THE ROLE OF VITAMIN E IN REDUCING BONE CANCER PAIN IN RAT MODEL OF BREAST CANCER-INDUCED BONE PAIN

NUR SAEIDA BINTI BAHARUDDIN

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by

NUR SAEIDA BINTI BAHARUDDIN

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LIST OF SYMBOLS

°C	Degree Celsius
α	Alpha
β	Beta
γ	Gamma
δ	Delta
µ	Micro
±	Plus-minus
%	Percentage
+	Positive
-	Negative
:	Ratio
<	Less than
x	Multiply
Δ	Delta
N	Total number of sample
n	Sample size

LIST OF ABBREVIATIONS

SPECT	Single Photon Emission Computed Tomography
CT	Computed Tomography
ER	Estrogen receptor
PR	Progesterone receptor
HER	Human Epidermal Receptor Factor
TNBC	Triple Negative Breast Cancer
BL	Basal-like
M	Mesenchymal
MSL	Mesenchymal Stem Like
IM	Immunomodulatory
LAR	Luminal Androgen Receptor
DNA	Deoxyribonucleic Acid
EMT	Epithelial Mesenchymal Transition
TGF	Transforming Growth Factor
FGF	Fibroblast Growth Factor
IGF	Insulin-Like Growth Factor
HGF	Hepatocyte Growth Factor
EGF	Epidermal Growth Factor
SMAD	*Caenorhabditis elegans* Sma genes, Drosophila Mad
MET	Mesenchymal to Epithelial Transition
Runx2	Runt-related Transcription Factor 2
MSX2	Homologous of Drosophila muscle segment homeobox gene
MMP	Matrix Metalloproteinase
PTHrP	Parathyroid Hormone-related Protein
M-CSF	Macrophage Colony Stimulating Factor
Ca	Calcium
MAPK	Mitogen-activated Protein Kinase
IL	Interleukin
RANK	Receptor Activator Nuclear Kappa B
RANKL	Receptor Activator Nuclear Kappa B Ligand
OPG	Osteoprotegerin

CIBP	Cancer-induced Bone Pain
NSAID	Non-steroidal Anti-inflammatory Drug
MOH	Ministry of Health
3D	Three dimensional
TC	Technetium
MDP	Methylene Diphosphonate
F	Fluorine
NaF	Sodium Fluoride
FDG	Fluorodeoxyglucose
H&E	Haematoxylin and Eosin
ELISA	Enzyme Linked Immunosorbent Assay
BALP	Bone-specific Alkaline Phosphatase
P1NP	Procollagen Type 1 N-Terminal Propeptide
TRAP 5b	Tartrate-resistant Acid Phosphatase 5b
1CTP	Type 1 Collagen C-terminal Peptide
qPCR	Real Time Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse Transcriptase Real Time Polymerase Chain Reaction
MRI	Magnetic Resonance Imaging
BMC	Bone Mineral Content
BV	Bone Volume
O^{-}_{2}	Superoxide Anion
OH	Hydroxyl Radical
ONOO	Peroxynitrite Radical
LOOH	Lipid Hydroperoxide
Nrf2	Nuclear Factor Erythroid 2-related Factor 2
Keap1	Kelch-like ECH-associated Protein 1
ERK	Extracellular Signal-regulated Kinase
JNK	Jun N-terminal Kinase
ATF	Alpha Tocopherol
TRF	Tocotrienol Rich Fraction
RPMI	Roswell Park Memorial Institute
FBS	Fetal Bovine Serum
CO_2	Carbon Dioxide

USM	Universiti Sains Malaysia
IACUC	Institutional Animal Care and Use Committee
SDR	Sprague Dawley Rats
SH	Sham
NC	Negative Control
ZA	Zoledronic Acid
mg/kg	Milligram per kilogram
Ml	Millilitre
g	Gram / Gravitational Acceleration
s	Seconds
min	Minutes
mCi	Millicurie
n	Sample Size
MBq	Megabecquerel
EDTA	Ethylenediaminetetraacetic Acid
RNA	Ribonucleic Acid
PBS	Phosphate Buffered Saline
RNase	Ribonuclease
M	Molar
HCl	Hydrochloric Acid
ml/g	Millilitre per gram
mM	Millimolar
SD	Reference Standards
Au	Absorbance Unit
SEM	Standard Error of Mean
SPSS	Social Package for Social Science
ANOVA	Analysis of Variance
pg/ml	Picogram per Millilitre
ng/ml	Nanogram per Mililitre
C_T	Cycle Threshold

LIST OF APPENDICES

PERANAN VITAMIN E DALAM MENGURANGKAN KESAKITAN TULANG KANSER MENGGUNAKAN MODEL TIKUS YANG KESAKITAN TULANG DIARUH KANSER PAYUDARA

ABSTRAK

Ketika ini, kesakitan tulang disebabkan kanser menghadapi kekurangan dalam segi pengurusan kesakitan yang berkesan, mengambilkira kesan sampingan yang tidak dikehendaki, ketidakberkesanan relatif, dan kesesuaian jenis rawatan. Kesakitan ini berpunca daripada sel kanser primer yang merebak ke dalam tulang, dan hadir bersama mekanisme kompleks yang melibatkan laluan keradangan dan neuropatik, menyebabkan komplikasi berkaitan skeletal seperti kepatahan tulang, pemampatan akar saraf dan juga hiperkalsemia malignan. Pencarian pilihan terapeutik alternatif bagi mengurangkan kesakitan tulang dan menyasarkan beberapa laluan mekanisme dapat membantu meningkatkan kelangsungan hidup pesakit kanser yang telah metastasis. Kajian ke atas vitamin E sebagai antioksidan telah terbukti dalam membantu kesihatan tulang, anti-radang, anti-kanser dan anti-metastatik, justeru dilihat berpotensi mengurangkan kesakitan tulang yang diaruh kanser. Kajian ini dilakukan bagi menilai peranan vitamin E dalam mengurangkan kesakitan tulang kanser dalam model tikus betina, yang kesakitan tulang diaruh kanser payudara. Model tikus dibahagikan secara rawak ke dalam lima kumpulan yang terdiri daripada 10 ekor tikus. Sel kanser payudara pada konsentrasi 1 x 10^6diaruh ke dalam tulang femur kiri ke atas empat kumpulan rawatan iaitu Kawalan Negatif (NC), Alfa Tokoferol (ATF), Fraksi Kaya Tokotrienol (TRF) dan Asid Zoledronik (ZA) manakala kumpulan Sham (SH) hanya dimasukkan medium kultur steril. Model tikus diberikan setiap hari 60 mg/kg alfa tokoferol untuk kumpulan ATF, 60 mg/kg fraksi kaya tocotrienol untuk

kumpulan TRF and minyak zaitun untuk kumpulan SH dan NC selama 21 hari. Kumpulan ZA diberi 0.1 mg/kg Zoledronic Acid selang seminggu. Penilaian tahap kesakitan dijalankan berselang 4 hari selepas prosedur pengaruhan sel kanser manakala berat model tikus ditimbang berselang seminggu. Kesemua tikus dimatikan pada hari ke 21, selepas prosedur pengimejan Single Photon Emission Computed Tomography (SPECT) menggunakan teknologi radionuklid. Spesimen tulang femur kemudiannya dikeluarkan dan dianalisa untuk histopatologi, ujian enzim-immuno dan juga kajian ekspresi gen. Data dinilai secara statistik menggunakan Statistical Product and Service Solutions (SPSS). Hasil kajian menunjukkan bahawa model tikus berjaya dihasilkan melalui kehadiran "uptake" tidak normal di dalam sistem rangka. Penilaian tahap kesakitan menunjukkan rawatan vitamin E terutamanya TRF mengurangkan kesakitan dengan kesan bererti terhadap stimuli mekanikal berbanding kumpulan NC. Hasil kajian histopatologi terhadap vitamin E juga menunjukkan kesan pengurangan skor yang bererti berbanding kumpulan NC. Selain itu, rawatan ATF menunjukkan kebolehan untuk merencat invasi sel kanser di dalam sum-sum tulang, manakala rawatan TRF mampu mencegah kerosakan tulang di bahagian korteks. Ujian enzim-imuno terhadap vitamin E terutamanya TRF berupaya untuk mengawal penanda metabolisme tulang hampir standing dengan kumpulan ZA. Kajian ekspresi gen mendapati bahawa rawatan TRF setanding dengan kumpulan ZA di mana rawatan ini mampu untuk mengawal proses pembentukan osteoklas dari sel darah, pengaktifan osteoklas, menghalang kerosakan sel tulang dan juga mengawal perembesan sitokin kanser metastatik. Selain itu, rawatan ATF berupaya untuk mengawal laluan RANKL/OPG yang berkait secara langsung dengan aktiviti penyerapan tulang. Secara kesimpulannya, rawatan TRF telah menunjukkan keberkesanan dan potensi sebagai rawatan terapeutik alternatif di dalam mengurangkan kesakitan tulang dan ia juga

mampu mengawal penanda metabolisme tulang serta merencat invasi sel kanser. Hasil kajian ini mengetengahkan potensi TRF berbanding ATF sebagai pilihan terapeutik untuk rawatan kesakitan tulang yang diaruhkan kanser.

THE ROLE OF VITAMIN E IN REDUCING BONE CANCER PAIN IN RAT MODEL OF BREAST CANCER-INDUCED BONE PAIN

ABSTRACT

Cancer-induced bone pain is currently facing inadequate pain management considering the unwanted side effects, relative ineffectiveness and suitability of medicine. The pain resulted from primary cancer that metastasized to bone comes with complicated mechanisms involving inflammatory and neuropathic pathway causing skeletal-related complications such as pathological bone fractures, nerve root compression and hypercalcemia of malignancy. Search for therapeutics alternative options to reduce pain and target few mechanism pathways could improve the overall survival for metastatic patients. Vitamin E as an antioxidant is widely published in bone health, anti-inflammatory, anti-cancer and anti-metastatic properties, thereby potentiate its ability in targeting cancer-induced bone pain. This study aimed to evaluate the role of vitamin E in reducing bone cancer pain in female rat model of breast cancer-induced bone pain. The rats were randomly divided into five groups of ten rats according to the experimental design. Breast cancer cell line, MDA-MB-231 at a concentration of 1×10^6 were induced into left femur of four groups namely Negative Control (NC), Alpha tocopherol (ATF), Tocotrienol Rich Fraction (TRF) and Zoledronic Acid (ZA) whereas Sham (SH) group was injected with culture media only. The rats were orally supplemented daily with 60 mg/kg alpha tocopherol for ATF group, 60 mg/kg Tocotrienol Rich Fraction for TRF group, and olive oil that serves as vehicle to SH and NC groups for 21 days. Whereas, the ZA group was dosed with 0.1 mg/kg Zoledronic Acid weekly. Pain assessment tests were carried out at four days' interval following post breast cancer induction, while body weight were recorded at

weekly intervals. The rats were sacrificed after 21 days, following radionuclide imaging via Single Photon Emission Computed Tomography-Computed Tomography (SPECT-CT). Bone specimens were dissected out and were analysed for histopathological evaluation, enzyme immunoassay biomarkers and gene expression studies. Data were analysed statistically using Statistical Product and Sevice Solutions (SPSS). The results showed that the animal model was successfully validated via the presence of abnormal uptake within skeletal system and the rat model generally displayed good health throughout the treatment by showing consistent weight gain. Pain assessment test demonstrated both vitamin E, specifically TRF supplement significantly alleviate pain perception in mechanical stimuli compared to NC group. Histopathological evaluation showed that both vitamin E demonstrated significant reduction in scoring value compared to NC group. Moreover, ATF supplement specifically prevent invasion in bone marrow whereas TRF supplement specifically prevent destruction of cortex area. Biomarker activity illustrated that vitamin E particularly TRF supplement group able to regulate the bone turnover activity comparable to ZA treatment group. Gene expression studies signify the role of vitamin E specifically TRF supplement comparable to ZA group in the ability to regulate osteoclastogenesis, osteoclast activation, preventing disruption of bone cells in bone and regulating the secretion of metastatic cancer cytokine. ATF supplement also showed efficacy in regulating the RANKL/OPG pathway that directly involve in bone resorption process. Thus, TRF supplement exhibited promising therapeutics target in alleviating the pain as well as regulating bone turnover activity and notably inhibited the cancer invasion. This finding addressed the beneficial potency of TRF compared to ATF as a therapeutic option in the management for cancer-induced bone pain.

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Breast cancer contributes to 11% mortality in Malaysia with incidence frequently occurred in women (Sung *et al.*, 2021) and almost 50% of breast cancer patients experienced relapse at common metastatic site such as skeletal system (Zhang *et al.*, 2018). Although the primary cancer does not derived from bone, breast cancer have a strong predilection to grow in bone microenvironment and thus causing bone cancer pain due to aberrant activity within bone homeostasis (Coelho *et al.*, 2016). Specifically, metastatic breast cancer cell breaks away from the primary site and travel through the bloodstream and invade bone microenvironment where it provides favourable localization for the growth of metastatic cancer cells (Melzer et al., 2017). Bone surfaces along with bone growth factors then promotes invasion, growth and proliferation for the metastatic cancer cell and the growth of cancer cells also promotes activation of higher bone resorption and bone formation activity (McGrath, 2011; Yates *et al.*, 2017). Bone metastasis affecting the normal bone homeostasis activity resulting in either weakened or excessively built-up bone thus increasing the pathological fracture within bone resulting in excruciating pain (Yates *et al.*, 2017). Therefore, these interaction between bone factors, bone microenvironment and metastatic cancer cells cause a vicious cycle leading to bone cancer pain that is currently incurable (Gollahon *et al.*, 2017; Kang *et al.*, 2019).

Alternative medicine provides an adjunct to the modern medicine which is currently in the mainstream within consumers. Compared to newly found and researched herbal medicine, vitamin goes a long way and has been safely established

as a dietary supplements (Combs Jr and McClung, 2016). Vitamin E is a fat soluble vitamin that generally act as antioxidant and can be classified into two main types which are tocopherols and tocotrienols that are composed of eight different compounds namely alpha, beta, gamma and delta classes (Colombo, 2010). They are synthesized by plants and thus are mainly found in plant products such as vegetable oils, seeds and nuts as well as germ portions of cereals (Watson and Preedy, 2008).

Vegetables oils particularly palm oil yield higher sources of tocotrienols thus, incorporating the benefit of palm oil, this study used vegetable suspension oil extracted and concentrated from red palm fruits, *Elaeis guineensis* produced by a Malaysian company, ExcelVite Sdn. Bhd. located in Perak. In contrast to tocotrienol, this study favour upon synthetically available tocopherol with purity ≥ 96% in liquid form. Few studies both in *in-vitro* and *in-vivo* reported the beneficial effect of tocotrienol and tocopherol as antioxidant in bone health activity as well as anti-metastatic and anticancer properties (Patacsil *et al.*, 2012; Kasai *et al.*, 2015; Ahmed and Sylvester, 2018; Kim et al., 2018).

In an effort to study the role of vitamin E in reducing bone cancer pain caused by breast cancer bone metastasis, a model of Sprague Dawley rats was used to mimic the bone cancer pain. The rat model of breast cancer-induced to bone via intraosseous injection in the femur was orally supplemented with both types of vitamin E; tocopherols and tocotrienols along with control treatment. During treatment, pain behaviour activity of rat model was monitored via pain assessment test held every 4 days until sacrifice. The presence of bone metastasis was validated via SPECT-CT imaging via radiotracer before bone sample collection. The *ex-vivo* bone samples were then used on histological analysis, ELISA and qRT-PCR analysis.

1.2 Problem Statement

The widespread use of synthetic drugs and modern medicine remains the single most serious complications following drug consumption. Current drug treatments are primarily focusing on commonly used analgesics where it is restricted by significant adverse side effects; nonsteroidal anti-inflammatory drugs (NSAIDS) with its toxicity to the hepatic system and cardiovascular adverse effects (Maturana et al., 2019), bisphosphonate with its renal toxicity (Fallon *et al.*, 2018) and opioids with common cases of dizziness and nausea (Kiguchi *et al.*, 2019). Along with analgesics, current treatment also fixated on the newly developed treatment; bone targeting therapy via established anti-resorptive agents such as bisphosphonate although the surveillance data reported contradictory results reported its inefficacies (Addison *et al.*, 2016; Hilton *et al.*, 2018). Furthermore, the use of bisphosphonate has been limited to patients with no history of kidney diseases as the drug have been reported to produce direct nephrotoxicity (Ott, 2015). In this regard, management of safe and established alternative medicine is necessary to overcome these problems.

1.3 Rationale of The Study

Breast cancer bone metastasis will eventually resulted in bone cancer pain that is significantly debilitate daily activities and functional status for patients (Tahara *et al.*, 2019). It is currently controllable with common analgesics although there is report on inadequate pain relief and unpleasant side effects due to under-managed pain control (Danson *et al.*, 2019). According to WHO, bone pain should be managed with NSAIDs followed by opioid (Anekar and Cascella, 2021) however in metastases bone pain, bone-targeted agents (BTAs) such as bisphosphonate should be highly considered (von Moos *et al.*, 2022) due to high relief of bone pain and might exert

antitumor effects (von Moos *et al.*, 2019). Despite that, Zoledronic Acid is not recommended for patients with renal impairment, pregnant and nursing women and in patients with non-corrected hypocalcemia (Lambrinoudaki *et al.*, 2008). Moreover, the usage of bisphosphonate can increase the risk of heart failures and skeletal system (Rubin *et al.*, 2020). Nevertheless, bisphosphonate is still the relevant choices for treatment and management of bone metastasis in cancer patients with no other risk of diseases (Gulati *et al.*, 2018).

Current research showed that bone cancer pain can be postulated via few mechanism of actions on pain sensitivity and thus targeting single site does not effectively reduce the pain (James *et al.*, 2020). A better understanding in molecular mechanism of neuropathic pain in breast cancer bone metastasis is required for development of more effective therapeutics option with less side effect. Alternative medicine such as vitamin E might provide a new pain management therapy considering its antioxidant properties that could target few mechanisms in pain signalling pathway. The role of vitamin E as potent antioxidant has been linked to pain signalling and anticancer activities. Few studies has reported that vitamin E actively altering the cancer cell lines *in-vitro* and *in-vivo* by reducing free radical effects (Zulkapli *et al*., 2017), and actively linked to pain signalling pathway (Chiricosta *et al.*, 2019). Therefore, using an animal model with breast cancer-induced bone pain might provide insight of the roles of vitamin E in alleviating bone cancer pain by investigating the mechanism of pain signalling pathway involved. In addition to that, this is the first study to demonstrate the role of vitamin E in bone cancer pain using rat model of breast cancer-induced to bone and thus, the study could lead to a better pain management therapy using alternative medicine in future.

1.4 Objectives of The Study

1.4.1 General Objective

To study the role of vitamin E in regulating bone cancer pain in rat model of breast cancer-induced bone pain

1.4.2 Specific Objectives

i. To assess the effects of alpha-tocopherol and tocotrienol rich fraction on bone cancer pain in rat model of breast cancer-induced bone pain.

ii. To elucidate the cancer invasion and progression activity following cancer cells inoculation in rat model of breast cancer-induced to bone.

iii. To evaluate the effect of alpha-tocopherol and tocotrienol rich fraction in regulating bone destruction within implantation site of breast cancer cell rat model.

iv. To determine the effect of alpha-tocopherol and tocotrienol rich fraction in bone formation and bone resorption properties in rat model of breast cancer-induced bone pain.

v. To validate the mechanism of alpha-tocopherol and tocotrienol rich fraction in bone cancer pain signalling pathway specific to RANKL/RANK/OPG pathway in reducing bone cancer pain in rat model of breast cancer-induced bone pain.

CHAPTER 2

LITERATURE REVIEW

2.1 Breast Cancer

Breast cancer has become one of the most overwhelming diseases with increasing new cases each year and is probably to continue rising. It is now the most common invasive cancer and has surpassed lung cancer with a difference in 0.3% as the highest cases globally in 2020 (Sung *et al.*, 2021). Figure 2.1 showed that breast cancer dominated at 17.3% of the number of new cases in Malaysia for 2020 in both sexes although male patients of breast cancer are low in incidence (Sung *et al.*, 2021).

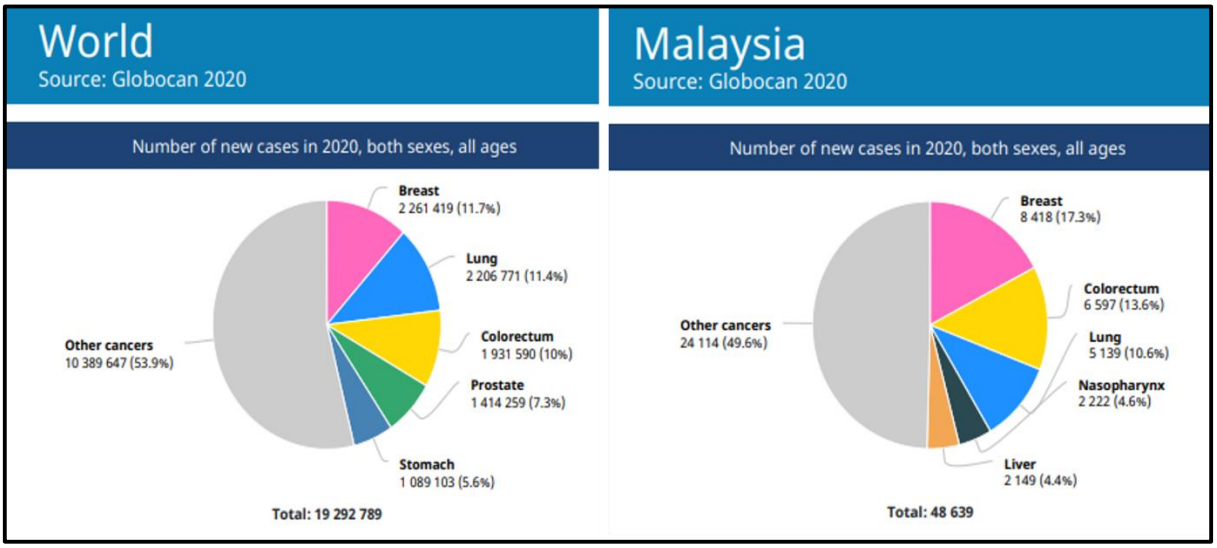

Figure 2.1 Breast cancer cases globally and Malaysia in 2020 for both sexes and all ages (Sung *et al.*, 2021).

Breast cancer specifically starts in breast ducts and some originated from gland and also breast tissue although it is not common and often produces lump in the area (Welsh, 2017). Breast cancer carcinogenesis is commonly linked with unbalanced cell growth, which follows the activation of the oncogenes and/or the deactivation of the tumour suppressor genes (Minari *et al*., 2016). The progression and development of breast cancer were always associated with a complex mechanism including the excessive proliferation and resistance to apoptosis (Aggarwal *et al*., 2014).

2.1.1 **Heterogeneity of Breast Cancer**

Breast cancer is a heterogeneous disease where certain tumour can be treated efficiently but the rest are aggressive and have poor survival rate with such effective treatment option is still in research (Birbrair, 2020; Sferrazza *et al.*, 2020). Currently, it is known that oestrogen plays a vital role in normal breast and also in the development and progression of breast cancer (Dhadlie *et al*., 2018). There are a few subtypes of breast cancer and they can be categorized into molecular classes via the receptor status of the breast cancer cells, oestrogen receptor (ER), progesterone receptor (PR) and human epidermal receptor factor-2 (HER-2) (Yanagawa *et al.*, 2012). Most breast cancer cases detected are ER positive (oestrogen-dependent) and less common are ER negative (oestrogen-independent) while the most aggressive and rarest type are the Triple Negative Breast Cancer (Raisner *et al*., 2019). Figure 2.2 shows schematic representation of the breast cancer subtypes based on their receptor status. TNBC is ER/PR negative and also HER-2 negative.

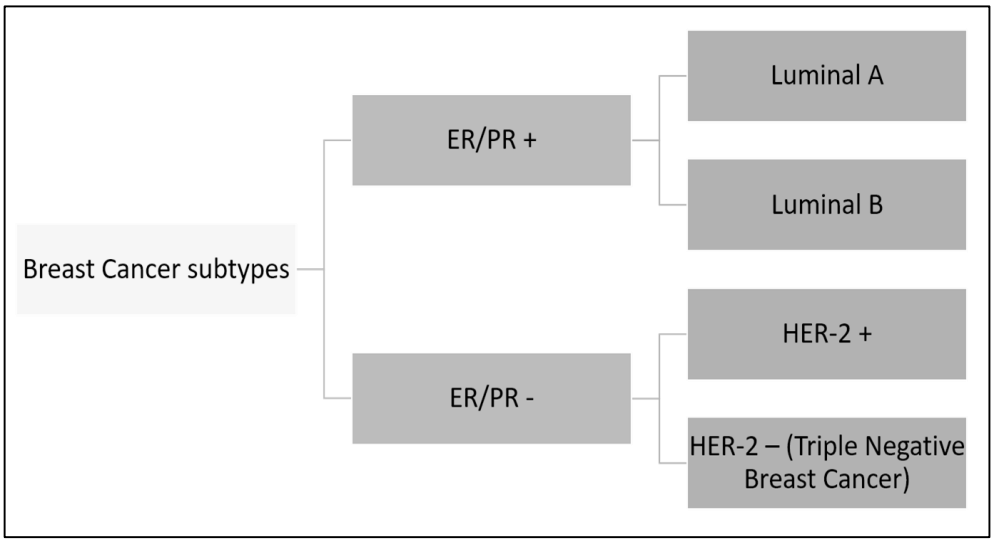

Figure 2.2 Schematic representation of breast cancer subtypes (Deng *et al*., 2020).

Belonging in each subtypes makes each have different risk factors for incidence, contrasting treatment option, varied progression of diseases and preferential sites for metastasis pathway (Perou *et al.*, 2000; Birbrair, 2020). Profiling of the breast cancer tissue subtypes can aid in effective treatment option, and accurate prognosis of the breast cancer subtypes will be useful in facilitating the best strategies to treat breast cancer patients (Platanias, 2006). Luminal A and luminal B subtypes are oestrogen-dependent breast cancer and it represents almost 40% and 30% of all breast cancer cases respectively (Dhadlie *et al*., 2018). They are categorized based on their Ki-67 proliferation index where Ki-67 is a protein in cells that increase as cells are preparing to divide into new cells. In general, higher proliferation index suggest a higher malignancy and implied a high-grade tumour (Yates *et al.*, 2017). Comparing both of these oestrogen-dependent subtypes, the luminal A is less aggressive and the tumour showed a low Ki-67 proliferation index while the luminal B subtypes has worse prognosis with high Ki-67 index (Yanagawa *et al.*, 2012; Inic *et al.*, 2014; Maisonneuve *et al.*, 2014).

Both subtypes frequently have lower scoring in histological grading, low degree of nuclear pleomorphism indicator and low mitotic activity with usually better prognosis although in luminal B subtypes, the histological score could be higher with invasive carcinomas (Welsh, 2017; Yates *et al.*, 2017). The recurrence and relapse rate in both of these subtypes are less than 10% compared to other subtypes and the treatment is mainly based on hormonal therapy (Deng *et al.*,, 2020). Contrarily, for oestrogen-independent breast cancer, the prognosis of this subtype is worse as they are insensitive to anti-oestrogens, higher histological scoring grade, higher recurrence and relapse rates with low survival (Singh and Orr, 2004). HER-2 is a member of four-member tyrosine kinases and encoded by the HER2 gene where overexpression of these gene can transform cells into malignant tumour, thus accelerating tumorigenesis (Burstein, 2005). Without specific treatment, HER-2 positive tumour were positively associated with larger tumour size and subsequently lower the survival rate for almost 20% of patients of this breast cancer subtype (Pathmanathan *et al.*, 2012).

Another subtype with oestrogen-independent and also HER-2 negative are called triple-negative breast cancer (TNBC). This subtype is a lot more difficult to cure as there is no specific protein for cancer drug targets. TNBC are also known as basal-like breast cancer and it represent about 20-30% of all breast cancer cases (Singh and Orr, 2004; Tan, 2018). Physical properties of this subtype under histological evaluations are poor tubule formation and presence of central necrotic and fibrotic zones, conspicuous lymphocytic infiltrate with high mitotic and proliferative index (Heitz *et al.*, 2009). In addition to that, the tumour size are larger and they exhibit high tumour grading score with mass-like lesion, round regular morphology deceiving as benignity and aggressive invasion which would explained poor prognosis and high mortality rates (Deng *et al.*, 2020).

There are 6 known subtypes under TNBC which are basal-like 1 (BL1), basal-like 2 (BL2), mesenchymal (M), mesenchymal stem like (MSL), immunomodulatory (IM) and luminal androgen receptor (LAR) (Deng *et al.*, 2020). BL1 and BL2 generally known as invasive ductal carcinoma and their expression of gene profile is basically instructed with deoxyribonucleic acid (DNA) damage and growth factor signalling. Both M and MSL exhibited increased expression of epithelial-mesenchymal transition (EMT) and growth factor which recently has been considered as the first step in metastatic cascade. In IM subtypes, high expression of immune cell signalling and thus aiding in favourable prognosis and lastly, the LAR is essentially the most distant among TNBC subtype where the gene profile is generally instructed in oestrogen-regulated genes although the main subtypes is ER-negative. LAR subtypes might explain why some of the TNBC showed responsiveness to hormonal therapy and partly responsive in chemotherapy (Harano *et al.*, 2018; Li *et al.*, 2018).

The 5-year survival rate for TNBC subtype is only 14% compared to the 55% survival rate for other subtype (Gonçalves *et al.*, 2018) and positive treatment lasted within one year before the cancer start to metastasize (Deng *et al.*, 2020). High mortality rate in this subtype is due to transcription factor that activated many pathways that regulate genes involved in cancer cell survival, invasion and also metastasis (Singh and Orr, 2004). Metastasis is a complex process involving the transformation of cells from epithelial to mesenchymal morphology in a pathway frequently found on ER-negative tumour cells (Deng *et al.*, 2020).

2.2 Metastasis Pathway

The process of malignant cancer cells expanding to metastasis occur in a series of cascade originating from accumulation of cells mutations where normal cells aggregate into a state of hyperplasia and subsequently detach from their surroundings (Birbrair, 2020). In genetic pathway of metastasis, down-regulation of cell adhesion receptors (E-cadherin) is responsible for cell adhesion and attachment while up-regulation of the receptors (N-cadherin) enhanced the cell motility (Shevde and King, 2007). The metastatic cells then release proteases which eventually degrade the extracellular matrix and thus become mobile along with addition of membrane metalloproteases which aid in physical pathway for cells to metastasize to other organs (Gollahon *et al.*, 2017). In general, the metastatic cells gains more mesenchymal phenotype via this complex process known as the epithelial to mesenchymal transition (EMT) and schematic figure of this pathway is presented in Figure 2.3. This process also may activate cell-cycle dysregulation genes and also inactivate normal apoptotic pathway (Melzer *et al*., 2017; Ma *et al.*, 2020).

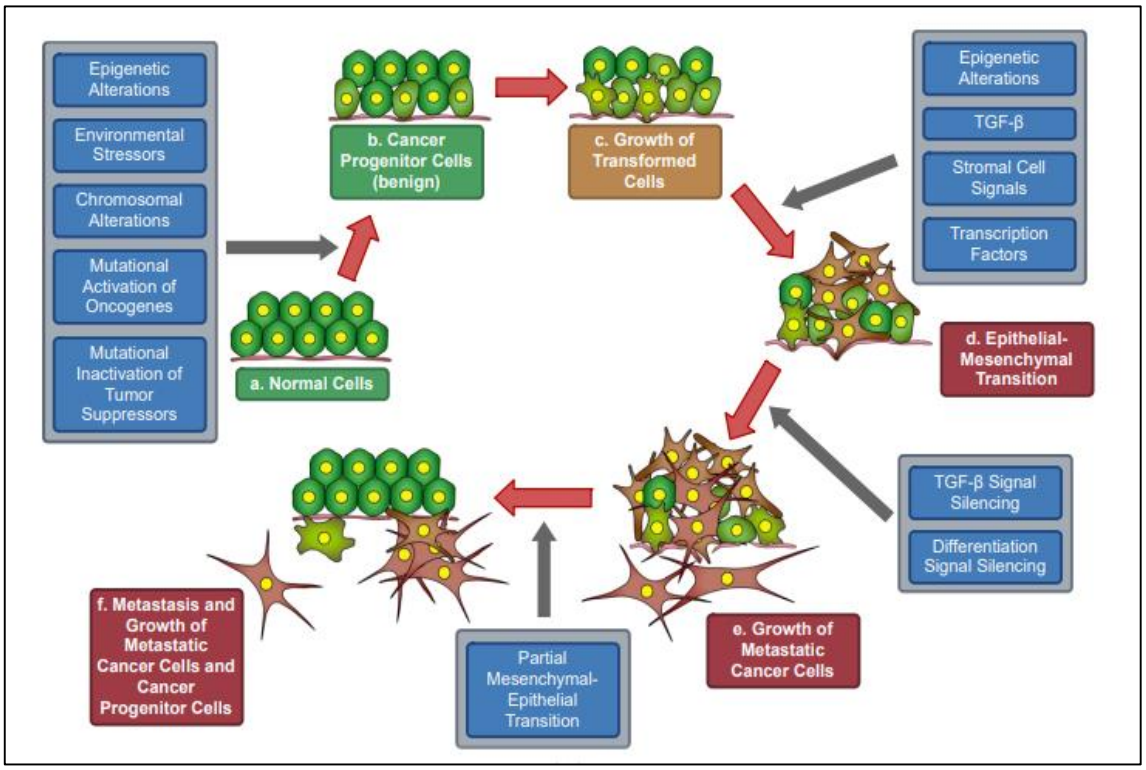

Figure 2.3 Pathway progression of metastatic cancer. Progression of normal cells to progenitor cells can be caused by several factors such as mutation and environmental. Transcription factors aided transformed cells to undergo EMT and eventually grow into metastatic cancer cells (Aggarwal *et al*., 2014).

2.2.1 **Epithelial Mesenchymal Transition**

Despite the role of EMT in normal cell development, EMT also present in pathological process particularly in cancer progression and metastasis (Xu, 2016). In cancer biology study, EMT are frequently expressed in many non-epithelial tumour cells such as leukaemia and melanoma causing confusion in prognosis (Fischer *et al.*, 2015). Tumour cells to metastasize into lymph, blood vessel and other organs must establish an EMT first to gain motility with mesenchymal characteristic in cells. Generally, after the epithelial tumour cells going through an EMT and gain contact with the lymphatic system, the epithelial tumour cells becomes more invasive resulting in dissipation of cancer cells to other organ (Fischer *et al.*, 2015).

The epithelial to mesenchymal transitions are related to down-regulated of epithelial cell adhesion molecule, E-cadherin and lower E-cadherin values has been linked to higher frequency of metastasis activity (Ablin and Mason, 2007). However, the role of EMT in cancer are complicated due to their multiple functions and are often non-redundant in which they might trigger primary cancer to metastasize on bone but sometimes negatively affecting the metastasis in other primary cancer (Fischer *et al.*, 2015).

2.2.2 EMT and Transforming Growth Factor β

Several factors that induced or regulated the EMT process has been outlined such as transforming growth factor β (TGF-β), fibroblast growth factor (FGF), insulin-like growth factor (IGF), hepatocyte growth factor (HGF) and epidermal growth factor (EGF) (Romagnoli *et al.*, 2012). In particular, TGF-β draws a broad purpose in cellular response and alterations in TGF-β signalling have resulted in many diseases including cancer (Hao *et al*., 2019). TGF- β is a multifunctional cytokine that has received loads of research study due to their vital role in cancer progression in metastasis pathway. Evidence show that increased TGF-β signalling plays a vital role in cancer progression and metastasis and also enhances angiogenesis of the tumour cells to provide an effective migratory mesenchymal cells (Xu, 2016). Mesenchymal phenotype induced by TGF-β also suppress the epithelial genes and thus promotes the expression of mesenchymal proteins and further increase the more migratory mesenchymal cells (Hao *et al*., 2019). Figure 2.4 show the signalling pathway where TGF-β involved in transition of epithelial cancer cells to mesenchymal cancer cells and thus promotes the metastasis process.

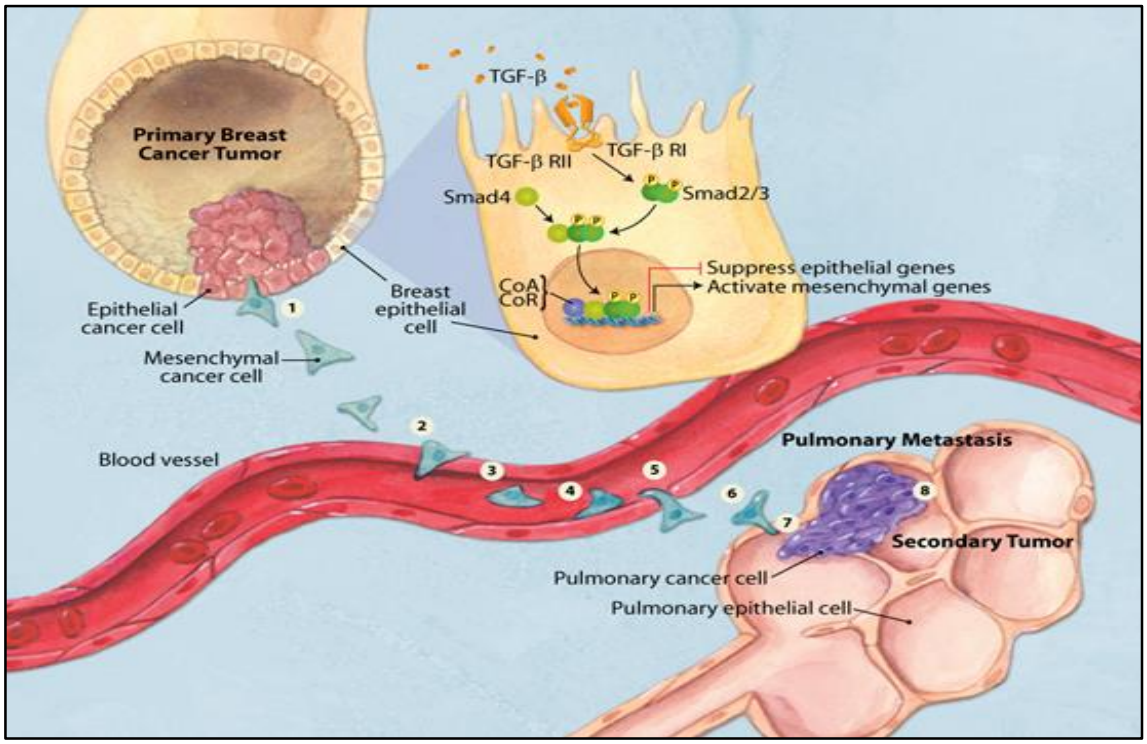

Figure 2.4 Breast cancer metastasis dependent on EMT and induced by TGF-β signalling (Gulati *et al.*, 2018)

Details signalling pathway in this complex process originated with TGF-β binding with type II TGF-β receptors which further stimulate the phosphorylation of Smad2 and Smad3. The phosphorylated Smad2 and Smad3 forms a trimer with Smad4 and then translocate to the nucleus where it activated the transcription factors, co-repressor, co-activators to suppress epithelial genes and activates mesenchymal genes which in turns regulates the expression of mesenchymal proteins (Buijs *et al.*, 2007; Tang *et al.*, 2009; Hao *et al.*, 2019). Highly motile mesenchymal cancer cells metastasize to other organs via a series of cascade which requires migration through lymphatic system, invasion to secondary tissue, reverse EMT or known as mesenchymal to epithelial transition (MET) and finally distal proliferation in the secondary tissue or organ such as lung and bone where lung provide ample glucose and bone contain large supply of growth factor for cancer cells to grow effectively (Romagnoli *et al.*, 2012).

2.2.3 **Transforming Growth Factor β and MDA-MB-231 Promotes Bone Metastasis**

Research on the relation between TGF-β signalling and breast cancer metastasis repeatedly utilizing the breast cancer cell line of MDA-MB-231 (human breast carcinoma cell line), a TNBC subtype with aggressive invasion characteristic. Metastatic study employing MDA-MB-231 showed that the cells capable of reallocation to greater distance and the cell displayed eminent motility after stimulated with TGF-β compared to without stimulation (Luwor *et al.*, 2015). Treatment with TGF-β in MDA-MB-231 cells activate the p38 that involved in apoptotic pathway and cancer-related pathway (Hedrick and Safe, 2017). Both studies showed that the role of TGF-β in metastasis cancer is linked to one another which makes it harder for treatment as there are too many interactions and pathway involved to target. In an *in-vivo* model, usage of MDA-MB-231 breast cancer cell lines were always directed towards breast cancer bone metastasis model specifically in rodents. The stimulation of TGF-β in breast cancer MDA-MB-231 bone metastasis model is able to activate the release of cytokines and other growth factors to maintain the metastatic process in bone microenvironment (Welsh, 2017; Birbrair, 2020). Implementation of this cell line in breast cancer bone metastasis model have been major contributor towards the understanding of the mechanism and complex process for this late stage metastasis.

Preclinical *in-vivo* model using MDA-MB-231 injected via intra-cardiac prompt to metastasize to bone with higher resorption activity in bone compared to using MCF7 breast cancer cell line (Lemma *et al.*, 2017). Moreover, breast cancer cell lines, MDA-MB-231 with an overexpressing PTHrP (growth factor) in bone metastatic model is shown to increase osteolytic bone metastasis and accelerate the bone degradation process (Yoneda *et al.*, 2001). Further study by Yoneda *et al.*, (2001) also

proved that MDA-MB-231 exclusively metastasize to bone with larger osteolytic lesion. Suitable environment in bone which is hypoxic facilitate the tumour growth and tumour invasion thus fluctuating the bone homeostasis (Coelho *et al.*, 2016; Lemma *et al.*, 2017). Generally, the cancer cells and bone microenvironment symphonize the vicious cycle of tumour growth and bone resorption.

2.2.4 Vicious Cycle of Bone Metastasis

The core process of bone metastasis started with dissemination of metastatic cancer cells within the bone microenvironment causing disruption of normal bone homeostasis in bone formation and bone resorption activity (Coelho *et al.*, 2016). High bone turnover activity induced by the binding of RANKL and RANK along with secretion of M-CSF causing the osteoclastogenesis activation. Specifically, RANKL that is secreted by the osteoblast binds to RANK that are expressed on osteoclast precursors to activate the osteoclast maturation and differentiation whereas M-CSF secreted by osteoblast are required for osteoclast activation (Takahashi *et al.*, 2014; Gollahon *et al.*, 2017). Following high resorption activity, mature osteoclast secretes TGF-β which in turn provides growth factors to the cancer cells to survive within bone microenvironment (Yin *et al.*, 1999). Progression and invasion of the cancer cells induces the secretion of cytokine factor such as PTHrP and IL-6 to stimulate the production of osteoblast thus continuing the vicious cycle (Gollahon *et al.*, 2017). In addition to that, OPG secreted by osteoblast could act as a decoy receptor inhibiting the binding of RANKL and RANK in osteoclastogenesis (Birbrair, 2020). Figure 2.5 showed the illustration of bone metastasis pathway.

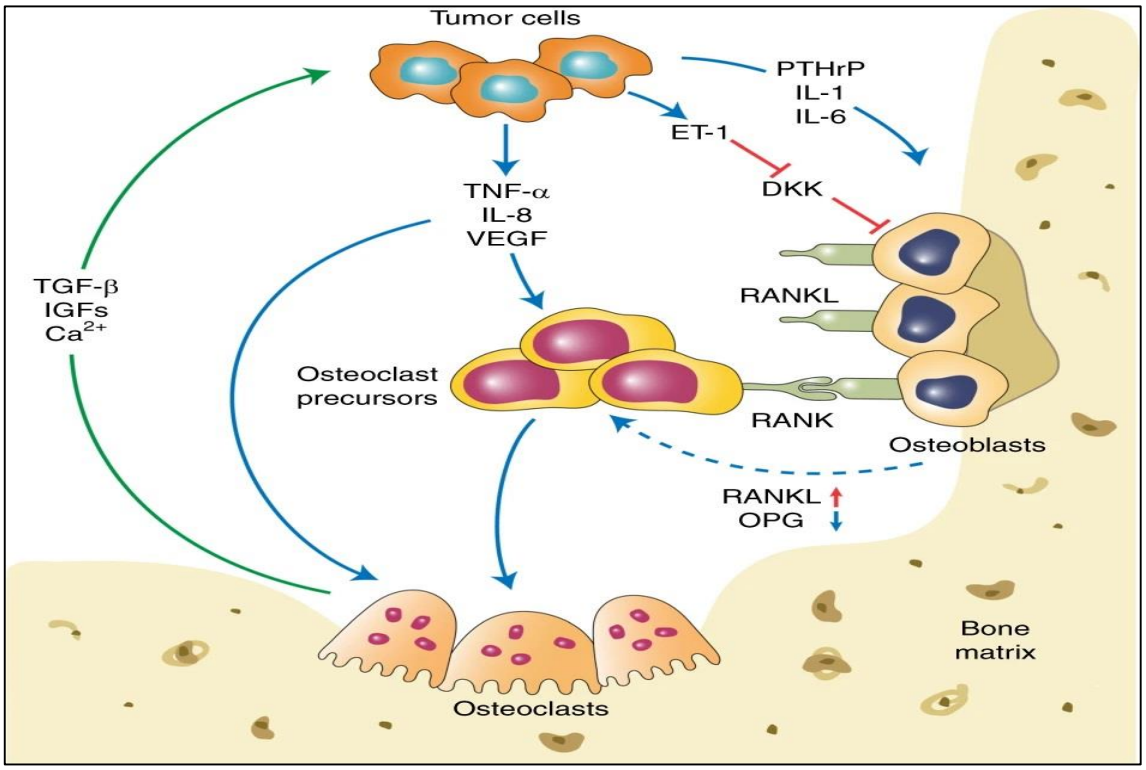

Figure 2.5 Vicious cycle of bone metastasis pathway (Jandial, 2013)

2.3 Bone Tissue

Bone is the main element of skeletal system with mineralized connective tissue that contains few important types of cells including osteoblasts, osteocytes and osteoclast where it is known as basic multicellular unit together (Burr and Allen, 2013). Osteoblasts are bone-producing cells derived from osteoprogenitor cells that is differentiated via bone morphogenetic protein (BMP) (Burr and Allen, 2013). They are small and single nucleated cells found nearly within periosteum and endosteum (Steele and Bramblett, 1988). Osteoblasts produce multi-fold of cell products such as osteocalcin, growth factors and osteoid which gradually become calcified and mineralized (Burr and Allen, 2013). As the mineral calcifies, these single cells are trapped within lacunae and functionally become the osteocyte or bone maintaining cells.

Osteocytes make up almost 90% of cells in bone tissue and they are vital for cell communication within bone tissue via canaliculi through the bone matrix. In addition to that, osteocytes have shown to regulate the bone homeostasis where abnormal osteocytes function could lead to bone brittleness (Birbrair, 2020). Osteoclasts are large with multinucleated cells that are related to bone resorption process where an injured bone is removed by dissolving enzymes and acids into bone minerals. Higher osteoclast activity can be seen within osteoporosis, bone metastasis and other bone related diseases (Burr and Allen, 2013).

2.4 Bone Microenvironment Facilitates Bone Metastasis

"Seed and Soil" theory demonstrate the interactions between cancer cell growth and bone microenvironment where bone possess highly mineralized and specialized connective tissue to facilitate the growth and survival of cancer cells (Coleman and Eccles, 2005; Segaliny *et al.*, 2019). Primarily the cancer cells secrete cytokines which stimulate the osteoclast-mediated bone destruction and also promoting cancer cells growth and survival. The bone destruction mechanism then releases growth factors that further stimulate tumour growth resulting in a cycle pattern of bone destruction and tumour proliferation (Kardamakis *et al*., 2009). Both cancer cells and bone cells may depend on similar signalling pathways and transcription factors to survive in bone microenvironment regarded as 'osteomimicry'. This anomaly enables the cancer cells go undetected by the immune system. For instance, the bone sialoprotein under Runx2 and MSX2 transcription factors expressed by metastatic breast cancer cells were also a regulators in osteoblast function (Koeneman *et al.*, 1999). Both activity of breast cancer metastatic cells and osteoblast cells stimulates the production of angiogenic factors and matrix metalloproteinase (MMP)

which then facilitate the cancer cells to bind into bone environment. Bone matrix which consists of growth factors such as transforming growth factors-β (TGF-β) and insulin-like growth factor-I (IGF-I) that are released by osteolysis can stimulate the tumour cell proliferation (Pratap *et al.,* 2005; Kardamakis *et al*., 2009).

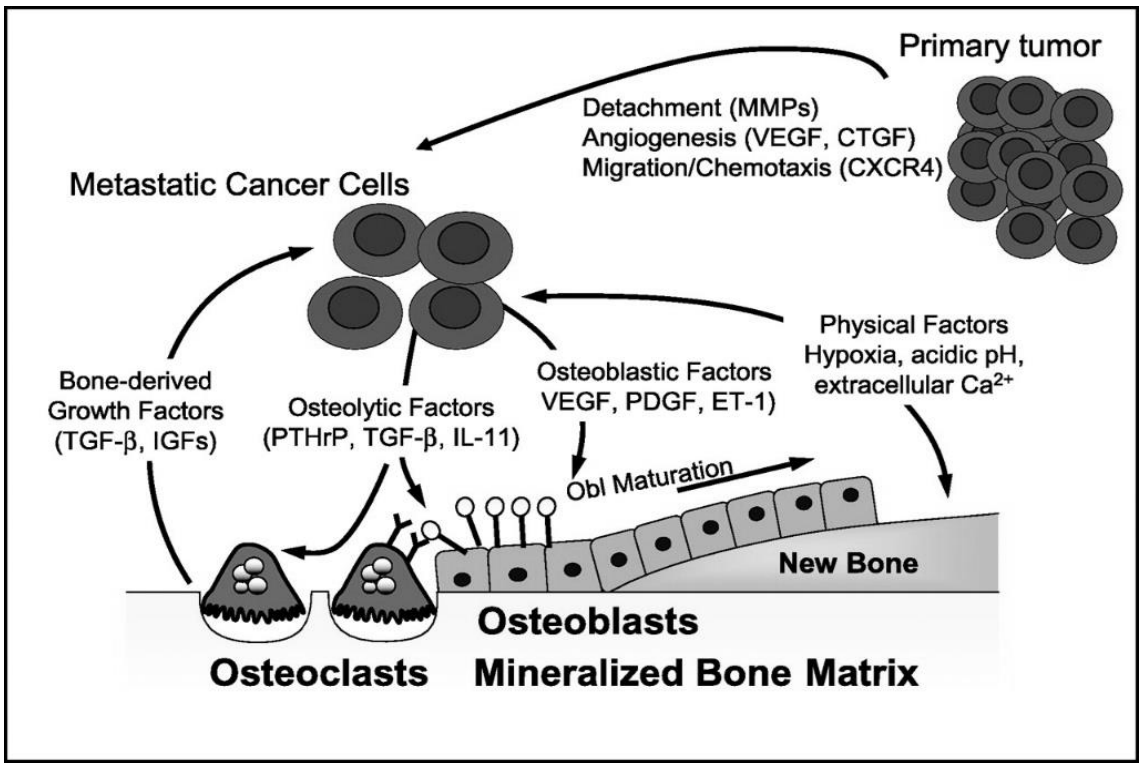

Figure 2.6 Cycle of bone metastasis and regulatory factors in tumour-bone microenvironment (Song *et al*., 2022)

Factors secreted by tumour cells were able to transform the bone microenvironment and thus generating a metastatic niche for colonization. Based on Figure 2.6 breast cancer cells secrete osteolytic factors such as PTHrP, TGF-β and Macrophage colony stimulating factors (M-CSF) as they attempt to mimic the osteolysis activity. Other clinical and preclinical data associate the high expression of PTHrP and M-CSF with bone metastasis and bone destruction (Park *et al.,* 2014; Gollahon *et al.,* 2017). When breast cancer cells invade bone microenvironment,

secretion of osteolytic factors activate osteoclast formation indirectly through osteoblast stimulation via regulator gene and thus resulting in bone resorption. Consequently, growth factors derived from bone caused proliferation to the cancer cells and in turn, stimulates growth and survival of cancer cells and causing more bone loss (Singh and Orr, 2004).

Increased expression of this osteolytic factors in bone metastasis might alter the responses to induction therapy and thus could be a potent therapeutic target for bone metastasis. Blocking the osteolytic factors such as PTHrP and TGF-β might turns into clinical benefit as few studies have shown that decreased expression of PTHrP could inhibit the osteolysis caused by human breast cancer cells (Huang *et al.*, 2014; Pitarresi *et al.*, 2019). Moreover, there are few potential target in this metastatic pathway as the interactions between each growth factors, cytokines and transduction pathway are closely related. For example, as osteolysis caused the releases of bone-derived growth factors such as TGF-β and IGF-β, the extracellular calcium (Ca_{2+}) concentrations from mitochondria also increased (Burr and Allen, 2013). The extracellular Ca_{2+} binds and activates Ca_{2+} pump which used by cancer cells for cell signalling. Few studies have used Ca_{2+} for potential target in cancer study (James *et al.*, 2020) and showed a promising effect.

Other potential target is during the binding of growth factors on cancer cells where it activates phosphorylation and further enabling signalling through pathways involving SMAD and MAPK pathway. In this signalling, cytokines such as M-CSF, IL-1, IL-6 and IL-18 with growth factors such as PTHrP and TGF-β were involved and could be targeted in therapeutic action (Deeble *et al.*, 2001; Harmer *et al.*, 2019). Blocking or supplementing compounds that decrease PTHrP expression was shown to

inhibit osteolysis and osteoclast activation *in-vitro* induced with breast cancer (Kunihiro *et al.*, 2019; Ponnapakkam *et al.*, 2019). Suppression of TGF-β signalling pathway inhibited invasion of TNBC cells subtype *in-vitro* where it could lead to a potential target of breast cancer bone metastasis (Amerizadeh *et al.*, 2019; Gong *et al.*, 2019; Khoshakhlagh *et al.*, 2019; Li *et al.*, 2019).

Within the literature, there has been much research surrounding the concepts of receptor activator of nuclear factor kappa-B (RANK) and its ligand (RANKL) with osteoprotegerin (OPG). A RANKL is a homo-trimer transmembrane protein expressed by osteoblast which is responsible for the bone formation while RANK is a receptor located on osteoclast precursors and mature osteoclast and provide signal for differentiation, activation and survival of osteoclast (Fohr *et al.*, 2003). OPG is an osteoclast regulatory protein expressed by osteoblast and inhibits RANKL and plays a specific role in inhibition of bone destruction (Preedy and Patel, 2017). Brief diagram as in Figure 2.7, pre-osteoclast or osteoclast precursor have the RANK receptor on their cell membrane and the osteoblast will express and release RANKL along with OPG. OPG will then inhibit and bind to RANKL as a negative regulator of RANKL. After RANKL is available and are able to bind to RANK receptors on osteoclast precursor, then it initiates the osteoclast precursors differentiate and activated into mature osteoclast (Fohr *et al.*, 2003).

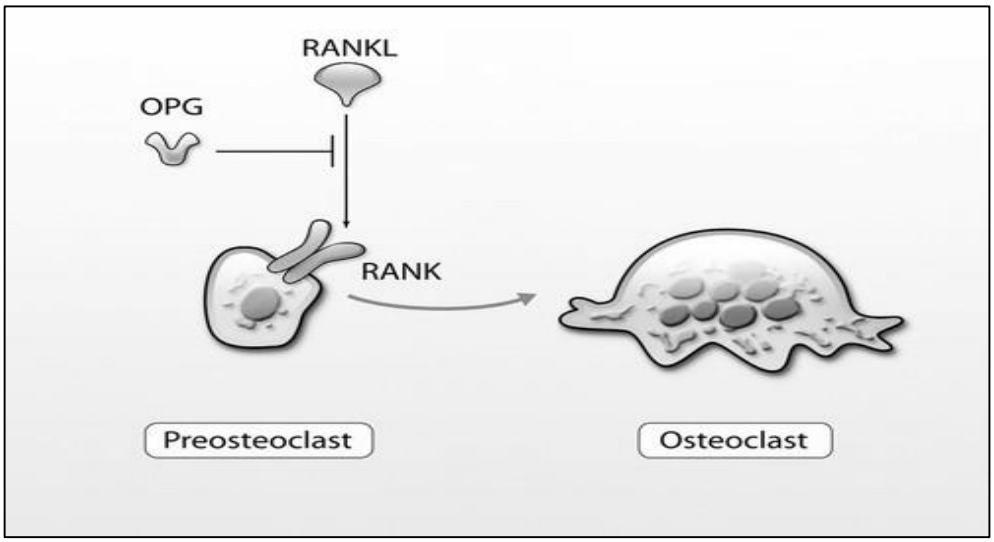

Figure 2.7 RANKL/RANK and OPG signalling pathway (Linda *et al*., 2011)

In bone metastasis, role of RANKL, RANK and OPG as a potential biomarker and therapeutic targets has been widely discussed (Gollahon *et al.*, 2017; Timotheadou *et al.*, 2017; Birbrair, 2020). Secretion of growth factors and cytokines by cancer cells stimulate the osteoblast which in turn expressed the release of RANKL that acts on receptor either the RANK receptor or OPG decoy receptor. During bone metastasis, other factor such as PTHrP and M-CSF coexisted and thus increase the secretion of RANKL to bind with its receptor RANK (Gollahon *et al.*, 2017). PTHrP were also believed to decrease the production of OPG and thus leading to higher production of activated osteoclast (Gollahon *et al.*, 2017).

Inhibition to overexpression of RANKL provide an insight to the treatment of breast cancer bone metastasis. Using an *in-vivo* animal model induced TNBC breast cancer subtypes, mainly MDA-MB-231 treated with RANKL blocking antibody showed a reduction in cell invasion and migration and as well as activity of osteoclastogenesis (Cuyàs *et al.*, 2017; Sousa *et al.*, 2018; Nakai *et al.*, 2019). The inhibition of RANKL may disrupts the metastatic capability of breast cancer and thus

improves the survival in animal model of breast cancer bone metastasis (Bao *et al.*, 2015). OPG is also a receptor and can reduce the osteoclast activation by binding to the RANKL. Despite the function as decoy receptor for further osteolysis in normal bone homeostasis, there is a new finding associating the OPG with breast cancer progression and bone metastasis (Elfar *et al.*, 2017; Kiechl *et al.*, 2017; Kang *et al.*, 2019; Rachner *et al.*, 2019; Shaker and Elbaz, 2020). The findings suggest that the mechanism of OPG and bone microenvironment are capable to inhibit bone resorption in normal bone however no apparent advantages exists in later part of breast cancer progression and invasion in the bone.

2.5 Cancer-Induced Bone Pain

The term of pain describes as an "displeasing experience within the system related to definite or uncertain damage experienced by the subject". Nociception is a process within neural system that encode the signals input leading to pain via nociceptors in the periphery and transmitted to the brain via spinal cord (Deuis *et al.*, 2017). The nociceptors detect any possible type of stimuli including mechanical, thermal and chemical stimuli that have potential causing tissue damage. The perception of pain is divided into three major stages. During the early and middle stage, sensitivity of pain signals was transmitted to the dorsal horn located in spinal cord from the periphery via the peripheral nervous system while during the last stage, the signals are then transmitted to the brain via central nervous system (Sinatra *et al.*, 2010). Herein, two pathways for signal transmission were involved which are ascending pathway and descending pathway. The first route transmitting sensory information goes upward from body towards the brain while the second route goes

downward carrying pain perception from brain to the reflex organ (Falk and Dickenson, 2014).

Cancer-induced bone pain (CIBP) is a chronic pain where the exact mechanism is complex and incompletely understood until now where the evidence on the efficacy of the treatment varied (Gulati *et al.*, 2018) as the pain occurring concurrently as mixed pain, such as acute and chronic within the neuropathic and inflammatory (Falk *et al*., 2014). The molecular mechanism and pathological behaviour of bone cancer pain have not been fully answered despite the survival rate for breast cancer bone metastasis patients were significantly decreased after diagnosis. The 5-year survival rate drops to 27% from 91% in patient with late stage distant metastasis compared to the stage I and stage II breast cancer and the life expectancy after diagnosis was 2-3 years (DeSantis *et al.*, 2019). In Malaysia, limited studies focusing on late stage cancer compared to early stage cancer and the survival rate for Malaysian in breast cancer bone metastasis patients were almost 20% and according to the ethnic studies, the highest survival rate of breast cancer patients were Chinese and Indian, compared to Malays women (Bhoo Pathy *et al.*, 2011; Ibrahim *et al*., 2012). In bone cancer pain, the pain may be problematic and cause difficulties towards the patients, however the possible reason for what may have caused the pain still causing some confusion in research communities. Figure 2.8 showed possible mechanism and stimulus for pain in distant metastasis. It was found that both acute pain and chronic pain were likely caused by the tissue damage, pathological fracture, treatment complications and spinal cord or nerve root compression although the prevalence of these symptoms are unclear (Peters *et al.*, 2005; Kardamakis *et al*., 2009; Yamaguchi *et al.*, 2015).