cells. This procedure is used sometimes instead of conventional surgery to treat internal and external tumors (Stokkel, M. P., *et al.,* 2002; Jafari, F., *et al.,* 2020; Geller, D. S., & Gorlick, R. 2010; Tan, M. L., *et al.,* 2009).

### 1.10. Problems associated with bone cancer

#### 1.10.1. Late-stage diagnosis

Management of bone cancer still remains a major challenge. This can be primarily accredited to the poor prognosis, due to which, in majority of the cases, it is not diagnosed until it spreads and reaches highly advanced metastatic stage (Stokkel, M. P., *et al.,* 2002; Jafari, F., *et al.,* 2020; Geller, D. S., & Gorlick, R. 2010; Tan, M. L., *et al.,* 2009). Consequently, most of the patients undergo an intensive and invasive treatment regime comprising of surgery, radiotherapy, or chemotherapy, or their combinations based on patient's performance status or disease stage. Furthermore, many patients also may have to undergo multiple lines of therapy as they develop chemoresistance to the agents used for the treatment of the disease (Stokkel, M. P., *et al.,* 2002; Jafari, F., *et al.,* 2020; Geller, D. S., & Gorlick, R. 2010; Tan, M. L., *et al.,* 2009).

#### 1.10.2. Tumor recurrence

The quality of life in bone cancer patients immensely depends on the success of the surgery. In spite of the recent advancements made in the treatment of bone cancer, the rate of local recurrence in non-metastatic bone cancer patients is as high as 46% (Stokkel, M. P., *et al.,* 2002; Jafari, F., *et al.,* 2020; Geller, D. S., & Gorlick, R. 2010;

Tan, M. L., *et al.*, 2009). Recurrent bone cancer can form either in the same bone in which primary cancer originated, or it can affect other tissues and organs, such as

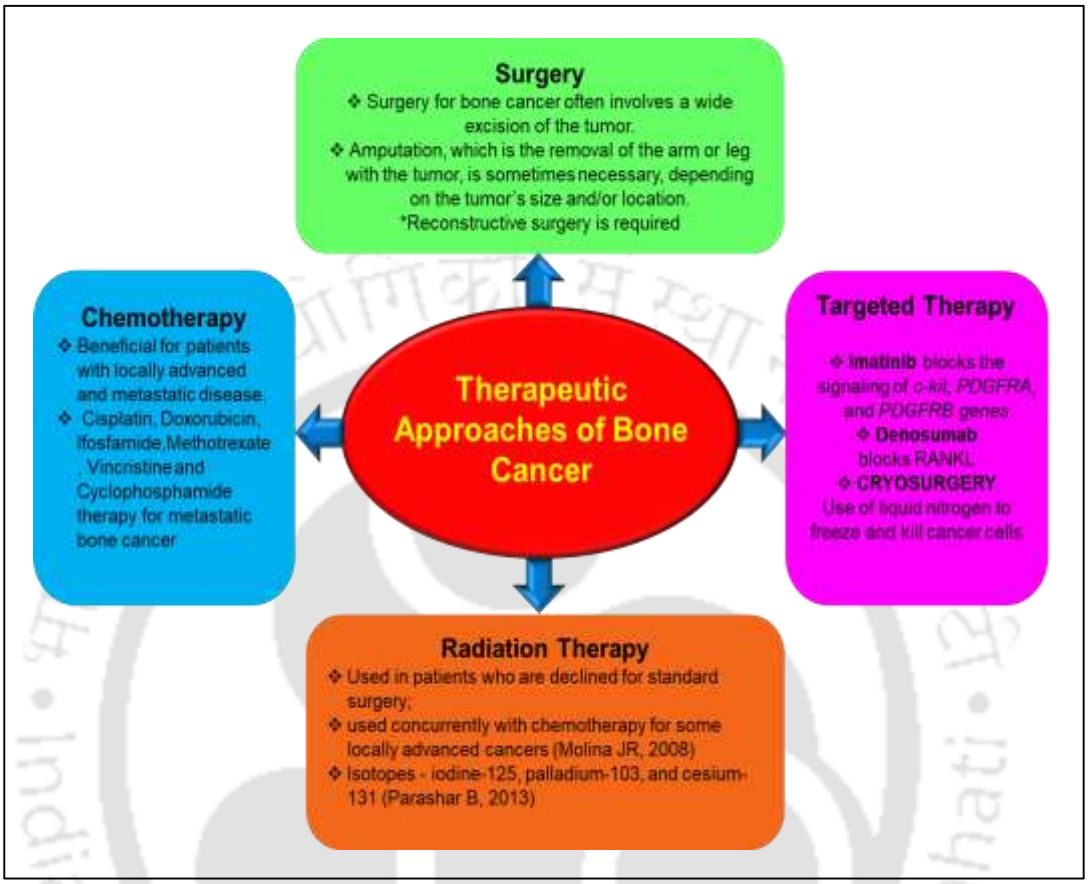


**Figure 1.3.**: The various therapeutic approaches of bone cancer

the lungs. In many cases, the cancer returns within two to three years after the patient complete an initial course of treatment (Stokkel, M. P., *et al.*, 2002; Jafari, F., *et al.*, 2020; Geller, D. S., & Gorlick, R. 2010; Tan, M. L., *et al.*, 2009).

### 1.10.3. Development of chemoresistance

Resistance to chemotherapeutic agents used for the treatment of bone cancer presents a major impediment in thriving the long-term after-effects of chemotherapies used for the treatment of bone cancer (Padmavathi, G., *et al.*, 2018b). Most bone cancer patients develop chemoresistance against the chemotherapeutics eventually, even after showing

a good initial response. Several traditional resistance mechanisms described in other solid tumors such as decreased uptake of the drug, mutation of cellular targets, alteration of drug targets, increased drug metabolism, drug inactivation etc. have also been found in several bone cancer studies (Padmavathi, G., *et al*., 2018b; Khatoon, E., *et al*., 2020).

### 1.11. TIPE family of proteins

The TIPE family of proteins have been identified lately and are involved in the control of immunity and tumorigenesis. This family consists of four members, viz., TIPE, TIPE1, TIPE2, and TIPE3. Even though all the four members of this family share remarkable sequence homology, i.e. ~54% sequence identity, they partake in the regulation of different cellular activities (Bordoloi, D., *et al.*, 2018; Padmavathi, G., *et al*., 2018a; Bordoloi, D., *et al.*, 2020).

Over the past few years, numerous studies have revealed that TIPE2 protein to be differentially expressed in different cells and tissues. Atypical expression of TIPE2 protein can lead to aberrant immune homeostasis and inflammatory responses and change the basic characteristics in cancer conditions (Bordoloi, D., *et al*., 2018; Padmavathi, G., *et al*., 2018). However, the importance of this protein in the development and progression of bone cancer is not yet completely understood. In consideration of the immeasurable values of TIPE2 in diagnosis, prognosis and treatment of various human diseases, unravelling the action of this protein would assist in understanding the bone tumor regulation and would serve as an early prognostic marker for this cancer.

### 1.11.1 TIPE2

The gene for the TIPE2 resides on human chromosome 1(1q21.2–1q21.3), and it was first discovered in autoimmune encephalomyelitis in the year 2008 (Gu, Z., *et al*., 2020; Sun, H., *et al.*, 2008; Zhang, G., *et al.*, 2010). The TIPE2 protein consisting of 184 amino acids is stated to exist mainly in the cytoplasm of the cell, and its expression analysis advocated that it pursued a tissue-specific expression pattern (Sun, H., *et al.*, 2008; Zhang, L., *et al.*, 2011; Li, Z., *et al.*, 2018; Gu, Z., *et al*., 2020). It is primarily discovered as a negative regulator of cellular and innate immunity, with substantial sequence homology with the other proteins of the same family (Bordoloi, *et al.*, 2018). A BLAST search of TIPE2 showed that TIPE2 retains 53% and 94% sequence identity with human TIPE and murine TIPE2, respectively. Similar to TIPE, it possesses an N-terminus death effector domain (DED) with 6 conserved α-helices (Sun, H., *et al.*, 2008; Freundt, E. C., *et al.*, 2008; Zhang, G., *et al.*, 2010). It was also reported to be highly expressed in the inflamed spinal cord but entirely lacking in the normal spinal cord (Sun, H., *et al.*, 2008; Zhang, G., *et al.*, 2010).

The TIPE2 crystal structure comprises of six antiparallel α –helices where, α5 helix contains a kink, owing to Pro153, which further splits it into two helices, viz., α5a and α5b, where α5b forms the helical bundle base. TIPE2 holds a cylindrical cavity located at the centre, which is mainly hydrophobic in nature; however, the outer surface of TIPE2 remains highly charged (**Figure 1.4.)**. The central cavity helps in cofactor binding, and in turn, plays a vital role in immune homeostasis. The basic structure of TIPE2 varies from that of DED; first of all, TIPE2 comprises 184 amino acids that are comparatively bigger than that of DED, and then, the topology of both TIPE2 and DED

are dissimilar. As a matter of fact, the topology of TIPE2 appears to be a mirror image of DED (Zhang *et al.*, 2009).

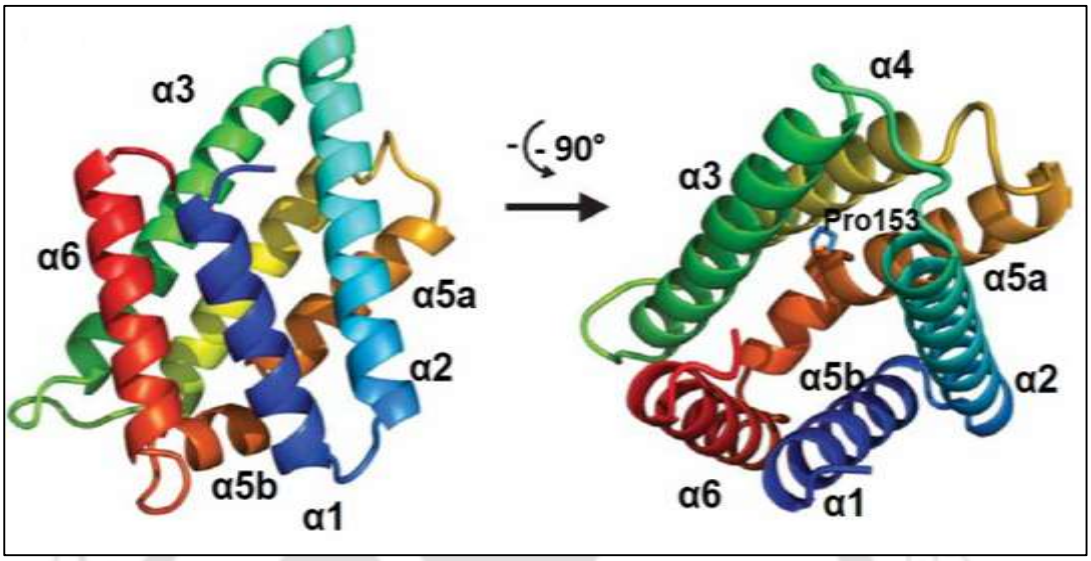


**Figure 1.4.: The structure of TIPE2. The structure of TIPE2 defines a previously uncharacterized fold that is different from the DED. It is shown in two perpendicular views. The six α-helices are rainbow colored. Pro153, which breaks α-5 into two helices is indicated at right (Figure adapted from Zhang, *et al.*, 2008).**

### 1.11.2. Expression pattern of TIPE2

TIPE2 is primarily expressed in myeloid and lymphoid tissues, whereas in other cell types, it is instigated by tumor necrosis factor α (TNF-α). TIPE2 is constitutively expressed in macrophages, T- and B-lymphocytes (Lou, Y., & Liu, S., 2011; Sun, H., *et al.*, 2008). In contrast to this, Zhang *et al.*, (2010) showed that TIPE2 protein is mainly detected in the T-cell zone but not in B cells of the germinal center and possesses a tissue-specific pattern of expression. In addition to the lymphatic tissues, TIPE2 is also expressed in various types of endocrine, somatic, and germ cells of the mice reproductive organs, which denotes that it may also play a crucial role in endocrine and reproductive activities (Li, Z., *et al.*, 2018; Zhang, G., *et al.*, 2010). Moreover, the human TIPE2 protein is expressed in several non-hematopoietic cells such as the ureter

and the bladder's transitional epithelial cells, brain neurons, the cervix and the esophagus squamous epithelial cells, and the appendix, colon, and stomach's glandular epithelial cells ( Zhang, L., *et al.*, 2011; Li, Z., *et al.*, 2018; Carmody, R. J., *et al.*, 2002; Lou, Y., & Liu, S., 2011). This protein was found to be overexpressed in ovarian adenocarcinoma cells, lung cancer cells and macrophage-derived cell lines; whereas weak or no expression was observed in astrocytoma, cervical carcinoma, chronic myelogenous leukemia, hepatocellular carcinoma (HCC), lung carcinoma and transitional cell carcinoma of the urinary bladder (Zhang, G., *et al.*, 2010).

### 1.11.3. Upstream regulators of TIPE2

A microRNA or miRNA is a small single-stranded non-coding RNA molecule that are master modulators of gene expression in various cellular and biological process and helps in post-transcriptional regulation of gene expression through RNA silencing (Rishabh, K., *et al*., 2021). The miRNA-21 targets NF-κB, which regulates the TIPE2 expression in a coding region-dependent manner. The miRNA-21 is highly expressed in activated T-cells and macrophages, while TIPE2 expression was downregulated. On the contrary, the T-cells deficient in TIPE2 are unresponsive to apoptosis. Thus, miR-21 prevents apoptosis of T-cells via the modulation of TIPE2 (Ruan, Q., *et al.*, 2014). Besides, OAS/RNaseL-expressing VACV recombinants infected HeLa human cervical cancer cells resulted in a remarkable enhancement of TIPE2 expression, signifying its part in viral-mediated immune responses (Domingo-Gil, E., *et al.*, 2010). Additionally, activating protein-1 (AP-1) was affirmed to be an essential porcine TIPE2 transcription, which has substantial sequence resemblance with human TIPE2, thus suggesting AP-1 to be also a controller of human TIPE2 (**Figure 1.5.)** (Li, A., *et al.*, 2010).

### 1.11.4. Downstream effectors of TIPE2

TIPE2 was found to interact with TGF-β-activated kinase 1 (TAK1), a modulator of immune signals, and influence its expression by obstructing the formation of the TAK1-TAK1-binding protein (TAB)-1-TAB2 complex (Oho, M., *et al.*, 2016). TIPE2 was also found to reduce the levels of NF-κB and AP-1 by binding with caspase-8. TIPE2 also deregulated the c-Jun N-terminal kinase (JNK), p38 MAPK, and NF-κB signaling pathways by decreasing the nuclear translocation of c-Jun, c-Fos and NF-κB, and also repressed IκBα degradation. Additionally, it was also found to control the levels of TNF-α (Sun, H., *et al.*, 2008). Besides, TIPE2 also regulated Rac1- signal transducer and activator of transcription (STAT)3 and ERK1/2 signaling pathways as it repressed the activation and nuclear translocation of STAT3 in a restenosis mice model and also suppressed the levels of cyclin-D1 and -D3 (Zhang, G., *et al.*, 2015). Additionally, this protein was also shown to be linked with the dysregulation of Wnt/β-catenin and PI3K/AKT signaling by reducing β-catenin, cyclin-D1, and c-myc, and inhibiting the phosphorylation of PI3K and AKT (Liu, Z. J., *et al.*, 2016; Lu, Q., *et al.*, 2016). TIPE2 also restricted the PI3K/AKT/ glycogen synthase kinase 3 beta (GSK3β)-mediated β-catenin signaling via inhibition of p-AKT, which ultimately led to the suppression of inhibitory phosphorylation of GSK3β which triggered the degradation and diminished the nuclear translocation of β-catenin (Wu, J., *et al.*, 2016). TIPE2 inhibited epithelial-mesenchymal transition (EMT) markers such as Snail1, Snail2/Slug, and Zeb1 through the deregulation of AKT/GSK3β pathway (Yin, H., *et al.*, 2017). In addition, TIPE2 triggered apoptosis in adjuvant arthritis fibroblast-like synoviocytes by ameliorating death receptor (DR5) expression, which results in activation of caspases and in turn suppressed NF-κB signaling (Shi, C., *et al.*, 2016).

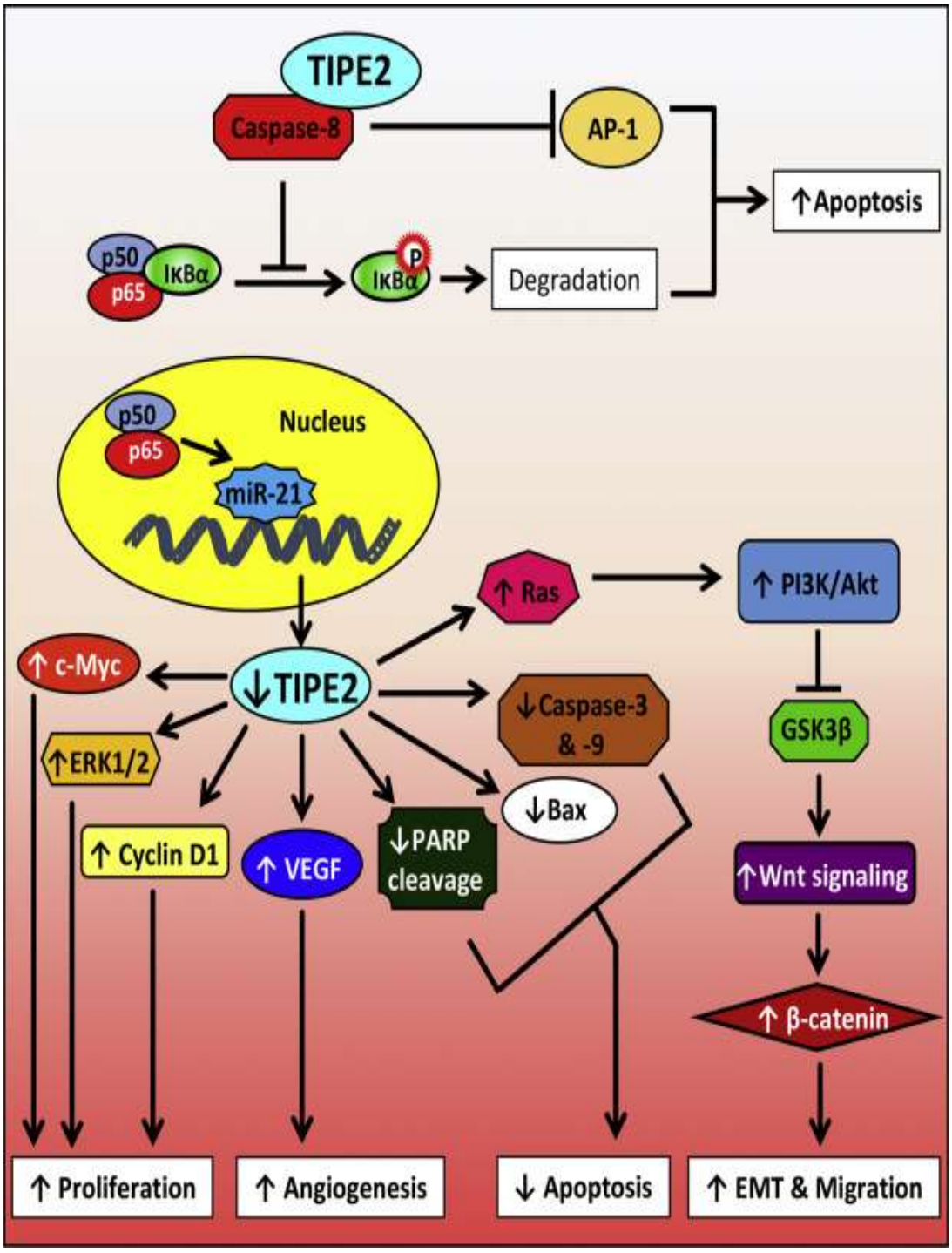


**Figure 1.5.: Downregulation of TIPE2 leads to the activation of survival and proliferative signals; induction of angiogenesis, invasion, migration, and metastasis; and inhibition of pro-apoptotic signals, thus resulting in tumorigenesis. ↑- upregulation; ↓- downregulation. (Padmavathi, *et al.*, 2018).**

Furthermore, it also augmented the expression of pro-apoptotic proteins Bax, caspase-3, caspase-9, and cleaved poly ADP ribose polymerase (PARP), and diminished the levels of anti-apoptotic proteins Bcl-xL, p-AKT, and p-ERK1/2 (Zhu, Y., *et al.*, 2016). Besides, it was also found to mediate anti-angiogenic activities via upregulating the expression level of VEGF (Suo, L., *et al.*, 2016). Lastly, TIPE2 enhanced the expression of p27 via the induction of interferon regulatory factor 4 signaling cascades (Peng, Y., *et al.*, 2016).

### 1.11.5. Functions of TIPE2

TIPE2 is a key regulator in maintaining immune homeostasis, including both innate and cellular immunity. It modulates Toll-like receptor (TLR) and T-cell receptor signaling negatively (Sun, H., *et al*., 2008; Freundt, E. C., *et al*., 2008). The loss of function of TIPE2 induced lethal inflammatory disorders in mice and systemic autoimmunity in humans, which suggested its crucial role in maintaining immune homeostasis (Zhang, G., *et al*., 2010). The knockout of TIPE2 induced splenomegaly, multi-organ inflammation, and early death in mice. Moreover, it also ensued in elevated levels of inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-12, TNF-α, and the anti-inflammatory cytokine IL-10 in the serum. Besides, the augmented levels of both CD4+ and CD8+ T-cell immune responses resulted in induced hypersensitivity reactions, leukocyte accumulation, and auto-inflammatory diseases (Sun, H., *et al*., 2008; Freundt, E. C., *et al*., 2008; Lou, Y., & Liu, S., 2011). Furthermore, the selective expression of TIPE2 inhibited hyper-responsiveness and sustained the immune homeostasis (Luan, Y. Y., *et al*., 2013; Sun, H., *et al*., 2008). The overexpression of TIPE2 also led to a reduced cell division, implying its role in the regulation of mitosis in certain specific conditions (Sun, H., *et al*., 2008). A study reported that TIPE2

negatively regulated inflammation by altering L-arginase metabolism from nitric oxide synthesis to urea during the host inflammatory response (Lou, Y., *et al*., 2014). Moreover, *in vitro* investigation of TIPE2 expression in normal BALB/C mouse CD4+CD25+ Tregs showed that TIPE2 is positively expressed. TIPE2 also exhibited an inhibitory and stimulatory effect on the T-cell receptor-mediated T lymphocyte and CD4+CD25+ Treg-mediated immunosuppression, respectively (Sun, H., *et al*., 2008; Luan, Y. Y., *et al*., 2011). Furthermore, TIPE2 facilitates Fas-induced apoptosis in immune cells by interacting with caspase-8 and suppressing the activation of NF-κB and AP-1 (Sun, H., *et al*., 2008). Further, studies demonstrated that deletion of TIPE2 negatively regulated the JNK and p38 MAPK signaling pathways and NF-κB activation. In contrast, TIPE2 expression did not affect the ERK signaling pathway (Sun, H., *et al*., 2008; Lou, Y., & Liu, S., 2011).

The crystal structure of TIPE2 displayed that its DED domain contains an extremely hydrophobic central cavity which facilitated the binding of cofactors (Lou, Y., & Liu, S., 2011; Zhang, X., *et al*., 2009). Besides, TIPE2 was also found to possess a significant role in the prevention of stroke. Overexpression of TIPE2 was predominantly observed in the microglia/macrophages of wild-type mice after cerebral ischemia. However, no such expression was observed in the neurons and astrocytes. The TIPE2 null mice exposed to middle cerebral artery occlusion displayed a very high infarction volume and neurological dysfunction with more lymphocytes, macrophages and neutrophils infiltration in the ischemic hemisphere. Therefore, these results suggested TIPE2 possess a significant neuroprotective effect on primary cerebral cell cultures (Zhang, Y., *et al*., 2012). Besides, TIPE2 also contributed to the polarization and migration (chemotaxis) of leukocytes (Fayngerts, S. A., *et al*., 2017). Moreover,

the knockdown of TIPE2 also enhanced phagocytosis and developed resistance against bacterial infections in the mice model (Wang, Z., *et al.*, 2012).

### 1.11.6. Role of TIPE2 in malignancy

TIPE2 acts as an antagonist of Ras, an oncogene regulating cell survival, proliferation, migration, and transformation (**Figure 1.6.)**. It binds to the Ras-interacting domain of the RalGDS proteins family, thereby averting Ras from forming an active complex with its effector proteins (Gus-Brautbar, Y., *et al.*, 2012). Further, TIPE2 hinders the activation of Ral and Akt and retains homeostasis between cell survival and death. Suppression of TIPE2 was found to enhance the expression of Ral and Akt, leading to an increase in cell migration and reduction in cell death, evincing that TIPE2 acted as a potential tumor suppressor (Gus-Brautbar, Y., *et al.*, 2012). In renal cell carcinoma (RCC) tissues, a significant upregulation in the expression of TIPE2 and downregulation in the expression of myxoma resistance protein 1 (MX1; a type I interferon-inducible gene) were witnessed when compared to normal controls. Moreover, the mechanistic role of TIPE2 in RCC pathogenesis was found to be correlated positively with TNM staging and negatively with MX1(Zhang, Z., *et al.*, 2013). Additionally, diminished levels of TIPE2 in patients suffering from primary HCC was found to be positively correlated with tumor invasion and metastasis. The ability of the protein in reducing tumor migration and invasion was ultimately attributed to its role in inhibiting Rac1 and reducing F-actin polymerization, matrix metalloproteinase (MMP)-9, and urokinase plasminogen activator (uPA) expression (Cao, X., *et al.*, 2013). In addition, elevated expression of TIPE2 lessened the effects of LPS-induced TNF-α on MMP-13/MMP-3 augmentation, cell migration, and ERK1/2, NF-κB activation, thereby suppressing TNF-α-induced HCC metastasis

(Zhang, Y. H., *et al.,* 2015). Downregulated TIPE2 expression was also reported to be involved in a mechanism of hepatitis C virus (HCV) induced HCC where HCV-encoded non-structural protein NS5A interacted and persuaded the degradation of TIPE2, leading to the development of HCC (Wang, Y., *et al.,* 2016). Likewise, a lesser level of TIPE2 expression was detected in glioma tissues and cells, and interestingly, overexpression of TIPE prevented migration, invasion, and EMT of glioma cells by repressing the hypoxia-induced elevated expression of the components of the Wnt/β-catenin cascade such as β-catenin, c-myc and cyclin D1 (Liu, Z. J., *et al.,* 2016). A similar role of downregulated TIPE2 was also evidenced in the case of prostate cancer, and restoration of its expression significantly prevented cell migration, invasion, and EMT through the inhibition of PI3K/Akt signaling (Lu, Q., *et al*., 2016). Furthermore, almost undetectable levels of TIPE2 was observed in gastric cancer cells compared to the normal gastric mucous epithelial cells and enforced elevation of the lost TIPE2 expression decreased migration and invasion of gastric cancer cells via lessening PI3K/Akt/GSK3β/β-catenin signaling regulated by TIPE2-mediated suppression of Akt, which ultimately led to the instigation of GSK3β expression (Wu, J., *et al.,* 2016). In accordance with these preclinical studies, it was observed that the expression of TIPE2 negatively correlated with the progression of gastritis to gastric cancer. Further, the TIPE2-mediated upregulation of p27 is attributed to the tumor-suppressing activity of TIPE2. In control paraneoplastic stomach tissues, the expression of TIPE2 was significantly enhanced compared to the cancer tissues. The low expression of TIPE2 in cancer tissues suggested its potential as a low abundance biomarker for gastric cancer progression.

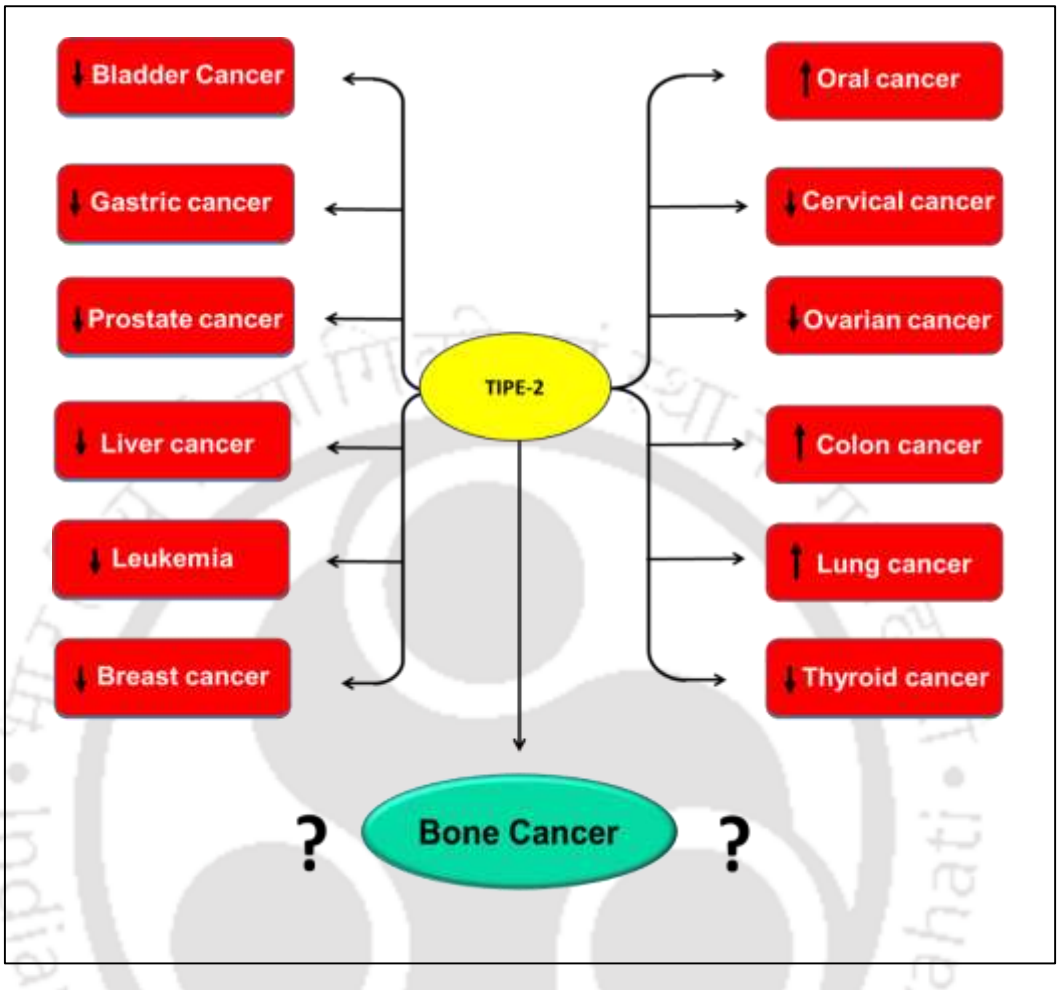


**Figure 1.6.: Expression of TIPE2 protein in different cancers.**
**↑- upregulation; ↓- downregulation.**

However, further validation with more number of tissue samples from normal individuals and cancer patients should be considered to avoid the possibility of false-positive and false-negative diagnosis (Peng, Y., *et al.*, 2016; Zhao, Q., *et al.*, 2015). In gastric cancer, TIPE2 induced anti-metastatic effects by modulating the levels of crucial EMT markers such as Snail1, Snail2/Slug, and Zeb1 in an AKT/GSK-3β signaling- and proteasome-dependent manner (Yin, H., *et al.*, 2017). Further, the overexpression of TIPE2 decreased the growth of the gastric cancer cells and induced apoptosis by activating the intrinsic apoptotic pathway and inhibiting the AKT and ERK1/2 signaling (Zhu, Y., *et al.*, 2016). In non-small-cell lung carcinoma (NSCLC), the expression of

TIPE2 was downregulated. Overexpression of TIPE2 in NSCLC cells decreased the colony formation, migration, and invasion of the cells. Further, it also significantly reduced the tumor invasiveness and angiogenesis via reduced activation of Rac1, F-actin polymerization, and VEGF expression. This overexpression of TIPE2 is negatively correlated with the metastasis of lymph node and advanced clinical stage in NSCLC (Li, Z., *et al.*, 2016; Li, Y., *et al.*, 2015). In breast cancer, the expression of TIPE2 is downregulated, and the overexpression of TIPE2 is associated with inhibition of tumor growth *in vitro* and *in vivo*. Moreover, TIPE2 also inhibited EMT, invasion and migration of breast cancer cells. TIPE2 also induced anti-tumorigenic activity via TIPE2-mediated inhibition of β-catenin, cyclin D1, c-Myc, p-p38, and p-AKT (Wang, K., *et al.*, 2017; Zhang, Z., *et al.*, 2016).

The gene delivery of TIPE2 also decreased the proliferation, tumor growth, and metastasis of breast cancer cells in preclinical settings by increasing the immune response in the spleen and tumor microenvironment via activated T and NK cells-induced enhanced production of interferon-gamma (IFN-γ) and TNF-α (Zhang, Z., *et al.*, 2017). In Non-Hodgkin's lymphoma (NHL), the expression of TIPE2 was upregulated in peripheral T lymphoma and diffuse large B-cell lymphoma (DLBCL). TIPE2 upregulation was significantly increased in germinal centre B-cell (GCB) type DLBCL than the non-GCB type, which suggested its clinical significance as a marker for improved prognosis (Hao, C., *et al.*, 2016). TIPE2 regulates the cross-talk between skin squamous cell carcinoma (SCC) and tumor-associated macrophages (TAMs). The downregulation of macrophage TIPE2 significantly regulated the phenotypic modification of SCC cells, and patients with high-TIPE2 TAMs are associated with poor overall 5-year survival (Li X., 2016). In contrast to other cancers, both colon cancer cells and tissues showed higher expression of TIPE2 than the normal controls

and was associated with lymph node metastasis. Contrastingly in colon cancer, the expression of TIPE2 was found to be higher than the normal controls in both the in vitro and the in vivo settings and was allied with lymph node metastasis. Further, the knockdown of TIPE2 after TLR4 activation elevated the levels of caspase-8 (Li, X. M., *et al*., 2014).

### 1.12. Importance of the study

The most threatening feature of bone cancer is that it hits the human body in the first 20 years of life. It contributes to about 6-7% of all pediatric cancers. Bone cancer mostly occurs in adolescence as there is maximum growth in the musculoskeletal system. The 5-year survival rate for the period of 2006- 2012 was estimated to be only 67.4%. The survival rate continued to reduce every year due to the increase in disease progression despite the advances made in the treatment of cancer. In most cases, bone cancer is diagnosed only at an advanced metastatic stage because of the lack of biomarkers. This added to the impediments of disease management. Besides, chemoresistance and tumor recurrence also pose a huge challenge in treating bone cancer and contributed to the poor prognosis in patients. Alterations in the expression levels of many proteins have been identified and shown to induce bone tumorigenesis. TIPE2 protein is a unique protein with a variety of functions. This protein is detected to have remarkable roles in the development and progression of various cancers. Yet, the mechanism of regulation of carcinogenesis by this protein remains vague.

Although the first TIPE2 protein was initially found in head and neck squamous cell carcinoma cell lines, the importance of this protein in the etiopathogenesis of bone cancer remains imprecise. Thus, resolving the action of this protein would facilitate in better understanding of the role of TIPE2 protein in bone tumor regulation, which would serve as an early prognostic marker for this cancer. As it is a well-known fact

that cytokines such as tumor necrosis factor (TNF)-α, TNF-β and receptor activator of NF-κB ligand (RANKL) play a critical role in bone remodelling and deregulation of this process leads to bone cancer; therefore, assessing the role of these cytokines in regulating the expression of TIPE2 would facilitate new understandings of the development and progression of bone cancer **(Figure 1.7.)**. Thus, deciphering the role of TIPE2 protein in bone cancer would help cancer biologists to develop novel and highly efficacious therapies with fewer or no adverse effects against this vicious disease and to increase the survival rate.

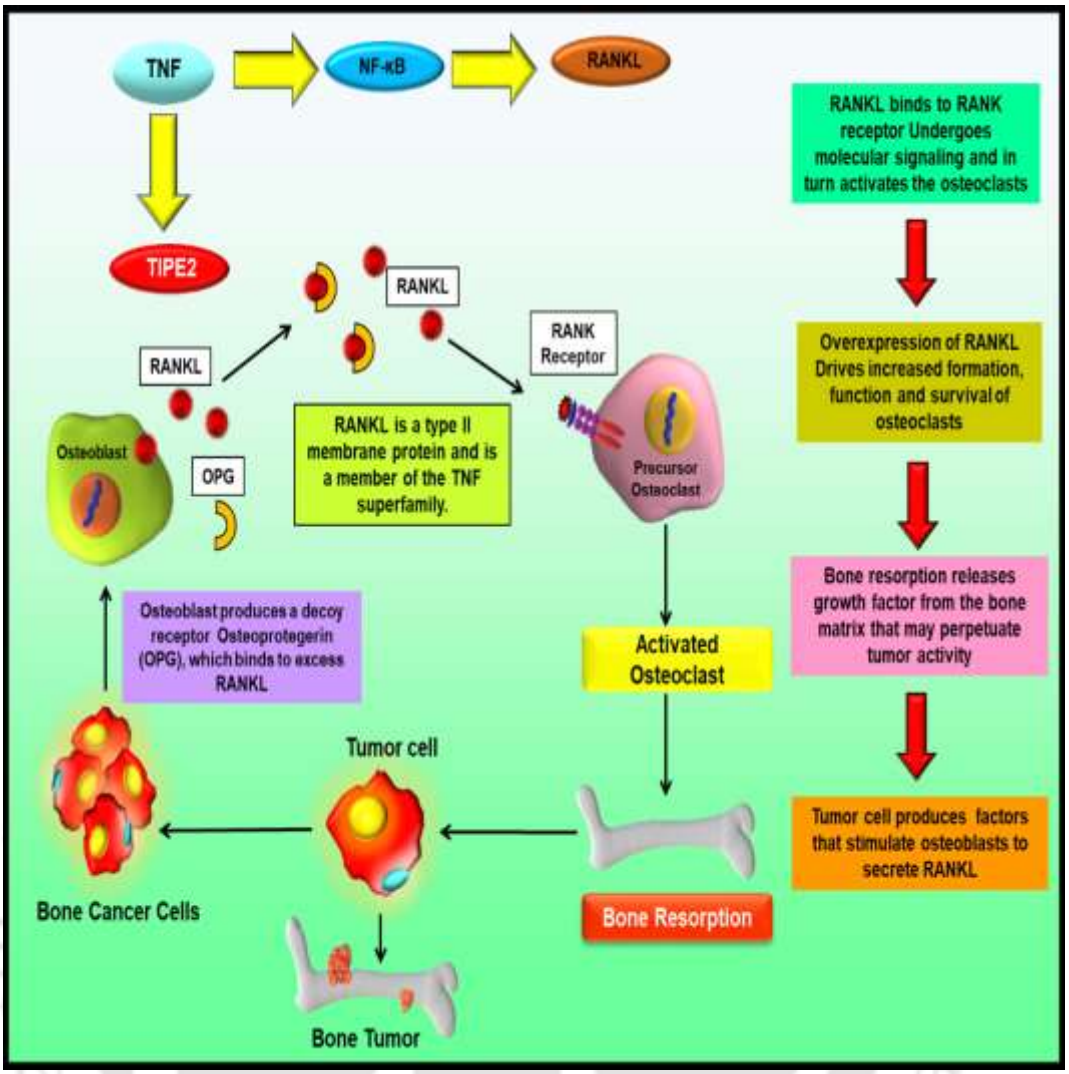


**Figure 1.7.: An outline of the hypothesis of the association of TIPE2 protein and cytokines in bone cancer.**

### 1.13. Objectives

The main objectives of this study are framed as follows:

1. To determine the expression of TIPE2 protein in bone cancer.

2. To examine the role of TNF-α, TNF-β and RANKL on TIPE2 in bone cancer.

3. To elucidate the role of TIPE2 in the regulation of different hallmarks of bone cancer

# Chapter 2

## *Expression of TIPE2 protein in bone cancer*

## 2.1. Introduction

Aforementioned, bone cancer is accountable for 3–5% of pediatric cancers and nearly 0.2% of all malignant neoplasms. Regardless of the remarkable advances made in the field of therapy and disease management, the prognosis of bone cancer still remains extremely poor. The poor prognosis with this disease is correlated to the fact that most of the cases of bone cancer are not recognized until their malignancy has reached an advanced metastatic stage (Brown, H. K., *et al.*, 2018; Ottaviani, G., & Jaffe, N., 2009; Lam, S. W., *et al.*, 2019; Szuhai, K., *et al.*, 2012). Thus, a detailed understanding of its pathogenesis and identification of novel biomarkers will significantly expand the diagnosis and treatment strategies for this malignancy. As mentioned earlier, TIPE2 protein plays a critical role in the modulation of different processes such as immunity, inflammation, tumorigenesis etc. and exerts varied expression patterns in different cancers and hence, they can be utilized as a potential biomarker (Bordoloi, D., *et al.*, 2018; Padmavathi, G., *et al.*, 2018). A thorough review of the literature revealed that the expression of this protein was studied in different cancers; however, its expression pattern in bone cancer is not studied till date. Henceforth, the present study was intended at assessing the expression of TIPE2 protein in the development and progression of bone cancer. Taken together, the expression analysis of this protein will provide us with an opportunity for understanding their exact role and their function as a diagnostic and prognostic biomarker along with a target to develop therapies against this lethal disease.

## 2.2. Materials and Methods:

### 2.2.1. Cell culture

HaCaT (human skin epithelial) and HOS (human bone cancer) cell lines were procured from National Centre for Cell Science (NCCS), Pune, India and the U2OS (human bone cancer) cell line was generously gifted by Prof. Renu Wadhwa, AIST, Japan. The HOS cells were maintained in Minimum Essential Medium (MEM) (MEM; Gibco™; Life Technologies, NY, USA), and HaCaT and U2OS cells were maintained in Dulbecco's Modified Eagle's Media (DMEM; Gibco™; Life Technologies, NY, USA). The MEM and DMEM media were added with 10% fetal bovine serum (FBS; Gibco®, NY, USA) and 1X Pen-Strep (Invitrogen, CA, USA). The cells were cultured and maintained at 37 °C in a CO2-regulated incubator (5% CO2 and 95% humidity).

### 2.2.2. Western blot analysis

The expression of TIPE2 in human normal skin epithelial cell line and bone cancer cell lines were determined by Western blot analysis. The total protein lysates were prepared using whole-cell lysis buffer containing 2 mM EDTA, 20 mM HEPES buffer, 0.1% (v/v) Triton-X100, 250 mM NaCl and protease inhibitors such as 1 mM PMSF, 2 µg/mL Aprotinin, 1 mM DTT, and 2 µg/mL Leupeptin hemisulfate. The concentration of the obtained proteins was evaluated using Bradford protein assay (Bio-rad, California, USA). Bovine serum albumin (BSA) was used as the standard for protein estimation. Further equal amounts of protein were resolved on a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel with 5X Laemmli Buffer (5% β-mercaptoethanol, 250 mM Tris-HCl, 30% Glycerol, 0.02% Bromophenol blue and 10% SDS) at a voltage of 70-100 V. After resolving, the proteins were transferred to nitrocellulose membranes, and

the protein transfer was checked by Ponceau-S stain (HiMedia). The membranes were blocked in 5% non-fat dry milk (Amulya, India) and then were probed with respective primary antibodies **(Table 2.1.)** overnight at 4 °C. The next day, membranes were washed with 1X TBST and incubated with horseradish peroxidase (HRP) – conjugated anti-rabbit or anti-mouse secondary antibodies. After 2 h incubation, the membranes were again washed with 1X TBST, and the blots were developed using Clarity Western ECL substrate (Bio-Rad, California, USA) and ChemiDoc™ XRS System (Bio-Rad, California, USA). The housekeeping gene α -tubulin was used as a loading control (Bordoloi, D., *et al*., 2020).

**Table 2.1. Details of the primary and secondary antibodies used for Western blot.**

| Name | Details | Dilutions used |
|---|---|---|
| Anti-TNFAIP8L2 antibody | ab110389; abcam®, Cambridge, USA | 1:4000 |
| Anti-α-Tubulin antibody | 2144S; Cell Signaling Technology, Massachusetts, USA | 1:2000 |

### 2.2.3. Tissue microarray:

The expression of TIPE2 protein in normal adjacent bone tissues and bone cancer tissues was evaluated by immunohistochemical analysis. For this purpose, tissue microarray (TMA) containing paraffin-embedded normal, OS and CS tissues (US Biomax, Inc Cat No. OS802, Derwood, USA) was used. The TMA slide consists of 80 tissues from different individuals: 50 cases of OS, 28 cases of CS and 2 cases of cancer adjacent normal bone tissue **(Figure 2.1. & Table 2.2.).** Each tissue core is 1.5mm in diameter and 5µm in thickness.

#### 2.2.3.1. Tissue microarray details

Bone cancer tissue array, including TNM, clinical stage and pathology grade, 80 cases

**Name**: OS802

**Description**: OS and CS microarray, containing 50 cases of OS, 28 cases of CS, and 2 cases adjacent normal tissue, single core per case Bone cancer tissue array, including TNM, clinical stage and pathology grade

**Cases**: 80

**Cores**: 80

**Row number**: 8

**Column number**: 10

**Core Diameter**: 1.5 mm

**Thickness**: 5 µm

**Table 2.2.** Bone cancer tissue array details

| Position | Sex | Age | Organ | Pathology | Stage | TNM | Type |
|---|---|---|---|---|---|---|---|
| A1 | F | 7 | Bone | OS of right femur | IIb | T2N0M0 | Malignant |
| A2 | F | 23 | Bone | OS of left knee | IIb | T2N0M0 | Malignant |
| A3 | F | 15 | Bone | OS of left knee | IIb | T2N0M0 | Malignant |
| A4 | M | 22 | Bone | OS of left femur | IIb | T2N0M0 | Malignant |
| A5 | F | 31 | Bone | OS of right upper arm | IIb | T2N0M0 | Malignant |
| A6 | M | 16 | Bone | OS of right femur | IIb | T2N0M0 | Malignant |
| A7 | F | 10 | Bone | OS of right tibia | IIb | T2N0M0 | Malignant |
| A8 | M | 10 | Bone | OS of left tibia | IIb | T2N0M0 | Malignant |
| A9 | F | 19 | Bone | OS of right tibia | IIb | T2N0M0 | Malignant |
| A10 | M | 14 | Bone | OS of right femur | IIb | T2N0M0 | Malignant |
| B1 | F | 13 | Bone | OS of left femur | IIb | T2N0M0 | Malignant |
| B2 | M | 19 | Bone | OS of left fibula | IIb | T2N0M0 | Malignant |
| B3 | F | 23 | Bone | OS of left femur | IIb | T2N0M0 | Malignant |
| B4 | M | 37 | Bone | OS of left femur | IIb | T2N0M0 | Malignant |
| B5 | M | 69 | Bone | OS of right thigh | IIb | T2N0M0 | Malignant |
| B6 | M | 39 | Bone | OS of right upper arm | IIa | T1N0M0 | Malignant |
| B7 | F | 37 | Bone | OS of right femur | IIa | T1N0M0 | Malignant |
| B8 | F | 31 | Bone | OS of left femur | IIb | T2N0M0 | Malignant |
| B9 | M | 28 | Bone | OS of left femur | IIb | T2N0M0 | Malignant |
| B10 | F | 57 | Bone | OS of left femur | IIa | T1N0M0 | Malignant |
| C1 | M | 17 | Bone | OS of left femur | IIb | T2N0M0 | Malignant |
| C2 | F | 12 | Bone | OS of left femur | IIa | T1N0M0 | Malignant |
| C3 | M | 18 | Bone | OS of thoracic spine | IIb | T2N0M0 | Malignant |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| C4 | M | 16 | Bone | OS of right femur | IIb | T2N0M0 | Malignant |
| C5 | M | 55 | Bone | OS of left knee | IIa | T1N0M0 | Malignant |
| C6 | M | 60 | Bone | OS of right tibia | IIb | T2N0M0 | Malignant |
| C7 | M | 34 | Bone | OS of right fibula | IIb | T2N0M0 | Malignant |
| C8 | F | 14 | Bone | OS of right femur | IIb | T2N0M0 | Malignant |
| C9 | M | 21 | Bone | OS of left femur | IIb | T2N0M0 | Malignant |
| C10 | F | 16 | Bone | OS of left femur | IIb | T2N0M0 | Malignant |
| D1 | M | 15 | Bone | OS of left femur | IIb | T2N0M0 | Malignant |
| D2 | F | 18 | Bone | OS of left fibula | IIb | T2N0M0 | Malignant |
| D3 | M | 15 | Bone | OS of right femur | IIb | T2N0M0 | Malignant |
| D4 | M | 19 | Bone | OS of left thigh | IIb | T2N0M0 | Malignant |
| D5 | M | 24 | Bone | OS of skull | IIb | T2N0M0 | Malignant |
| D6 | M | 19 | Bone | OS of left tibia | IIb | T2N0M0 | Malignant |
| D7 | F | 40 | Bone | OS of left leg | IIb | T2N0M0 | Malignant |
| D8 | F | 28 | Bone | OS of right femur | IIb | T2N0M0 | Malignant |
| D9 | M | 15 | Bone | OS of right femur | IIb | T2N0M0 | Malignant |
| D10 | M | 16 | Bone | OS of right femur | IIb | T2N0M0 | Malignant |
| E1 | M | 15 | Bone | OS of right femur | IIb | T2N0M0 | Malignant |
| E2 | F | 17 | Bone | OS of right tibia | IIb | T2N0M0 | Malignant |
| E3 | M | 19 | Bone | OS of right femur | IIb | T2N0M0 | Malignant |
| E4 | F | 38 | Bone | OS of left scapula | IIa | T1N0M0 | Malignant |
| E5 | M | 31 | Bone | OS of left humerus | IIb | T2N0M0 | Malignant |
| E6 | M | 10 | Bone | OS of left femur | IIb | T2N0M0 | Malignant |
| E7 | F | 13 | Bone | OS of left femur | IIb | T2N0M0 | Malignant |
| E8 | F | 62 | Bone | OS of left thigh | IIb | T2N0M0 | Malignant |
| E9 | F | 13 | Bone | OS of right femur | IIb | T2N0M0 | Malignant |
| E10 | M | 13 | Bone | OS of left tibia | IIb | T2N0M0 | Malignant |
| F1 | M | 14 | Cartilage | CS of right femur | | | Malignant |
| F2 | M | 35 | Cartilage | Dedifferentiation CS of left femur | | | Malignant |
| F3 | F | 31 | Cartilage | CS of right occipital | | | Malignant |
| F4 | F | 27 | Cartilage | CS of left femur | | | Malignant |
| F5 | M | 16 | Cartilage | CS of tibia | | | Malignant |
| F6 | F | 13 | Cartilage | CS of left tibia | | | Malignant |
| F7 | F | 16 | Cartilage | CS of left femur | | | Malignant |
| F8 | M | 28 | Cartilage | Dedifferentiation CS of left foot sole | | | Malignant |
| F9 | F | 48 | Cartilage | CS of femur | | | Malignant |
| F10 | F | 64 | Cartilage | CS of back (sparse) | | | Malignant |
| G1 | M | 38 | Cartilage | Dedifferentiation CS of left hip bone | | | Malignant |
| G2 | M | 37 | Cartilage | CS of left ilium | | | Malignant |
| G3 | M | 52 | Cartilage | CS of sacroiliac | | | Malignant |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| G4 | F | 47 | Cartilage | CS of left shoulder | | | Malignant |
| G5 | M | 52 | Cartilage | CS of shoulder | | | Malignant |
| G6 | M | 30 | Cartilage | Mesenchymal CS of leg | | | Malignant |
| G7 | M | 74 | Cartilage | CS of right hand middle finger | | | Malignant |
| G8 | M | 22 | Cartilage | CS of left thigh | | | Malignant |
| G9 | M | 50 | Cartilage | CS of left thigh | | | Malignant |
| G10 | M | 41 | Cartilage | CS of femur | | | Malignant |
| H1 | F | 39 | Cartilage | CS of right scapula | | | Malignant |
| H2 | M | 40 | Cartilage | CS of bone | | | Malignant |
| H3 | M | 59 | Cartilage | CS of right pollicis | | | Malignant |
| H4 | F | 12 | Cartilage | CS of right femur | | | Malignant |
| H5 | M | 50 | Cartilage | CS of L3 pyramis | | | Malignant |
| H6 | M | 35 | Cartilage | CS of left ilium | | | Malignant |
| H7 | M | 50 | Cartilage | CS of right femur | | | Malignant |
| H8 | M | 42 | Cartilage | CS of ankle | | | Malignant |
| H9 | M | 79 | Bone | Cancer adjacent normal bone tissue | | | NAT |
| H10 | M | 14 | Bone | Cancer adjacent normal bone marrow tissue (sparse) | | | NAT |

(Table 2.2. adapted from https://www.biomax.us/OS802).

**2.2.3.2. Immunohistochemistry**

Histostain-Plus IHC Kit, HRP, broad-spectrum (Invitrogen, Cat. No. 859043) and Metal enhanced DAB Substrate Kit (Invitrogen, Cat No. 34065) were used for immunostaining the tissue microarray. Immunohistochemistry (IHC) was performed as per the manufacturer's protocol: i.e., deparaffinization, rehydration, peroxidase quenching, blocking, primary antibody incubation, secondary antibody-peroxidase conjugate incubation, the addition of DAB chromogen and counterstaining with hematoxylin. Finally, the slide is dehydrated and mounted with a coverslip using D.P.X. mountant (Merck, USA). The primary antibodies anti-TIPE2 antibody (Cat. No. ab110389) was obtained from abcam® and used in the dilution of 1:50 for immunohistochemical analysis (Bordoloi, D., *et al*., 2020).

#### 2.2.3.3. Scoring

The immunostained microarray was analyzed under Olympus light microscope. Tissues stained brown were considered as positive for the presence of antigen of interest and given a score according to the staining intensity and the number of positive cells. The score for the percentage of positive cells is scaled from 0 to 4+ and staining intensity is scaled from 1 to 3 (Shiao, Y. H., 2000; Bordoloi, D., *et al*., 2020; Charafe-Jauffret, E., *et al.*, 2004; McDonald, J. W., & Pilgram, T. K. 1999; Monisha, J., *et al.*, 2018) (**Table 2.3**.).

**Table 2.3.** 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 | | |

### 2.2.4. Statistical analysis

Student's *t*-test was performed to determine the statistical significance. *p* value <0.05 was considered statistically significant.

## 2.3. Results and Discussion

### 2.3.1. Expression of TIPE2 in bone cancer cell lines

The first aim of this study was to evaluate the expression of TIPE2 in two bone cancer cell lines. The expression of TIPE2 was determined in both normal skin epithelial (HaCaT) and bone cancer (HOS and U2OS) cell lines. Our Western blot results demonstrated that TIPE2 was overexpressed in both HOS and U2OS cell lines compared to HaCaT cells (**Figure 2.1.**). It is the first study that reports the expression

of TIPE2 in different bone cancer cell lines. Our results are supported by a few previous findings as well. In line with our findings, Bordoloi and the group showed that TIPE2 is overexpressed in lung cancer cells and mediates its action through the regulation of AKT/mTOR/NF-κB signaling cascade (Bordoloi *et al*., 2019). Furthermore, in RCC tissues, a drastic increase of TIPE2 expression and downregulation of MX1 were observed compared to the normal controls. Likewise, TIPE2 expression was positively linked with TNM staging and negatively with MX1 expression, denoting its role in RCC pathogenesis (Zhang, Z., *et al*., 2013). Altogether, the results obtained indicated that overexpression of TIPE2 could be plausibly involved in the positive regulation of bone carcinogenesis.

### 2.3.2. Expression of TIPE2 in bone cancer tissue samples

We have also evaluated the expression of TIPE2 in commercially available bone cancer tissue microarray slide **(Figure 2.2.A).** The tissue samples are of patients of different pathologies and age groups. The TMA slide mainly consists of tissues of two types of bone cancer, i.e. OS and CS. Expression studies of this protein show distinguished variability in different cancers. Hence, here we attempted to analyze the expression of TIPE2 protein (based on different bone cancer types and age groups) in bone cancer tissues compared with respect to normal adjacent tissues. The total score (Q) of IHC is considered as the expression score of a particular protein for a tissue.

#### 2.3.2.1. Overexpression of TIPE2 in human bone cancer tissues

In this chapter, we have analyzed the expression of the TIPE2 protein in bone cancer tissues compared to normal adjacent bone tissues. Our analysis revealed that TIPE2 is

significantly upregulated in bone cancer tissues compared to normal bone tissues **(Figure 2.2. (A) and 2.2. (B))**. TIPE2, a negative modulator of innate as well as cellular

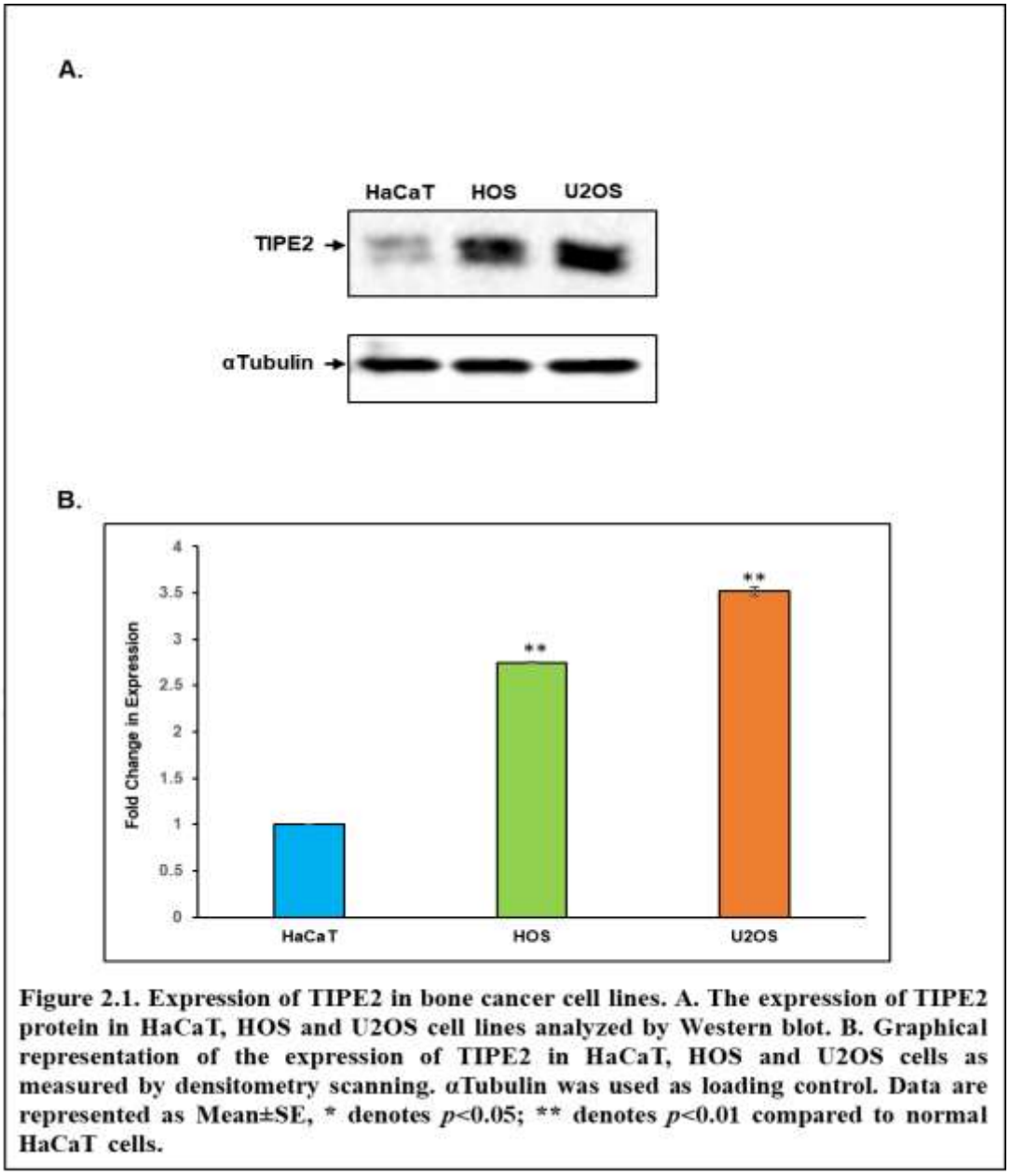


**Figure 2.1. Expression of TIPE2 in bone cancer cell lines. A. The expression of TIPE2 protein in HaCaT, HOS and U2OS cell lines analyzed by Western blot. B. Graphical representation of the expression of TIPE2 in HaCaT, HOS and U2OS cells as measured by densitometry scanning. αTubulin was used as loading control. Data are represented as Mean±SE, * denotes $p<0.05$; ** denotes $p<0.01$ compared to normal HaCaT cells.**

immunity, was reported to be drastically upregulated in malignant lung tissues than the normal tissues (Bordoloi, D., *et al.*, 2019). Likewise, Hao and colleagues reported an increased expression of TIPE2 in both DLBCL and peripheral T-cell lymphoma (Hao, C., *et al.*, 2016). In line with our findings, another study carried out on the expression of TIPE2 in NSCLC displayed that TIPE2 expression level was highly ameliorated in

NSCLC tumor tissues when compared with the adjacent normal tissues (Li, Z., *et al.*, 2016). Contrary to the findings mentioned above, a study showed TIPE2 to be suppressed in human breast cancer cells, and tissues and its forced expression inhibited the proliferation of tumor cells and the xenograft growth (Wang, K., *et al.*, 2017). Moreover, its expression was also found to be remarkably less in glioma cells (Liu, Z. J., *et al.*, 2016). Another study demonstrated that TIPE2 repressed the metastasis of gastric cancer cells via downregulation of β-catenin signaling pathway (Wu, J., *et al.*, 2016). Thus, altogether, it can be hypothesized that upregulation of TIPE2 might be involved in the malignant transformation of bone tissues.

**2.3.2.2. Expression analysis of TIPE2 in OS and CS**

Aforementioned, bone cancer accounts for 3–5% of pediatric cancers cases and includes three major subtypes: (1) OS (2) CS and (3) ES (Brown, H. K., *et al.*, 2018; Ottaviani, G., & Jaffe, N., 2009; Lam, S. W., *et al.*, 2019; Szuhai, K., *et al.*, 2012). The expression of TIPE2 protein in both OS and CS with respect to normal bone tissues was analyzed. Expression analysis of TIPE2 protein in normal adjacent, OS and CS tissue samples revealed that TIPE2 is overexpressed in both OS and CS tissues compared to the normal adjacent tissues, with more definite upregulation in CS type (**Figure 2.3.**). Bone tumors are classified on the basis of the presence of tumor osteoid. OSs, possesses tumor osteoid and, in the absence of evident tumor osteoid, were considered either as fibrosarcoma, CS, spindle cell sarcoma, unspecified or pleomorphic sarcoma, or giant cell sarcoma. However, this current definition is not satisfactory because it fails to recognize few examples of chondroblastic, fibroblastic, and anaplastic OS having no evident tumor osteoid.

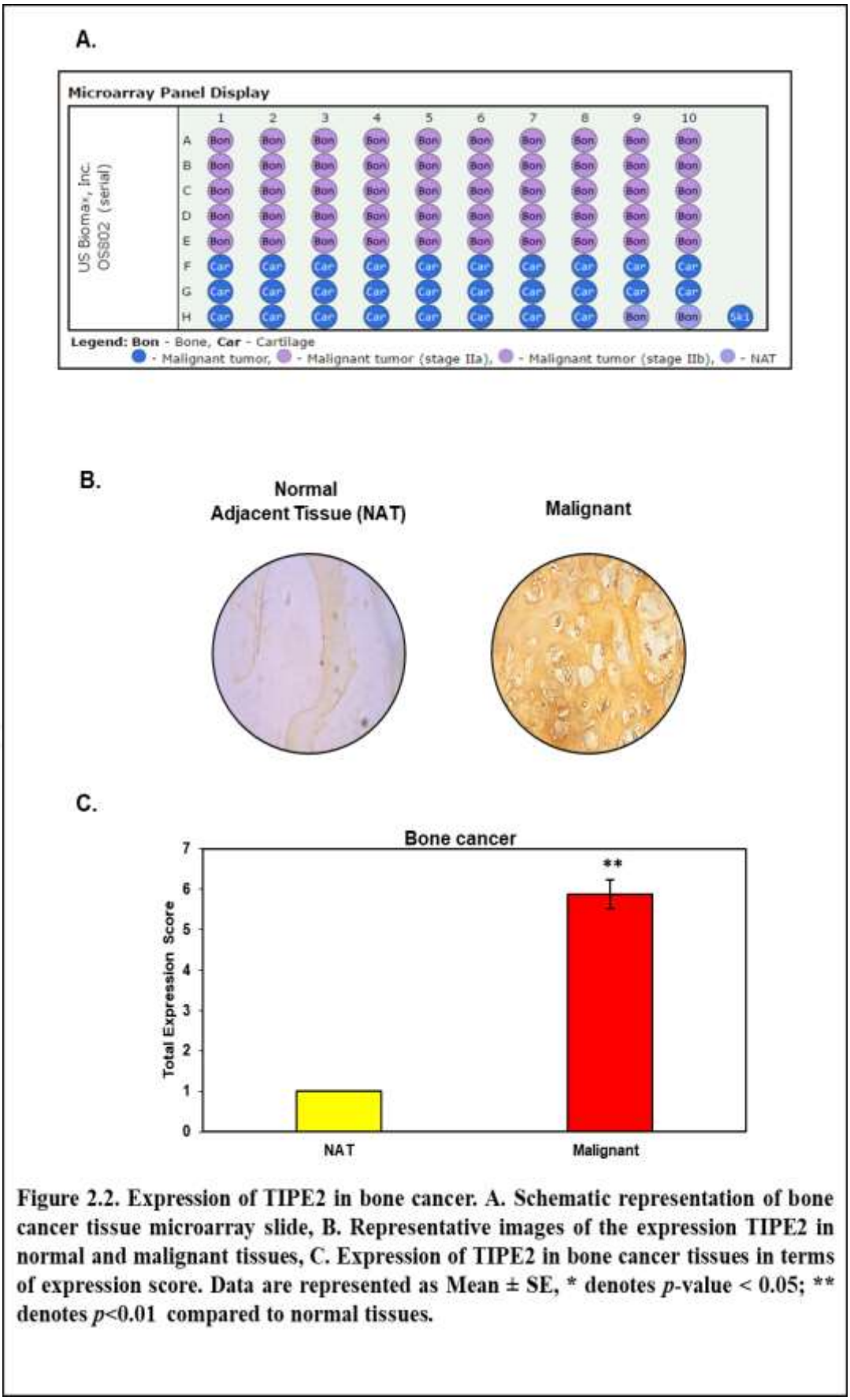


**Figure 2.2. Expression of TIPE2 in bone cancer. A. Schematic representation of bone cancer tissue microarray slide, B. Representative images of the expression TIPE2 in normal and malignant tissues, C. Expression of TIPE2 in bone cancer tissues in terms of expression score. Data are represented as Mean ± SE, * denotes *p*-value < 0.05; ** denotes *p*<0.01 compared to normal tissues.**

The histochemical and cytochemical studies have demonstrated that the presence of alkaline phosphatase indicated osteosarcomatous character (Whelan, J., *et al.,* 2012; Whelan, J. S., & Davis, L. E. 2018; Sanerkin, N. G., 1980). The tumor cells in OS, whether chondroblastic, osteoblastic, fibroblastic, or anaplastic, holds abundant alkaline phosphatase, while this enzyme is scanty or absent in CS and fibrosarcoma. Hence, it is proposed that these bone sarcomas are best classified based on the origin of the constituent tumor cells and their ALP content: OS, a malignant tumor of osteoblasts (ALP positive); CS, a malignant tumor of chondroblasts (ALP negative); and fibrosarcoma, a malignant tumor of fibroblasts (ALP negative) (Sanerkin N. G., 1980). Bone sarcomas are 8- to 10-folds uncommon than that of soft-tissue sarcoma; however, they present wide-ranging and critical challenges for the patients and all those involved in their care (Whelan, J. S., & Davis, L. E. 2018). Therefore, the increased expression of TIPE2 protein in OS and CS might possess a correlation in the malignant transformation of OS and CS tissues which need to be elucidated.

#### 2.3.2.3. Expression analysis of TIPE2 in bone cancer tissues of different age grouped patients

As mentioned earlier, bone cancer contributes to about 0.2% of all malignant neoplasms, and in adults, CS makes up more than 40% of primary bone cancers, followed by OS (28%), chordoma (10%), ES (8%), and fibrosarcoma (4%). In children and teenagers, OS accounts for 56% and Ewing tumors, around 34% of the total cases and occur more frequently than CS. CS mainly occurs in adults, with an average age of diagnosis at 51 (Evola, F. R., 2017; Weber, K., *et al.,* 2008; von Eisenhart-Rothe, R., *et al.,* 2011; Shweikeh, F., *et al.,* 2014; Palmerini, E., *et al.,* 2020; Ricotta, F., *et al.,* 2020; Lam, S. W., *et al.,* 2019). Apart from the risk factor abundance, differential

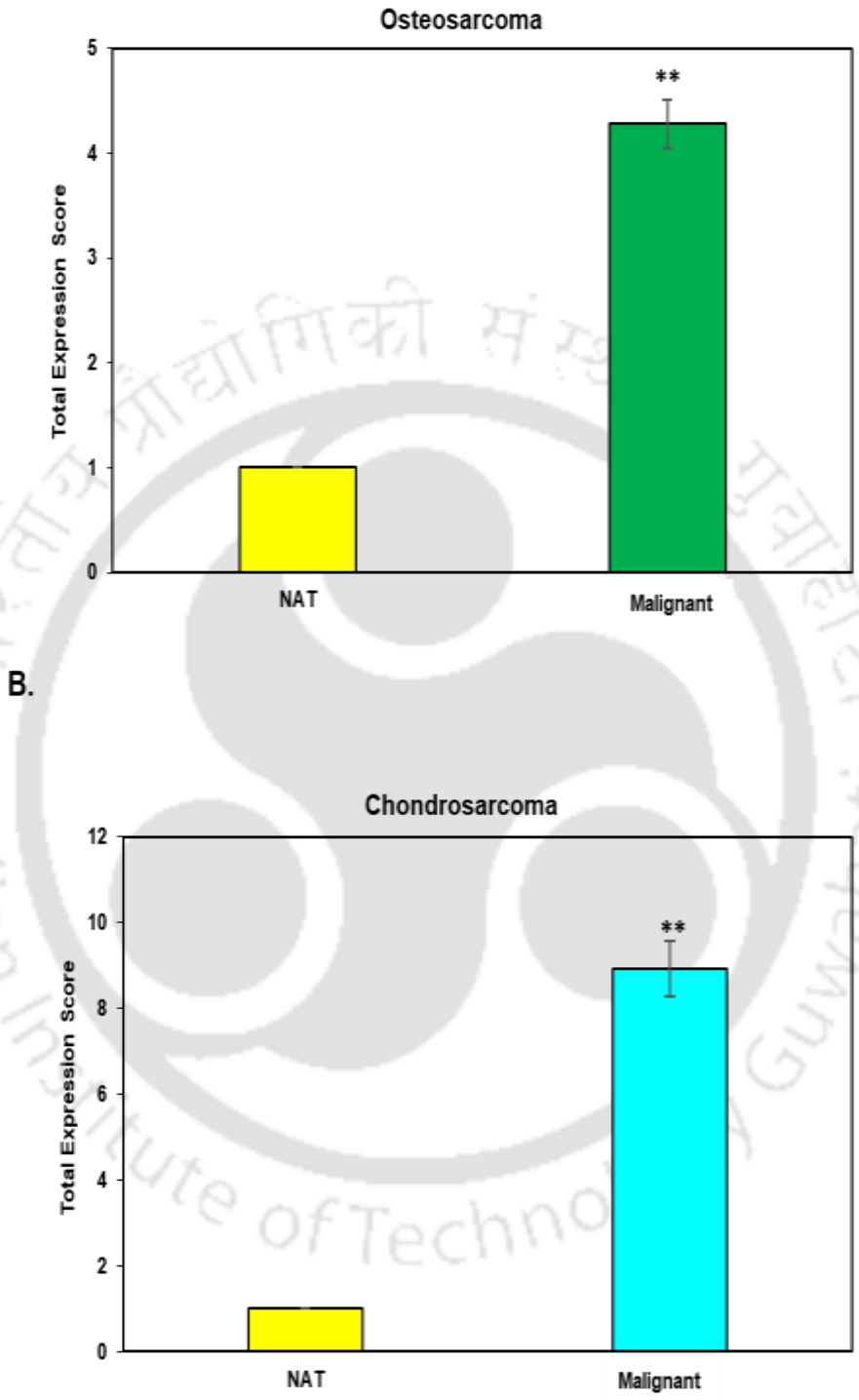


**Figure 2.3. The expression score of TIPE2 protein in different bone cancer types. A. The expression of TIPE2 protein in osteosarcoma tissues. B. Expression of TIPE2 protein in chondrosarcoma tissues. Data are represented as Mean ± SE, * denotes *p*-value < 0.05; ** denotes *p*<0.01 compared to normal adjacent tissues.**

regulation of molecular pathways among different age groups might also be the reason for this increased cancer risk. However, there would be a certain connotation between carcinogen exposure and dysregulation of essential signaling pathways.

The incidence of OS is bimodal, with an age-adjusted incidence of 4 per 1 million in patients younger than age 25 years or older than age 59 years, but fewer than 2 per 1 million among people in the age of 25 to 59 years. It is known that globally OS incidence peaks in boys at the age between 15 to 19 years and in girls at the age of 10 to 14 years, which corresponds with the age of puberty. Similarly, a second incidence peak occurs in the elderly (age ≥ 75 years). There are many controversies as to the significance of age within the range of the first incidence peak as a prognostic factor for survival in patients with OS. It certainly appears to be a less influential factor than in ES, and this may co-relate with pubertal status and a complex interplay of sex, which impacts the drug metabolism and experience of toxicity, along with psychosocial factors, which affects the administered-dose intensity (Janeway, K. A., *et al.*, 2012; Collins, M., *et al.*, 2013; McTiernan, A., *et al.*, 2012; Whelan, J. S., & Davis, L. E. 2018).

Therefore, here we attempted to analyze the age-wise difference in the expression of TIPE2 protein in bone cancer tissues (**Figure 2.4.**). The expression of TIPE2 protein in patients of different age groups ranging from 5 to 75 years was evaluated, and it was observed that TIPE2 protein showed a similar pattern of expression with respect to age. We observed that the TIPE2 protein was highly upregulated from early childhood to adolescence compared to the normal adjacent bone tissues. We also perceived that the expression level of TIPE2 protein was found to be high till later age. The maximum expression is seen in the age between 45 to 54 years of age. In OS, the maximum

expression was observed in the age group of 5 to 14 years of age. In the case of CS, we observed the maximum expression score in the age group of 45 and above. This may be due to the fact that CS, occurs mainly in adults with an average age of diagnosis at 51 and constitutes more than 40% of primary bone tumors, followed by OS at 28% whereas in children and teenagers, OS makes up to 56% of the total bone cancer cases. The expression of TIPE2 was in line with the bone cancer statistics mentioned above. Overall, the expression of TIPE2 was augmented in various age groups of bone cancer compared to normal adjacent bone tissues. Therefore, our data show the involvement of TIPE2 in bone cancer pathogenesis. Nevertheless, additional studies with a larger sample size (number of patient samples) are requisite to confirm these findings.

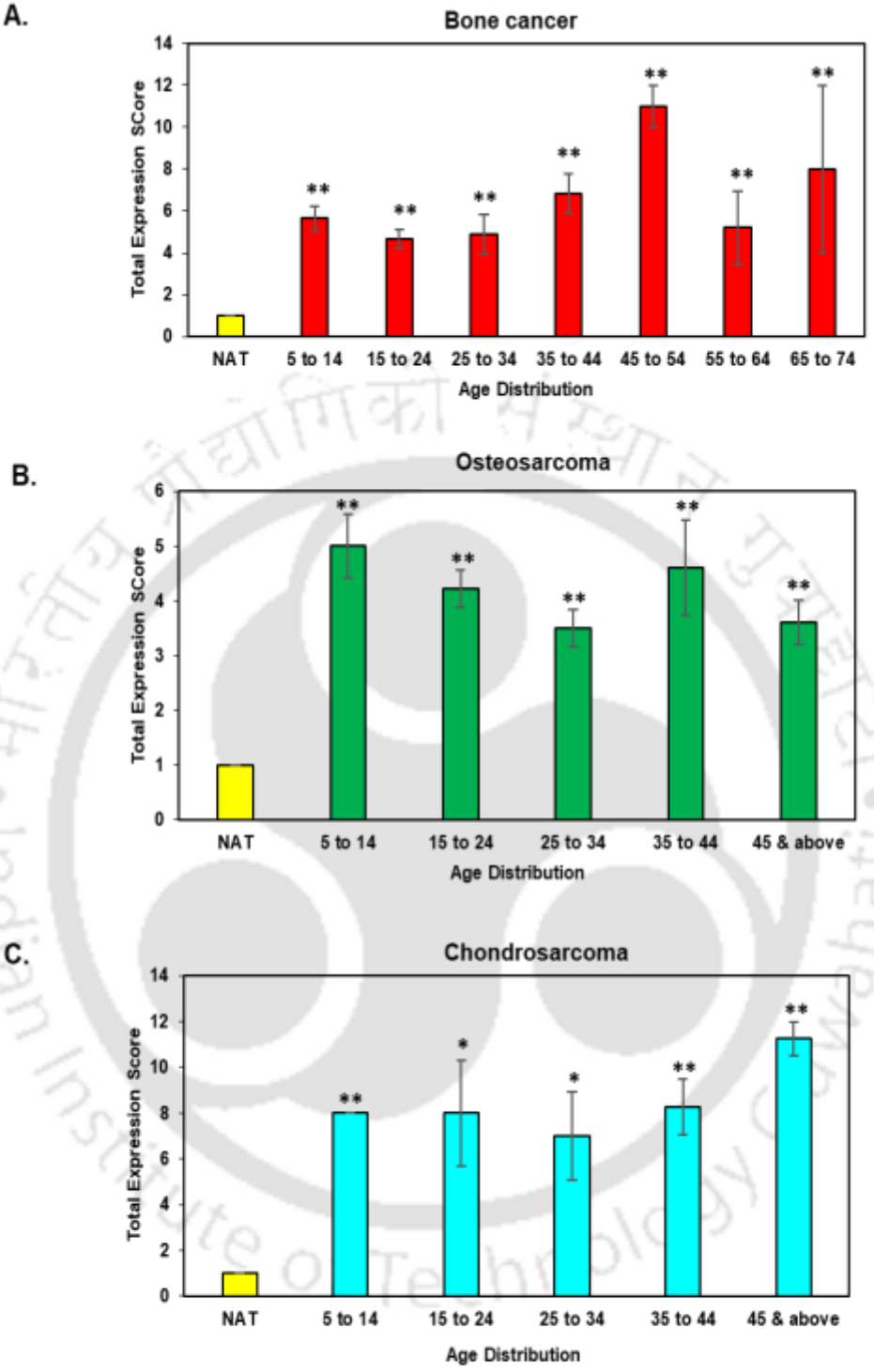


**Figure 2.4. Expression of TIPE2 protein in bone cancer tissues of patients of different age groups. A. Graphical representation of age wise expression of TIPE2 in bone cancer tissues compared to non-malignant bone tissues i.e. 5y to 14y, 15y to 24y, 25y to 34y, 35y to 44y, 45y to 55y, 56y to 64y, 65y to 75y. B. Graphical representation of age wise expression of TIPE2 in osteosarcoma tissues. C. Graphical representation of age wise expression of TIPE2 in chondrosarcoma tissues. Data are represented as Mean ± SE, * denotes *p*-value < 0.05, **=*p*<0.01 compared to normal adjacent tissues.**

## 2.4. Conclusion

In this chapter, we aimed at evaluating the expression of TIPE2 protein in different bone cancer cell lines and bone cancer tissues, its subtypes, and in different age groups of bone cancer patients. Determining the implication of the expression of TIPE2 with these factors would be highly beneficial in ascertaining whether TIPE2 can serve as a predictive biomarker for bone cancer. Interestingly, our results showed that TIPE2 was strongly involved in the positive regulation of bone carcinogenesis and was found to be significantly overexpressed in both HOS and U2OS cell lines and overall bone cancer cases compared to normal HaCaT cell line and normal adjacent tissues, respectively. It is noteworthy that TIPE2 was found to be augmented in both OS and CS. This chapter also indicates that TIPE2 plays an important role in bone carcinogenesis based on its expression profile in tissues of different age groups; this may co-relate with the pubertal status and complex interplay of sex, which impacts drug metabolism, the toxicity level, and the psychosocial factors which affect the administered-dose intensity. Broadly, it can be concluded that overexpression of TIPE2 may be strongly associated with poor clinical outcomes of bone cancer patients, thus denoting its potential as a prognostic factor and novel therapeutic target in bone cancer.

Nonetheless, additional investigation is requisite with a larger sample size to validate our findings. Moreover, more in-depth studies on the molecular mechanisms involving TIPE2 protein in the pathogenesis of bone cancer are necessary for a better understanding of this chronic illness, as well as to offer new avenues for effective treatment of this malignant neoplasm.

# Chapter 3

## *Evaluating the role of TNF-α, TNF-β and RANKL on TIPE2 in bone cancer*

### 3.1. Introduction

In the earlier chapter, we have shown that TIPE2 is strongly associated with the positive regulation of bone carcinogenesis. Our results also showed that TIPE2 is significantly upregulated in HOS and U2OS bone cancer cell lines than the normal cell line HaCaT. Another finding from the previous chapter that TIPE2 is overexpressed in bone cancer tissues compared to normal adjacent tissues as determined by immunohistochemistry further strengthened our hypothesis. Therefore, these findings provide a solid basis for the presence of a positive association between TIPE2 and bone carcinogenesis.

An increasing line of evidence has supported the belief that chronic inflammation triggers tumorigenesis (Wang, X., & Lin, Y., 2008). Inflammation contributes to osteolysis, which causes bone pain and skeletal instability. The skeletal integrity basically relies on the bone homeostasis attained by means of the stable function of the bone cells. In a healthy individual, the bone resorption by osteoclasts and bone formation by osteoblasts are lifelong events that are delicately balanced (Abu-Amer Y., 2009; Walsh, N. C., *et al.,* 2005; Hagemann, T., *et al.,* 2007). Under pathological conditions such as inflammatory osteolysis, osteoporosis, and bone tumors, the bone homeostasis is compromised, which causes enhanced osteoclast activity, ultimately resulting in bone loss. The penalties of localized focal bone erosions and overall bone-weakening range from bone pain to bone fractures, hypercalcemia condition, and other mineral disparities that erode skeletal stability. Theoretically, inflammatory and metastatic factors largely takeover the bone cells and the signaling cascades from their basally balanced state and intimidate them into a continuously fueled hyperactive state to begin debilitating osteolysis (Abu-Amer, Y., 2009; Walsh, N. C., *et al.,* 2005; Hagemann, T., *et al.,* 2007). It is a well-established fact that tumor necrosis factor

(TNF) is a multifunctional cytokine that acts as an important mediator in various cellular events, for instance, cellular proliferation, survival, growth, differentiation, and death. TNFs are secreted as a pro-inflammatory cytokine by the inflammatory cells, which may participate in inflammation-associated carcinogenesis (Kunnumakkara, A. B., *et al.,* 2019; Kunnumakkara, A. B., *et al.,* 2020). TNF mediates its biological functions by initiating distinct signaling pathways such as NF-κB and c-Jun N-terminal kinase (JNK). The NF-κB signaling pathway is one of the chief cell survival pathways that mainly functions by apoptosis inhibition; at the same time, sustained JNK activation promotes apoptosis by increasing the expression of pro-apoptotic genes, which ultimately results in cell death (Kunnumakkara, A. B., *et al.,* 2019; Kunnumakkara, A. B., *et al.,* 2020). The crosstalk between the JNK and NF-κB is crucial in establishing the cellular outcomes in TNF response. In cancer, TNF acts as a double-dealer where, on the one hand, it can act as an endogenous tumor promoter, and on the other hand, it could serve as a cancer killer (Kunnumakkara, A. B., *et al.,* 2019; Kunnumakkara, A. B., *et al.,* 2020).

A growing body of evidence suggested that TNF-α and TNF-β are pro-inflammatory cytokines that play a vital role in chronic diseases, including cancer. Lymphotoxin-α, commonly known as TNF-β, is a structural homolog to TNF-α and exerts its activity through the activation of NF-κB signaling pathway similar to TNF-α, thus contributing to cancer progression (Buhrmann, C., *et al.,* 2020). Moreover, TNF-α and TNF-β have been reported to partake in numerous cell processes such as survival, proliferation, differentiation, etc., and maybe implicated in inflammation-related carcinogenesis through the activation of NF-κB (Wang, X., & Lin, Y., 2008; Balkwill, F., *et al.,* 2006; Drutskaya *et al.,* 2010). The TNF superfamily primarily comprises 19 ligands and 29

receptors. Amongst the ligands, the TNF-α and TNF-β, mainly generated by the macrophages and the lymphocytes are the vital regulators of airway inflammation and are robustly associated with tumorigenesis (Aggarwal, B. B., *et al*. 2012; Seifart, C., *et al.*, 2005). TNF mainly binds to two receptors, namely TNF receptor-1 (TNFR-1), also known as p55 receptor, and TNFR-2, commonly called as p75 receptor.

Over the years, the TNF-TNFR-1signaling cascade has been extensively studied. In most of the cell types, TNFR1 is constitutively expressed, while the TNFR2 expression is highly regulated. The ligand-receptor interaction between TNF and TNFR-1 causes the TNFR-1 to form a homotrimer which activates TNFR-associated death domain (TRADD), which in turn further recruits the downstream adaptor proteins, which include receptor-interacting protein (RIP), TNFR-associated factor 2 (TRAF-2), and Fas-associated death domain (FADD) which ultimately results in regulation of essential downstream molecules that stimulates the activation of several signaling pathways such as NF-κB, MAPKs, and apoptosis mediated cell death (Wang, X., & Lin, Y., 2008). The mechanism that defines the dominant effect, whether it has to induce tumorigenesis or cell death, has not been fully clarified.

It is well evinced that in the bone microenvironment, TNF-α exerts multiple effects on bone cells. For instance, TNF-α augments the synthesis of proteolytic enzymes such as matrix metalloproteinases (MMPs) and plasminogen activators, and of cytokines such as IL-6, IL-8, and monocyte-macrophage colony-stimulating factor (M-CSF) in these bone cells but then TNF-α prevents collagen and DNA synthesis and osteocalcin gene expression in osteoblasts (Nanes, M. S., *et al*., 1989; Nanes, M. S., *et al*., 1991; Kuno, H., *et al*., 1994; Panagakos, F. S., & Kumar, S., 1994; Chaudhary, L. R., *et al*., 1992; Ishimi, Y., *et al*., 1990; Passeri, G., *et al*., 1994; Elford, P. R., *et al*., 1987; Felix, R., *et*

*al*., 1989). A systematic meta-analysis of genetic variants associated with OS susceptibility revealed that the TNF-α gene contributed to OS susceptibility (Wang, X., & Liu, Z., 2018). Furthermore, it has been reported that TNF-α produced by the host macrophages mainly functions to sustain the osteosarcoma cells in an undifferentiated state which is requisite for tumor progression (Mori, T., *et al*., 2014).

The RANKL/RANK/osteoprotegerin (OPG) signaling cascade was identified in the late-1990s as an essential regulator of bone remodelling (Infante, M., *et al*., 2019; Yasuda, H., *et al.*, 1998a; Lacey, D. L., *et al.*, 1998; Boyce, B. F., & Xing, L., 2007). Before this discovery, it was believed that osteoclast formation was controlled by factors that are expressed by the osteoblast or the stromal cells. Moreover, it was not predicted that members of the TNF superfamily of ligands and receptors were involved and would have widespread roles beyond bone remodelling. Moreover, the RANKL/RANK signaling modulates the formation and development of multinucleated osteoclasts from the precursor osteoclast cells, and their activation assists in normal bone modelling or remodelling (Boyce, B. F., & Xing, L., 2007). This system mainly comprises three chief signaling molecules, i.e. the cytokine RANKL, the receptor RANK, and the soluble decoy receptor OPG. RANKL was earlier identified as TNF-related activation-induced cytokine (TRANCE), which belongs to the TNF superfamily and is mainly expressed by the bone marrow stromal cells, osteocytes and the osteoblasts (Boyce, B. F., & Xing, L., 2007; Boyce, B. F., & Xing, L., 2008; Wong, B. R., *et al.*, 1999). RANKL is a homotrimeric transmembrane protein that is usually expressed on the activated T cells and the osteoblasts and also released as a secretory protein (Boyce, B. F., & Xing, L., 2008). RANKL interacts with its receptor, RANK, on the surface of the precursor osteoclast cells and triggers the fusion of these cells into

multinucleated cells, which ultimately helps them to differentiate into mature osteoclasts (Lacey, D. L., *et al.,* 1998; Dougall, W. C., *et al.,* 1999; Arai, F., *et al.,* 1999). Further, the mature osteoclast cells adhere to the bone surface and stimulate bone resorption by releasing acid and lytic enzymes such as tartrate-resistant acid phosphatase (TRAP), cathepsin K, etc. (Lacey, D. L., *et al.,* 1998; Boyle, W. J., *et al.,* 2003).

OPG, an atypical member of the TNFR family, is mainly expressed by the osteoblasts and the bone marrow stromal cells as it functions as a soluble decoy receptor lacking a transmembrane domain (Boyce, B. F., & Xing, L., 2007; Yasuda, H., *et al.,* 1998b). The OPG has 500-fold higher binding affinity towards RANK than RANKL (Nelson, C. A., *et al.,* 2012). Henceforth, OPG averts RANKL from binding to its receptor RANK, which hinders osteoclastogenesis and protects the bone from excessive osteoclast-mediated resorption (Nakagawa, N., *et al.,* 1998; Lacey, D. L., *et al.,* 2000). In addition, the RANKL/RANK axis has been recognized as an essential signaling pathway involved in various mechanisms beyond bone homeostasis (Walsh, M. C., & Choi, Y. 2014). Aforementioned, TIPE2 is a member of the TIPE/TNFAIP8/Oxi-α family, and this family of protein is significantly induced by TNF-α in different tumor cells (Kumar, D., *et al.,* 2000). As there is a lack of information on the effect of TNF-α, TNF-β, and RANKL on TIPE2 expression in bone cancer, in this chapter, we attempt to decipher whether these three pro-inflammatory cytokines have any effect on the expression of TIPE2 in bone cancer cells, which would provide better insight on the function of TIPE2 in bone carcinogenesis.

## 3.2. Materials and methods

### 3.2.1. TNF-α, TNF-β and RANKL

The TNF-α and TNF-β were gifted by Prof. B. B. Aggarwal, Inflammation Research Center, San Diego, USA, and a stock solution of 200ng/ml was prepared using DMEM media. Human RANKL/TRANCE/TNFSF11 (hRANKL) (Cat no. #5312) was bought from Cell Signaling Technology (CST), Danvers, Massachusetts, USA.

### 3.2.2. Cell culture

The details of the cell lines, cell culture media, and supplements used for our study have been mentioned in Chapter 2. Please refer to Chapter 2, Section 2.1.1. Cell culture.

### 3.2.3. MTT assay

To examine the effect of TNF-α, TNF-β and RANKL on the proliferation of human bone cancer cells MTT assay was carried out. For this, the HOS and U2OS cells were harvested, and 2000 cells were seeded in 96-well plates per well and incubated for 24 h in a CO2 incubator at 37°C. Different concentrations of TNF-α (0, 0.05, 0.1, and 0.2 nM), TNF-β (0, 0.05, 0.1, and 0.2 nM), and RANKL (0, 0.05, 0.1, 0.5, 1, 5, and 10 ng/ml) were added to the bone cancer cells after 24 h incubation was over,. The MTT assay was carried out at 0 and 24 h. At each time point, 10µl of 5mg/ml of MTT (Sigma-Aldrich, Missouri, USA) was added to each well and incubated for 2h. The culture medium was discarded after the incubation period was over, and 100µl of DMSO (Merck, Darmstadt, Germany) was added in all the wells followed by incubation for 1h at room temperature (RT) to dissolve the MTT-formazan product. Lastly, the absorbance was measured using a microplate reader (TECAN Infinite 200 PRO multimode reader, Switzerland) at 570 nm. The effect of TNF-α, TNF-β, and RANKL treatment on the proliferation was calculated by normalizing the 24 h absorbance value

with 0 h while considering the absorbance of untreated control as 100% (Bordoloi, D., *et al*., 2020).

### 3.2.4. Colony formation assay

To evaluate the effect of TNF-α, TNF-β, and RANKL on the clonogenic potential of HOS and U2OS bone cancer cells, the colony formation assay was performed. Briefly, HOS and U2OS bone cancer cells were seeded at a density of 1000 cells/2ml/well in a 6-well plate. After 24 h incubation, different concentrations of TNF-α (0 and 0.1nM), TNF-β (0 and 0.1nM), and RANKL (0 and 0.05 ng/ml) were added to the bone cancer cells and incubated for 24 h. After that, the media from all the wells were replaced, cells were further allowed to grow for 10 days with replacing of media as required. At the end of the 10th day, the plates were washed, and the colonies were fixed with 70% ethanol. The colonies were washed again with IX PBS and then stained using 0.01% (w/v) crystal violet (SRL Pvt. Ltd., Mumbai, India). Gentle washing was done again for the removal of the excess stain. The images of each well were captured, and the survival fraction was calculated using the formula:

PE, Plating efficiency = (Number of colonies counted/ Number of cells plated) × 100

SF, Survival fraction = (PE of treated sample/ PE of control) × 100 (Bordoloi, D., *et al*., 2020; Monisha, J. *et al*., 2018).

### 3.2.5. PI-FACS assay

To determine the effect of TNF-α, and TNF-β on cell death of HOS and U2OS bone cancer cells, the PI- fluorescence-activated cell sorting (FACS) assay was performed. For this, 5 X $10^4$ cells/2ml/well were seeded in 6-well plate and incubated for 24 h, followed by treatment with (0 and 0.1nM) TNF-α and (0 and 0.1nM) TNF-β for 72 h.

After 72 h of incubation, the media from the wells were collected in labelled 5x77mm polystyrene test tubes. The adhered cells were washed with 1X PBS, trypsinized and then collected in their respective tubes. The cell suspension was then centrifuged at 4000 rpm for 10 mins in $4^0$C. After centrifugation, the supernatant was discarded, and the pellet was washed with 1ml 1X PBS and centrifuged again at 4000 rpm for 10 mins, and the step was repeated twice. Lastly, the pellet was suspended in 495 µl of PBS, and 5 µl of Propidium iodide (PI) (Sigma-Aldrich, Missouri, USA) dye was added. The cells were then analysed in flow cytometer (BD FACS Celesta™, Becton-Dickinson, New Jersey, USA) (Aswathy, M., *et al*., 2021).

**3.2.6. Live/Dead assay**

To further confirm the effect of TNF-α, and TNF-β on cell death of HOS and U2OS bone cancer cells, the Live/Dead assay using an inverted fluorescence microscope was performed. Briefly, $2x10^3$ cells were seeded in a 96-well plate and incubated for 24 h, followed by treatment with (0 and 0.1nM) TNF-α and (0 and 0.1nM) TNF-β for 72 h at 37°C. After the incubation time was over, the cells were then stained with the Live/Dead reagent (5 µM ethidium homodimer and 5 µM calcein-AM) and incubated at 37°C for 20 min. Cells were analyzed under an inverted fluorescence microscope (Olympus, Japan) (Aswathy, M., *et al*., 2021).

**3.2.7. Migration Assay**

To evaluate the effect of TNF-α, TNF-β, and RANKL on the migratory potential of HOS and U2OS bone cancer cells compared to the untreated control, wound healing assay was carried out. For this, HOS and U2OS cells were seeded at a density of $5x10^5$ cells/2 mL/well in 6 well plates and allowed them to form a monolayer. The medium was then replenished with a serum-free MEM and DMEM medium for HOS and U2OS

cells, respectively, to inhibit cell proliferation and incubated 6-8 h for adaptation. After serum starvation, a wound was formed across the monolayer using a 100 µL micro tip. The detached cells were then washed using 1X PBS and then treated with the indicated concentration of TNF-α, TNF-β, and RANKL. Thereafter, fresh serum-free MEM and DMEM medium for HOS and U2OS cells were added to the cells, respectively. Finally, migration of the cells was assessed by analyzing the difference in the area of the scratched wounds using an inverted microscope (Nikon T1-SM, Japan). Images were captured at different time intervals and then examined using Image J software (Bordoloi, D., *et al*., 2020; Monisha, J. *et al*., 2018).

### . 3.2.8. Western Blot

To determine the effect of TNF-α, TNF-β and RANKL on the expression of TIPE2 in bone cancer cell lines HOS and U2OS, was analyzed by Western blot. In brief, after treatment of HOS and U2OS cells with different concentrations of TNF-α (0 and 0.1nM), TNF-β (0 and 0.1nM) and RANKL (0 and 0.05 ng/ml) for 24h. The Western blot was performed as per the protocol mentioned in Chapter 2, Section 2.2, Subsection 2.2.2. Western blot analysis. The housekeeping gene α -tubulin was used as a loading control (Bordoloi, D., *et al*., 2020; Monisha, J. *et al*., 2018).

### 3.2.9. Statistical analysis

Statistical analysis was performed using Student's *t*-test, and *p*-value < 0.05 was denoted as statistically significant.

## 3.3. Results and discussion

### 3.3.1. Effect of TNF-α, TNF-β, and RANKL on the proliferation and survival of human bone cancer cells.

Aforesaid, pro-inflammatory cytokines such as TNF-α and TNF-β are mainly produced by the macrophages and the lymphocytes, respectively and play substantial roles in airway inflammation and tumorigenesis (Aggarwal, B. B., *et al*. 2012; Seifart, C., *et al.*, 2005). TNF acts as a key mediator of inflammation-induced oncogenesis (Gong, K., *et al*., 2021). Even though TNF-α has been reported to mediate its anticancer effect by inducing cancer cell death, it has been demonstrated to improve cell survival, proliferation, angiogenesis, and metastasis in most of the cancer types that are resistant to TNF-α-triggered cell death (Wang, X., & Lin, Y., 2008; Ooppachai, C., *et al.*, 2019). In that case, TNF-α interacts to its receptor and stimulates different molecular pathways such as NF-κB, and MAPKs which in return enhance cancer cell survival and progression via augmentation of anti-apoptotic [eg. survivin, X-linked inhibitor of apoptosis protein (XIAP), B-cell lymphoma 2 (Bcl-2)], proliferative (e.g. cyclin D and cyclin B1), invasive (e.g. MMP-9), and angiogenic [e.g. VEGF and cyclooxygenase-2 (COX-2)] proteins (Subkamkaew, C., *et al.*, 2019; Wang, X., & Lin, Y., 2008;). Endogenous cytokine TNF-α is known to act as a potent mutagen due to its capability to cause DNA damage via the induced reactive oxygen species (ROS) generation. The secretion of TNF-α by the tumor cells in the tumor microenvironment helps in cancer cell survival by inducing NF-κB associated anti-apoptotic proteins (Landskron, G., *et al*., 2014; Woo, C. H., *et al*., 2000; Hussain, S. P., *et al*., 2003). TNF-β is a structural homolog to TNF-α and exerts its activity through the activation of NF-κB signaling pathway similar to TNF-α (Buhrmann, C., *et al*., 2020; Buhrmann, C., *et al*., 2019b; Buhrmann, C., *et al*., 2018; Buhrmann, C., *et al*., 2019a). Recent findings strongly

suggested the involvement of the RANKL as the key regulator of bone remodelling in bone oncology (Navet, B., *et al*., 2018; Okamoto K., 2021). Therefore, to determine the effect of TNF-α, TNF-β, and RANKL on the cell proliferation of HOS and U2OS bone cancer cells, MTT assay was performed. MTT assay is known to measure the quantity of living cells. The insoluble violet-blue formazan generated through the reduction of MTT tetrazolium salt by mitochondrial dehydrogenases helps to determine the percentage of live cells. The HOS and U2OS cells were treated with increasing concentrations of TNF-α, TNF-β, and RANKL. All three pro-inflammatory cytokines, i.e., TNF-α, TNF-β, and RANKL, induced the proliferation rate of both the cancer cell lines (**Figure 3.1.**). However, maximum percentage proliferation was observed at a concentration of 0.1nM TNF-α, 0.1nM TNF-β, and 0.05ng/ml RANKL. This study indicates that TNF-α, TNF-β, and RANKL plays an important role in inducing proliferation of the bone cancer cells

In addition to enhanced proliferation, an upsurge in survival fraction is also a principal characteristic of cancer cells. Thus, to evaluate the effect of TNF-α, TNF-β, and RANKL on the survival of HOS and U2OS cells, colony formation assay was performed **(Figure 3.2.)**. This assay helps in evaluating the clonogenic potential or the ability of a cell to proliferate indefinitely and retain its reproducibility to generate a colony or a clone that can, in turn, give the measure of cell survival fraction (Munshi, A., *et al.*, 2005). The findings of our study revealed that treatment of TNF-α, TNF-β and RANKL in HOS and U2OS cells resulted in the increased clonogenic potential of both the cell lines compared to untreated control, implying that TNF-α, TNF-β, and RANKL are involved in increasing the survival of bone cancer cells.

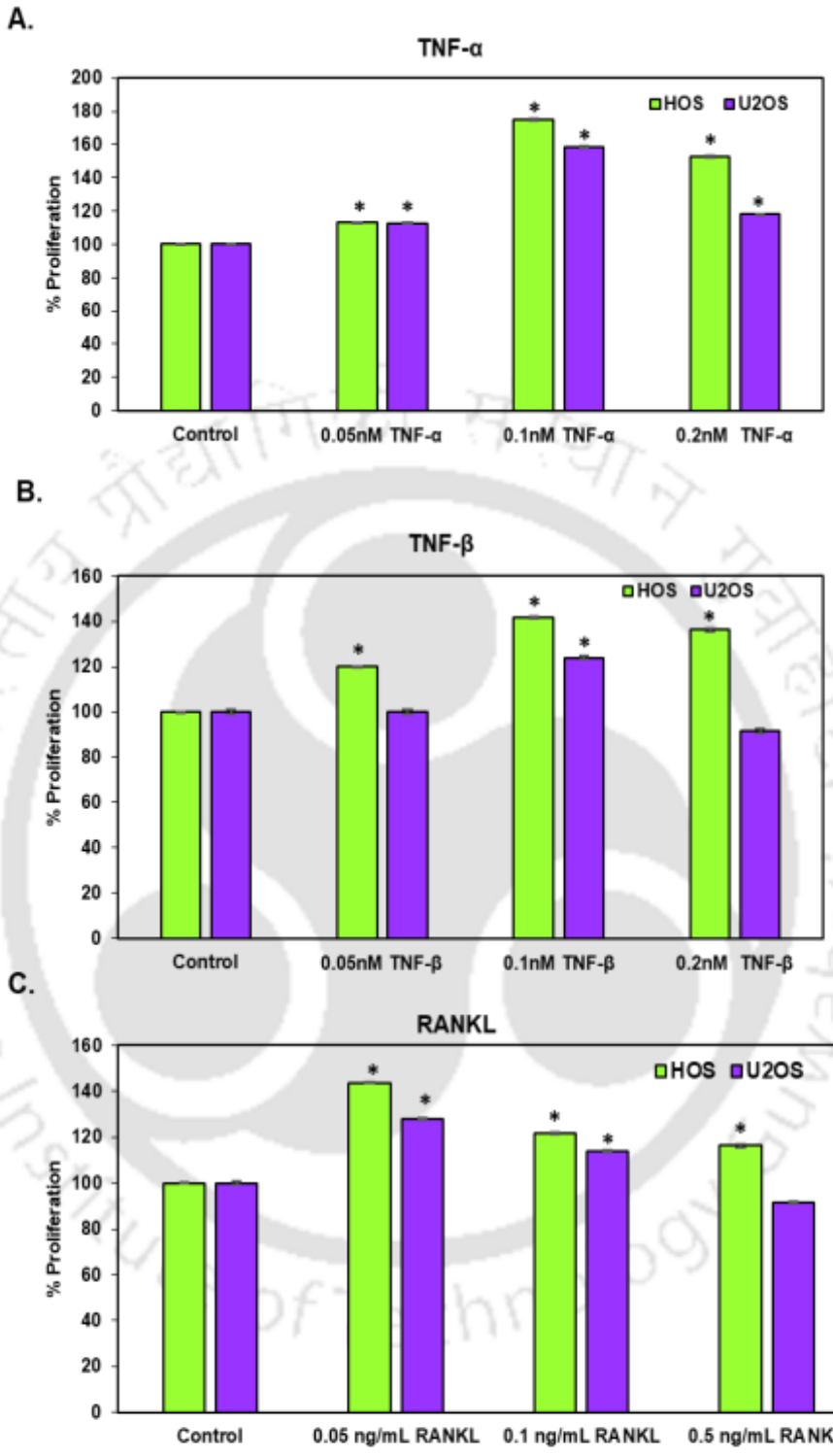


**Figure 3.1. Effect of TNF-α, TNF-β and RANKL on the cell proliferation of HOS and U2OS cells. Percent proliferation in A. TNF-α, B. TNF-β, and C. RANKL treated HOS and U2OS cells evaluated using MTT assay. Data are represented as Mean±SE, * denotes $p$ <0.05 compared to untreated control.**

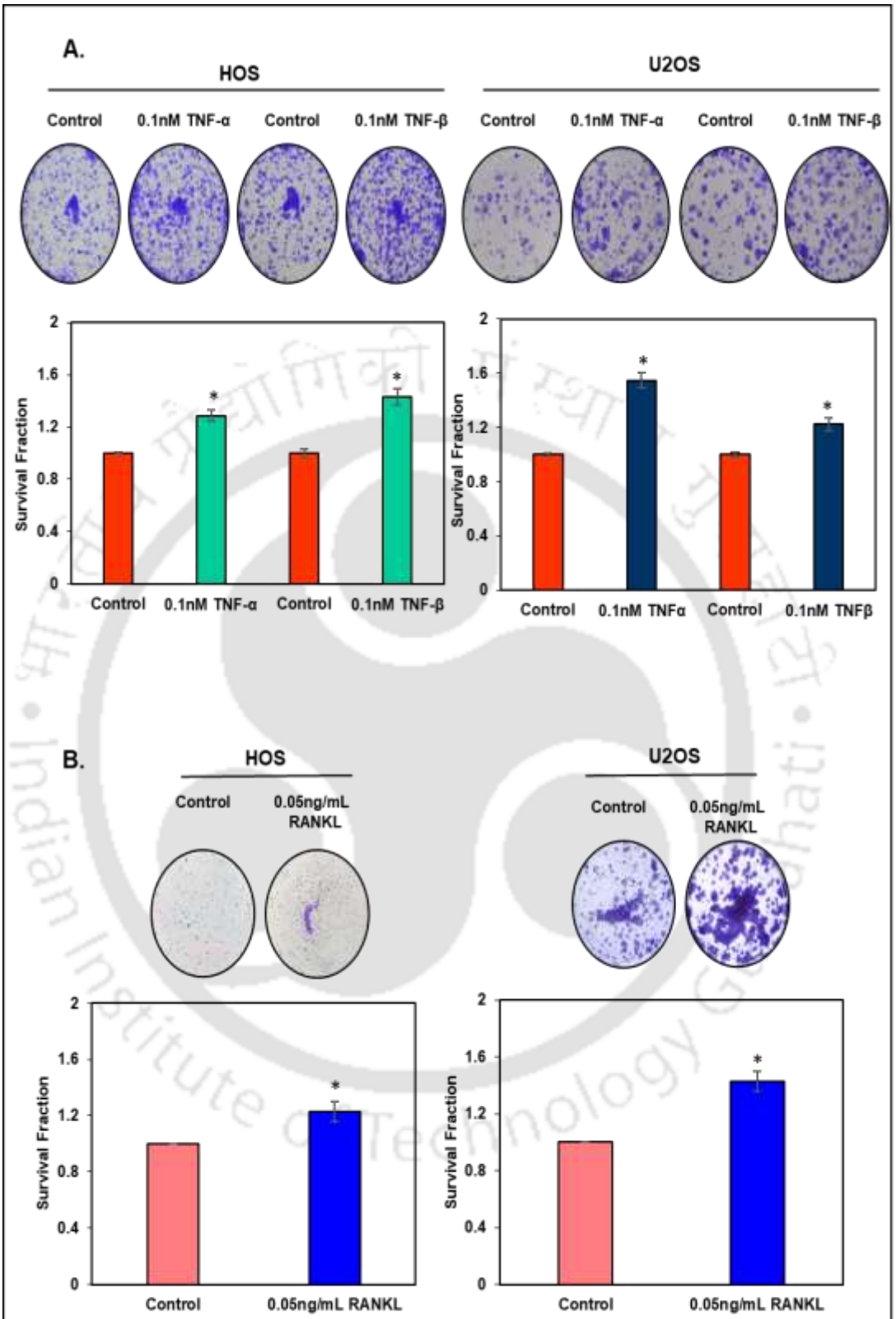


**Figure 3.2. Effect of TNF-α, TNF-β and RANKL on the cell survival of HOS and U2OS cells. Survival fraction in A. TNF-α and TNF-β, and B. RANKL treated HOS and U2OS cells were evaluated using colony forming assay. Data are represented as Mean±SE, * denotes $p$ <0.05 compared to untreated control.**

### 3.3.2. Effect of TNF-α, TNF-β, and RANKL on cell death of human bone cancer cells.

It is a well-known fact that TNF-α and TNF-β prompt apoptosis in different cell types. TNFR-1 acts as a death receptor and signals the cells to die. It internally signals the Complex I to form Complex II consisting of TRADD, RIP, FADD, and caspase-8 (Wang X., 2001; Wang, X., & Lin, Y., 2008). Auto-activation of caspase-8 triggers the activation of executor caspases-3, and -7, and the endonucleases, which lead to DNA fragmentation, destruction of cell components and apoptotic cell death. This death receptor-mediated apoptosis pathway is also known as the extrinsic apoptosis pathway (Wang X., 2001; Wang, X., & Lin, Y., 2008). Additionally, TNF can also induce the mitochondria-mediated (intrinsic) apoptosis pathway. The cleavage of Bid by caspase-8 produces tBid, which travels to the mitochondria and results in loss of mitochondrial membrane potential, and release of cytochrome c (Cyt c). Furthermore, Cyt c interacts with the apoptotic protease activating factor 1(Apaf-1) and pro-caspase-9 to form apoptosome, which ultimately causes caspase-9-mediated activation of the executor caspases that leads to apoptotic cell death (Wang X., 2001; Wang, X., & Lin, Y., 2008). Therefore, to determine the effect of TNF-α and TNF-β on the cell death of HOS and U2OS bone cancer cells, PI-FACS assay using flow cytometry and Live/Dead assay using inverted fluorescence microscopy were performed **(Figure 3.3.)**.

Flow cytometry offers a quick and consistent method to quantify viable cells in a cell suspension. Assessment of cell viability is vital while evaluating the response to cytotoxic drugs or certain carcinogens, or other environmental factors. Moreover, it is necessary to identify dead cells in a cell suspension in order to avoid them from the analysis (Cheung, M., *et al*., 2021; Vitelli, M., *et al*., 2021; Bonilla, D. L., *et al*., 2021).

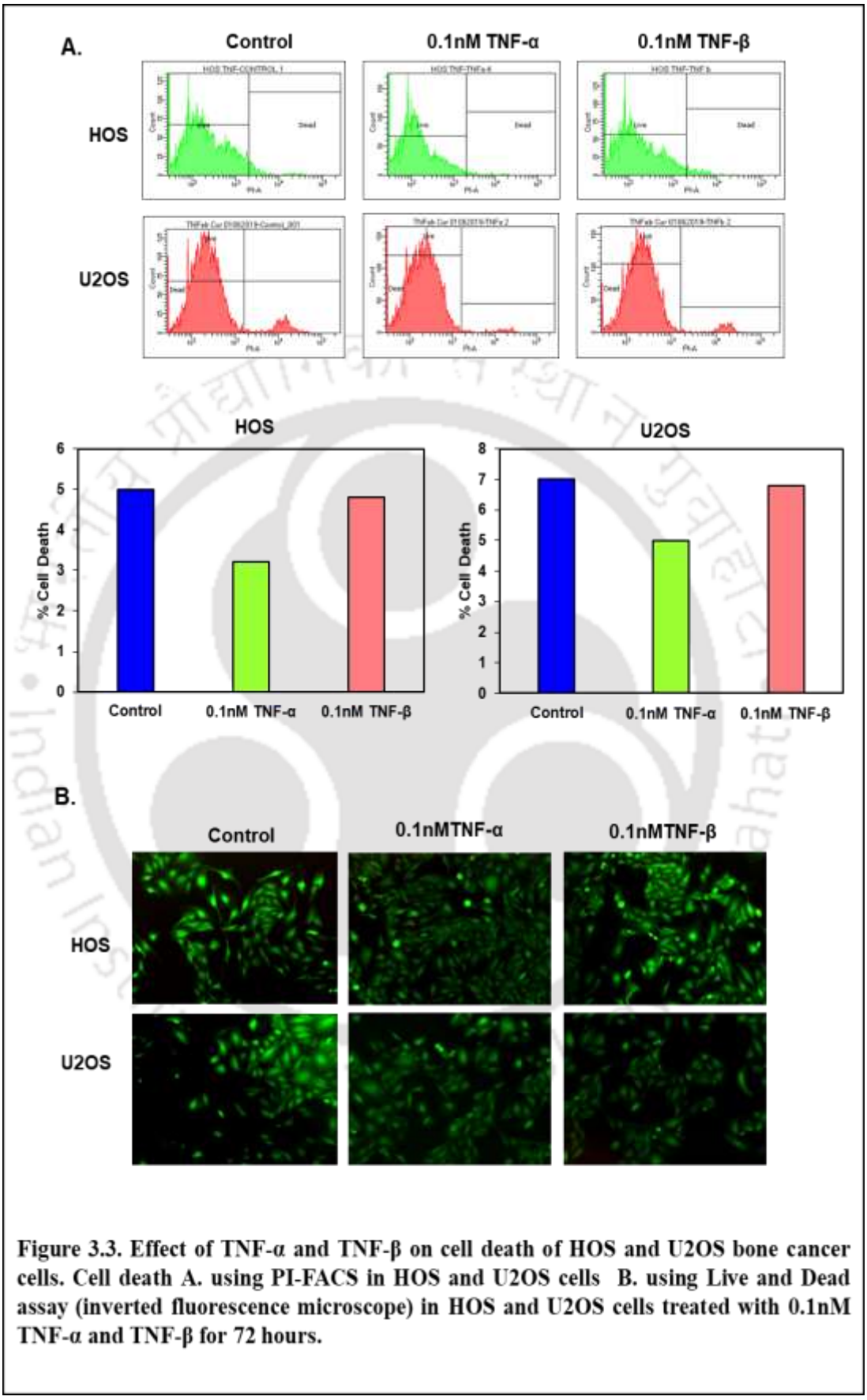


**Figure 3.3. Effect of TNF-α and TNF-β on cell death of HOS and U2OS bone cancer cells. Cell death A. using PI-FACS in HOS and U2OS cells B. using Live and Dead assay (inverted fluorescence microscope) in HOS and U2OS cells treated with 0.1nM TNF-α and TNF-β for 72 hours.**

The flow cytometry analysis showed that TNF-α and TNF-β do not induce cell death in HOS and U2OS bone cancer cells even after 72 h of treatment.

In order to determine whether TNF-α and TNF-β could induce cell death of bone cancer cells, Live/Dead assay was performed, which estimates the intracellular esterase activity and plasma membrane integrity. Calcein-AM, a non-fluorescent polyanionic dye is taken by the live cells, and as a result, it generates intense green fluorescence by enzymatic (esterase) conversion. Additionally, the ethidium homodimer enters the cells with damaged or ruptured membranes and interacts with the nucleic acids, which produces a bright red fluorescence in the dead cells (Bratosin, D., *et al*., 2005; Mitrofan, L., *et al*., 2005).

To further affirm the effect of TNF-α and TNF-β in HOS and U2OS bone cancer cells, the live and dead fluorescence staining assay was employed. **(Figure 3.3. (B))** shows the live/dead of HOS and U2OS bone cancer cells stained with green and red dye. The results demonstrated that TNF-α and TNF-β treated bone cancer cells exhibited negligible cytotoxic effects similar to the untreated control cells. It is interesting to note that TNF-α and TNF-β significantly induced proliferation and survival fraction in HOS and U2OS bone cancer cells as shown previously; however, both TNF-α and TNF-β did not induce any significant cell death in both the cell lines. Henceforth, these results are consistent with the results of MTT and PI-FACS analysis. Altogether, TNF-α and TNF-β were found to regulate bone carcinogenesis positively by inducing cell proliferation and survival and not causing any cytotoxicity.

### 3.3.3. Effect of TNF-α, TNF-β and RANKL on the migration potential of human bone cancer cells.

As mentioned previously, the majority of bone cancer patients are presented with a highly advanced stage of the disease. The ability of the bone cancer cells to invade and migrate to nearby cells is robustly related to their high metastatic potential (Riquelme, M. A., *et al.*, 2020; Okamoto, K., 2021; Coleman, R. E., *et al*., 2020). During metastasis, the primary tumor cells invade and migrate nearby tissues and as a consequence, form secondary tumor sites. Therefore, in order to know the effect of TNF-α, TNF-β, and RANKL on the migration of HOS and U2OS bone cancer cells, migration assay was carried out **(Figure 3.4.)**. The results indicated that the treatment of 0.1nM TNF-α, 0.1nM TNF-β, and 0.05ng/ml RANKL compared to their untreated control, displayed increased migration potential of the bone cancer cells. The effects of TNF-α, TNF-β and RANKL on the migration potential of different cancers were stated earlier as well. For example, similar to our results, Wolczyk, D., *et al.*, reported that TNF-α, a key inflammatory cytokine in the tumor microenvironment, promoted breast cancer cell migration via activation of the MAPK/ERK signaling pathway (Wolczyk, D., *et al.*, 2016). Another study revealed that TNF-α induced colon cancer migration and invasion potential by boosting trophoblast antigen 2 (TROP-2) expression via the ERK1/2 signaling pathway (Zhao, P., & Zhang, Z., 2018). Both the exogenous and the macrophage-produced TNF augmented the EMT, which explained that the progression of carcinoma was related to the attainment of an invasive phenotype. Besides, TNF enhanced the invasiveness of cancer cells through inducing MMP-2, -3, -9, -12 or α2β1 integrin (Wang, X., & Lin, Y., 2008). Altogether, TNF-α, TNF-β, and RANKL were found to enhance the migration potential of bone cancer cells effectively.

### 3.3.4. Effect of TNF-α and TNF-β on the expression of TIPE2 in HOS and U2OS bone cancer cells.

Immune cells can respond to inflammatory stimulations, secreting pro-inflammatory cytokines belonging to the TNF family, for example, TNF-α and TNF-β, which play an essential role in chronic diseases including cancer (Aggarwal, B. B., *et al*., 2012; Seifart, C., *et al.,* 2005). Even though TNF-α has been described to mediate its anticancer effect by enhancing cancer cell death, The TNF-α has been demonstrated to augment cell survival, proliferation, angiogenesis, and metastasis in most cancer cells that are resistant to TNF-α-induced cell death (Ooppachai, C., *et al.,* 2019). Furthermore, TNF-α binds to its receptor and instigates the regulation of various signaling cascades, including NF-κB, and MAPKs which causes improved cancer cell survival, proliferation, migration, invasion and angiogenesis (Wang, X., & Lin, Y., 2008; Subkamkaew, C., *et al.,* 2019). TNF-β, the closest structural homolog to TNF-α, was discovered around 35 years ago (Aggarwal, B. B., *et al.,* 1985; Aggarwal, B. B., *et al.,* 1984) and it has come to attention that TNF-β may stimulate NF-κB activation in cancer cells with a similar potency to TNF-α, and thus ameliorates the proliferation, invasion and malignancy of cancer cells (Buhrmann, C., *et al.,* 2018; Buhrmann, C., *et al.,* 2019a; Buhrmann, C., *et al.,* 2019b). The RANKL/RANK binding is vital for normal bone homeostasis. Upon binding of RANKL to its receptor RANK, it enhances osteolytic bone resorption, a process that occurs in excess when tumor cells are present in bone. RANKL was formerly identified as TRANCE and belongs to the TNF superfamily (Boyce, B. F., & Xing, L., 2008). TNF-α can bind to TNFR1 and TNFR2, activating NF-κB signaling pathway and inducing the expression of TIPE family proteins, including TIPE2. Nevertheless, TIPE2 exhibited differential expression patterns and seems to play diverse roles in different cells and tissues. Previous studies suggested that TIPE2 acts as a regulator in inflammatory responses and immune homeostasis. TIPE2 protein was found to be overexpressed in ovarian adenocarcinoma

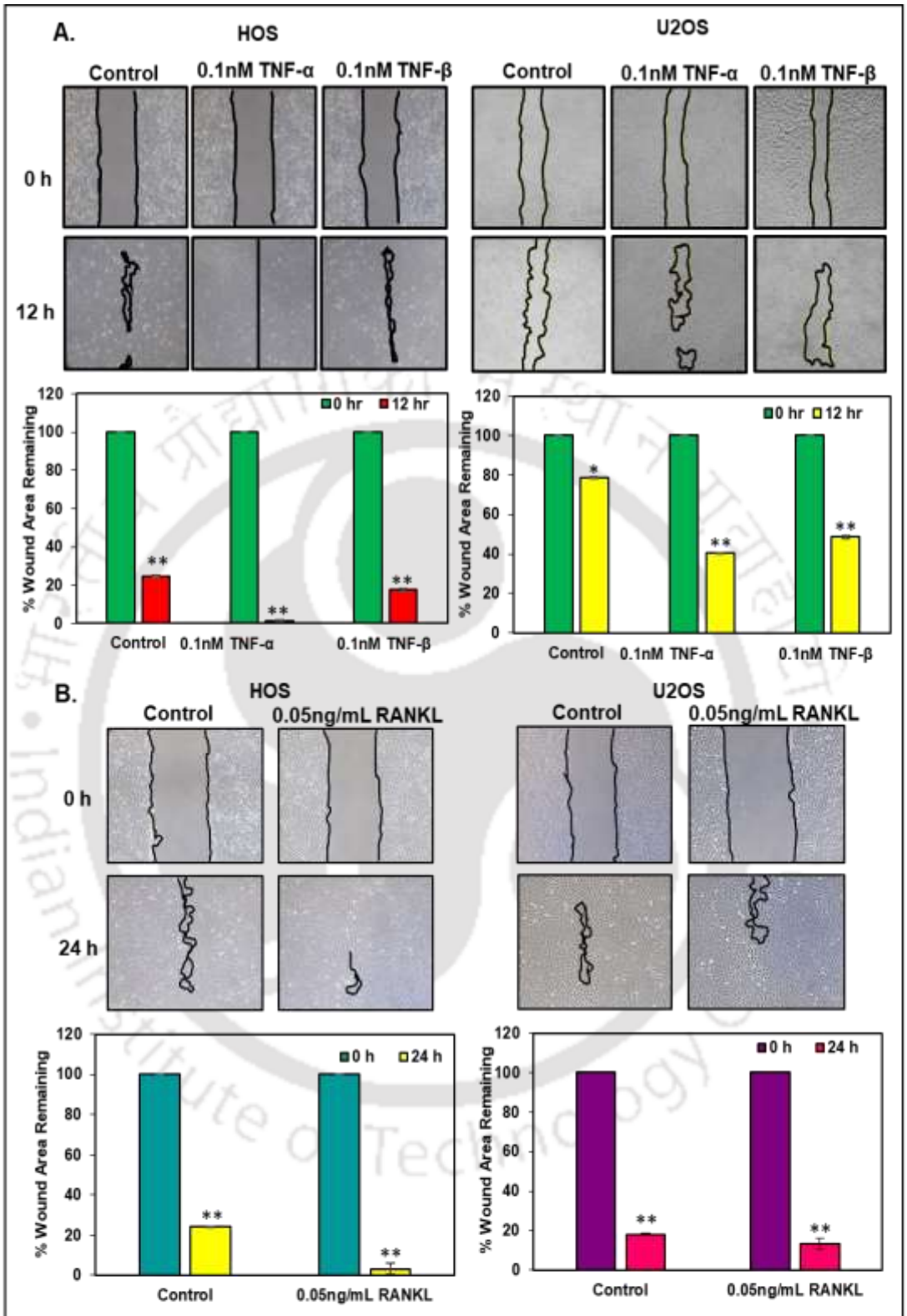


**Figure 3.4. Effect of TNF-α, TNF-β and RANKL on the migration potential of HOS and U2OS cells. Percentage wound area remaining in A. TNF-α and TNF-β, and B. RANKL treated HOS and U2OS cells was evaluated using wound healing assay (ImageJ software). Data are represented as Mean±SE, * denotes $p$ <0.05; ** denotes $p$<0.01 compared to untreated control.**

cells, lung cancer cells and macrophage-derived cell lines; whereas weak or no expression was observed in astrocytoma, cervical carcinoma, chronic myelogenous leukemia cell lines, HCC, lung carcinoma and transitional cell carcinoma of the urinary bladder (Zhang, G., *et al.*, 2010).

So, examining the effect of TNF-α, TNF-β and RANKL on the expression of TIPE2 would help us understand whether TIPE2 has any role in TNF- associated bone carcinogenesis. For this purpose, HOS and U2OS cells were treated with 0.1 nM of TNF-α and TNF-β; and 0.05ng/ml of RANKL for 24h, and then the expressions of TIPE2 was analyzed using Western blot **(Figure 3.5.)**. Our findings showed that the protein level of TIPE2 was augmented from its basal level upon treatment with the TNF-α, TNF-β, and RANKL. Many studies confirmed that the TNF members mediate the activation of NF-κB and its associated genes, which triggers cancer cell survival and metastasis (Gaur, U., & Aggarwal, B. B., 2003; Ooppachai, C., *et al.*, 2019). Hence, our results proved the involvement of TIPE2 in TNF-mediated bone carcinogenesis.

### 3.4. Conclusion

This study, for the first time, reports that there exists a correlation between TNF-α, TNF-β, and RANKL and the regulation of TIPE2 in human bone cancer. Multifunctional cytokine TNF partakes in diverse cellular events such as cell survival, proliferation, differentiation, and death. TNF is primarily secreted by the inflammatory cells as a pro-inflammatory cytokine and leads to inflammation-associated carcinogenesis. The crosstalk between the NF-κB and JNK is crucial for the assessment of the cellular outcomes in response to TNF. With respect to cancer, TNF acts as a double-dealer. On the one hand, TNF can act as an endogenous tumor promoter because TNF stimulates cancer cells' growth, proliferation, invasion and metastasis, and tumor

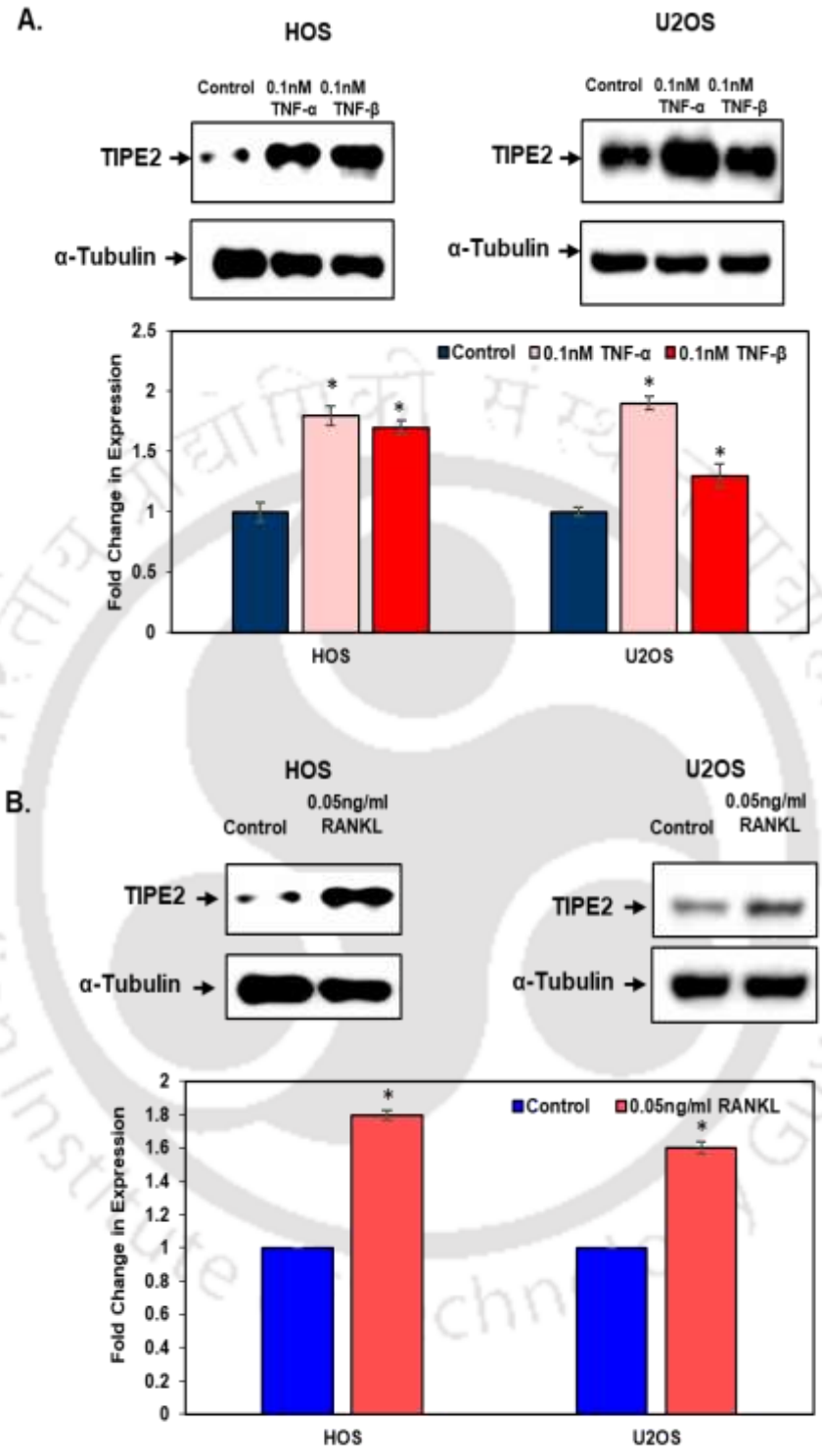


**Figure 3.5. Effect of TNF-α, TNF-β and RANKL on the expression of TIPE2 protein in HOS and U2OS cells. Expression at translational level is measured by Western blot method. The expression of TIPE2 in HOS and U2OS cells treated with A. 0.1nM concentration of TNF-α and TNF-β, B. 0.05ng/mL RANKL for 24 hours is shown. Data are mean + SE. *=$p$<0.05.**

angiogenesis. On the other hand, TNF could also induce cell death and kill cancer cells. Furthermore, increasing lines of evidence have demonstrated the important role of pro-inflammatory cytokines such as TNF in the development of bone cancer. Therefore, in this chapter, we discussed the effect of TNF-α, TNF-β, and RANKL on the proliferation, viability, cell death, colony formation, and migration of bone cancer cells. We found that TNF-α, TNF-β, and RANKL induced proliferation, colony formation, and migration potential of HOS and U2OS bone cancer cells and did not induce cell death in the cancer cells. Furthermore, in this chapter, we discussed the effect of TNF-α, TNF-β and RANKL and their relationship with the TIPE2 protein. Our study has shown that treatment of bone cancer cells with TNF-α, TNF-β and RANKL significantly upregulated TIPE2 expression. Nevertheless, our results provide only a preliminary indication of the involvement of TIPE2 protein in TNF-α, TNF-β and RANKL induced bone carcinogenesis. Therefore, mechanistic studies are obligatory to decipher the upstream regulators as well as downstream targets of TIPE2 in TNF-α, TNF-β and RANKL induced bone carcinogenesis.

# Chapter 4

## *Role of TIPE2 protein in the regulation of different hallmarks of bone cancer*

## 4.1. Introduction

In the preceding chapters, we have shown that TIPE2 protein was overexpressed in bone cancer tissues compared to normal bone tissues and are involved in the positive regulation of bone carcinogenesis. Further, treatment with the pro-inflammatory cytokines such as TNF-α, TNF-β and RANKL induced proliferation, colony formation and migration potential of HOS and U2OS bone cancer cells. Further, the upregulation of TIPE2 upon treatment of bone cancer cells with TNF-α, TNF-β and RANKL indicated the involvement of TIPE2 in bone carcinogenesis. From these results, it is evident that TIPE2 is one key molecule involved in the development and progression of TNF-induced bone cancer. However, to elucidate the exact role of TIPE2 protein and its downstream targets, we have silenced the TIPE2 protein in bone cancer cell lines. Notably, siRNA or shRNA mediated silencing of this protein was found to influence cell growth, proliferation, invasion and metastasis of other cancer types as evinced by a few studies carried out thus far, which are discussed in the first chapter. Therefore, in the current chapter, we silenced the expression of TIPE2 protein in bone cancer cell lines HOS and U2OS using siRNA-mediated knockdown and determined the effect of gene knockdown on the proliferation, survival fraction, cell cycle progression, autophagy, EMT, invasion and migration of HOS and U2OS cells *in vitro*. Also, we identified the upstream and downstream targets of TIPE2 protein involved in the regulation of bone carcinogenesis. In addition, we found their involvement in TNF-α, TNF-β and RANKL mediated bone carcinogenesis and the underlined mechanism of action.

## 4.2. Materials and Methods

### 4.2.1 Cell culture

The details of the cell lines, cell culture media and supplements used for our study have been mentioned in Chapter 2. Please refer Chapter 2, Section 2.1.1. Cell culture.

### 4.2.2. siRNA-mediated gene knockdown

For disrupting the TIPE2, TNF-α and NF-κB gene, we used siRNA mediated gene-editing method. The HOS and U2OS cells were seeded in a 6 well plate. Upon reaching 70% confluency, the cells were transiently transfected with TIPE2 (10pmol/µl) (Eurofins Genomics, Germany) mixed with 5x universal siMAX siRNA buffer (Eurofins genomics, Germany) using transfection reagent lipofectamine RNAiMax (Invitrogen, CA, USA) according to manufacturer's protocol. The total protein lysates were prepared using whole-cell lysis buffer 48h post-transfection. Gene silencing was analyzed by western blot.

**Table 4.1. siRNA target sequences.**

| Gene | siRNA target sequence |
|---|---|
| TNFAIP8L2 (TIPE2) | CAGGTCCTTGATCACGCGCT |
| NF-κB | GCGACAAGGUGCAGAAAGAdTdT |
| Tnf Alpha (TNF-α) | pGUCUCAGCCUCUUCUCAUUCCUGct |
| Scramble | GCACTCACATCGCTACATCA |

### 4.2.3. MTT assay

The effect of TIPE2 knockdown on the proliferation of human bone cancer cells was determined with the help of an MTT assay. Briefly, the scrambled siRNA transfected cells (represented as SCR), TIPE2 knockdown cells (represented as siTIPE2), were seeded at a density of $2X10^3$ cells/well in 96 well plates and incubated for 24 h at 37°C in a $CO_2$ incubator. The MTT assay was performed as per the protocol mentioned previously in Chapter 3, Section 3, Subsection 3.2.3. MTT assay. The inhibition in proliferation caused due to the knockdown of TIPE2 was then calculated by normalizing the absorbance value of 24 h with 0 h while considering the absorbance of SCR as 100%.

Further, the effect on the proliferation of TNF-α, TNF-β and RANKL treated TIPE2 knockdown cells were also evaluated using this assay, in which after 24 h incubation of the seeded cells in 96 well plates, TNF-α (0.1nM), TNF-β (0.1nM) and RANKL (0.05 ng/ml), were added to the SCR as well as siTIPE2 cells. The MTT assay was performed at 0 and 24 h after the addition of TNF-α, TNF-β and RANKL and the same procedure as mentioned previously in Chapter 3, Section 3, Subsection 3.2.3. MTT assay was followed. Finally, the percentage of proliferation of TNF-α, TNF-β and RANKL treated knockdown cells were calculated by normalizing the absorbance value of 24 h with 0 h while considering the absorbance of SCR as 100%.

### 4.2.4. Colony formation assay

The clonogenic potential of TIPE2 knockdown HOS and U2OS cells were determined with the help of colony formation assay. Briefly, the scramble and siTIPE2 cells were seeded in 6-well plates at low density (~1000 cells per well). The colony formation

assay was performed as per the protocol mentioned previously in Chapter 3, Section 3, Subsection 3.2.4. Colony formation assay.

Additionally, the effect on the clonogenic potential of TNF-α, TNF-β and RANKL treated TIPE2 knockdown cells was also evaluated with the help of this assay, in which after 24 h incubation of the seeded cells in 6 well plates, TNF-α (0.1nM), TNF-β (0.1nM) and RANKL (0.05 ng/ml), were added to the SCR as well as siTIPE2 cells and the same procedure as mentioned previously in Chapter 3, Section 3, Subsection 3.2.3. Colony formation assay was followed.

### 4.2.5 Cell cycle analysis

The effect of knockdown of TIPE2 on the cell cycle progression of HOS and U2OS cells were determined by flow cytometry assisted cell cycle analysis. In short, the SCR, and siTIPE2 cells were plated in 6 well plates at a density of 2,00,000 cells/2 mL/well and allowed to divide for 24 h. At the end of 24 h, the cells were collected by trypsinization, washed with 1X PBS and fixed with 75% ethanol without forming clumps overnight at -20 °C. Thereafter, the ethanol used for fixing was removed by centrifugation, and the cells were washed with 1X PBS and stained with Propidium Iodide (PI)/RNase solution for 20 min in the dark. Following the 20 mins incubation, the cells were analyzed by flow cytometer (BD FACSCelesta™, Becton-Dickinson, New Jersey, USA), and the percentage of cells in each phase of the cell cycle was analyzed using FCS express software.

### 4.2.6. Immunocytochemistry for evaluation of autophagy and EMT

The effect of knockdown of TIPE2 on the autophagy and EMT of HOS and U2OS cells were determined using immunocytochemistry. Approximately, $6x10^4$ TIPE2

knockdown and scrambled control cells were seeded individually on glass coverslips in a 12-well plate. After 60-70% confluency was achieved, the cells were washed and fixed with 3.7% formaldehyde for 20 mins. This step was followed by hydration of the cells using 1X PBS. Subsequently, the cells were permeabilized for 10 mins with 1X PBST (PBS containing 0.2% TritonX) and incubated for 10 mins with 2% BSA prepared in 1XPBS containing few drops of Triton-X for blocking the probable non-specific binding sites. Subsequently, the cells were incubated with primary antibody, i.e., anti-LC3 polyclonal antibody (5 µg/mL, Life technologies, Ref L10382) at 4 °C. Next, the cells were washed with 1X PBST and incubated with Alexa Fluor® 594 conjugated goat anti-rabbit IgG secondary (Invitrogen, CA, USA, dilution 1: 2000) for 45 mins at room temperature. The cells were again washed and counterstained with DAPI dihydrochloride (Hi Media, India) (0.01 µg/ml) for 1 min. The coverslips containing the cells were then dried and mounted on a glass slide using D.P.X. mountant (Merck). After overnight incubation in the dark, the slides were visualized using an upright microscope (Olympus, Japan). The images were captured and analyzed using Cell Sens software (Olympus, Japan).

The same procedure as mentioned above was followed for the estimation of EMT in TIPE2 knockdown and scrambled control bone cancer cells. It is a single-step immunocytochemical staining process as the antibodies used are conjugated to human Snail-NL557 and Vimentin-NL493 (R&D systems, USA). The intensity of the signals depicting vimentin and Snail was measured in both TIPE2 knockdown and scrambled control bone cancer cells using Cell Sens software (Olympus, Japan).

### 4.2.7. Invasion assay

Invasion is a critical step in tumor metastasis. Therefore, the invasive potential of HOS and U2OS cells after knockdown of TIPE2 was analyzed using a Boyden chamber assay. Briefly, SCR and siTIPE2 cells were serum-starved for 18h before seeding in transwell migration chambers. 24-well, 8mm pore transwell inserts (Cat No. 354480, Corning, USA) pre-coated with Matrigel were used for this assay. After serum starvation, the cells were trypsinized and then $5x10^4$ cells were seeded in 500µl of serum-free medium in the upper chamber of the transwell insert. Thereafter 750µl of MEM and DMEM medium containing 10% FBS was added as a chemo-attractant to the lower chamber for HOS and U2OS cells, respectively. Cells were then incubated for 24 h at 37°C in a $CO_2$ regulated incubator. The non-invading cells on the membrane's upper surface were then scraped off with cotton swabs. The invaded cells present at the bottom of the transwell insert were fixed in 70% ethanol and then stained with 0.01 % (w/v) crystal violet. Stained cells were visualized under an inverted microscope (Nikon Eclipse TS100, Japan), and images were captured using a Nikon 500 camera. The membrane was then dissolved using 1% SDS solution at 37°C for 1 h, and absorbance was read at 595nm using a microplate reader (Molecular Devices, SpectraMax, iD3 multimode reader, Austria).

### 4.2.8. Migration assay

This assay was performed to evaluate the migration potential of HOS and U2OS cells after the knockdown of TIPE2 compared to the scrambled control. For this, siTIPE2 and SCR cells were seeded at a density of $5x10^5$ cells/well in 12 well plates. The migration assay was performed as per the protocol mentioned previously in Chapter 3, Section 3, Subsection 3.2.7. Migration Assay.

Furthermore, migration assay was also performed to analyze the effect of TNF-α, TNF-β and RANKL on the migration potential of TIPE2 knockdown cells. For that, after serum starvation followed by scratching of the wound, TNF-α (0.1nM), TNF-β (0.1nM) and RANKL (0.05 ng/ml) were added to the SCR as well as siTIPE2 cells and then the migration of the cells was evaluated as the same procedure mentioned earlier in Chapter 3, Section 3, Subsection 3.2.7. Migration Assay.

### 4.2.9. Western blot

Western blot analysis was carried out for the confirmation of TIPE2 knockdown in HOS and U2OS cell lines. Further, it was also done to determine the expression of the upstream and the downstream targets. For this purpose, briefly, SCR and siTIPE2 cells were lysed, and the total protein lysates were prepared. The Western blot was performed as per the protocol mentioned previously in Chapter 2, Section 2.2, Subsection 2.2.2. Western blot analysis. In case of phospho (p) antibodies, blocking was done using 5% BSA in TBST. The housekeeping gene α-tubulin was used as the loading control. Further, to determine the expression of different targets in TNF-α, TNF-β and RANKL treated TIPE2 knockdown cells, Western blot was carried out in which cells were lysed after 24 h of treatment with TNF-α (0.1nM), TNF-β (0.1nM) and RANKL (0.05 ng/ml) and the same procedure was followed thereafter. The housekeeping gene GAPDH was used as the loading control.

**Table 4.2. Details of the primary and secondary antibodies used for Western blot.**

| Name | Details | Dilutions used |
|---|---|---|
| Anti-TNFAIP8L2 antibody | ab110389; abcam®, Cambridge, USA | 1:4000 |

| | | |
|---|---|---|
| Anti-α-Tubulin antibody | 2144S; Cell Signaling Technology, Massachusetts, USA | 1:2000 |
| Anti-GAPDH antibody | 2118S; Cell Signaling Technology, Massachusetts, USA | 1: 2000 |
| Anti-Phospho- NF-κB p65 (Ser536) antibody | 3033P; Cell Signaling Technology, Massachusetts, USA | 1: 5000 |
| Anti- NF-κB p65 antibody | 8242P; Cell Signaling Technology, Massachusetts, USA | 1: 4000 |
| Anti-TNF-α antibody | 3707S, Cell Signaling Technology, Massachusetts, USA | 1:1500 |
| Anti-TNF- β antibody [AT15A3] | ab100844, abcam®, Cambridge, USA | 1:2000 |
| Anti-TNF-R1 antibody | 3736, Cell Signaling Technology, Massachusetts, USA | 1:2000 |
| Anti-TNF-R2 antibody | 3727, Cell Signaling Technology, Massachusetts, USA | 1:2000 |
| Anti-RANKL antibody | ab45039, abcam®, Cambridge, USA | 1:2000 |
| Anti-p53 antibody | 2524T; Cell Signaling Technology, Massachusetts, USA | 1: 1000 |
| Anti-p21 antibody | 10-7526; ABGENEX Pvt. Ltd., Odisha , India | 1:1000 |
| Anti-LC-3B antibody | 2775S; Cell Signaling Technology, Massachusetts, USA | 1: 1000 |
| Anti- SQSTM1 - p62 antibody | 8025T,Cell Signaling Technology, Massachusetts, USA | 1: 2000 |
| Anti-COX-2 antibody | 12282P; Cell Signaling Technology, Massachusetts, USA | 1: 2000 |
| Anti-survivin antibody | 2808BC; Cell Signaling Technology, Massachusetts, USA | 1: 2000 |
| Anti-Runx2 antibody | 12556S, Cell Signaling Technology, Massachusetts, USA | 1: 2000 |
| Anti-caspase-3 antibody | 20-1039; ABGENEX Pvt. Ltd., Odisha, India | 1: 2000 |
| Anti-CDK2 antibody | 2546; Cell Signaling Technology, Massachusetts, USA | 1: 2000 |
| Anti-CDK6 antibody | 3136; Cell Signaling Technology, Massachusetts, USA | 1: 2000 |
| Anti-cyclin D3 antibody | 2936; Cell Signaling Technology, Massachusetts, USA | 1: 2000 |
| Anti-cyclin A2 antibody | 4656T, Cell Signaling Technology, Massachusetts, USA | 1:2000 |
| Anti-cyclin E2 antibody | 4132; Cell Signaling Technology, Massachusetts, USA | 1: 2000 |
| Anti-MMP-2 antibody | 4022S; Cell Signaling Technology, Massachusetts, USA | 1: 2000 |

| | | |
|---|---|---|
| Anti-MMP-9 antibody | 13667P; Cell Signaling Technology, Massachusetts, USA | 1: 1000 |
| Anti-E-Cadherin antibody | 3195S, Cell Signaling Technology, Massachusetts, USA | 1:1000 |
| Anti-N-Cadherin antibody | 13116; Cell Signaling Technology, Massachusetts, USA | 1: 2000 |
| Anti-TWIST1 antibody | 46702; Cell Signaling Technology, Massachusetts, 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

Statistical analysis was performed using Student's *t*-test. All the data are represented as Mean±SE. $p$-value < 0.05 was denoted as statistically significant.

## 4.3. Results and discussion

In this chapter, we determined the role of TIPE2 on the regulation of various cancer hallmarks. At first, the knockdown of TIPE2 in HOS and U2OS cell lines was carried out separately. Subsequently, we studied the effect of the knockdown of TIPE2 on the proliferation, survival, cell cycle progression, EMT, invasion, migration and autophagy of HOS and U2OS bone cancer cells. Further, we identified the upstream and downstream molecular targets of TIPE2. We have also studied the effect on the proliferation, survival and migration of TNF-α, TNF-β and RANKL treated TIPE2 knockdown cells and also the underlined mechanism of action of TIPE2 in TNF-α, TNF-β and RANKL induced bone carcinogenesis.

### 4.3.1. Confirmation of knockdown of TIPE2

The knockdown of TIPE2 in HOS and U2OS human bone cancer cells were done with the help of siRNA method of gene editing. Among two siRNA targets provided for TIPE2, transfection with target 2 siRNA generated successful knockdown clones for TIPE2. The knockdown was confirmed by Western blot analysis (**Figure 4.1.**). The scrambled control and the TIPE2 knockdown cells were used for various studies.

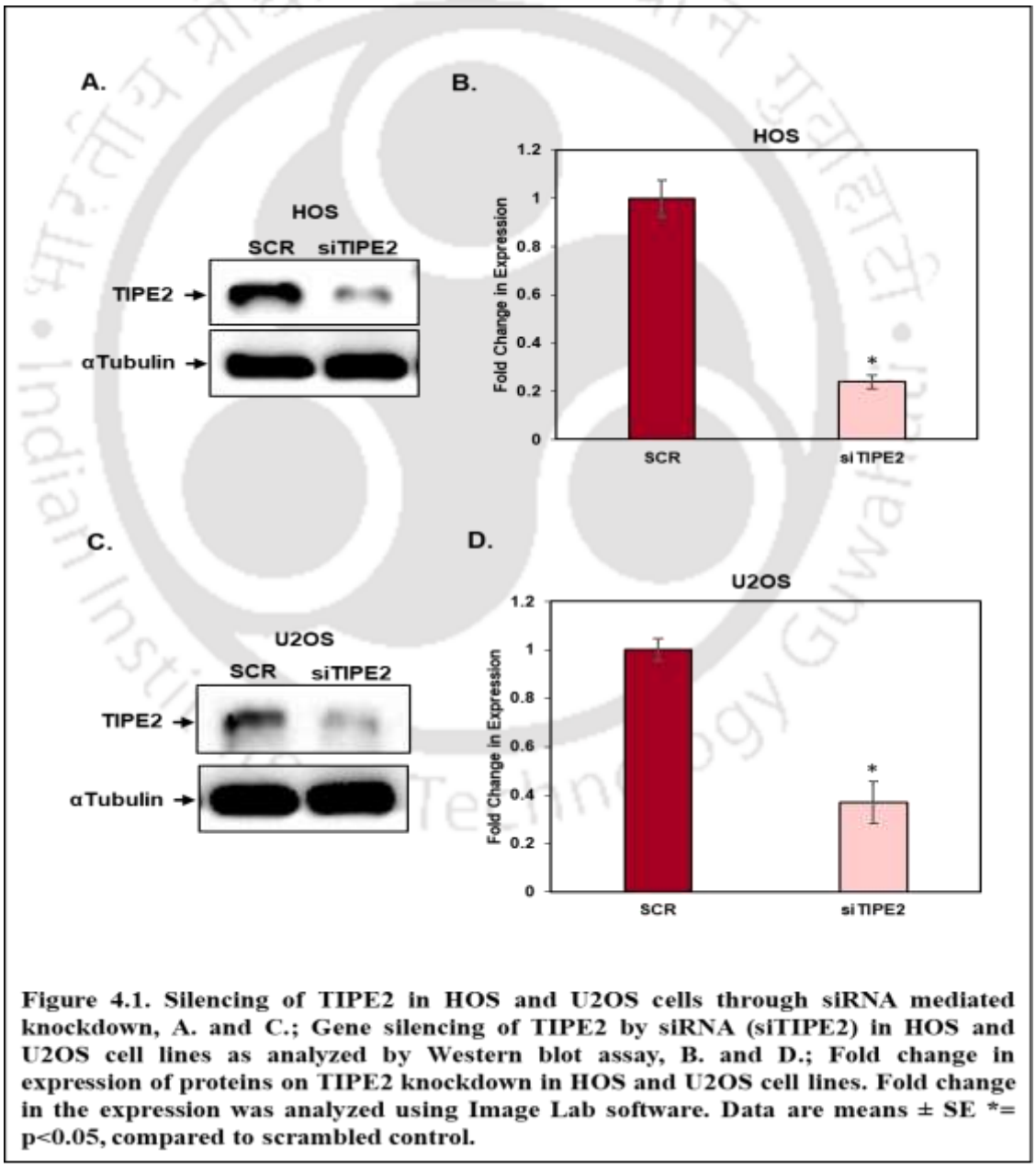


**Figure 4.1. Silencing of TIPE2 in HOS and U2OS cells through siRNA mediated knockdown, A. and C.; Gene silencing of TIPE2 by siRNA (siTIPE2) in HOS and U2OS cell lines as analyzed by Western blot assay, B. and D.; Fold change in expression of proteins on TIPE2 knockdown in HOS and U2OS cell lines. Fold change in the expression was analyzed using Image Lab software. Data are means ± SE *= $p<0.05$, compared to scrambled control.**

**4.3.2. The effect of TIPE2 knockdown on the proliferation and survival of bone cancer cells.**

Augmented proliferation and survival, suppressed apoptosis are some of the main characteristics presented by cancer cells which are attained through modulation of diverse signaling cascades (Kurgan, N., *et al.*, 2017). Therefore, we determined the effect of knockdown of TIPE2 on the proliferation of human bone cancer cells with the help of MTT assay. Our results showed that knockdown of TIPE2 resulted in significant reduction in proliferation compared to scrambled control (**Figure 4.2. (A)**). The percentage inhibition of proliferation of TIPE2 knockdown HOS and U2OS cells were found to be significantly downregulated compared to the scrambled control cells after 24 h. This is the first report that showed the participation of TIPE2 in enhancing the proliferation of bone cancer cells. Our results are supported by a few previous findings as well. In line with our findings, Bordoloi, D., and group showed that knockout of TIPE2 led to the reduction in proliferation of lung cancer cells, and it was through the regulation of AKT/mTOR/NF-κB signaling cascade (Bordoloi, D., *et al.*, 2019). Furthermore, in RCC tissues, a significant increase of TIPE2 expression and downregulation of MX1 were observed compared to the normal controls. Moreover, TIPE2 expression was positively correlated with TNM staging and negatively with MX1 expression, denoting the function of TIPE2 in RCC pathogenesis (Zhang, Z., *et al*., 2013). However, few studies also reported contrasting results than our study. For instance, TIPE2 was shown to be downregulated in human breast cancer, and its overexpression inhibited the proliferation of tumor cells and tumor xenograft growth (Wang, K., *et al.*, 2017). In addition, suppression of TIPE2 enhanced the Ral expression and AKT activation, and in turn, resulted in increased cell migration and reduced cell

death, showing that TIPE2 is a potential tumor suppressor (Gus-Brautbar, Y., *et al.*, 2012). Altogether, the results obtained are in accordance with our previous findings, and it can be said that TIPE2 is plausibly involved in the positive regulation of bone carcinogenesis.

Aforementioned, apart from enhanced proliferation, an upsurge in survival is also a primary characteristic of cancer cells. Hence, to determine the effect of the knockdown of TIPE2 on the survival of HOS and U2OS cells, colony formation assay was performed. This assay determines the clonogenic potential of the cancer cells, which can be demarcated as the ability of a cell to proliferate endlessly and hold its reproducibility to give rise to a large colony which in turn gives the measure of the cell survival fraction (Munshi, A., *et al.*, 2005). The results of our study revealed that knockdown of TIPE2 resulted in the reduced clonogenic potential of HOS and U2OS cells compared to scrambled control implying that TIPE2 is involved in increasing the survival of bone cancer cells (**Figure 4.2. (B)**). In accordance with our results, knockdown of TIPE2 was found to reduce the colony formation ability of colon cancer cells (Miao, Z., *et al.*, 2012). Furthermore, a study carried out by our research group have shown that knock out of TIPE2 in lung cancer resulted in a drastic reduction in the colony-forming ability of lung cancer cells (Bordoloi, D., *et al.*, 2019). However, overexpression of TIPE2 resulted in an inhibitory effect on the clonogenic potential of human NSCLC cells in a study conducted by Li and group, in contrast, our results revealed the opposite effect (Li, Z., *et al.*, 2016).

In order to unravel the mechanism of action of TIPE2 in the proliferation and survival of bone cancer cells, it is imperative to analyze the association of TIPE2 in the modulation of the signaling molecules involved in proliferation and survival. For this

purpose, expression analysis of various proliferation and survival-related proteins were carried out using Western blot (**Figure 4.2. (C)**). Our results demonstrated that knockdown of TIPE2 resulted in downregulation of the expression of apoptosis regulatory protein survivin and upregulated the expression of caspase-3. Survivin is a member of the inhibitors of apoptosis protein (IAP) family and is involved in the inhibition of apoptosis (Erkanli, S., *et al.,* 2007). The caspase-3 protein is a prototypical apoptotic executioner which is activated by either caspase-8 or caspase-9, and upon activation, it can cleave several structural and regulatory proteins, which are critical for cell survival and maintenance (Parrish, A. B., *et al.,* 2013). Runt-related transcription factor 2 (Runx2) belongs to the Runx family, which contains the DNA-binding domain runt, and is crucial for osteoblast differentiation and maturation of the chondrocytes. As the osteoblast differentiation takes place, Runx2 is weakly expressed in uncommitted mesenchymal cells, and its expression is amplified in preosteoblasts, which reaches the maximal level in immature osteoblasts, and is again suppressed in mature osteoblasts. Runx2 enhances the proliferation of osteoblast progenitors (Komori, T., 2019). Therefore, reduced expression of Runx2 in TIPE2 knockdown cells might be one reason for the decreased proliferation. Further, knockdown of TIPE2 resulted in the suppression of COX-2, which plays an essential role in cellular growth, differentiation and inflammation (Erkanli, S., *et al.,* 2007). Taken together, our results suggest that TIPE2 is responsible for the positive regulation of bone cancer cell proliferation and survival.

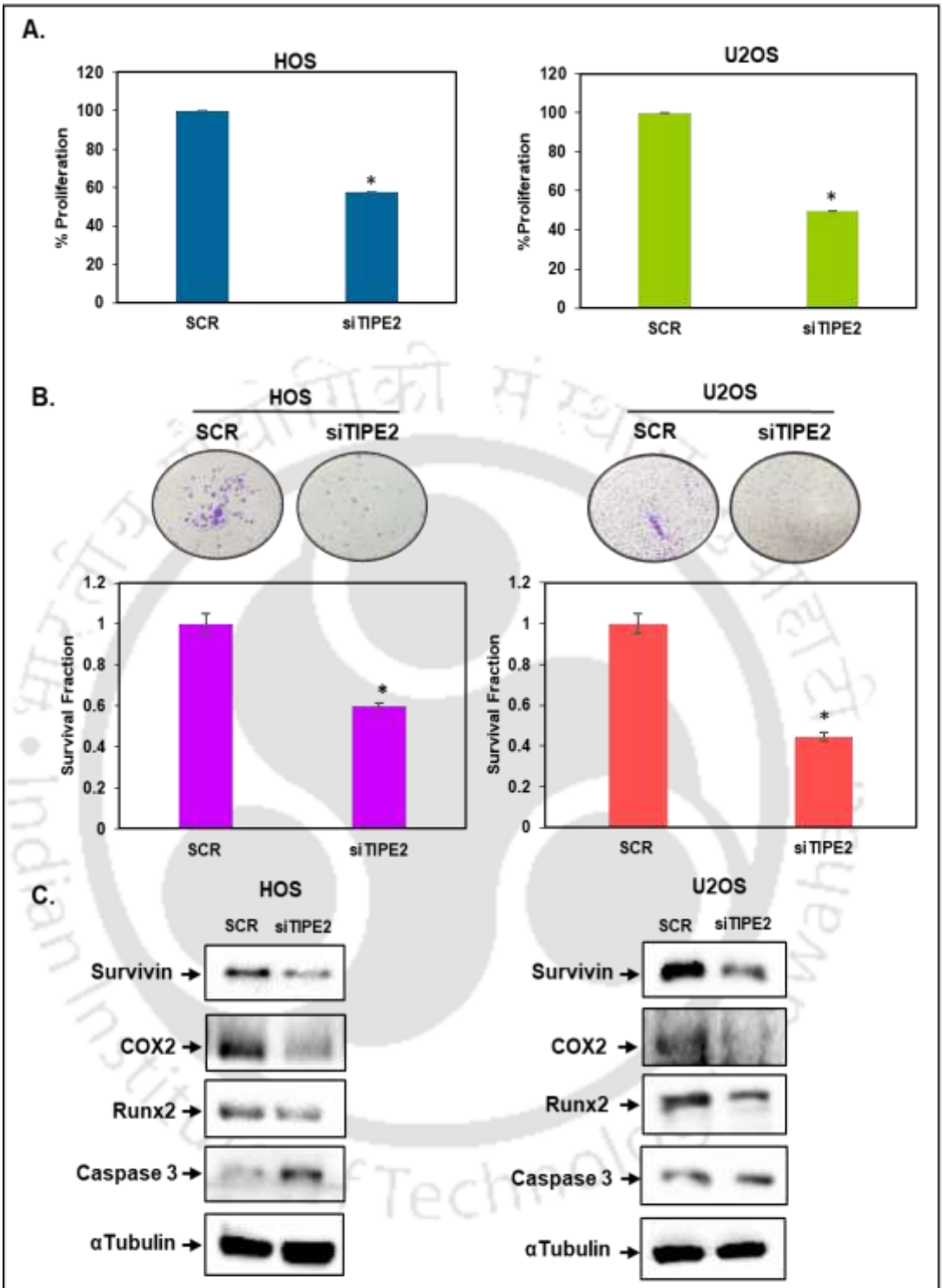


**Figure 4.2 Effect of siRNA mediated knockdown of TIPE2 on proliferation and survival of bone cancer cells A. Graphical representation of effect of knockdown of TIPE2 on proliferation (MTT assay) in HOS and U2OS cells in comparison to the SCR cells B. Representative image and graphical representation of effect of knockdown of TIPE2 on survival fraction of HOS and U2OS cells (Colony forming assay) C. Effect of TIPE2 knockdown on the proteins involved in cell survival and proliferation analyzed by Western blotting. (*$p$ value ≤ 0.05; **=$p$<0.01).**

### 4.3.3 The effect of TIPE2 protein on bone cancer cell proliferation was mediated through regulation of the cell cycle progression

After proving TIPE2 protein to modulate bone cancer cell proliferation, we further analysed if this control of proliferation is mediated through the regulation of cell cycle. Therefore, we examined the effect of silencing of TIPE2 on the cell cycle progression by analysing the percentage of cell distribution in each phase of the cell cycle using flow cytometry and compared the results with the cell cycle distribution in SCR cells (**Figure 4.3. (A), 4.3. (B)**). Analysis of the cell cycle data with FCS express software (BD FACS Celesta™, Becton-Dickinson, New Jersey, USA) revealed that knockdown of TIPE2 results in accumulation of cells at the S phase and a subsequent reduction in G2/M and G0/G1 phases in the HOS cell line. Further, TIPE2 knockdown in the U2OS cell line induced G0/G1 and G2/M phase arrest, unlike the HOS cells (Figure 4.3A and D). Hence, it is confirmed that TIPE2 protein control bone cancer cell proliferation through the regulation of cell cycle progression. Interestingly, as this protein was found to act on different phases of the cell cycle, a possible difference in the molecular targets of TIPE2 protein is suggested in cell cycle regulation. The tumor suppressor protein p53 partakes in the regulation of important biological functions such as metabolic homeostasis, immune functions and cell death, which are meticulously related to several diseases such as tumors, infection, metabolic disorders, and neurodegeneration. It is now well established that the p53 gene is found to be mutated in nearly 50% of human cancer cells (Xu, Z., *et al.,* 2021). p53 regulates a safeguard mechanism that averts the accumulation of abnormal cells and their transformation by controlling DNA repair, cell death, cell cycle progression, or senescence (Lahalle, A., *et al.,* 2021). Cyclin A2 is a crucial modulator of the cell cycle that activates the kinases that particip-

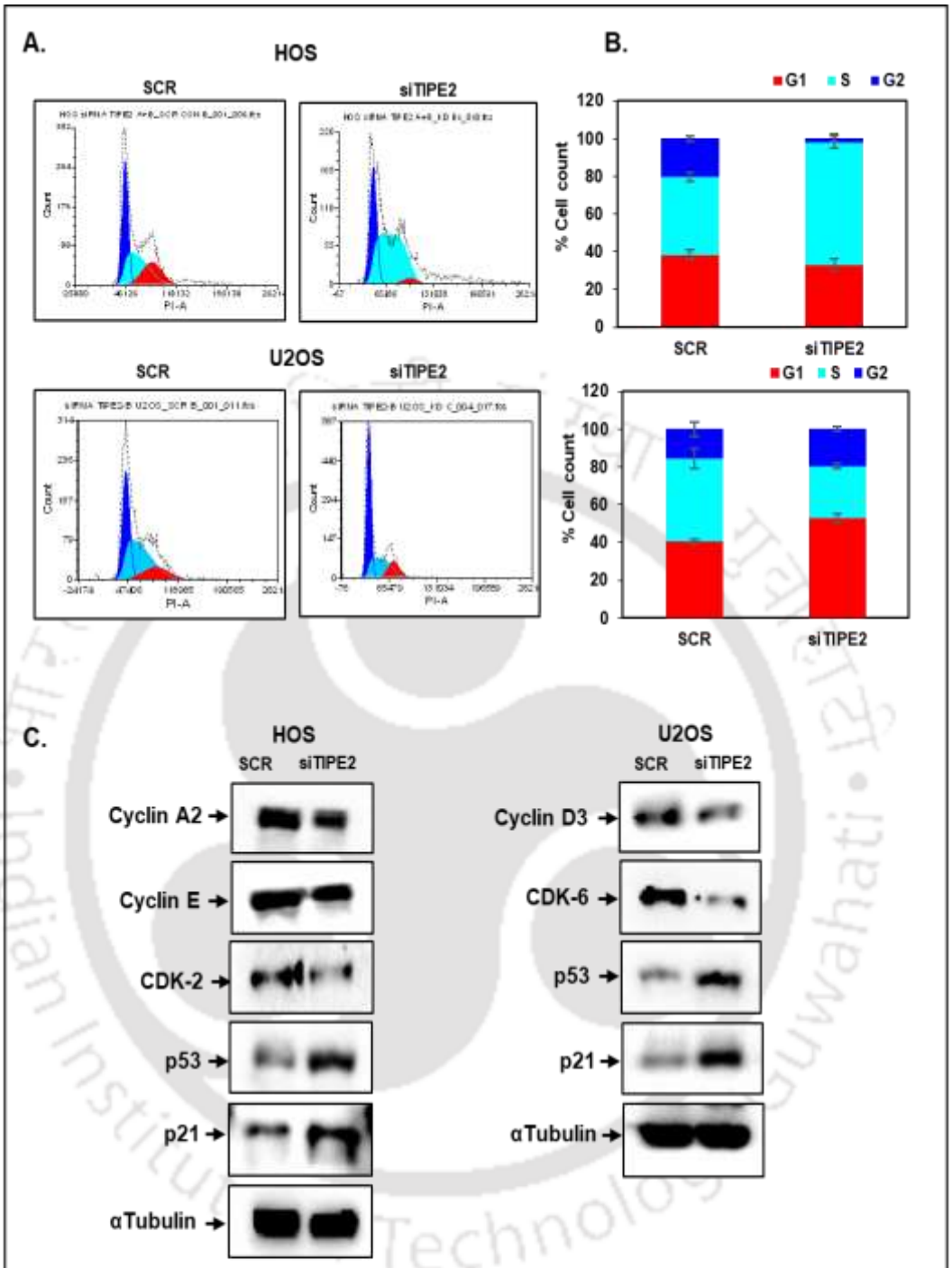


**Figure 4.3. The effect of knockdown of TIPE2 on the cell cycle regulation and its associated proteins in bone cancer cells. A. Cell cycle distribution determined by flow cytometry analysis in TIPE2 knockdown and scrambled control cells, B. Graphical representation of percentage of TIPE2 knockdown cells and scrambled control cells at different stages of cell cycle. C. Representative Western blot image of the cell cycle regulatory proteins in TIPE2 knockdown compared with scrambled control cells. Data are represented as Mean±SE, *denotes $p$ <0.05 compared to scrambled control.**

-ate in the control of S phase of the cell cycle and mitotic entry (Loukil, A., *et al.*, 2015). Cyclin E is expressed in the late G1 phase until the end of the S-phase of the cell cycle and limits the passage of cells through the restriction point, which marks a "point of no return" for cells from G1 into S-phase. The cyclin E binds and activates the kinase CDK-2, and the cyclin E/CDK-2 complexes initiate a cascade of events that leads to the expression of S-phase specific genes (Möröy, T., & Geisen, C. 2004). Cyclin D3 can act as a critical regulator to the differentiation and proliferation of cancerous cells (Wang, B., *et al*., 2019). In the G1 stage, cyclin D proteins are overexpressed and interact with CDK-4 and CDK-6; thereby, it regulates the physiological progression from G1- to S-phase (Ding, Z. Y., *et al.*, 2019). It has been stated that p53 instigates G1-phase arrest by the transcriptional activation of p21, which binds to cyclin E/CDK-2 and cyclin D/CDK-4 complexes (Chen, J., 2016). Notably, our study showed that silencing of TIPE2 led to the upregulation of p53. TIPE2 knockdown in the HOS cell line was found to repress cyclin E, cyclin A2, and CDK2 and upregulate p21 and p53 protein expression, which lead to the arrest of the cells at the S-phase of the cell cycle. Besides, in the U2OS cell line, knockdown of TIPE2 resulted in suppression of G1-specific cell cycle regulatory protein cyclin D3 and CDK-6 and enhanced expression of p21 and p53 proteins (**Figure 4.3. (C))**. Therefore, it has been proven that reduction in the proliferation of bone cancer cells acquired by silencing of oncogenic TIPE2 protein is mediated through inhibition of cell cycle progression as the accumulation of cells in S and G2/M or G0/G1 phase and consequent decrease in other phases of the cell cycle takes place.

### 4.3.4. TIPE2 protein also enhances autophagy in bone cancer cells.

Autophagy is a well-established conserved catabolic cellular mechanism that acts on the intracellular components for degradation and recycles the protein and organelles to generate biomolecules like nucleotides, amino acids, sugars, fatty acids, and ATP to survive under stress conditions, such as hypoxia and nutrient deprivation (Maiti, A., & Hait, N. C., 2021; Yu, L., *et al.,* 2018; Cheon, S. Y., *et al.,* 2018). Mounting evidence suggests that autophagy plays an intricate role in cancer. Autophagy helps in conserving physiological tissue homeostasis by several mechanisms, including improving metabolic/redox homeostasis and sustaining stemness by averting senescence; as a result, it inhibits malignant transformation (Mathew, R., *et al.,* 2009; García-Prat, L., *et al.,* 2016). Autophagy acts as both pro-metastatic and also as an anti-metastatic factor. In the early stages of tumor metastasis, autophagy has been shown to be related to anti-metastasis via the limitation of cancer necrosis and inflammatory responses (Salah, F. S., *et al.,* 2016; Michaud, M., *et al.,* 2011).

Conversely, recent studies have advocated that autophagy endorses tumor progression, survival, and colonization in different cancers. It is activated in the central part of the solid tumors, where the cells are exposed to enormous stressful conditions, such as hypoxia and nutrient deprivation. Autophagy facilitates the cancer cells to overcome these stress conditions. Autophagy helps to cope with the high metabolic demands of proliferating tumors by reutilizing intracellular components to supply metabolic substrates. Therefore, one more aspect of autophagy is to contribute towards tumor-cell survival by augmenting the nutrient supply to meet metabolic demands providing them stress tolerance (Maiti, A., & Hait, N. C., 2021; Yu, L., *et al.,* 2018; Cheon, S. Y., *et al.,* 2018). The membrane-bound microtubule-associated protein chain 3 (LC3) is one of the most specific biomarkers of autophagy. In mammals, LC3B has broad tissue

specificities and is extensively used in autophagy-related studies. Though Lazova and group demonstrated that autophagy plays a major role in cancer progression, the function of LC3B in primary tumors remains debatable because autophagy has also been shown to contribute to the suppression of tumorigenesis (Chen, S., *et al.*, 2013; Qu, X., *et al.*, 2003). It is well known that p62 is a classical receptor of autophagy. Several studies have demonstrated that the physiological role of p62 is to control osteoclastogenesis and bone remodelling (Durán, A., *et al.*, 2004). It is a multifunctional protein present throughout the cell and is involved in the regulation of signal transduction pathways. It participates in the proteasomal degradation of ubiquitinylated proteins. The proteasome inhibition can impose proteotoxic stress that can activate autophagy through p62 phosphorylation (Moscat, J., & Diaz-Meco, M. T., 2012). Therefore, to access the effect of TIPE2 knockdown on the autophagy of HOS and U2OS bone cancer cells, we performed the immunocytochemistry. Our study showed that TIPE2 knockdown considerably increased autophagy of both HOS and U2OS cells, as demonstrated by the increase in the intensity of LC3B expression in TIPE2 knockdown cells compared to the scrambled control (**Figure 4.4**. **(A)**). Similarly, we observed an increase in the expression of LC3B and p62 protein expression in TIPE2 knockdown cells compared to the scrambled control using the Western blot method (**Figure 4.4**. **(B)**). This indicated that TIPE2 is involved in increasing the autophagy in bone cancer cells. In line with our results, TIPE2 overexpression diminished autophagy by decreasing the level of expression of p-Smad-2, p-Smad-3, and TGF-β in rectal adenocarcinoma cells; on the other hand, TIPE2 knockdown showed opposite effects (Wu, D. D., *et al.*, 2019). Henceforth, this is the first report that showed that TIPE2 might have a significant role in autophagy in bone cancer cells.

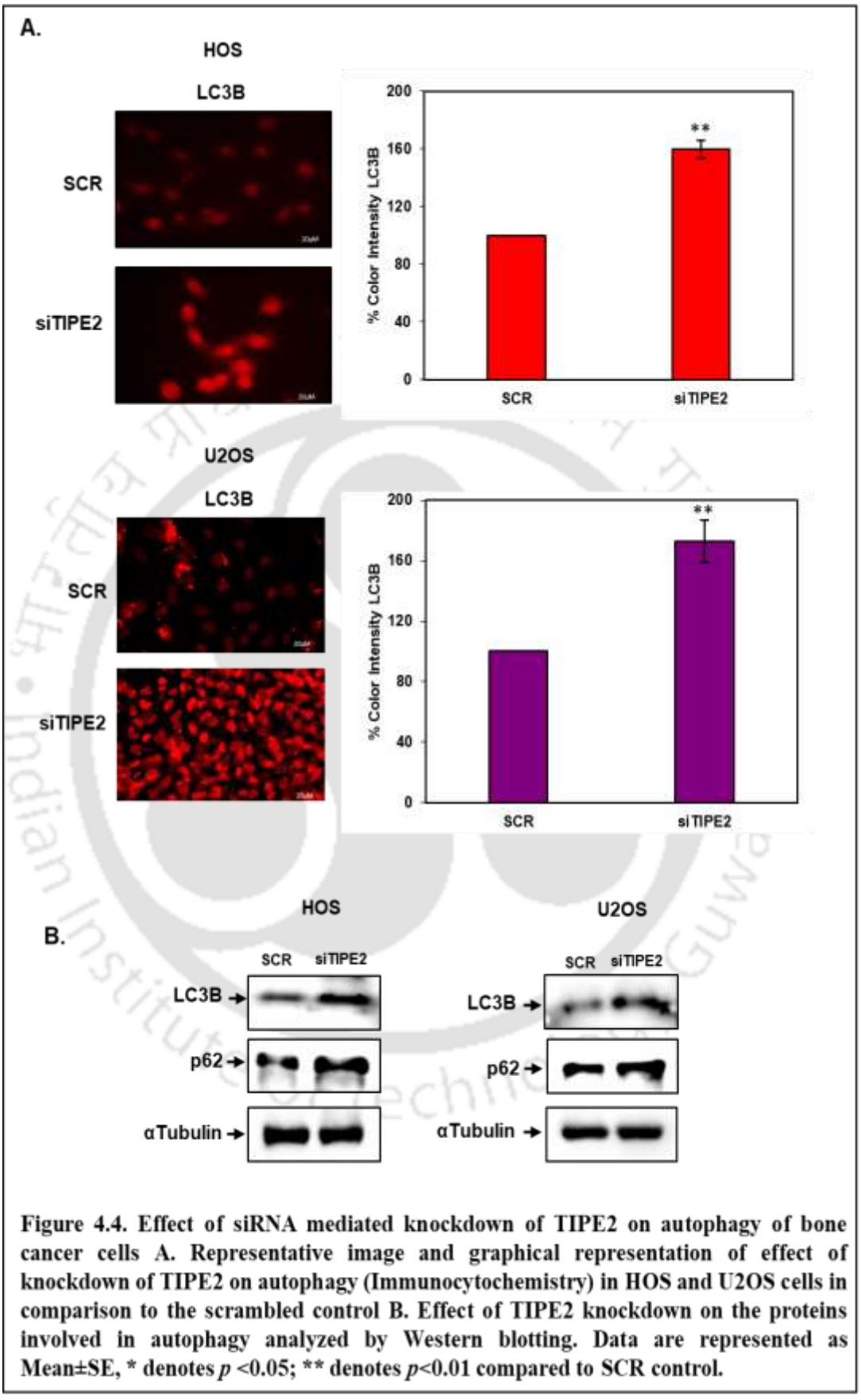


**Figure 4.4. Effect of siRNA mediated knockdown of TIPE2 on autophagy of bone cancer cells A. Representative image and graphical representation of effect of knockdown of TIPE2 on autophagy (Immunocytochemistry) in HOS and U2OS cells in comparison to the scrambled control B. Effect of TIPE2 knockdown on the proteins involved in autophagy analyzed by Western blotting. Data are represented as Mean±SE, * denotes $p$ <0.05; ** denotes $p$<0.01 compared to SCR control.**

### 4.3.5. Silencing of TIPE2 protein altered the EMT of bone cancer cells.

Aforesaid, most bone cancer cases are diagnosed at a late-stage or metastatic stage. Bone cancer metastasis involves enhanced motility, survival in circulation, and the ability to form new tumors by exhibiting epithelial to mesenchymal transition (EMT) and enhanced survival and migratory signals (Liu, F., *et al*., 2020; Jayarangaiah, A., & Theetha Kariyanna, P., 2020). From our previous findings, it is evident that TIPE2 has a crucial role in inducing bone cancer cell proliferation, and survival, as silencing of TIPE2 remarkably downregulated these processes. Hence, to determine whether TIPE2 is involved in the EMT of bone cancer cells, we performed EMT immunocytochemistry assay. EMT is a physiological process that indicates a change of phenotype from an epithelial, polarized cell to a cell that displays mesenchymal characteristics, aiding cellular plasticity and cancer cells' adaptability (Escalante, P. I., *et al.,* 2021; Le Magnen, C., *et al.,* 2018; Vessoni, A. T., *et al.,* 2020; Lu, W., & Kang, Y., 2019). The reverse process, also known as mesenchymal-epithelial transition (MET), may also occur in cancerous cells. For instance, EMT may allow the migration of cancer cells from the primary tumor site to a metastatic focus, where a new population of cancer cells originate from the tumor propagating cells via MET (Escalante, P. I., *et al.,* 2021; Le Magnen, C., *et al.,* 2018; Vessoni, A. T., *et al.,* 2020; Lu, W., & Kang, Y., 2019). The activation of EMT also helps in the development of cancer stem cell (CSC) from cancer cells. This CSCs are characterized mainly by stem cell phenotype, low differentiation capability, low proliferation rate and an inherent resistance to several chemotherapeutic agents (Escalante, P. I., *et al.,* 2021; Le Magnen, C., *et al.,* 2018; Vessoni, A. T., *et al.,* 2020; Lu, W., & Kang, Y., 2019). EMT is a process where there is a shift from the expression of the epithelial marker, E-cadherin, to the improved

expression of mesenchymal markers, including N-cadherin and vimentin; these changes occur mainly at the transcriptional level due to the activity of EMT transcription factors, such as Slug, Snail, and Twist1 (Nieto, M. A., *et al.*, 2016; Zhang, Y., *et al.*, 2019). The result of this assay revealed that silencing of TIPE2 significantly inhibited the EMT in both HOS and U2OS cell lines (**Figure 4.5. (A)**). The immunocytochemistry analysis revealed that the mesenchymal markers, i.e. vimentin and snail, were downregulated in TIPE2 knockdown cells compared to the scrambled control. Thus, the TIPE2 protein is involved in the regulation of EMT of bone cancer cells. Furthermore, western blot analysis of proteins related to EMT of cancer cells was carried out. Our results demonstrated that silencing of TIPE2 resulted in repressed expression of EMT regulatory proteins, for instance, N-cadherin, and Twist1 and increased expression of E-cadherin in both HOS and U2OS cell lines (**Figure 4.5. (B)**). However, in contrary with our results, TIPE2 was found to inhibit the invasion and migration of endometrial cells by targeting β-catenin to reverse EMT in adenomyosis condition (Liu, Y., *et al.*, 2020). Additionally, forced expression of TIPE2 obstructs gastric cancer metastasis through the reversal of EMT (Yin, H., *et al.*, 2017). Another study revealed that TIPE2 overexpression hindered the invasion and migration of breast cancer cells by preventing the EMT phenotype (Wang, K., *et al.*, 2017). Similarly, in prostate cancer, TIPE2 overexpression led to the suppression of EMT process and, in turn, inhibited invasion/ migration (Lu, Q., *et al.*, 2016). Hence, this is the first report that showed that TIPE2 might have a significant role in sustaining EMT in bone cancer cells.

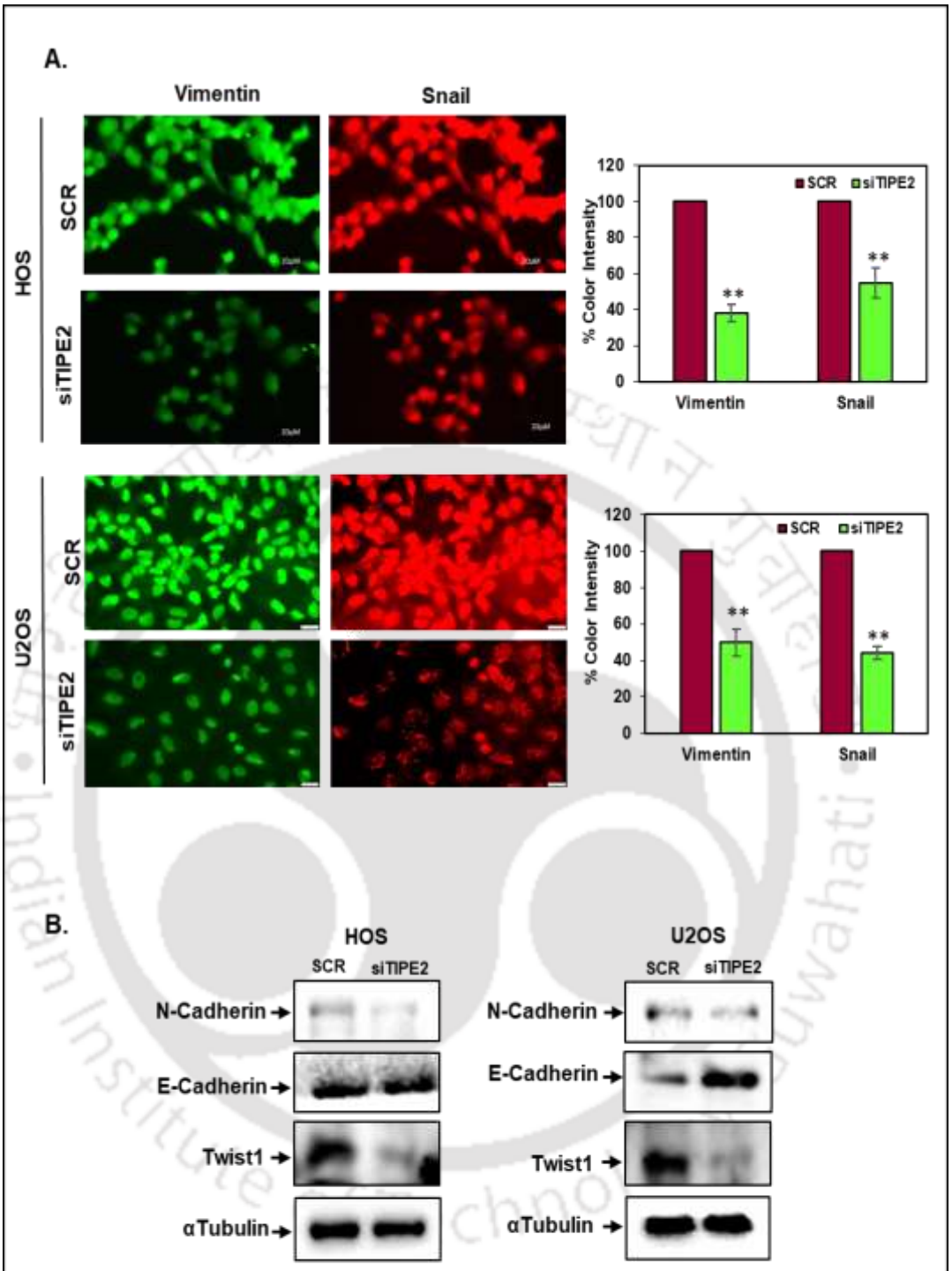


**Figure 4.5. Effect of siRNA mediated knockdown of TIPE2 on EMT of bone cancer cells A. Representative image and graphical representation of effect of knockdown of TIPE2 on EMT (Immunocytochemistry) in HOS and U2OS cells in comparison to the scrambled control B. Effect of TIPE2 knockdown on the proteins involved in EMT analyzed by Western blotting. Data are represented as Mean±SE, * denotes $p$ <0.05; ** denotes $p$<0.01 compared to SCR control.**

**4.3.6. TIPE2 protein plays a crucial role in the regulation of invasion and migration of bone cancer cells.**

As previously mentioned, in bone cancer, the majority of the patients are presented with a highly advanced stage of the disease. The ability of bone cancer cells to migrate and invade the nearby cells is strongly related to their high metastatic potential. In metastasis, primary tumor cells migrate and invade the nearby tissues and thus form secondary tumor sites. An increasing line of evidence advocated that diverse signaling molecules are present in the tumor microenvironment, which play a significant role in modulating the migratory properties of the cancer cells (Luanpitpong, S., *et al.*,2010). Therefore, it is indispensable to determine whether TIPE2 has any role in the invasion and migration of bone cancer cells. Numerous epigenetic and genetic alterations in cancer cells cause activation of different signaling cascades, enhancing growth, survival, invasion and migration of cancer cells (Rao, G., *et al.,* 2017). Invasion to neighboring tissues by the cancer cells, which occurs either locally or distally through metastasis, is considered as one among the most critical hallmarks of cancer. It is mainly mediated via interactions between tumor and extracellular matrix and cancer-related fibroblasts (Haney, S., *et al*., 2018; Hanahan, D., & Weinberg, R. A., 2011). Therefore, we have performed Boyden chamber assay to determine whether siRNA mediated knockdown of TIPE2 have any effect on the invasive potential of human bone cancer cells. We observed that the number of cells that invaded to the lower part of the transwell insert were remarkably less in TIPE2 knockdown HOS and U2OS cells compared to scrambled control cells (**Figure 4.6. (A)**). Thus, TIPE2 is involved in the regulation of the invasive potential of bone cancer cells. The effect of TIPE2 on the invasive potential of different cancers was studied by different groups. Multiple studies

reported that TIPE2 suppresses cell invasiveness in different cancers such as in human rectal adenocarcinoma cells (Wu, D. D., *et al.*, 2019); papillary thyroid cancer (Jia, W., *et al.*, 2018); esophageal carcinoma (Zhu, L., *et al.*, 2018); osteosarcoma (Deng, B., *et al.*, 2015); lung cancer (Li, Z., *et al.*, 2016); gastric cancer (Wu, J., *et al.*, 2016) and HCC (Cao, X., *et al.*, 2013). These results are in contrast to our finding and thus suggest that TIPE2 possesses tumorigenic potential in bone cancer cells.

In order to establish the involvement of TIPE2 in disease progression, migration assay/wound healing assay was carried out. The results showed that the silencing of TIPE2 inhibited the migration potential of bone cancer cells effectively (**Figure 4.6. (B)**). In case of HOS scrambled control, almost complete healing of the wound was observed at 24 h, whereas in TIPE2 knockdown U2OS cell cells, slightly less healing in the wound was observed. As the wound area remaining was more compared to the scrambled control cells at 24 h time point, we can conclude that TIPE2 regulates the migratory potential of the bone cancer cells (Figure 4.4). In line with our studies Bordoloi, *et al.*, have shown that knock out of TIPE2 in lung cancer resulted in a drastic reduction in the migration potential of lung cancer cells (Bordoloi, D., *et al.*, 2019). However, contrary to our results, TIPE2 overexpression was demonstrated to exert an inhibitory effect on the migration potential of the lung and prostate cancer cells (Li, Z., *et al.*, 2016; Lu, Q., *et al.*, 2016).

In order to explore the mechanism of action of TIPE2 in the migration and invasion of bone cancer cells, it is vital to analyze the association of TIPE2 in the modulation of the signaling molecules involved in invasion and migration. For this purpose, Western blot analysis of proteins related to invasion and migration of cancer cells were carried out (Figure 4.4). Our results demonstrated that knockdown of TIPE2 resulted in

downregulation of the expression of invasion and migration regulatory proteins MMP-9 (**Figure 4.6.(C)**). During metastasis, tumor cells are involved in numerous interactions with the extracellular matrix (ECM). MMPs play an imperative role in tissue remodelling during several physiological processes, such as morphogenesis, embryogenesis, wound repair and angiogenesis. MMPs have been regarded as major critical molecules assisting tumor cells during metastasis. Several studies over the years have concluded that there has been a strong association between MMPs, ECM degradation and cancer cell invasion. MMPs are a family of zinc-dependent ECM remodelling endopeptidases that have the ability to degrade many components of the ECM. Increased expression of MMP-2 and MMP-9 is frequently associated with augmented cell invasion, migration and cancer metastasis (Cabral-Pacheco, G. A., *et al.*, 2020; Luo, Y., *et al.*, 2009; Hofmann, U. B., *et al.*, 2000; Deryugina, E. I., & Quigley, J. P., 2006). Altogether, in addition to the involvement of TIPE2 in the proliferation and survival, it is also found to be responsible for the modulation of invasion and migration of bone cancer cells exemplifying their potential in the progression and metastasis of bone cancer cells and their immense therapeutic implications.

### 4.3.7. Effect of TIPE2 knockdown on the bone cancer cell survival, proliferation, autophagy, EMT, invasion and migration was mediated through downregulation of NF-κB signaling axis.

The findings of our previous studies suggest TIPE2 to have a profound role in the promotion of bone cancer cell proliferation, survival, autophagy, EMT, invasion and migration. Notably, there are different signaling molecules/pathways associated with cancer hallmarks (Li, J., & Mansmann, U. R, 2014). In other words, modulation of these

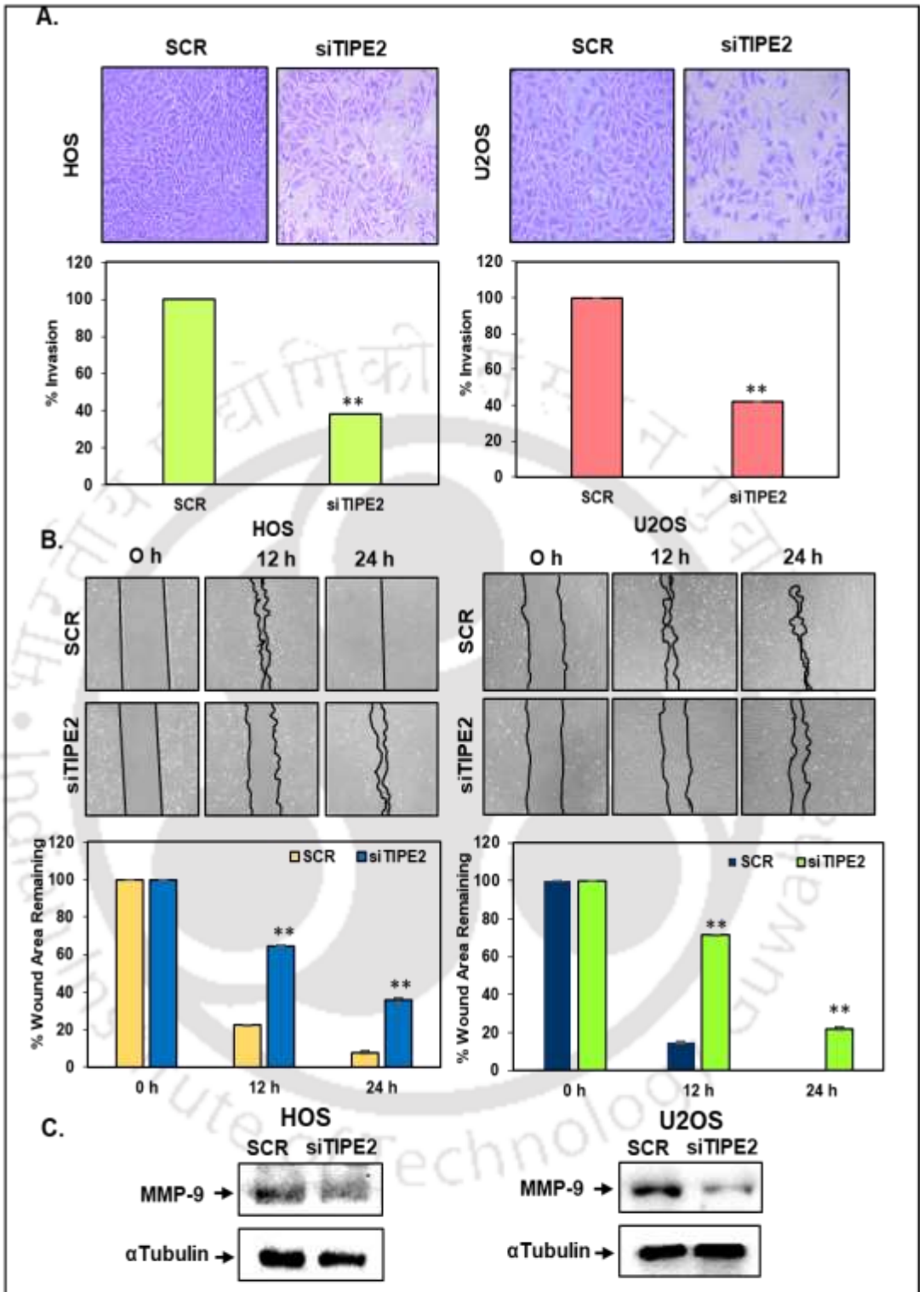


**Figure 4.6. Effect of siRNA mediated knockdown of TIPE2 on invasion and migration of bone cancer cells A. Representative image and graphical representation of effect of TIPE2 knockdown on EMT (Matrigel Invasion Assay) in HOS and U2OS cells B. Representative image and graphical representation of effect of TIPE2 knockdown on migration potential of HOS and U2OS cells C. Effect of TIPE2 knockdown on the proteins involved in migration analyzed by Western blotting. Data are mean + SE. *=$p$<0.05, **=$p$<0.01.**

pathways impact cancer cell growth, proliferation, survival, invasion, migration etc. Therefore, it is essential to know the involvement of these signaling molecules/pathways to decipher the exact molecular mechanism of action of TIPE2 in human bone cancer cells. As mentioned in the earlier sections, our findings revealed that knockdown of TIPE2 resulted in regulating the proteins involved in cell growth, survival, proliferation, cell cycle progression and regulation of apoptosis. For instance, we found that survivin, Runx2, COX2, cyclin D3, cyclin A, cyclin E2, CDK-6 and CDK-2 were found to be downregulated, whereas p53, p21 and caspase-3 were found to be upregulated in TIPE2 knockdown bone cancer cell lines. Further, silencing of TIPE2 induced the expression of LC3B and p62, which are essential in inducing autophagy in cancer cells. Moreover, we have also confirmed that TIPE2 knockdown causes the reversal of EMT and reduced invasion and migration through the repression of the expression of N-cadherin, Twist-1, MMP-2 and MMP-9 proteins and augmentation of E-cadherin protein. To decipher the underlined molecular mechanism of action of TIPE2 mediated bone cancer pathogenesis, we studied the expression of different upstream and downstream signaling molecules involved in bone cancer (**Figure 4.7.**). As mentioned in the previous chapter, TNF-α and TNF- β augmented various cellular events such as cell proliferation, survival, and migration of bone cancer cells and induced the expression of TIPE2 protein upon treatment with TNF-α and TNF-β. It is well established that TNF mediates its biological functions by mainly activating the NF-κB signaling pathway. TNF mainly binds to two receptors, namely TNFR-1 and TNFR-2. Constitutive activation of the TNF-TNFR-1 signaling cascade leads to the aberrant activity of NF-κB signaling pathway. Therefore, we wanted to determine whether TIPE2 mediated bone cancer has any involvement with this signaling axis. The results showed that silencing of TIPE2 did not affect the crucial

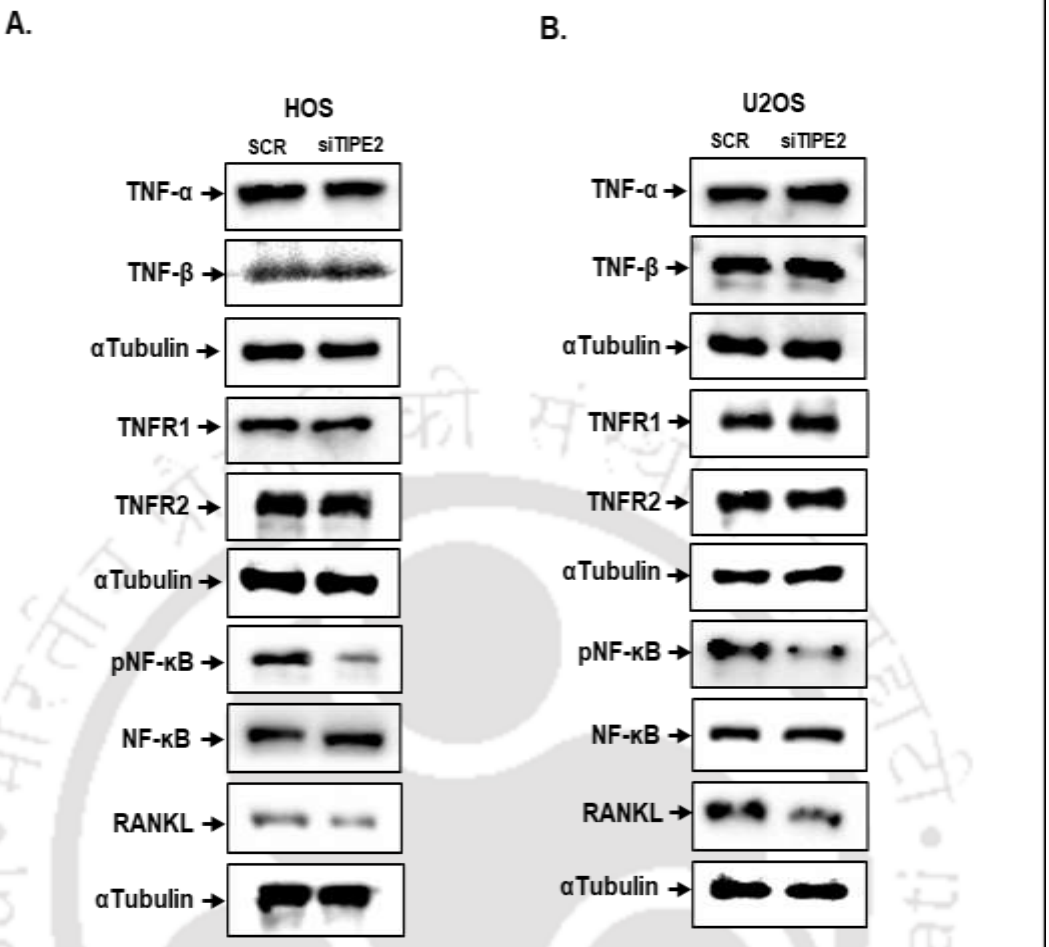


**Figure 4.7. Effect of siRNA mediated knockdown of TIPE2 on the upstream and downstream signaling molecules of TIPE2 A. HOS B. U2OS cells. α-tubulin was used as loading control.**

components of the TNF-TNFR-1 pathway i.e. TNF-α, TNF-β, TNFR-1 and TNFR-2. Further, the knockdown of TIPE2 downregulated the expression of p-NF-κB$^{S536}$ and RANKL notably. As the expression of TNF-α, TNF-β, TNFR-1, and TNFR-2 remain unaltered upon silencing of TIPE2 in bone cancer cells, we can conclude that these signaling molecules might be present upstream of the TIPE2 protein. Moreover, the knockdown of TIPE2 suppressed the expression of NF-κB, which suggests that NF-κB is present downstream of TIPE2. Thus, TIPE2 is found to activate the NF-κB signaling pathway, which contributes to the pathogenesis of bone cancer. Altogether, TIPE2

mediated induction of proliferation, survival, autophagy, EMT, invasion and migration was mediated through the activation of the NF-κB signaling pathway. Noteworthy, this is the first report which shows the involvement of NF-κB signaling axis in TIPE2 mediated bone tumorigenesis.

### 4.3.8. Silencing of TNF-α and NF-κB

The knockdown of TNF-α and NF-κB in HOS and U2OS human bone cancer cells were done with the help of siRNA method of gene editing. The siRNA targets provided for both TNF-α and NF-κB, generated successful knockdown clones. We performed Western blot for the confirmation of knockdown of TNF-α and NF-κB in HOS and U2OS cell lines (**Figure 4.8.**).

### 4.3.9. Silencing of TNF-α and NF-κB to determine the upstream and downstream target of TIPE2

In order to further evaluate the upstream and downstream target of TIPE2, the TNF-α and NF-κB were silenced using siRNA mediated knockdown in both HOS and U2OS cells and the expression of TIPE2 was determined. Knockdown of TNF-α and NF-κB and the expression of TIPE2 post-knockdown was analyzed by western blot analysis from isolated protein lysates 48h post-transfection. Previously, we have shown that silencing of TIPE2 in bone cancer cells did not alter the expression of TNF-α. However, the knockdown of TNF-α in HOS and U2OS cell lines, resulted in significant downregulation in the expression of TIPE2 protein (**Figure 4.8. (A)**). Therefore, we can confirm that TNF-α is present upstream of the TIPE2 protein.

Additionally, we have also shown that silencing of TIPE2 in bone cancer cells significantly suppressed the expression of NF-κB protein. On the contrary, the

knockdown of NF-κB in HOS and U2OS cell lines did not alter the expression of TIPE2 protein. Thus, we can confirm that NF-κB is a downstream target of the TIPE2 protein (**Figure 4.8. (B)**). Hence, our findings, together with the previous studies, confirm that the TNF-α induced bone carcinogenesis is mediated through TIPE2, which regulates the expression of several downstream molecules involved in proliferation, survival, autophagy, EMT, invasion and migration of bone cancer cells through the activation of the NF-κB signaling pathway (**Figure 4.9.)**.

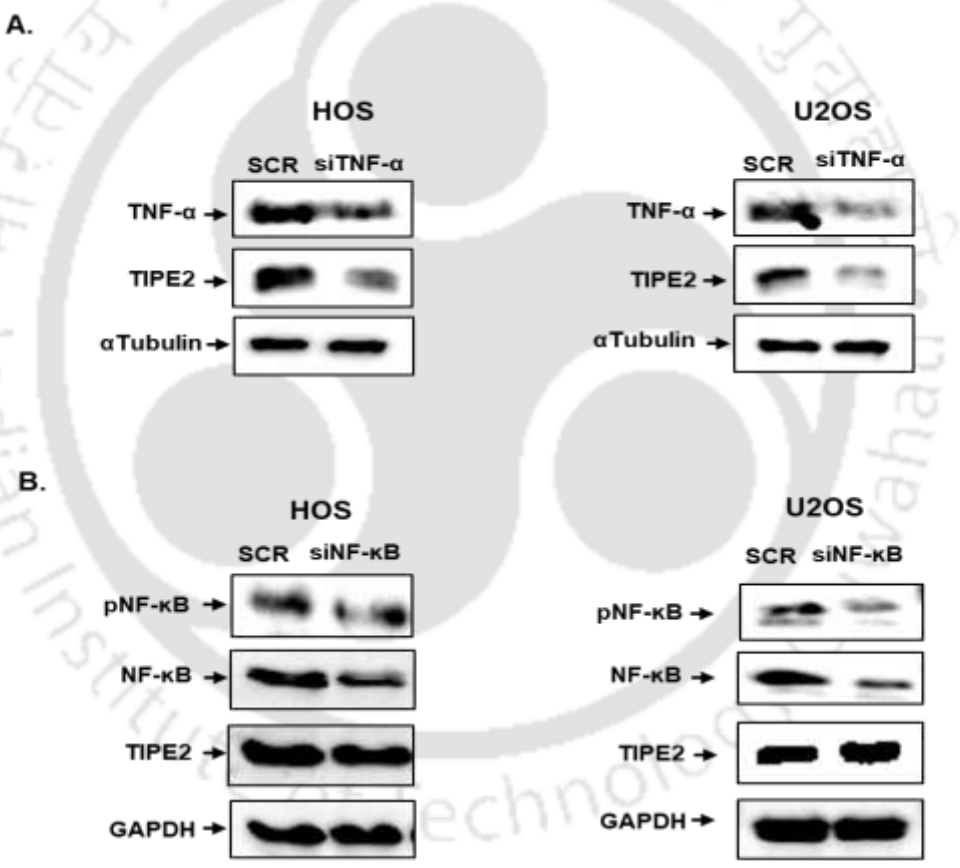


**Figure 4.8. Effect of siRNA mediated knockdown of TNF-α and NF-κB on the expression of TIPE2. A. Representative Western blot image of TNF-α knockdown and its effect on the expression of TIPE2 protein in HOS and U2OS cells. B. Representative Western blot image of NF-κB knockdown and its effect on the expression of TIPE2 protein in HOS and U2OS cells.**

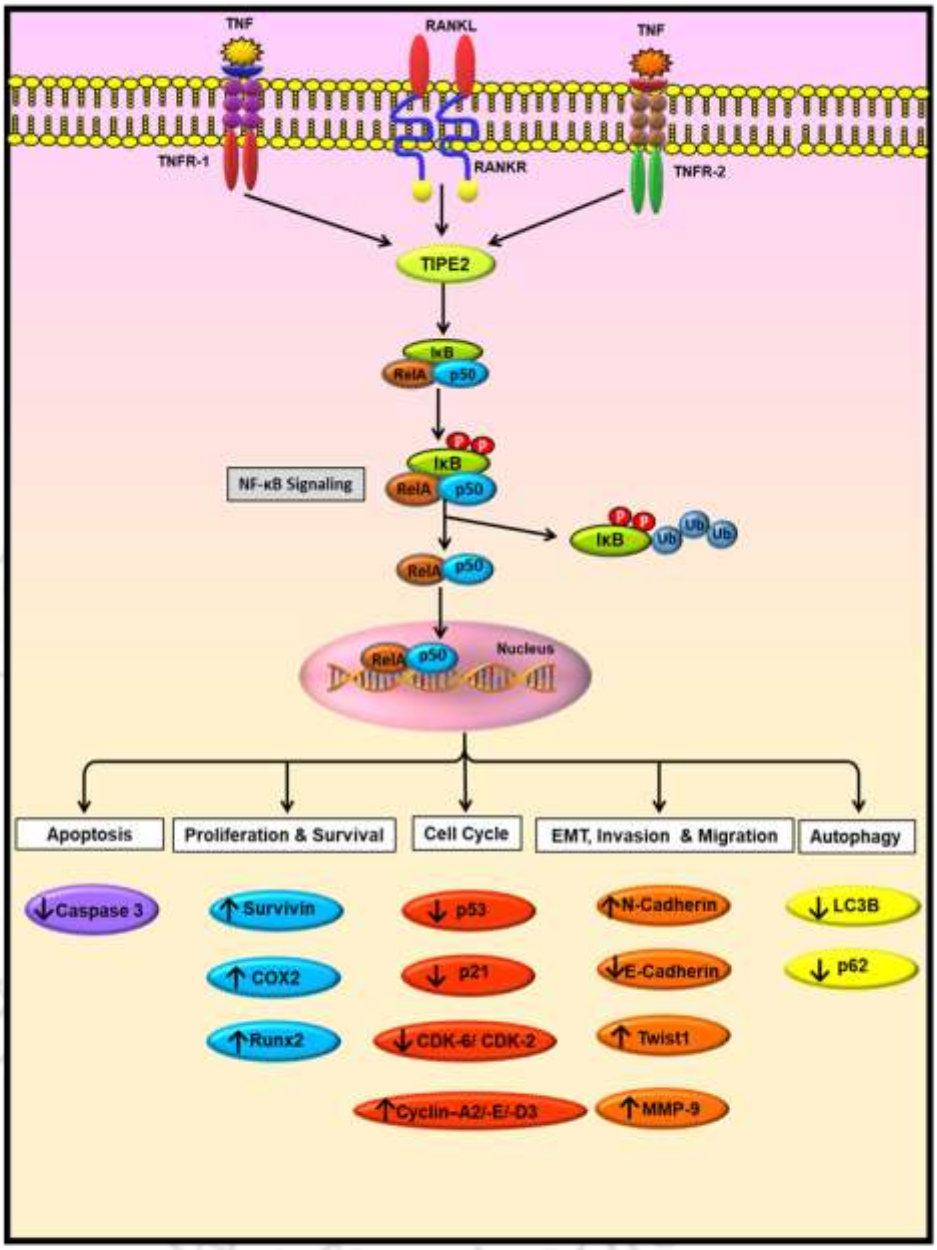


**Figure 4.9. Upstream and downstream targets of TIPE2. TIPE2 induces proliferation, survival, EMT, invasion, and migration and suppresses autophagy through the regulation of NF-κB signaling cascade.**

### 4.3.10. Role of TIPE2 in TNF-α, TNF-β and RANKL mediated bone carcinogenesis

Several epidemiological and clinical data revealed that chronic inflammation might cause cancer development and progression. TNF-α is a multifunctional pro-inflammatory cytokine that endogenously endorses tumor by connecting inflammation and carcinogenesis. Likewise, other studies have also shown the involvement of TNF in different cancer hallmarks such as cell transformation, survival, proliferation, invasion, angiogenesis, and metastasis (Wang, X., & Lin, Y., 2008). TNF-α employs its tumor-inducing effect through activation of the key signaling pathways such as NF-κB (Wang, X., & Lin, Y., 2008). TNF-β has also recently gained more attention as it may activate NF-κB in cancer cells with a potency similar to that of TNF-α, consequently enhancing cancer cells' proliferation, invasion, and metastasis (Buhrmann, C., *et al.*, 2020). Like TNF-α, TNF-β also promotes proliferation, survival, invasion, migration, and colony formation in colorectal and ovarian cancer (Buhrmann, C., *et al.*, 2019b). In addition, RANKL interacts with its receptor, RANK on the surface of the precursor osteoclast cells and triggers the fusion of these cells into multinucleated cells, which ultimately helps them to differentiate into mature osteoclasts (Lacey, D. L., *et al.*, 1998; Dougall, W. C., *et al.*, 1999; Arai, F., *et al.*, 1999). Further, the mature osteoclast cells adhere to the bone surface and stimulate bone resorption by secreting acid and lytic enzymes (Lacey, D. L., *et al.*, 1998; Boyle, W. J., *et al.*, 2003). Disbalance of bone homeostasis, associated with malfunctioning of RANK/RANKL/OPG system underlies the oncological processes such as the destruction of bone, metastasis development, tumor progression (Kushlinskiĭ, N. E., *et al.*, 2014). In the previous chapters, we have discussed that TIPE2 might be involved in TNF-α, TNF-β and

RANKL mediated bone carcinogenesis. We found that TNF-α, TNF-β and RANKL induced proliferation, colony formation and migration potential of HOS and U2OS bone cancer cells and did not induce cell death in the bone cancer cells. We have also discussed the effect of TNF-α, TNF-β and RANKL and their relationship with the TIPE2 protein. Our study has shown that treatment of bone cancer cells with TNF-α, TNF-β and RANKL significantly upregulated the TIPE2 expression. From the above findings, it is substantiated that TIPE2 is involved in the TNF-α, TNF-β and RANKL - mediated proliferation, survival, and migration of bone cancer cells. Therefore, to further validate our previous findings, we exposed the TIPE2 knockdown HOS and U2OS cells with TNF-α, TNF-β and RANKL and examined its effect on the proliferation, survival and migration of the cells. Also, the effect on the expression of the associated proteins that are involved in the proliferation, survival, invasion and metastasis of bone cancer cells was studied.

#### 4.3.10.1. Effect of TNF-α, TNF-β and RANKL on the proliferation and survival of TIPE2 knockdown bone cancer cells

As mentioned earlier, TNFs enhanced the survival, proliferation, angiogenesis, and metastatic potential in most cancer cells by binding to its receptor and activating several molecular pathways such as NF-κB, and MAPKs (Subkamkaew, C., *et al.*, 2019; Wang, X., & Lin, Y., 2008). TNF-induced NF-κB activation helps cells evade apoptosis by inhibition of JNK and upregulation of survival factors, including XIAP and survivin. TNF functions by activating IκB kinase (IKK), which in turn degrade IκB, thereby allowing NF-κB to enter the nucleus and activate the expression of its downstream anti-apoptotic targets (Wang, X., *et al.*, 2007). The RANKL/OPG ratio in the blood is increased in high-grade osteosarcoma, leading to the establishment of a vicious cycle

between pathological bone remodelling and osteosarcoma growth (Navet, B., *et al.*, 2018). There is evidence of the involvement of RANK/RANKL/OPG system in the pathogenesis of bone tumors (Kushlinskiĭ, N. E., *et al.*, 2014). Therefore, to elucidate these cytokines' effect on the proliferation of TIPE2 knockdown cells treated with TNF-α, TNF-β and RANKL, MTT assay was performed (Figure 4.9). Our results showed a significant decrease in the proliferation of TIPE2 knockdown cells treated with TNF-α and TNF-β compared to their respective SCR cells. Similarly, RANKL treated TIPE2 knockdown cells also displayed a significantly reduced proliferation rate compared to its scrambled control (**Figure 4.10.)**.

Furthermore, to determine the effect of these cytokines on the survival fraction of the TIPE2 knockdown cells, we performed the colony-forming assay. Our findings revealed that TNF-α, TNF-β and RANKL treated TIPE2 knockdown cells displayed a lesser colony-forming ability compared to their respective SCR (**Figure 4.11. and Figure 4.12.)**. These results indicate that knockdown of TIPE2 tremendously reduced the clonogenicity of the cytokines treated bone cancer cells. Also, the number of colonies formed on the TNF-α, TNF-β and RANKL treated scrambled controls are relatively higher than their respective untreated scrambled controls. This may be due to the enhancement of the cell's clonogenic potential induced by TNF-α, TNF-β and RANKL treatment. However, in TNF-α, TNF-β and RANKL treated TIPE2 knockdown cells, the number of colonies does not show a prominent increase as compared to the respective untreated TIPE2 knockdown cells. Collectively, this is the first report that indicates that TIPE2 is involved in the positive regulation of TNF-α, TNF-β and RANKL associated growth and survival of bone cancer cells.

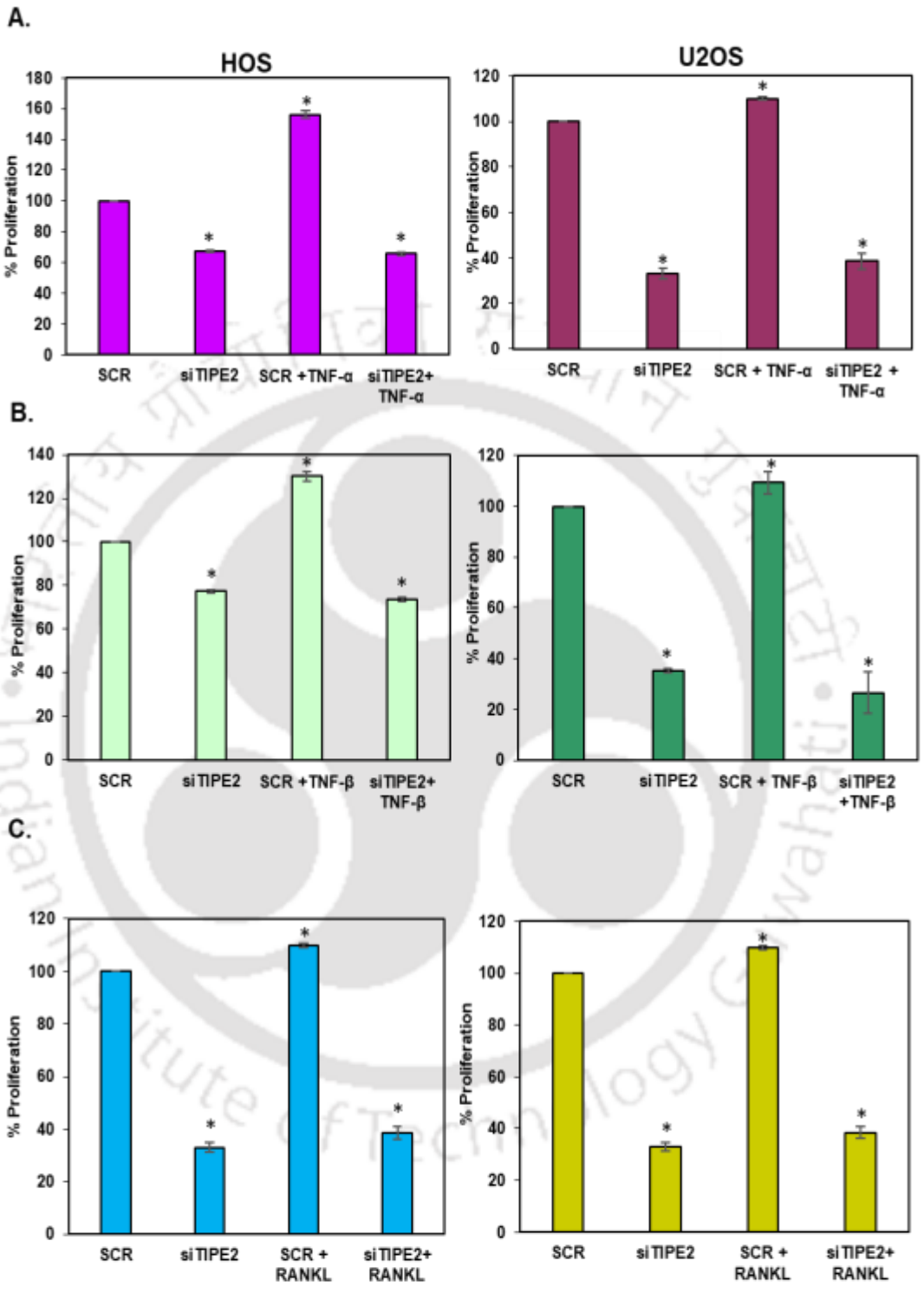


**Figure 4.10. Effect of TNF-α, TNF-β and RANKL treatment on siRNA mediated knockdown of TIPE2 in HOS and U2OS cells. Graphical representation of effect of A. TNF-α, B. TNF-β and C. RANKL treatment on the proliferation of TIPE2 knockdown bone cancer cells (MTT assay) compared to their SCR control. Data are mean + SE. *=$p$<0.05.**

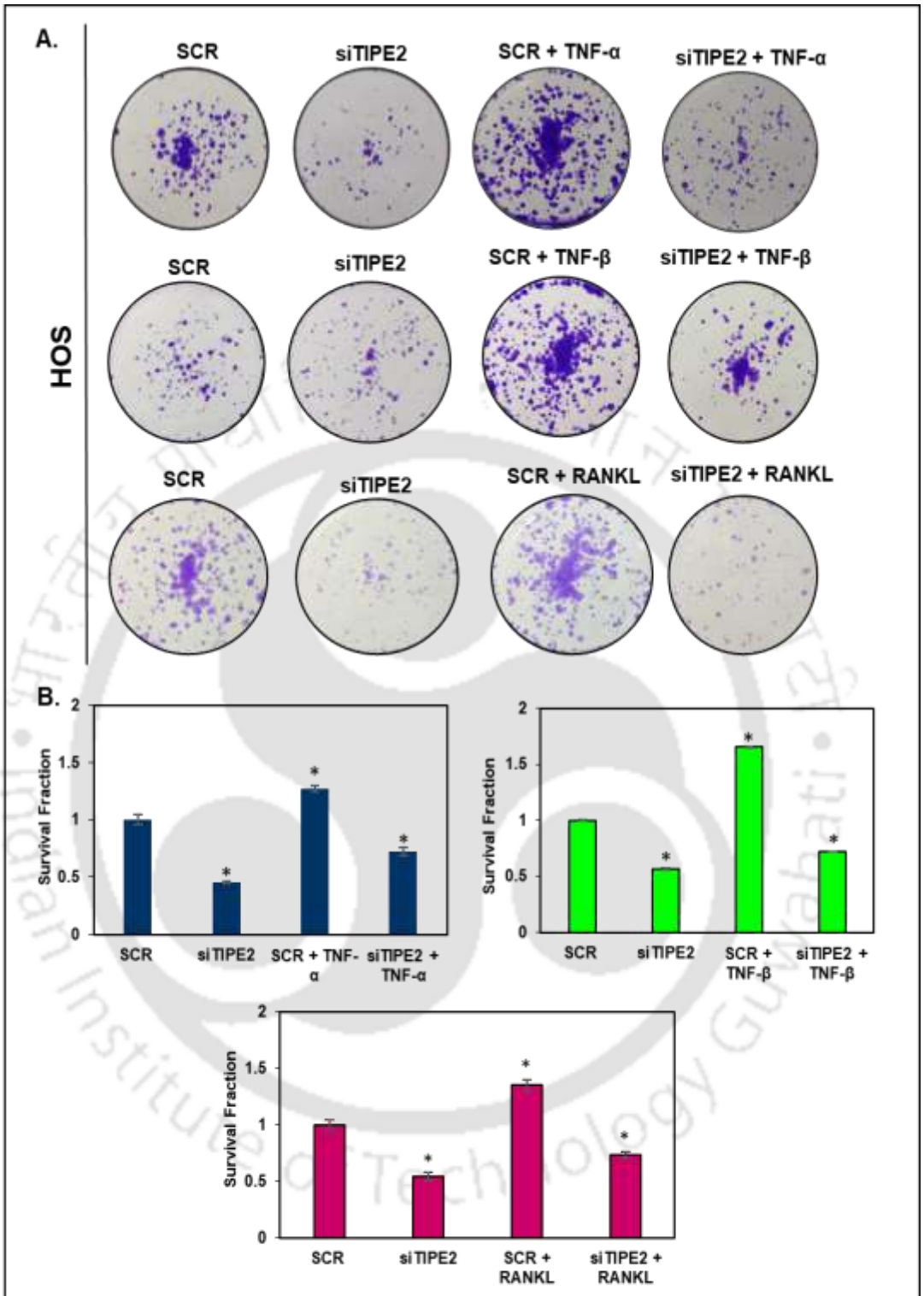


**Figure 4.11. Effect of TNF-α, TNF-β and RANKL treatment on colony forming ability of TIPE2 knockdown HOS cells. A. Representative image of the effect of TNF-α, TNF-β and RANKL treatment on the colony forming ability of TIPE2 knockdown HOS cells (Colony forming assay). B. Graphical representation of the effect of TNF-α, TNF-β and RANKL treatment on the survival fraction of TIPE2 knockdown HOS cells. Data are mean + SE. *=$p$<0.05.**

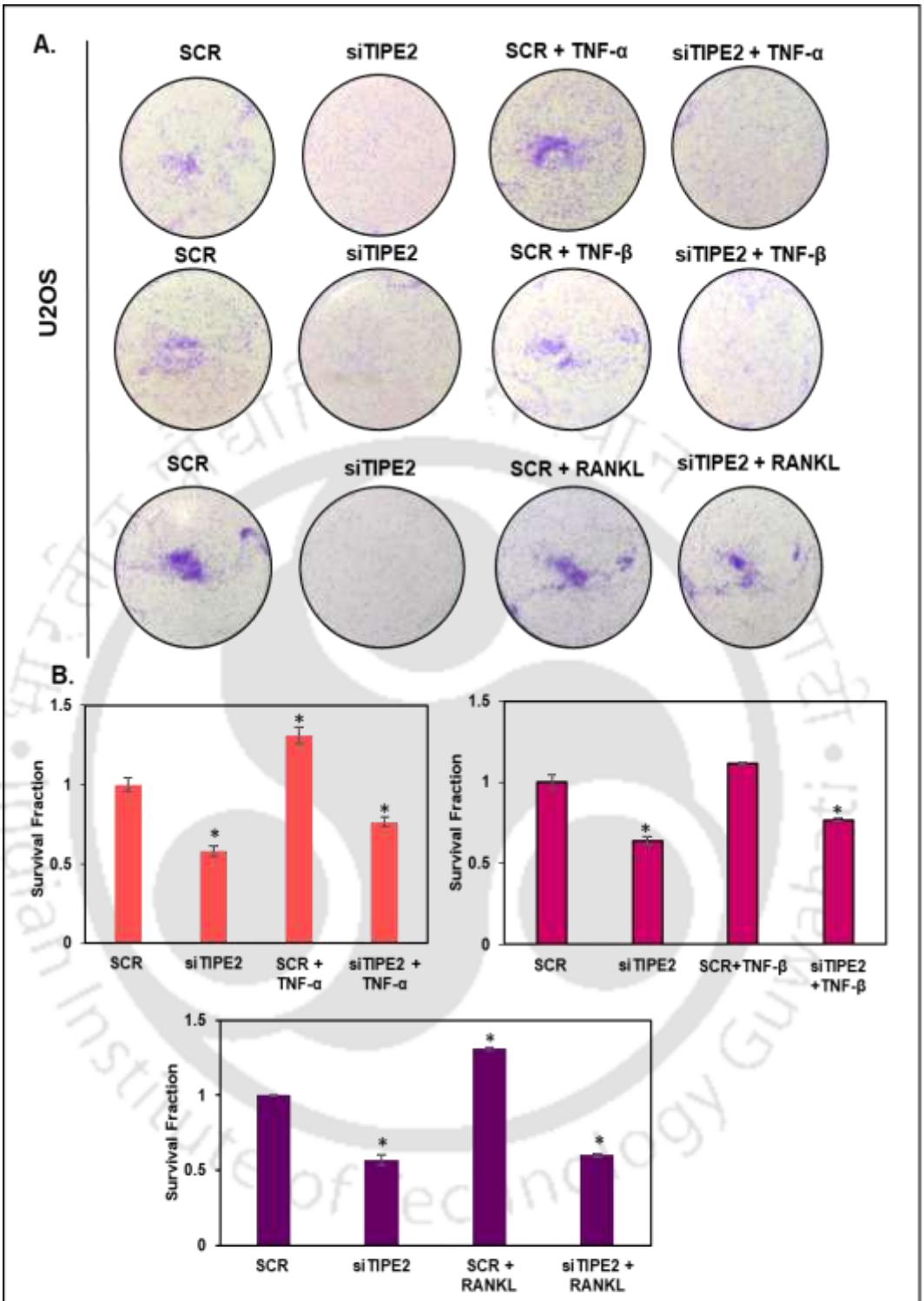


**Figure 4.12. Effect of TNF-α, TNF-β and RANKL treatment on colony forming ability of TIPE2 knockdown U2OS cells. A. Representative image of the effect of TNF-α, TNF-β and RANKL treatment on the colony forming ability of TIPE2 knockdown U2OS cells (Colony forming assay). B. Graphical representation of the effect of TNF-α, TNF-β and RANKL treatment on the survival fraction of TIPE2 knockdown U2OS cells. Data are mean + SE. *=$p$<0.05.**

### 4.3.10.2. Effect of TNF-α, TNF-β and RANKL on the migration potential of TIPE2 knockdown bone cancer cells

As mentioned previously, patients with bone cancer are diagnosed at the advanced or metastatic stage of the disease. The ability acquired by bone cancer cells to migrate and invade nearby cells is strongly linked with their high metastatic potential (Riquelme, M. A., *et al.*, 2020; Okamoto, K., 2021; Coleman, R. E., *et al*. 2020). We have also shown that besides promoting survival and growth, TNF-α, TNF-β, and RANKL also mediate the migrations of cancer cells. Therefore, to study whether TIPE2 is involved in TNF-α, TNF-β and RANKL mediated bone cancer migration, wound healing assay was carried out (**Figure 4.13. and Figure 4.14.**). Our findings revealed that after exposure of both TIPE2 knockdown cells and scrambled control cells to TNF-α and TNF-β for 24h; the TIPE2 knockdown cells exhibited a significantly large area of wound remaining, whereas, in scrambled control, complete healing of the wound was observed after 24h possibly due to TNF mediated enhancement of bone cancer cell migration. Similarly, TIPE2 knockdown cells treated with RANKL displayed a considerably large area of wound remaining, whereas there was almost complete healing of the wound in RANKL treated scrambled control after 24h, which may be due to enhanced migration of the cells in the wound area induced by RANKL. However, post TIPE2 knockdown, the migratory potential of the bone cancer cells significantly reduced even after treatment with TNF-α, TNF-β and RANKL. Hence, our finding ascertained that TIPE2 plays an invaluable role in TNF-α, TNF-β and RANKL - mediated migration and metastasis of bone cancer cells.

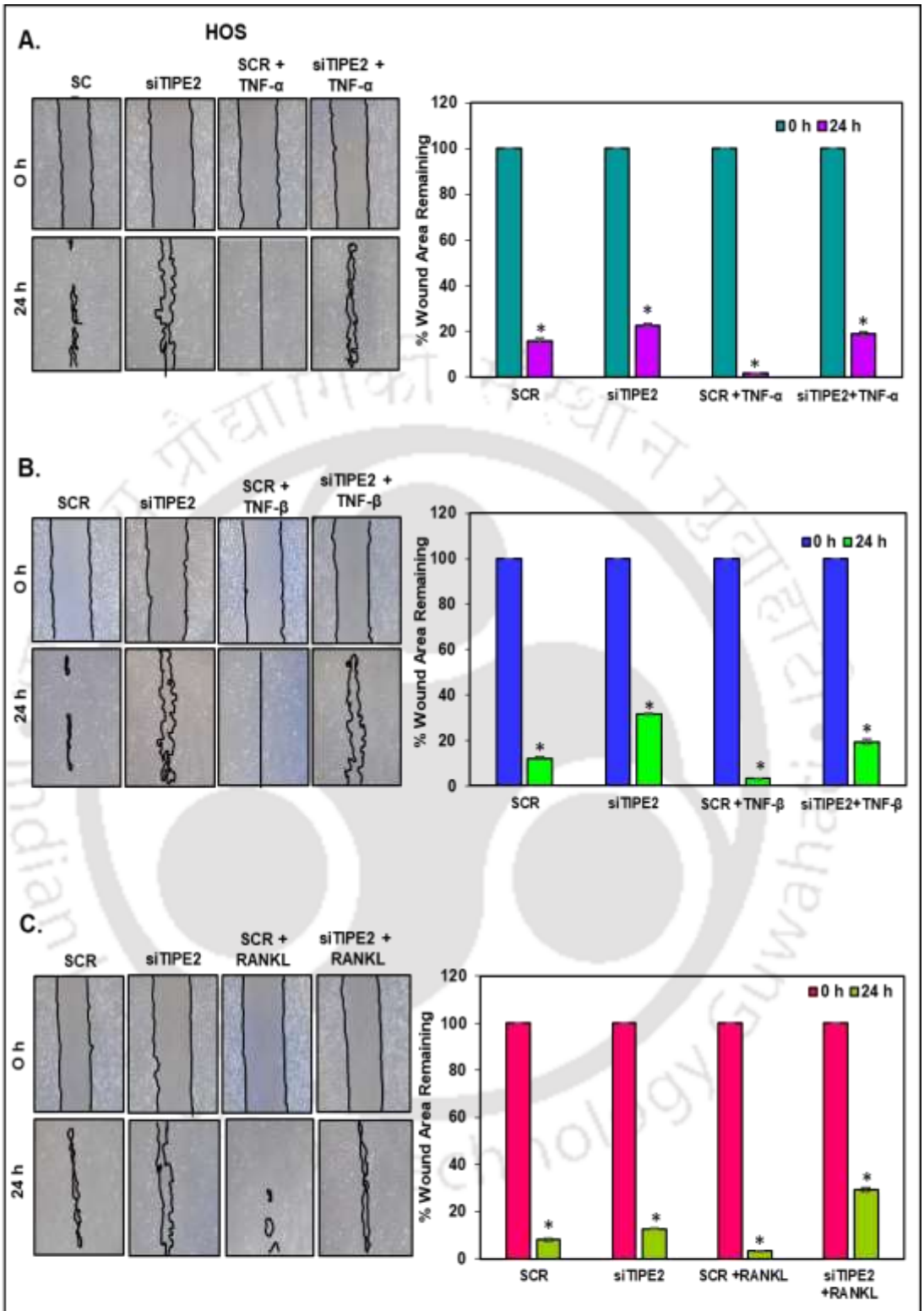


**Figure 4.13. Effect of TNF-α, TNF-β and RANKL treatment on the migration potential of TIPE2 knockdown HOS cells. Representative image and graphical representation of the effect of A. TNF-α, B. TNF-β and C. RANKL treatment on the migration potential of TIPE2 knockdown HOS cells (Wound healing assay). Data are mean + SE. *=$p$<0.05.**

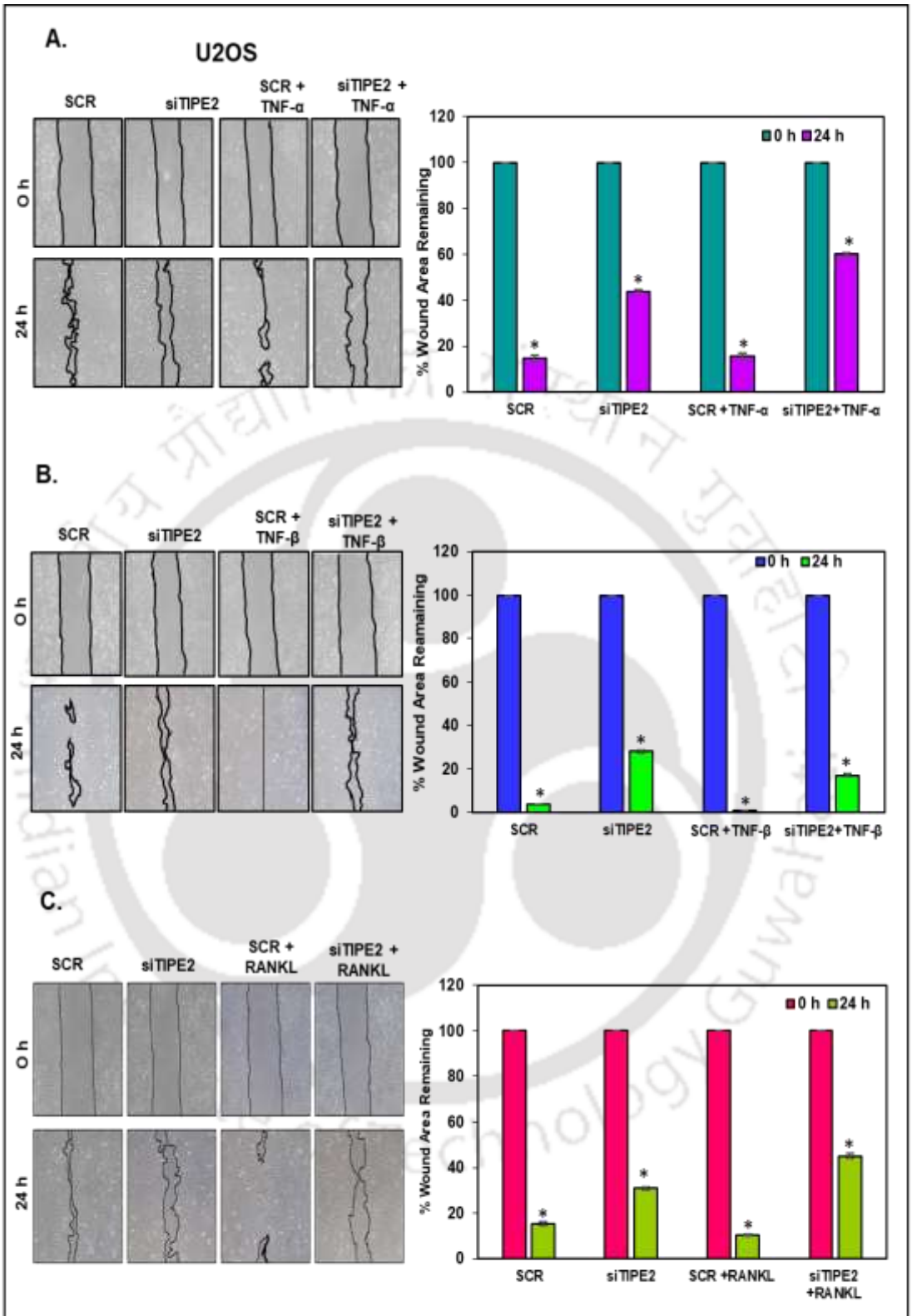


**Figure 4.14. Effect of TNF-α, TNF-β and RANKL treatment on the migration potential of TIPE2 knockdown U2OS cells. Representative image and graphical representation of the effect of A. TNF-α, B. TNF-β and C. RANKL treatment on the migration potential of TIPE2 knockdown U2OS cells (Wound healing assay). Data are mean + SE. *=$p$<0.05.**

**4.3.10.3. Effect of TNF-α, TNF-β and RANKL on the modulation of different molecular pathways in TIPE2 knockdown bone cancer cells**

Widespread research has acknowledged the imperative role of chronic inflammation in tumor initiation and progression. This process is facilitated by the action of inflammatory mediators, comprising of mainly cytokines and chemokines such as TNFs to aid in tumor growth, survival, angiogenesis, invasion and metastasis, and retain a cancer-promoting inflammatory environment (Peng, H. Y., *et al.,* 2020). Further, abnormal TNF expression inside the tumor microenvironment has been linked to augmented invasion, migration, and metastasis of the cancer cells (Mocellin, S., & Nitti, D., 2008). From the above findings, it is substantiated that TIPE2 is involved in the TNF-α, TNF-β and RANKL-mediated proliferation, survival, and migration of bone cancer cells. Thus, to unravel the molecular mechanism of TIPE2 in facilitating TNF-α, TNF-β and RANKL -mediated bone cancer pathogenesis, we performed Western blot analysis to study the expression level of various proteins involved in various malignant processes. Our results showed that TNF-α (**Figure 4.15.**), TNF-β (**Figure 4.16.**) and RANKL (**Figure 4.17.**) treated TIPE2 knockdown cells displayed downregulation of survivin and Runx2 proteins and upregulation of the p21 and p53 protein compared to the TNF-α, TNF-β and RANKL treated scrambled control cells. These proteins are involved in cell growth, survival, proliferation, cell cycle progression and regulation of apoptosis. We have also found that TNF-α, TNF-β and RANKL treated TIPE2 knockdown cells exhibited downregulation in the expression of proteins involved in EMT, invasion, and migration, including N-cadherin and MMP-2 compared to TNF-α, TNF-β, and RANKL treated scrambled control. As it is well evinced that TNF activates vital molecular pathways involved in inflammation-related

carcinogenesis, including the NF-κB pathway (Wang, X., & Lin, Y., 2008; Drutskaya, M. S., *et al.,* 2010). TNFs have been found to induce cancer cell invasion, angiogenesis and metastasis by activation of the NF-κB pathway (Tang, D., *et al.,* 2017; Wang, Z., *et al.,* 2017; Mu, H. Q., *et al.,* 2020). It is well-known fact that aberrant or constitutive NF-κB activation has been implicated in many human malignancies, including bone cancer (Li, W., *et al.,* 2020; Feng, Z. M., & Guo, S. M., 2016). In this study, downregulation of p-NF-κB$^{S536}$ observed in TNF-α, TNF-β, and RANKL treated TIPE2 knockdown cells compared to scrambled control cells treated with the same. Collectively, this is the first report that shows the involvement of TIPE2 in TNF-α, TNF-β and RANKL-mediated proliferation, survival, and migration of bone cancer via modulation of NF-κB signaling pathway and its regulated gene products.

### 4.4. Conclusion

In this chapter, we investigated the role of TIPE2 in the inflection of essential hallmarks of cancer and its accentuated molecular mechanism in bone carcinogenesis. To achieve this, siRNA mediated gene knockdown was performed. Knockdown of TIPE2 in HOS and U2OS bone cancer cells was confirmed with the help of Western blot. Upon confirmation, we analyzed the effect of knockdown of TIPE2 on the proliferation, survival, cell cycle arrest, epithelial to mesenchymal transition (EMT), autophagy, invasion and migration of HOS and U2OS bone cancer cells with the help of different assays such as MTT assay, colony formation assay, cell cycle arrest assay, autophagy immunocytochemistry, EMT immunocytochemistry, wound healing assay and matrigel invasion assay. The results showed that knockdown of TIPE2 caused marked inhibition in the proliferation and clonogenic potential and arrested the HOS and U2OS cells in

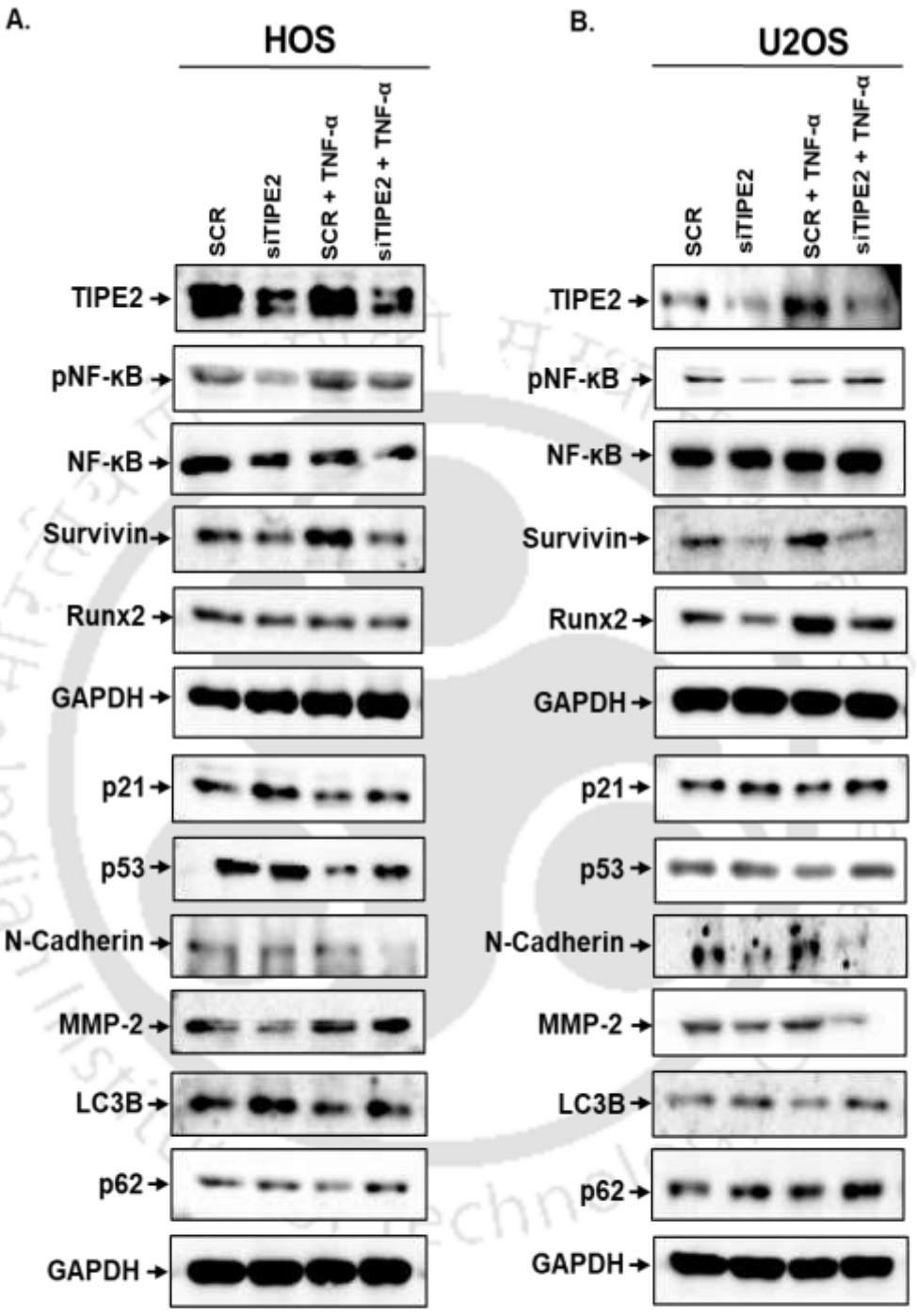


**Figure 4.15. Effect of TNF-α treatment on the on the downstream signaling molecules of TIPE2 in siRNA mediated TIPE2 knockdown of A. HOS and B. U2OS cells.**

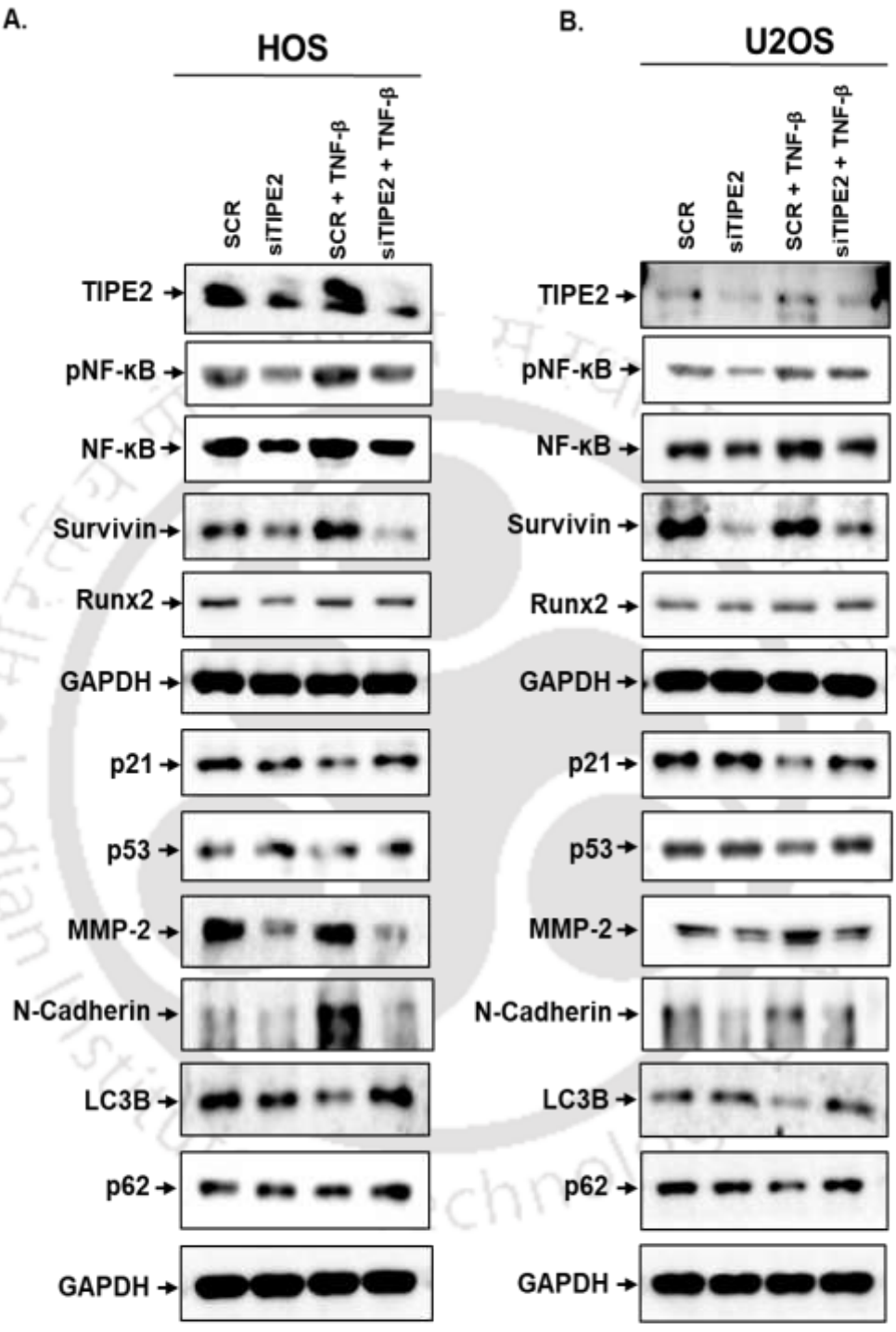


**Figure 4.16. Effect of TNF-β treatment on the on the downstream signaling molecules of TIPE2 in siRNA mediated TIPE2 knockdown of A. HOS and B. U2OS cells.**

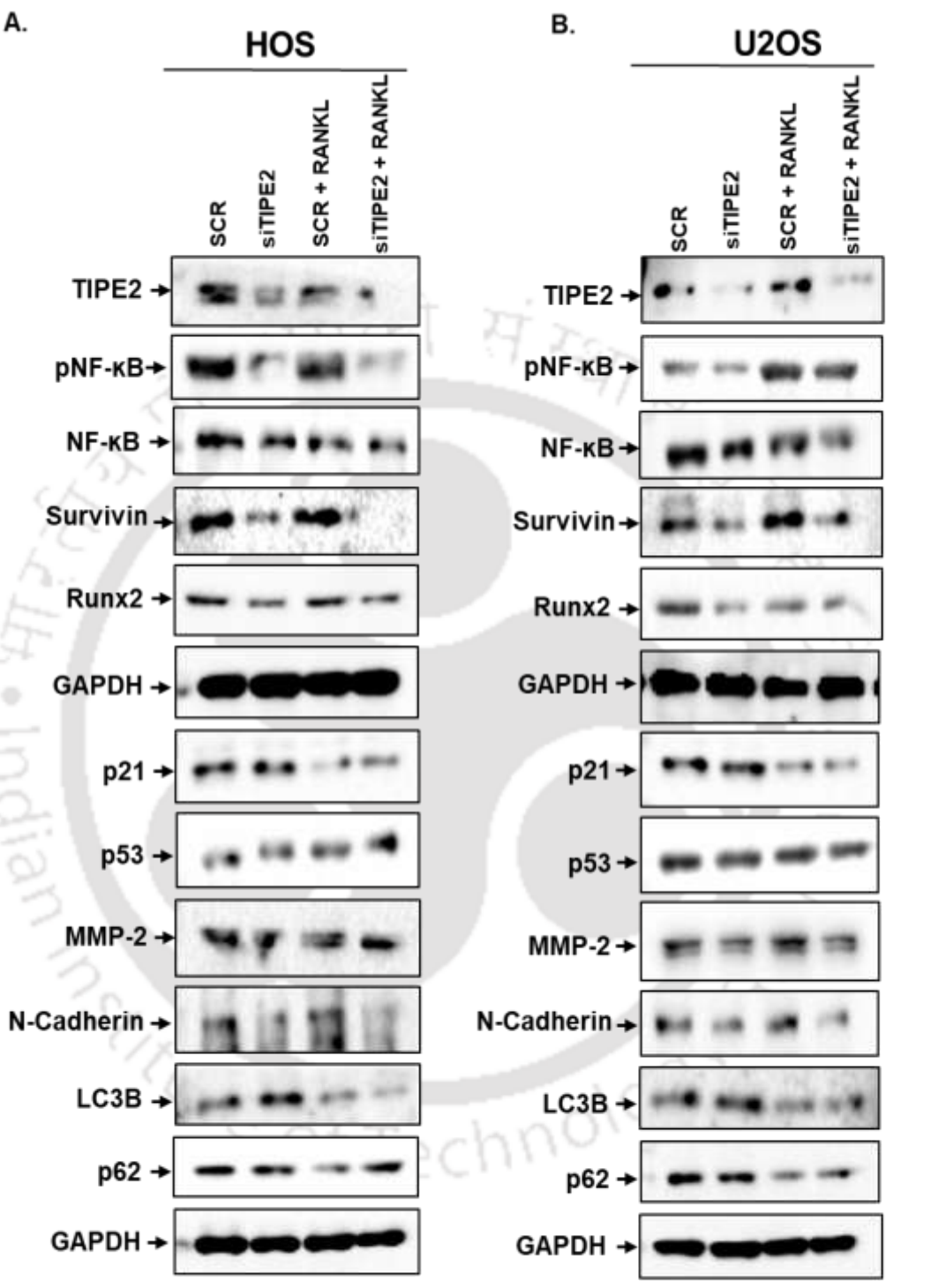


**Figure 4.17. Effect of RANKL treatment on the on the downstream signaling molecules of TIPE2 in siRNA mediated TIPE2 knockdown of A. HOS and B. U2OS cells.**

G1- and S-phase, respectively. Also, TIPE2 knockdown induced autophagy as shown by autophagy immunocytochemistry. Further, knockdown of TIPE2 resulted in the inhibition of the migration of bone cancer cells as evinced by wound healing assay. Moreover, knockdown of TIPE2 caused inhibited invasion of bone cancer cells as suggested by matrigel invasion assay. In addition, knockdown of TIPE2 resulted in the modulation of different proteins involved in growth, proliferation, survival, apoptosis regulation, invasion, angiogenesis, migration and metastasis, such as COX-2, survivin, Runx-2, caspase-3, cyclin D3, cyclin A2, cyclin E, CDK-2, MMP-2, MMP-9, E-Cadherin, N-Cadherin, and Twist-1. In addition, TIPE2 protein was also found to exert its function through regulation of autophagy, as LC-3B and p62 was found to be upregulated in TIPE2 knockdown cells. Furthermore, regulation of the expression of p53 and p21 by TIPE2 also indicates its part in controlling the different hallmarks of cancer. Thus, this protein was found to have a crucial role in the pathogenesis of bone cancer. To elucidate the role of TNF-α and find its relationship with the expression of TIPE2 protein, we performed siRNA mediated knockdown of TNF-α in HOS and U2OS cells and found its effect on the expression of TIPE2 protein. We found that the knockdown of TNF-α reduced the expression of the TIPE2 protein significantly. Therefore, we can confirm that TNF-α is present upstream of the TIPE2 protein. Upon confirming this, we have also validated the role of the oncogenic protein TIPE2 in TNF-α, TNF-β and RANKL induced bone carcinogenesis. Our results showed for the first time that TIPE2 is involved in the positive regulation of TNF-α, TNF-β and RANKL induced proliferation, survival and migration of bone cancer cells. In addition, TIPE2 was found to exert their effect through modulation of NF-κB signaling axis. As siRNA mediated knockdown of NF-κB did not exert any effect on the expression of TIPE2 protein, thus confirming that TIPE2 is upstream of NF-κB and mediates its actions

through regulation of NF-κB signaling pathway. Moreover, the involvement of NF-κB signaling and NF-κB regulated gene products in TIPE2 knockdown bone cancer cells treated with TNF-α, TNF-β and RANKL were observed. Taken together, TIPE2 protein was found to have a profound role in the development and progression of bone cancer and also in TNF-α, TNF-β and RANKL induced bone carcinogenesis. Nevertheless, further investigations in the in vivo and clinical settings are essential for validating the in vitro.

# Chapter 5

## Discussion, Conclusion and Future Prospects

## 5.1. Discussion and Conclusion

Bone cancer represents one of the most common pediatric cancer across the globe. It is a rare malignant tumor of the bone originating from the primitive mesenchymal cells. As mentioned, there are multiple subtypes, with OS, CS, and ES, being the most common. In adults, CS accounts for 40%, and OS accounts, nearly 28% of the primary bone cancers, whereas, in children and teenagers, OS (56%) occurs more frequently than CS. They are commonly aggressive and require early diagnosis, which requires imaging and tissue biopsy. Surgical excision remains the cornerstone of curative treatment, with chemotherapy and radiotherapy often used in conjunction (Evola, F. R., 2017; Weber, K., *et al.*, 2008; von Eisenhart-Rothe, R., *et al.*, 2011; Shweikeh, F., *et al.*, 2014; Palmerini, E., *et al.*, 2020; Ricotta, F., *et al.*, 2020; Lam, S. W., *et al.*, 2019). Similar to other cancers, the most prevailing treatment strategies for bone cancer are surgery, radiotherapy and chemotherapy or a combination of these. Chemotherapy is used as neoadjuvant (preoperative chemotherapy) and adjuvant (postoperative chemotherapy) therapy emerging as a standard treatment option for bone cancers. However, these treatment strategies possess many drawbacks. Apart from their low efficacy, these chemotherapeutic drugs are also found to be associated with detrimental side effects. Withal, the use of chemotherapeutics against metastatic bone cancer is invariably related to several glitches; including the development of chemoresistance and rapid postoperative recurrences spurning the overall disease-free survival (Brown, H. K., *et al.*, 2018; Ottaviani, G., & Jaffe, N., 2009; Pantano, F., *et al.*, 2015; Picci, P., 2007). Even though significant advancements have been made in the field of therapy for the management of bone cancer, the prognosis for patients with bone cancer remains extremely poor. The poor prognosis allied with this disease is due to the late-stage

diagnosis as it unveils negligible signs and symptoms at the early stages (Brown, H. K., *et al.*, 2018; Ottaviani, G., & Jaffe, N., 2009; Pantano, F., *et al.*, 2015; Picci, P., 2007). By and large, bone cancer's low survival rates can be fundamentally accredited to the propensity for early spread, lack of adequate and effective biomarkers for early diagnosis and prognosis and the ineffectiveness of existing bone cancer therapies. Hence, there is an indispensable need to find novel biomarkers and targets for this lethal disease that would benefit the patients to overcome the above-mentioned problems.

Increasing line of evidence suggested that TIPE family of proteins presents a novel group of proteins that acted as the modulators of tumorigenesis, inflammation, and cell death and were also found to perform diverse other cellular activities. TIPE2 is mostly found in the cell's cytoplasm and is composed of 184 amino acids. It primarily partakes in the negative regulation of innate and cellular immunity and plays a pivotal role in retaining immune homeostasis. Although TIPE2 was initially identified as an aberrantly expressed gene in the inflamed spinal cord of mice having autoimmune encephalomyelitis, it was later found to be expressed in different cell types, for instance, neurons in the brain and brainstem, squamous epithelial cells in the esophagus and cervix, glandular epithelial cells in the appendix, colon, and stomach, hepatocytes, and transitional epithelial cells in the ureter and bladder (Bordoloi, D., *et al.*, 2018; Padmavathi, G., *et al.*, 2018; Bordoloi, D., *et al.*, 2019). Markedly, various preclinical studies carried out to enlighten TIPE2 protein's role have affirmed its importance in the modulation of inflammatory responses and tumorigenesis. Besides, the expression analyses in clinical samples also showed TIPE2 to be deregulated in different malignancies (Bordoloi, D. *et al.*, 2018). Therefore, we studied the role of TIPE2 proteins in the development and progression of bone cancer through a comprehensive

analysis of its expression, function and linked mechanism of action. Interestingly, the expression of TIPE2 protein presented striking variability in different cancers (Bordoloi, D. *et al.*, 2018). Therefore, firstly, we analyzed the expression of TIPE2 protein in human tissue samples with the help of bone cancer tissue microarray containing tissues of different bone cancer types, pathologies, age groups and sexes along with normal adjacent bone tissues. Our results showed TIPE2 to be significantly overexpressed in bone cancer tissues compared to normal bone tissues. In accordance with our study, Bordoloi, D. *et al.*, 2019, showed TIPE2 to be significantly overexpressed in lung cancer tissues. In line with our findings, a study conducted by Hao and associates demonstrated augmented expression of TIPE2 in both peripheral T-cell lymphoma and diffused large B-cell lymphoma (Hao *et al.*, 2016). Furthermore, Li and colleagues suggested TIPE2 to be overexpressed in NSCLC tumor tissues than the adjacent normal tissues (Li, Z., *et al.*, 2016). However, few studies reported that TIPE2 displays lesser expression in breast cancer, glioma and gastric cancer (Wang, K., *et al.*, 2017; Liu, Z. J., *et al.*, 2016; Wu, J., *et al.*, 2016). Consequently, the findings of our analysis offer a preliminary basis for considering TIPE2 to have a role in the positive regulation of bone cancer pathogenesis. We further analyzed the expression of this family of proteins in both OS and CS bone cancer types where TIPE2 displayed enhanced expression in both OS and CS tissues compared to normal tissues, with more pronounced augmentation in the CS type. In addition, we analyzed the expression in tissue samples of patients of different age groups. We ascertained that the TIPE2 protein was inflated from early childhood to adolescence compared to the normal bone tissues. We also noticed that the TIPE2 protein expression level was high till later age. In fact, the maximum expression was observed in the age group of 45 to 54 years. Furthermore, in OS, the maximal expression of TIPE2 was discerned at the age group of 5 to 14 years

of age. However, in the case of CS, we observed the maximum expression score at the age group of 45 and above. This may be due to the fact that CS, occurs mainly in adults with an average age of diagnosis at 51 and constitutes more than 40% of primary bone tumors in adults, followed by OS at 28% whereas, in children and teenagers, OS makes up to 56% of the total bone cancer cases. Thus high expression of TIPE2 in the tissue samples of lower age group patients of OS compared to higher age grouped ones gives us an implication of the involvement of TIPE2 protein in the predominant occurrence of OS in children and teenagers. This may relate to a complex interplay of sex and pubertal status, which impacts drug metabolism, the experience of toxicity, and the psychosocial factors. Overall, it can be concluded that overexpression of TIPE2 may be closely associated with poor clinical outcomes of bone cancer patients, thus implying its potential as a prognostic factor and novel therapeutic target in bone cancer.

Our findings provided a strong basis for the existence of a positive association between TIPE2 and bone carcinogenesis. Mounting evidence has supported the notion that chronic inflammation evokes tumorigenesis (Wang, X., & Lin, Y., 2008). Inflammation confers to osteolysis, which causes bone pain and attenuates skeletal instability. The bone homeostasis is compromised under the pathological conditions such as inflammatory osteolysis, osteoporosis, and bone tumors, which causes increased osteoclast activity, ultimately resulting in bone loss (Abu-Amer Y., 2009; Walsh, N. C., *et al.*, 2005; Hagemann, T., *et al.*, 2007). It is well-evinced that TNF is a multifunctional cytokine that acts as an important regulator of various cellular events such as cell proliferation, survival, differentiation, and death. The inflammatory cells secrete TNFs as pro-inflammatory cytokines that may be involved in inflammation-associated carcinogenesis (Kunnumakkara, A. B., *et al.*, 2019). TNF exerts its biological activities by triggering distinct signaling pathways such as NF-κB pathway.

This pathway is one of the chief cell survival pathways that mainly functions by inhibiting apoptosis (Kunnumakkara, A. B., *et al.,* 2020). RANKL was formerly identified as TRANCE, and it belongs to the TNF superfamily. RANKL is mainly expressed by the bone marrow stromal cells, osteocytes and osteoblasts (Boyce, B. F., & Xing, L., 2007; Boyce, B. F., & Xing, L., 2008; Wong, B. R., *et al.,* 1999). Additionally, the RANKL/RANK/OPG signaling cascade acts as an essential regulator of bone remodelling (Yasuda, H., *et al.,* 1998a; Lacey, D. L., *et al.,* 1998; Boyce, B. F., & Xing, L., 2007). This signaling cascade modulates the formation and development of multinucleated osteoclasts from the precursor osteoclast cells, and their activation assists in normal bone modelling or remodelling (Boyce, B. F., & Xing, L., 2007).

We predicted a strong association between TIPE2 and TNF-mediated tumorigenesis. No study to date has reported the effect of these pro-inflammatory cytokines in the regulation of the expression of TIPE2 in bone carcinogenesis. Therefore, we evaluated the role of TNF-α, TNF-β and RANKL on the proliferation, viability, cell death, colony formation and migration of bone cancer cells. We found that TNF-α, TNF-β and RANKL induced proliferation, colony formation and migration potential of HOS and U2OS bone cancer cells and did not induce cell death in the cancer cells. Since the treatment of TNF-α, TNF-β and RANKL were involved in augmentation of proliferation, colony formation and migration potential of bone cancer cells, therefore, analyzing the effects of TNF-α, TNF-β and RANKL on the expression of TIPE2 would help us understand whether TIPE2 has any role in TNF- associated bone carcinogenesis. Our results showed that the expression of TIPE2 was upregulated from its basal level upon treatment with the TNF-α, TNF-β and RANKL. Hence, our results for the first time evidenced the involvement of TIPE2 in TNF-mediated bone carcinogenesis.

In order to decipher the exact role of TIPE2 protein and their upstream and downstream targets, we disrupted the expression of TIPE2 with the help of siRNA-mediated gene-editing method. Subsequently, we determined the effect of TIPE2 knockdown in bone cancer cells on different hallmarks of cancer. Further, we determined their upstream and downstream targets, which are involved in the pathogenesis of bone cancer. Notably, siRNA or shRNA mediated silencing of TIPE2 protein was already performed for different cancers, resulting in the modulation of cell growth, proliferation, invasion and metastasis of the cancer cells as evinced by a few studies (Bordoloi, D. *et al.*, 2018, Padmavathi *et al.*, 2018). After generating successful knockdown clones for TIPE2 and their subsequent confirmation through Western blot, we determined the effect of TIPE2 knockdown on the proliferation, survival, autophagy, EMT, migration and invasion of bone cancer cells. Knockdown of TIPE2 resulted in significantly decreased proliferation of HOS and U2OS cells compared to scrambled control. Along with proliferation, enhanced survival is also a major hallmark of cancer cells. We determined the effect of the knockdown of TIPE2 on the survival of HOS and U2OS cells using colony formation assay. We observed that knockdown of TIPE2 resulted in the reduced clonogenic potential of HOS and U2OS cells compared to scrambled control implying that TIPE2 is involved in increasing the survival fraction of bone cancer cells. This is the first report that showed the participation of TIPE2 in enhancing the proliferation and survival fraction of bone cancer cells. Moreover, our results are supported by a few previous findings as well. In line with our findings, Bordoloi and group showed that knockout of TIPE2 led to the reduction in proliferation of lung cancer cells, and it was through the regulation of AKT/mTOR/NF-κB signaling cascade (Bordoloi, D., *et al.*, 2019). Furthermore, in RCC tissues, a significant increase of TIPE2 expression and downregulation of MX1 were observed compared to the normal controls. Moreover,

TIPE2 expression was positively correlated with TNM staging and negatively with MX1 expression, denoting the function of TIPE2 in RCC pathogenesis. In accordance with our results, knockdown of TIPE2 was found to reduce the colony formation ability of colon cancer cells (Miao, Z., *et al.,* 2012). Furthermore, a study carried out by our research group have shown that knock out of TIPE2 in lung cancer resulted in a drastic reduction in the colony-forming ability of lung cancer cells (Bordoloi, D., *et al.*, 2019). Altogether, the results obtained are in accordance with our previous findings, and it can be said that TIPE2 is plausibly involved in the positive regulation of bone carcinogenesis.

In order to unravel the mechanism of action of TIPE2 in the proliferation and survival of bone cancer cells, it is imperative to analyze the association of TIPE2 in the modulation of the signaling molecules involved in proliferation and survival. For this purpose, expression analysis of various proliferation and survival-related proteins were carried out using Western blot. Our results demonstrated that knockdown of TIPE2 resulted in downregulation of the expression of apoptosis regulatory protein survivin and upregulated the expression of caspase-3. Survivin is involved in the inhibition of apoptosis, whereas the caspase-3 protein is an apoptotic protein, and upon activation, it can cleave several structural and regulatory proteins, which are critical for cell survival and maintenance (Zhou, C., *et al.*, 2018). Runx2 is crucial for osteoblast differentiation and maturation of the chondrocytes. It enhances the proliferation of osteoblast progenitors (Komori, T., 2019). Therefore, reduced expression of Runx2 in TIPE2 knockdown cells might be one reason for the decreased proliferation. Further, knockdown of TIPE2 resulted in the suppression of COX-2, which plays an essential role in cellular growth, differentiation and inflammation (Erkanli, S., *et al.,* 2007).

Overall, our results suggest that TIPE2 is responsible for the positive regulation of bone cancer cell proliferation and survival.

After confirming TIPE2 protein to regulate bone cancer cell proliferation, we further analysed if this control of proliferation is mediated through the regulation of cell cycle. The cell cycle analysis revealed that knockdown of TIPE2 results in accumulation of cells at the S phase and a subsequent reduction in G2/M and G0/G1 phases in the HOS cell line. Additionally, TIPE2 knockdown in the U2OS cell line induced G0/G1 and G2/M phase arrest, unlike the HOS cells. Interestingly, as this protein was found to act on different phases of the cell cycle, a probable difference in the molecular targets of TIPE2 proteins is proposed in cell cycle regulation. For this purpose, expression analysis of various cell cycle-related proteins were carried out using Western blot. Notably, our study showed that silencing of TIPE2 led to the upregulation of p53 and p21 in both the cell lines. Also, TIPE2 knockdown in bone cancer cells was found to repress other cell cycle regulatory proteins such as cyclin E, cyclin A2, and CDK-2, in HOS cell line and cyclin D3 and CDK-6 in U2OS cell line. The tumor suppressor protein p53 regulates the abnormal cells by controlling DNA repair, cell death, cell cycle progression, or senescence (Lahalle, A., *et al.,* 2021). Cyclin A2 activates the kinases that partakes in the control of S-phase of the cell cycle and mitotic entry (Loukil, A., *et al.,* 2015). Cyclin E limits cells' passage through the restriction point, which marks a "point of no return" for cells from G1 into S-phase. The cyclin E binds and activates the kinase CDK-2, and the cyclin E/CDK-2 complexes initiate a cascade of events that leads to the expression of S-phase specific genes (Möröy, T., & Geisen, C. 2004). Cyclin D3 can act as a critical regulator to the differentiation and proliferation of the cancerous cells and their physiological progression from G1- to S-phase (Ding,

Z. Y., *et al.*, 2019). Furthermore, p53 triggers G1-phase arrest through the activation of p21, which binds to cyclin D/CDK-4 and cyclin E/CDK-2 complexes (Chen, J., 2016). Hence, it has been verified that reduction in the proliferation of bone cancer cells attained by silencing of oncogenic TIPE2 protein is regulated through suppression of cell cycle progression as the accumulation of cells in G2/M or G0/G1 and S phase. Mounting evidence advocates that autophagy plays a complex role in cancer. The modulation of autophagy plays dual roles in tumor suppression and promotion in different cancers. Our study showed that TIPE2 knockdown substantially augmented autophagy in both the HOS and U2OS cell lines, as demonstrated by the enhanced intensity of LC3B expression. In the same way, we observed an increase in the expression of LC3B as well as p62 protein expression in TIPE2 knockdown cells compared to the scrambled control using the Western blot method. LC-3B is a structural protein found in autophagosomal membranes and is considered a marker for autophagy (Chen, *et al.*, 2018). The p62 protein controls osteoclastogenesis and bone remodelling and contributes in the proteasomal degradation of ubiquitinylated proteins (Durán, A., *et al.*, 2004). The proteasome inhibition can enforce proteotoxic stress, which can stimulate autophagy via p62 phosphorylation (Moscat, J., & Diaz-Meco, M. T., 2012). This shows that TIPE2 is involved in increasing the autophagy in bone cancer cells. In line with our results, in rectal adenocarcinoma cells, the overexpression of TIPE2 weakened autophagy by reducing the level of expression of p-Smad-3, p-Smad-2, and TGF-β (Wu, D. D., *et al.*, 2019). Altogether, this is the first report that presented that TIPE2 might have an important role in autophagy in bone cancer cells.

Aforementioned, maximum number of bone cancer cases are diagnosed at a late-stage or metastatic stage. Cancer metastasis involves improved motility, survival in

circulation, and the capability to form new tumors by presenting EMT and better survival and migratory signals (Liu, F., Ke, J., & Song, Y., 2020; Jayarangaiah, A., & Theetha Kariyanna, P., 2020). In EMT, there is a shift from the expression of E-cadherin to the induced expression of mesenchymal markers, such as N-cadherin and vimentin; these changes occur largely at the transcriptional level due to the activity of transcription factors associated with EMT, such as Snail, Slug and Twist1 (Zhang, Y., *et al.*, 2019; Nieto, M. A., *et al.*, 2016). Silencing of TIPE2 considerably inhibited the EMT in both HOS and U2OS cell lines. The immunocytochemistry analysis displayed that the mesenchymal markers, i.e. vimentin and snail, were repressed in TIPE2 knockdown cells compared to the scrambled control. Hence, the TIPE2 protein is involved in the regulation of EMT of bone cancer cells. Furthermore, Western blot analysis of proteins related to EMT of cancer cells demonstrated that silencing of TIPE2 resulted in downregulated expression of EMT regulatory proteins, such as N-cadherin, and Twist1 and upsurged expression of E-cadherin in both HOS and U2OS cell lines. However, contrary to our results, TIPE2 was found to inhibit the migration and invasion of endometrial cells, gastric cancer cells, breast cancer cells and prostate cancer cells through the reversal of EMT (Liu, Y., *et al.*, 2020; Yin, H., *et al.*, 2017; Wang, K., *et al.*, 2017; Lu, Q., *et al.*, 2016).

The acquired ability of bone cancer cells to invade and migrate to nearby cells is strongly linked with their extremely high metastatic ability (Coleman, R. E., *et al.*, 2020). Upon analyzing the effect of TIPE2 on the migration and invasion of bone cancer cells, we found that silencing of TIPE2 inhibited the migration potential of bone cancer cells effectively. Further, TIPE2 was found to induce the invasiveness of bone cancer cells as well. Similar to our findings, Bordoloi, D., *et al.*, have shown that knock out

of TIPE2 in lung cancer resulted in a drastic reduction in the migration potential of lung cancer cells (Bordoloi, D., *et al.,* 2019). Our results established that knockdown of TIPE2 resulted in suppressed expression of migration and invasion regulatory proteins MMP-9. During metastasis, tumor cells are involved in numerous interactions with the extracellular matrix (ECM). It is well established that there has been a strong association between MMPs, ECM degradation and cancer cell invasion. MMPs are ECM remodelling endopeptidases that can degrade many components of the ECM. Increased expression of MMP-2 and MMP-9 is frequently associated with augmented cell migration, invasion and cancer metastasis (Cabral-Pacheco, G. A., *et al.,* 2020; Luo, Y., *et al.,* 2009; Hofmann, U. B., *et al.,* 2000; Deryugina, E. I., & Quigley, J. P., 2006). Collectively, TIPE2 is found to be involved not only in proliferation, survival, autophagy and EMT but also in the migration and invasion of bone cancer cells exemplifying their critical role in the progression and metastasis of bone cancer and hence their immense therapeutic implications.

The findings of our previous studies suggested TIPE2 played a crucial role in the bone cancer cell proliferation, survival, autophagy, EMT, invasion and migration. Therefore, to decipher the underlined molecular mechanism of action of TIPE2 mediated bone cancer pathogenesis, we studied the expression of different upstream and downstream signaling molecules involved in bone cancer. It is well established that TNF mediates its biological functions by mainly activating the NF-κB signaling pathway. TNF mainly binds to two receptors, namely TNFR-1 and TNFR-2. Constitutive activation of the TNF-TNFR-1 signaling cascade leads to the aberrant activity of NF-κB signaling pathway. Our results showed that the silencing of TIPE2 did not affect the crucial components of the TNF-TNFR-1 pathway, i.e. TNF-α, TNF-β, TNFR-1 and TNFR-2.

Further, the knockdown of TIPE2 downregulated the expression of NF-κB, p-NF-κB$^{S536}$ and RANKL notably. As the expression of TNF-α, TNF-β, TNFR-1, and TNFR-2 remain unaltered upon silencing of TIPE2 in bone cancer cells, we can conclude that these signaling molecules might be present upstream of the TIPE2 protein. Thus, TIPE2 is found to activate the NF-κB signaling pathway, which contributes to the pathogenesis of bone cancer. Altogether, TIPE2 mediated induction of proliferation, survival, autophagy, EMT, invasion and migration was mediated through the activation of the NF-κB signaling pathway. Noteworthy, this is the first report which shows the involvement of NF-κB signaling axis in TIPE2 mediated bone tumorigenesis.

Furthermore, to confirm the upstream and downstream targets of TIPE2, the TNF-α and NF-κB were silenced using siRNA mediated knockdown of both HOS and U2OS cells, and the expression of TIPE2 was determined. Earlier, we have shown that silencing of TIPE2 in bone cancer cells did not alter the expression of TNF-α. However, the knockdown of TNF-α in HOS and U2OS cell lines resulted in significant downregulation in the expression of TIPE2. Therefore, we can confirm that TNF-α is present upstream of the TIPE2 protein. On the one hand, we have shown that silencing of TIPE2 in bone cancer cells suppressed NF-κB protein expression. On the other hand, the knockdown of NF-κB in HOS and U2OS cell lines did not alter the expression of TIPE2 protein. Thus, we can confirm that NF-κB is a downstream target of the TIPE2 protein. Hence, TNF-α induced bone carcinogenesis is mediated through TIPE2, which regulates the expression of several downstream molecules involved in proliferation, survival, autophagy, EMT, invasion and migration of bone cancer cells through the activation of the NF-κB signaling pathway.

To further strengthen this finding, we have discussed the possible involvement of TIPE2 in TNF-α, TNF-β, and RANKL associated bone carcinogenesis. TNFs have been reported to induce survival, proliferation, angiogenesis, and metastasis in most cancer cells by binding to its receptor and activating various molecular pathways such as NF-κB (Wang, X., & Lin, Y., 2008; Subkamkaew, C., *et al.*, 2019). We have already shown that TIPE2 might be involved in TNF-α, TNF-β and RANKL mediated bone carcinogenesis. We found that TNF-α, TNF-β and RANKL induced proliferation, colony formation and migration potential of HOS and U2OS bone cancer cells and did not induce cell death in the cancer cells. We have also discussed the effect of TNF-α, TNF-β and RANKL and their relationship with the TIPE2 protein. Our study has shown that treatment of bone cancer cells with TNF-α, TNF-β and RANKL significantly upregulated the TIPE2 expression. To validate the same, we treated the TIPE2 knockdown HOS and U2OS cells with the pro-inflammatory cytokines, i.e. TNF-α, TNF-β and RANKL and subsequently analyzed its effect on the proliferation, survival and migration of the bone cancer cells. Further, the impact on the expression of its associated downstream targets was also studied. Our results showed a significant reduction in the proliferation, survival fraction and migration potential of TIPE2 knockdown cells compared to their respective SCR cells even after treatment with the cytokines. Overall, this proved that TIPE2 is a key molecule involved in TNF-α, TNF-β and RANKL-mediated proliferation, survival and migration of bone cancer cells. Next, using Western blot, we analyzed the molecular mechanism of TIPE2 in facilitating TNF-α, TNF-β and RANKL-mediated bone cancer pathogenesis. Our results showed that TNF-α, TNF-β and RANKL treated TIPE2 knockdown cells displayed downregulation of survivin and Runx2 proteins and upregulation of the p21 and p53 proteins compared to the TNF-α, TNF-β and RANKL treated scrambled

control cells. These proteins are involved in cell growth, survival, proliferation, cell cycle progression and regulation of apoptosis. We have also found that TNF-α, TNF-β and RANKL treated TIPE2 knockdown cells exhibited downregulation in the expression of proteins involved in EMT, invasion, and migration such as N-cadherin and MMP-2 compared to TNF-α, TNF-β and RANKL treated scrambled control cells. It is well known that TNF activates vital molecular pathways involved in inflammation-related carcinogenesis, including the NF-κB pathway (Wang, X., & Lin, Y., 2008; Drutskaya *et al.*, 2010). Also, aberrant or constitutive NF-κB activation has been implicated in many human malignancies, including bone cancer (Li, W., *et al.*, 2020; Feng, Z. M., & Guo, S. M., 2016). Furthermore, downregulation of p-NF-κB$^{S536}$ was observed in TNF-α, TNF-β and RANKL treated TIPE2 knockdown cells compared to that of scrambled control cells treated with the same. Collectively, this is the first report that shows the involvement of TIPE2 in TNF-α, TNF-β and RANKL-mediated proliferation, survival, and migration in bone cancer cells via modulation of NF-κB signaling pathway and its regulated gene products. Altogether, TIPE2 protein was found to have profound role in the development and progression of bone cancer and particularly in cytokines-induced bone cancer, and henceforth specific targeting of them will provide tremendous prospect in newer therapeutic interventions in bone cancer.

### 5.2. Limitations and future prospects of the study

The present study establishes a strong correlation between TIPE2 protein and bone carcinogenesis, specifically cytokines induced bone carcinogenesis. Nonetheless, there are a few limitations associated with the study and hence needs a further valuation.

Firstly, the expression of TIPE2 protein in bone cancer and normal bone tissues was determined using immunohistochemical analysis of TMA slides containing only 50

cases of osteosarcoma, 28 cases of chondrosarcoma, and 2 cases of adjacent normal tissue, single core per case of bone cancer. These tissue cores are very small in size, i.e. each core is of 1.5 mm in diameter and 5 μm in thickness which confines our analysis to a smaller area. This creates a major concern as in cancer; each cell might have several molecular alterations and differ from each other even though they belong to the same tissue. Further, the 80 tissues are categorized based on age, sex, organ, disease pathology, TNM, stages and type, and the number of tissues in each category differ remarkably, restricting our search of differential expression to only a few categories leaving the knowledge on the expression of TIPE2 protein in other categories such as different age groups, gender, organs, disease pathology and differentiation status highly unexplored. Additionally, the TMA slide comes with only stage IIa and stage IIb patient samples. IHC analysis with tissues of different stages and grades will give a better idea about the differential expression of TIPE2 protein in different stages and grades of bone cancer. Therefore, keeping these limitations in mind, more extensive analysis can be performed in future, including more number of bigger tissue samples with a significant number of tissues in each aforementioned categories. Besides, the expression of TIPE2 protein can be determined in the tissues of different *in vivo* bone cancer models as well. Further, in the current study, factors like the history of the patients, details of therapies and their response to the treatment, chemoresistance and tumor recurrence status, and disease-free and overall survival profiles etc., were not included. Henceforth, analysing the change in the expression of TIPE2 protein with respect to these factors will provide a better understanding of the clinical significance of TIPE2 protein and their possible diagnostic, therapeutic and prognostic values.

Secondly, we studied the association of TIPE2 in cytokines-mediated bone carcinogenesis using TNF-α, TNF-β and RANKL. Aforementioned, RANKL/RANK/OPG signaling cascade is an essential regulator of bone remodelling and plays a critical role in bone tumorigenesis. Therefore, a more detailed study on the association of TIPE2 with RANKL/RANK/OPG signaling cascade and its role in osteoclastogenesis and osteoblastogenesis will deliver a comprehensive understanding of the involvement of TIPE2 in cytokines mediated bone carcinogenesis.

Thirdly, we could not study the role of TIPE2 protein in the chemoresistant cells. As the development of chemoresistance and tumor recurrence are the two major setbacks in cancer patients and are responsible for poor survival and recovery of bone cancer patients, therefore, the effect of standard chemotherapeutic drugs on the expression of TIPE2 protein and the effect of silencing of TIPE2 on the response to chemotherapy could also be assessed in future to determine the chemoresistant and chemosensitizing potential of TIPE2 protein in bone cancer. In the present study, we established the correlation of TIPE2 protein with bone cancer through in vitro assays performed in only two cell lines. Therefore, it is indispensable to validate our results in multiple cell lines and in animal models and clinical settings before proceeding with the development of novel diagnostic, therapeutic and prognostic methods.

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**Abbreviations**

| | |
|---|---|
| ADM: | Adriamycin |
| ALP: | Alkaline phosphatase |
| AP-1: | Activating protein-1 |
| BS: | Bone scintigraphy |
| BSA: | Bovine serum albumin |
| CDDP: | Cisplatin |
| CDK: | Cyclin-dependent kinases |
| CDKI: | Cyclin-dependent kinase inhibitor |
| COX-2: | Cyclooxygenase-2 |
| CS: | Chondrosarcoma |
| CSC: | Cancer stem cell |
| CST: | Cell Signaling Technology |
| CT: | Computed tomography |
| CXCR-4: | C-X-C chemokine receptor type 4 |
| DED: | Death effector domain |
| DLBCL: | Diffuse large B-cell lymphoma |
| DMEM: | Dulbecco's Modified Eagle's Media |
| DOX: | Doxorubicin |
| ECM: | Extracellular matrix |
| EGFR: | Epidermal growth factor receptor |
| EMT: | Epithelial–mesenchymal transition |
| ES: | Ewing sarcoma |
| ESFTs: | ES family of tumors |
| FACS: | Fluorescence-activated cell sorting |
| FADD: | Fas-associated death domain |
| FAK: | Focal adhesion kinases |
| FGFR1: | Fibroblast growth factor receptor 1 |
| GCB: | Germinal centre B-cell |
| GSK3β: | Glycogen synthase kinase 3 beta |

| | |
|---|---|
| h: | Hour |
| HCV: | Hepatitis C virus |
| HIF-1α: | Hypoxia inducible factor-1 subunit alpha |
| HMO: | Hereditary multiple osteochondromas |
| HME: | Hereditary multiple exostoses |
| HRP: | Horseradish peroxidase |
| IAP: | Inhibitors of apoptosis protein |
| IFOS: | Ifosfamide |
| IHC: | Immunohistochemistry |
| IKK: | IκB kinase |
| IL: | Interleukins |
| JNK: | Jun N-terminal kinase |
| LC3: | Microtubule–associated protein chain 3 |
| LDH: | Lactose dehydrogenase |
| MAPK: | Mitogen-activated protein kinases |
| MDM2: | Mouse double minute 2 homolog |
| MEM: | Minimum Essential Medium |
| miRISC: | miRNA-induced silencing complex |
| mL: | Milliliter |
| mM: | Milimolar |
| MMP: | Metalloproteinases |
| MRI: | Magnetic resonance imaging |
| mTOR: | Mechanistic target of rapamycin |
| MTX: | Methotrexate |
| MX1: | Myxoma resistance protein 1 |
| NF-κB: | Nuclear factor kappa light chain enhancer of activated B cells |
| ng: | nanogram |
| NHL: | Non-Hodgkin's lymphoma |
| NSCLC: | Non-small cell lung carcinoma |

| | |
|---|---|
| OPG: | Osteoprotegerin |
| OS: | Osteosarcoma |
| PARP: | Poly ADP ribose polymerase |
| PBS: | Phosphate buffer saline |
| PET: | Positron emission tomography |
| PI: | Propidium iodide |
| PI3K: | Phosphoinositide 3-kinase |
| PKC: | Protein kinase C |
| PTEN: | Phosphatase and tensin homolog |
| RANKL: | Receptor activator of nuclear factor kappa-B ligand |
| RB: | Retinoblastoma |
| RCC: | Renal cell carcinoma |
| RIP: | Receptor-interacting protein |
| ROS: | Reactive oxygen species |
| RTS: | Rothmund-Thomson syndrome |
| Runx2: | Runt-related transcription factor 2 |
| SCC: | Skin squamous cell carcinoma |
| SCR: | Scramble |
| SDS: | Sodium dodecyl sulfate |
| SEER: | Surveillance, Epidemiology and End Results |
| shRNA: | Short hairpin RNA |
| STAT3: | Signal transducer and activator of transcription 3 |
| TAK1: | TGF-β-activated kinase 1 |
| TAMs: | Tumor-associated macrophages |
| TGF-α: | Transforming growth factor alpha |
| TIPE2: | Tumor necrosis factor-α-induced protein 8-like 2 |
| TLR: | Toll-like receptor |
| TMA: | Tissue microarray |
| TNF: | Tumor necrosis factor |
| TNFAIP8/TIPE: | Tumor necrosis factor-α-induced protein 8 |

| | |
|---|---|
| TNFR-1: | TNF receptor-1 |
| TRADD: | TNFR-associated death domain |
| TRAF-2: | TNFR-associated factor 2 |
| TRANCE: | TNF-related activation-induced cytokine |
| TRAP: | Tartrate-resistant acid phosphatase |
| uPA: | Urokinase plasminogen activator |
| VEGF: | Vascular endothelial growth factor |
| WHO: | World Health Organization |
| XIAP: | X-linked inhibitor of apoptosis protein |
| µg: | Micogram |
| µl: | Microliter |
| µM: | Micromolar |

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1. Gupta, M.K., Sanjana, S., Chiranjivi, A.K., **Banik, K**., Girisa, S., Kunnumakkara, A.B., Dubey, V.K., Rangan, L. **(2021)**. Anti-inflammatory and anti-tyrosinase activity of 3,5-dihydroxy-4',7-dimethoxyflavone isolated from the leaves of Alpinia nigra**. Phytomedicine Plus,** 2021/7/21, 100097.

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3. ***Aswathy, M**., ***Banik, K**., *Parama, D., Sasikumar, P., Harsha, C., Joseph, A.G., Sherin, D.R., Thanathu, M.K., Kunnumakkara, A.B., Vasu, R.K. **(2021).** Exploring the Cytotoxic Effects of the Extracts and Bioactive Triterpenoids from *Dillenia indica* against Oral Squamous Cell Carcinoma: A Scientific Interpretation and Validation of Indigenous Knowledge. **ACS Pharmacology & Translational Science** 4(2), 834–847 **(*Equal Authorship).**

4. Bordoloi, D., Padmavathi, G., **Banik, K**., Khwairakpam, A. D., Harsha, C., Girisa, S., Buhrmann, C., Shakibaei, M., Kunnumakkara, A.B. **(2021).** Human tumor necrosis factor-alpha-induced protein eight-like 1 exhibited potent anti-tumor effect through modulation of proliferation, survival, migration and invasion of lung cancer cells. **Molecular and Cellular Biochemistry**. Epub ahead of print. PMID: 33895911.

5. Bordoloi, D., **Banik, K.,** Vikkurthi, R., Thakur, K. K., Padmavathi, G., Sailo, B. L., Girisa, S., Chinnathambi, A., Alahmadi, T. A., Alharbi, S. A., Buhrmann, C., Shakibaei, M., & Kunnumakkara, A. B. **(2020)**. Inflection of Akt/mTOR/STAT-3 cascade in TNF-α induced protein 8 mediated human lung carcinogenesis. **Life sciences**, 262, 118475.

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7. Singh, A. K., Roy, N. K., Bordoloi, D., Padmavathi, G., **Banik, K.,** Khwairakpam, A. D., Kunnumakkara, A. B., & Sukumar, P. **(2020).** Orai-1 and Orai-2 regulate oral cancer cell migration and colonization by suppressing Akt/mTOR/NF-κB signalling. **Life sciences**, 261, 118372. https://doi.org/10.1016/j.lfs.2020.118372

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9. Bordoloi, D., Monisha, J., Roy, N. K., Padmavathi, G., **Banik, K.,** Harsha, C., Wang, H., Kumar, A. P., Arfuso, F., & Kunnumakkara, A. B. **(2019)**. An Investigation on the Therapeutic Potential of Butein, A Tretrahydroxychalcone Against Human Oral

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**16.** Bordoloi, D., Harsha, C., Padmavathi, G., **Banik, K**., Sailo, B.L., Khwairakpam, A. D., Thakur, K. K., Chinnathambi, A., Alahmadi, T. A., Alharbi, S.A., Kunnumakkara, A.B. **(2021)**. Loss of TIPE3 reduced the proliferation, survival and migration of lung cancer cells through inactivation of Akt/mTOR/NF-κB/STAT-3 signaling cascade (Manuscript under review).

**17.** Thakur, K. K., **Banik, K**., Verma, E., Daimary, U. D., Chinnathambi, A., Alahmadi, T.A., Alharbi, S.A., Nair., M. S., Rajalakshmi, D. S., Sujatha, H.P., Kunnumakkara, A.B. (2021). Development of a Novel STAT3 Inhibitor from Hedychium coronarium for the Prevention and Treatment of Lung Cancer. (Manuscript under preparation).

**18.** Thakur, K.K., Kumar, A., **Banik, K**., Verma, E., Khatoon, E., Choudhary, H., Sethi, G., Gupta, S.C., Kunnumakkara, A.B. **(2021).** Long Non-coding RNAs in Triple-Negative Breast Cancer-A new frontier in regulation of tumorigenesis. **Journal of Cellular Physiology.** PMID: 34105151.

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Cervical Cancer', In Cancer Cell Chemoresistance and Chemosensitization. **World Scientific Publications (2018)**.

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**54.** Padmavathi, G., Bordoloi, D., **Banik, K.**, Singh, A.K. and Kunnumakkara, A.B.' Mechanism of Chemoresistance in Bone Cancer and Different Chemosensitization Approaches', In Cancer Cell Chemoresistance and Chemosensitization. **World Scientific Publications (2018)**.

**55.** Khwairakpam, A.D., Javadi M, **Banik, K.**, Harsha, C., Sharma A, Bordoloi, D. and Kunnumakkara, A.B.'Chemoresistance in Brain Cancer and Different Chemosensitization Approaches', In Cancer Cell Chemoresistance and Chemosensitization. **World Scientific Publications (2018)**.

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**63.** Padmavathi, G., Monisha, J., **Banik, K.**, Harsha, C., Bordoloi, D. and Kunnumakkara, A.B.' Rearrangements involving ETS family of genes and their role in different cancers', In Fusion Genes and Cancer, **World Scientific Publications**. 2017, pp. 147-162. **(2017)**.

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**66.** Padmavathi, G., Bordoloi, D., **Banik, K.,** and Kunnumakkara, A.B.' Cancer Biomarkers: Important Tools for Cancer Diagnosis and Prognosis', In Next generation point-of-care biomedical sensors technologies for cancer diagnosis", **Springer (2018)**.

## Abstracts Presented in Conferences:

1. **Kishore Banik**, Ajaikumar B. Kunnumakkara. Gave oral presentation on the topic "Studies on the Role of Cytokine- Induced Proteins in Bone Cancer" at the DAILAB PIKNIKH (Platform for Innovating KNowledge to International Know How) Series XXXX, organized by AIST, Tsukuba, Japan and Indian Institute of Technology Guwahati on 22nd February, 2020.

2. **Kishore Banik**, Ajaikumar B. Kunnumakkara. An Investigation on the Role of Novel Akt/mTOR Inhibitor against Oral Squamous Cell Carcinoma. at the 4th International Conference on Nutraceuticals and Chronic Diseases 2019, organized by Indian Institute of Technology Guwahati and Society for Nutraceuticals and Chronic Diseases, September, 23rd -25th, 2019.

3. **Kishore Banik**, Ajaikumar B. Kunnumakkara. "TIPE Family of Proteins as Novel Biomarker in Bone Cancer" at 2nd Workshop on "Recent Advances in Cancer Research" RACR-2019 organized by Indian Institute of Technology Guwahati, March, 28, 2019.

4. **Kishore Banik**, Ajaikumar B. Kunnumakkara. "TIPE Family of Proteins as Novel Biomarker in Bone Cancer" at Indo-Japan Symposium on "Recent Advances in Biomedical Research" RABR-2019 jointly organized by Indian Institute of Technology Guwahati and National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan, March, 26-27, 2019.

5. **Kishore Banik**, Ajaikumar B. Kunnumakkara. "Development of TNFAIP8 Family of Proteins as Novel Biomarker in Bone Cancer" at Research Conclave 2019, Indian Institute of Technology Guwahati, Assam, India, March 14-17, 2019.

6. **Kishore Banik**, Ajaikumar B. Kunnumakkara. "Differential Expression of TNFAIP8 Family of Proteins in Bone Cancer Tissues" 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 National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan, October 14-21, 2018.

7. **Kishore Banik**, Harsha, Choudhary, Devivasha Bordoloi, Ajaikumar B. Kunnumakkara. "Anticancer activity of Hondapara leaf extract against oral squamous cell carcinoma" at 3rd International Conference on "Nutraceuticals and Chronic Diseases" organized by Sri Rama Himalaya University and Society for Nutraceuticals and chronic diseases in Rishikesh, Uttarakhand, India, 14-16, September, 2018.

8. **Kishore Banik**, Harsha Choudhary, Devivasha Bordoloi, Ajaikumar B. Kunnumakkara "Anticancer activity of Elephant Apple leaf extract against Oral Cancer" at National conference on "Ethno-medicine and Traditional Health Practices In North-East Region of India" organized by National Institute of Pharmaceutical Education and Research (NIPER) Guwahati, Assam, India, 25th August, 2018.

9. **Kishore Banik**, Harsha Choudhary, Devivasha Bordoloi, Monisha, Javadi , Nand Kishor Roy, Ajaikumar B. Kunnumakkara "Investigation of the Anticancer Potential of Karambel on Oral Squamous Cell carcinoma", Trends in BIOCHEMICAL & BIOMEDICAL Research Advances and Challenges (TBBR-2018), Department of Biochemistry, Institute of Science, Banaras Hindu University, Varanasi, India, 13th – 15th February, 2018.

10. **Kishore Banik**, Harsha Choudhary, Devivasha Bordoloi, Ajaikumar B. Kunnumakkara, "Indian Catmon: A potent anticancer agent against human oral squamous cell carcinoma" 2nd INCD-2017.

11. **Kishore Banik**, Harsha Choudhary, Devivasha Bordoloi, Monisha, J.avadi, Ajaikumar B. Kunnumakkara 'Anticancer activity of Outenga (Elephant Apple) against Oral Cancer' Research Conclave, Indian Institute of Technology Guwahati, Assam, India, 2017.

12. **Kishore Banik**, Harsha Choudhary, Monisha, J.avadi, Ajaikumar B. Kunnumakkara 'Global Cancer Statistics' Research Conclave, Indian Institute of Technology Guwahati, Assam, India, 2017.

13. Harsha, C., **Kishore Banik**, Roy, N.K., Bordoloi, D., Khwairakpam, A.D. and Kunnumakkara, A.B.. Gold nanoparticles (GNPs) synthesized from Elephant apple preferentially kills cancer cells, First International Conference on Nutraceuticals and Chronic Diseases, Kerala, India, 2016.

15. **Kishore Banik**, Harsha Choudhary, Devivasha Bordoloi, Monisha, J.avadi, Ajaikumar B. Kunnumakkara 'An Evaluation of the Anticancer effect of Dillenia indica against Oral Cancer, Research Conclave, Indian Institute of Technology Guwahati, Assam, India, 2016.

16. **Kishore Banik**, Harsha Choudhary, Devivasha Bordoloi, Ajaikumar B. Kunnumakkara Investigation of anticancer activity of Dillenia indica on Oral Cancer, Translational Cancer Research-2016 (TCR-2016), Ahmedabad, India, 2016.

## Conferences, Workshops and Trainings Attended:

1. Attended the 1st Departmental Biotech Retreat, organized by Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati on 21st December, 2019.
2. Participated at the 4th International Conference on Nutraceuticals and Chronic Diseases 2019, organized by Indian Institute of Technology Guwahati and Society for Nutraceuticals and Chronic Diseases, September, 23rd -25th, 2019.
3. Participated at 2nd Workshop on "Recent Advances in Cancer Research" RACR-2019 organized by Indian Institute of Technology Guwahati, sponsored by Department of Biotechnology, Government of India, March, 28, 2019.
4. Participated at Indo-Japan Symposium on "Recent Advances in Biomedical Research" RABR-2019 jointly organized by Indian Institute of Technology Guwahati and National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan, March, 26-27, 2019.
5. Participated at Research Conclave 2019, organized by Indian Institute of Technology Guwahati, Assam, India, March, 14-17, 2019.
6. Attended "MAHE- AIST 3 days' workshop on Epigenomics" jointly organized by School of Life Sciences, MAHE, Manipal and AIST, Tsukuba, Japan, at Manipal University, Manipal, Karnataka, February, 20-22, 2019.
7. Participated in 8 days Workshop on "Introduction to basic and advanced biomedical approaches for enhancing QOL in ageing societies" under the Sakura Exchange Program in Science administered by Japan Science and Technology Agency at National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan, October 14-21, 2018.
8. 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.
9. Participated in Indo-Japan symposium on "Hope from Herbs: Research Based Care and Cure Potentials" jointly organized by IIT Guwahati and AIST, Japan, held on 8th May, 2017.
10. Participated in a 12-day advanced research training workshop on 'Understanding Human Disease and Improving Human Health Using Genomics-Driven Approaches' sponsored by Department of Biotechnology, Ministry of Science and Technology, India and organized by National Institute of Biomedical Genomics, Kalyani, Kolkata from 27th February-10th March, 2017.
11. Participated in 9th TCS Annual Event & Flow Cytometry Workshop on 'Flow Applications in Basics, Applied and Clinical Biology (FABACTCS-2016)' organized by Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati from 3rd – 5th November, 2016.
12. Participated in the First International Conference on "Nutraceuticals and Chronic Diseases" jointly organized by Society for Translational Cancer Research and Indian Institute of Technology Guwahati in Cochin, Kerala, India, from 9th -11th September, 2016.

13. Participated in a 5-day research training workshop on 'Understanding Human Disease and Improving Human Health Using Genomics-Driven Approaches' sponsored by Department of Biotechnology, Ministry of Science and Technology, India and organized by National Institute of Biomedical Genomics, Kolkata and Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur held during May 9$^{th}$ -13$^{th}$, 2016 and Selected for the advanced level workshop.
14. Participated in the National conference on 'Recent Developments in Medical Biotechnology and Structure Based Drug Designing' organized by Department of Biosciences and Bioengineering, IIT Guwahati, India, held on 6$^{th}$ & 7$^{th}$ December, 2015.
15. Participated in Symposium Cum Workshop on "Advances in Computational Biology and Computer Aided Drug Design" organized by Bioinformatics Infrastructure Facility (BIF), Department of Biosciences and Bioengineering, held on 24$^{th}$ -26$^{th}$ June, 2015.
16. Participated in a 5-day national course on 'Theoretical and Practical aspects of Cancer Research' conducted under the Technical Education Quality Improvement Programme sponsored by the Ministry of Human Resource Development, Govt. of India, from February 4$^{th}$ – 8$^{th}$, 2015.
17. Participated in the National Conference on 'Recent Advances in Cancer Biology and Therapeutics' organized by Department of Biotechnology, IIT Guwahati, India, held on 5$^{th}$ December, 2014.
18. Participated in the International Conference on 'Disease Biology and Therapeutics', organized by IASST Guwahati held during December 3-5, 2014.

## Awards and Achievements:

1. Received 'Best Poster Award' for the paper entitled "TIPE Family of Proteins as Novel Biomarker in Bone Cancer" at Indo-Japan Symposium on "Recent Advances in Biomedical Research" RABR-2019 jointly organized by Indian Institute of Technology Guwahati and National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan, March, 26-27, 2019.
2. Received travel grant and selected to join an invitation program carried under the framework of Japan-Asia Youth Exchange Program in Science (Sakura Exchange Program in Science) administered by Japan Science and Technology Agency at National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan, October 14-21, 2018.
3. Received "Second Prize In Poster Presentation Category" for the paper entitled "Anticancer activity of Elephant Apple leaf extract against Oral Cancer" at National conference on "Ethno-medicine and Traditional Health Practices In North-East Region of India" organized by National Institute of Pharmaceutical Education and Research (NIPER) Guwahati, Assam, India, 25$^{th}$ August, 2018.
4. Received 'Best Poster Award' for the paper entitled "Indian Catmon: A potent anticancer agent against human oral squamous cell carcinoma" at the Second International conference on Nutraceuticals and chronic diseases 2017 (INCD-2017), Goa, India, 2017.