Role of DNA Methylation in Skeletal Metastasis and Bone Remodeling: Diagnostic, Prognostic and Therapeutic Implications

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A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Acknowledgements

First and foremost, I would like to thank my supervisor Dr. Shafaat A. Rabbani for giving me the opportunity to join his lab. His prudent guidance helped me to stay focussed throughout my PhD program. He provided a very positive environment to develop scientific knowledge and gain research skills. His energy and outlook on life inspires me to follow his footsteps.

I would also like to thank:

My advisory committee members: Dr. Hugh Bennett, Dr. Andrew Bateman, Dr. Geoffery N. Hendy for their support and insightful research advice. Special thanks to Dr. Hugh Bennett for recommending a good lab with my research interest.

Our collaborators: Dr. Moshe Szyf and Dr. David Cheishvili for the Epigenetics research part of my project. Dr. Szyf provided valuable suggestions regarding my project and manuscripts. Dr. Cheishvili gave technical expertise and generous help in data analysis.

My friends and colleagues: Ani Arakelian, Loan Nguyen Yamamoto, Aimee Lee and Amirtha for their friendship and support throughout my PhD. Their love and affection made Montreal a home away from home. Ani's research skills helped me fine tune my experiments. Special thanks to Loan for translating the thesis abstract to French.

Finally, I would like to thank my parents and little sister for their love and constant encouragement to pursue my career. And last but not the least, my husband Kiran K. Chatrathi for his love and understanding.

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Preface and contribution of authors

This thesis is presented in a manuscript based format according to McGill University guidelines for thesis preparation. I wrote the entire thesis and my supervisor Dr. Shafaat A. Rabbani revised it. The thesis includes 5 chapters: Chapter 1- literature review, Chapter 2-4 – one published article and two other articles under revision and Chapter 5 – general discussion.

Publications:

I. Peer-reviewed:

1. S-adenosylmethionine blocks osteosarcoma cell proliferation and invasion in vitro and tumor metastasis in vivo: therapeutic and diagnostic clinical applications. **Parashar S**, Cheishvili D, Arakelian A, Hussain Z, Tanvir I, Khan HA, Szyf M, Rabbani SA. Cancer Med. 2015 May; 4(5):732-44

I performed all the research work with the exception of immunohistochemistry analysis which was performed by Dr. Tanvir and Dr. Hussain. Dr. Khan performed preliminary *in vitro* experiments for SAM dose analysis. Dr. Cheishvili helped in all the bioinformatics analysis and Ani Arakelian helped in the animal experiments presented in the manuscript. This study was supervised by Dr. Shafaat Rabbani. I also wrote the manuscript which was further revised by Dr. Shafaat Rabbani and Dr. Moshe Szyf.

II. Manuscript in Preparation:

2. DNA methylation signatures of breast cancer in peripheral T-cells. **Parashar S**, Cheishvili D, Arakelian A, Rabbani SA.

I performed all the research work presented in the manuscript. Dr. Cheishvili helped in all the bioinformatics analysis presented in the manuscript. The first draft was written by me. Minor revisions were recommended by Dr. Shafaat Rabbani.

3. Identification and Validation of an Epigenetic Signature of Osteoporosis in Post-Menopausal Women. **Parashar S**, Cheishvili D, Arakelian A, Rabbani SA.

I performed all the research work presented in the manuscript. Dr. Cheishvili helped in all the bioinformatics analysis presented in the manuscript. The first draft was written by me. Minor revisions were recommended by Dr. Shafaat Rabbani.

In addition to the manuscript presented in this thesis, I contributed to following article:

Pharmacological methyl group donors block skeletal metastasis in vitro and in vivo. Shukeir N, Stefanska B, **Parashar S**, Chik F, Arakelian A, Szyf M, Rabbani SA. Br J Pharmacol. 2015 Jun; 172(11):2769-81.

Abbreviations:

ALP	Alkaline phosphatase
AML	Acute myeloid leukemia
ATP	Adenosine triphosphate
BMP	Bone morphogenetic protein
BSP	Bone sialoprotein
cDNA	Complementary DNA
Coll A1	Collagen type I, Alpha I
CpG	Cytosoine-guanine dinucleotide
CXCL12	C-X-C motif chemokine 12
CXCR4	C-X-C chemokine receptor type 4
DNA	Deoxyribose nucleic acid
DNMT	DNA methyl transferase
ECM	Extracellular matrix
EGFR	Epithelial growth factor receptor

EMT	Epithelial to mesenchymal transition
EWAS	Epigenome-wide association studies
EXOC7	Exocyst Complex Component 7
FDA	Food and drug administration
FGF	Fibroblast growth factor
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HER2	human epidermal growth factor receptor 2
i.v.	intravenous
IGF-1	Insulin-like growth factor 1
IPA	Ingenuity pathway analysis
MAPK-ERK	Microtubule associated protein kinase-extracellular signal-regulated kinases

M-CSF	Macrophage-colony stimulating factor
MDS	Myelodysplastic syndrome
mg	Milligram
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OCN	Osteocalcin
OPG	Osteoprotegrin
OS	Osteosarcoma
OSX	Osterix
PAI1	Plasminogen activator inhibitor 1
PAI2	Plasminogen activator inhibitor 2
PBS	Phosphate buffered saline
PCGF3	Polycomb Group Ring Finger 3

PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFA	Platelet-derived growth factor alpha
PDGFR	Platelet-derived growth factor receptor
PHGDH	3-Phospoglycerate Dehydrogenase
PPi	Pyrophosphate
qPCR	Real time PCR
RANK	Receptor activator of nuclear factor kappa- B
RANKL	Receptor activator of nuclear factor Kappa-B ligand
RB1	Retinoblastoma protein
RUNX2	RUNX- related transcription factor 2
SAH	S-adenosylhomocysteine

SAM	S- adenosylmethionine
SCID	Severe combined immunodeficiency
SEM	Standard error of mean
SERPINS	Serine protease inhibitors
SMAD4	SMAD family member n°4
SP7	Transcription factor Sp7
STAT3	Signal transducer and activator of transcription 3
TGF-β	Transforming growth factor beta
TP53	Tumor protein p53
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor
VEGFR	Vascular endothelial growth factor receptor

Abstract:

Breast and prostate cancer are two most common malignancies associated with skeletal metastasis which affect the quality of life and result in high rate of mortality. Various lifestyle choices, genetic disposition and epigenetic factors can favour the process of cancer initiation and progression. Early detection of epigenetic changes can lead to early diagnosis and enhance treatment options for cancer patients to block skeletal metastasis and reduce cancer associated morbidity and mortality.

In order to understand the role of epigenetics in cancer progression, I utilised two human osteosarcoma (OS) cell lines and treated them with a DNA methylating agent S-adenosylmethionine (SAM) and its inactive analogue s-adenosylhomocysteine (SAH) as a control. Treatment with SAM reduced the expression of tumor promoting genes and significantly decreased the proliferation and migration of tumor cells. In the xenograft model of OS where LM& cells were inoculated via tail vein and directly into tibia, both lung and bone metastases were significantly reduced in the experimental group as compared to the control group of animals. To determine the epigenetic alterations in OS, I utilized SAM treated LM7 cell line and performed epigenome wide association studies (EWAS). The results showed significant differential methylation in genes involved in tumor progression and bone remodelling. The results were further validated by immunohistochemcial analysis of the identified genes on human OS tissue samples.

Cancer immunosurveillance is a well-known mechanism by which immune system keeps a check on transformed cancer cells. We hypothesized that DNA methylation signature of breast cancer can be found in peripheral cells of the immune system. Towards these goals I employed T-cell DNA samples obtained from the blood of breast cancer patients. The DNA methylation signatures present in breast cancer patients were compared to an age matched cohort of normal women by EWAS analysis. The results showed significant differential methylation in genes involved in the phosphatase and tensin homolog (PTEN), p53 and retinoic acid receptor (RAR) activation pathways in breast cancer patients.

I also investigated the DNA methylation pattern in post-menopausal women with osteoporosis, which is the most common bone disease associated with high incidence of morbidity. Whole blood DNA samples from osteoporotic women and age matched normal women were utilised for EWAS analysis. Results from these studies showed differential methylation of several genes involved in transcriptional regulation, angiogenesis, apoptosis and immune checkpoint pathways.

Collectively these studies demonstrate an intricate relationship between epigenetic modifications in cancer progression and bone remodelling. The results presented pave the way for development of novel epigenetic based diagnostic tools and better treatment of metastatic cancers.

Résumé :

Le cancer du sein et de la prostate sont fréquemment associés aux métastases osseuses qui affectent grandement la qualité de vie des patients atteints et augmentent ainsi le taux de mortalité. Le mode de vie, la prédisposition génétique et les facteurs épigénétiques jouent un rôle important dans le développement et la progression du cancer. Un dépistage rapide des changements épigénétique permet un diagnostic précoce et des choix plus efficaces dans le traitement des métastases osseuses afin de réduire les taux de mobilité et mortalité.

Le rôle de l'épigénétique dans la progression du cancer a été étudié chez deux lignées cellulaires humaines d'ostéosarcome (OS) traitées avec un agent de méthylation de l'ADN, S-Adenosylmethionine (SAM) ou avec un analogue inactif S-Adenosylhomocysteine (SAH) comme contrôle. Le traitement au SAM réduisait l'expression des gènes favorisant la formation des tumeurs et diminuait de façon significative la prolifération et la migration des cellules tumorales.

De plus, l'étude *in-vivo*, à l'aide d'un modèle de tumeur xénogreffée, où les souris sonts inoculées avec des métastases osseuses et pulmonaires, soit directement dans le tibia ou par la veine de la queue, a démontré une réduction significative des métastases dans le groupe traité au SAM comparé au groupe contrôle. Pour déterminer les altérations épigénétiques dans l'ostéosarcome métastasique, les cellules métastasiques dérivées de l'ostéosarcome humain (LM7) sont traitées au SAM et analysées avec la méthode de l'association épigénétique à l'échelle du génome (EWAS). Les résultats ont mis en évidence

de la présence d'une méthylation différentielle significative au niveau des gènes impliqués dans la progression tumorale et le renouvellement osseux. Les gènes identifiés sont aussi validés par la suite par les analyses immunohistochimiques sur les échantillons de l'ostéosarcome métastasique humains.

L'immunosurveillance est un mécanisme bien connu qui permet au système immunitaire de surveiller la transformation des cellules cancéreuses. Nous avons postulé que la méthylation de l'ADN des gènes associés spécifiquement au cancer du sein pourrait être détectée dans les cellules du système immunitaire périphérique. Afin de confirmer notre hypothèse, les échantillons de l'ADN des lymphocytes T, isolés à partir du sang des patientes atteintes du cancer du sein, ont été analysés par EWAS en comparaison avec un groupe contrôle de femmes de même âge. Les résultats obtenus chez les patientes atteintes du cancer du sein ont démontré qu'il y avait bien une méthylation différentielle significative des gènes impliqués dans les voies de signalisation des suppresseurs de tumeurs (PTEN), impliquant les phosphatases et les homologues de tensin, de p53 et de l'activation du récepteur de l'acide rétinoïde (RAR).

Le phénomène de la méthylation de l'ADN a été aussi étudié chez les femmes ménopausées souffrant d'ostéoporose, une maladie osseuse fréquemment associée à un indice élevé de morbidité. L'analyse EWAS des échantillons de l'ADN, isolés du sang des femmes de même âges avec ou sans ostéoporose, a démontré qu'il y avait une méthylation différentielle de plusieurs gènes impliqués dans les processus tels que la régulation de la transcription,

l’angiogenèse, l’apoptose ainsi que les points de contrôle des voies de régulation du système immunitaire.

L’ensemble des études de cette thèse démontre une relation très complexe entre les modifications épigénétique, observées dans la progression du cancer, et les processus de renouvellement osseux. Les résultats présentés ouvrent la voie au développement de nouveaux outils de diagnostiques à base épigénétique et à un meilleur traitement de cancers métastatiques.

Chapter 1: Literature Review

Cancer metastasis to distant organs is the major cause of cancer associated mortality. The primary tumor can be surgically resected, however the metastatic tumors are much more diverse and continue to be a challenge to treat effectively [1]. While recent advances in neo-adjuvant chemotherapy and surgery have improved the long-term survival rates of patients without metastatic disease, patients who exhibit metastasis continue to respond poorly to chemotherapy and have a poor prognosis[1]. This poor response to therapy is also associated with a high incidence of drug toxicity, and efforts to change chemotherapeutic regimens has yielded limited success with no improvement in outcome [2]. Therefore, it is crucial to understand the molecular mechanism of tumor metastasis for early diagnosis, predict prognosis and identify new targets for the development of innovative, targeted and effective therapeutic strategies.

Primary tumors are different from metastatic tumors, where prognosis and tissue morphology vary excessively [3]. A high degree of genetic heterogeneity is found in the primary tumor microenvironment [4]. Various cells such as endothelial cells, fibroblasts and extracellular matrix components are present in the tumor vicinity, which interact with each other and the tumor cells [4]. To control normal tissue homeostasis, the extracellular matrix is subjected to constant remodeling. Disruption of the extracellular matrix structure and its components is known to support various pathological conditions and metastatic cancers [5]. Specific enzymes called proteases are responsible for extracellular matrix degradation, [6, 7]. Matrix

metalloproteinases (MMPs) and serine proteases like urokinase plasminogen activator (uPA) regulate tissue processing, but in the event of cancer the normal functioning of this system is deregulated. The metastatic cascade triggers with the disruption of extracellular matrix and invasion of tumor cells from the primary site to the surrounding matrix. Intravasation of malignant cancer cells into the blood circulation is the first step in this process [8]. It is furthered by decisive steps like migration and extravasation [9]. Thereafter, adhesion to a favorable microenvironment. The metastatic soil - helps in establishing a niche for secondary tumor growth [10].

Various proteases are known to regulate the process of tumor cell proliferation, migration, adhesion, angiogenesis and apoptosis. These proteases are categorized into five classes namely, aspartic, cysteine, serine, threonine and metalloproteinases based on the amino acids and metal ions involved in the proteolytic action [11]. Proteases regulate tumor growth by targeting specific growth factors and cytokines which in turn activate various intracellular signaling pathways involved in tumor progression [12]. This interaction of proteases, growth factors and cytokines are further elaborated in the next section with the focus on their role in the development and progression of bone metastasis.

1.1 Bone metastasis:

The current treatment of a primary solid tumor involves surgery, radiation therapy, chemotherapy, or a combination of these approaches. The effectiveness of this treatment and the disease outcome depend on the metastatic status of tumor. The localized tumor can be resected and controlled surgically. But the metastatic tumor which has spread to a different site is difficult to treat and has a poor prognosis. Metastatic progression may take place through lymph nodes, locoregional growth, embolus formation and dissemination to distant organs, and bone metastasis. Approximately 70% of cancer patients suffer from bone metastasis [13]. Factors like bone pain, fractures, hypercalcemia and spinal cord compression reduce the quality of life and survival for these cancer patients [14].

The bone microenvironment is composed of several cell types including bone-forming osteoblasts, bone resorbing osteoclasts and several other cell types. Chemokines secreted by the bone microenvironment attract circulating tumour cells through their actions on cell-surface receptors present on the tumour cells. Colonization of bone by tumor cells results in a vicious self-sustaining cycle whereby factors secreted by tumour cells deregulate proper osteoblast and/or osteoclast proliferation and maturation leading to factors being released by the bone matrix, which are tumour proliferative [15]. Several factors have been implicated in the establishment and maintenance of the above-mentioned cycle and they include the transforming growth factor-β (TGF-β)/bone morphogenetic proteins (BMP) axis, the receptor activator of

nuclear factor kappa B (NF-κB) ligand (RANKL) axis and the WNT signalling pathway. Moreover, a number of signalling pathways have been implicated in the development and progression of prostate cancer and noteworthy among those is the pathway involving the transcription factor signal transducer and activator of transcription 3 (STAT3), which has significant effects on cell survival, angiogenesis and gene expression regulation. The eventual imbalance between osteoblasts and osteoclasts results in either bone forming (osteoblastic), bone resorbing (osteoclastic) or mixed lesions.

Anti-resorptive therapy is the current treatment for patients with metastatic bone disorders which includes bisphosphonates and humanized antibody ("Denosumab") against RANKL. Tumor cells take advantage of continuous bone remodelling and distort this process for overproduction of bone-resorbing and angiogenic factors which promote tumor growth. High blood flow in the red bone marrow aids in the process. Tumor cells have the ability to induce osteoclast differentiation; which in turn results into osteolytic lesions in breast cancer patients [16]. The nitrogen containing bisphosphonate "Zometa" is being used extensively as an adjuvant therapy for bone metastasis [16]. More recently "Denosumab" has gained attention and is being used for osteoporosis treatment in postmenopausal women. But the effects of Denosumab on cancer progression remain to be established.

1.2 Breast cancer:

Selecting treatments tailored to individual patients is a critical step in breast cancer therapy, where prognosis and tissue morphology varies extensively [17]. Numerous classifications based on histopathology and genetic profiles of breast tumors are known today [18]. Depending on tissue or site of origin, breast cancers can be categorized into ductal and lobular carcinoma; whereas characteristics like hormone responsiveness and genetic signatures place breast cancers into specific subtypes like Estrogen/Progesterone receptor positive or human epidermal growth factor receptor 2 (HER2)/neu positive tumors [19].

1.2a Invasion and Metastasis- The Mechanism:

Invasion and metastasis are two multistep processes which aggravate the disease outcome. The process of metastasis begins with invasion of tumor cells into the surrounding matrix. The malignant tumor cells intravasate into the blood circulation and migrate to distant sites[9]. Upon finding favourable microenvironment, tumor cells adhere to the new site and establish secondary metastatic tumor. [10]. Breast cancer associated-bone metastasis is most likely to occur as bone microenvironment provides growth factors like TGF-β, Insulin-like growth factor 1(IGF-1), Fibroblast growth factor (FGF), Platelet-derived growth factor (PDGF) and BMPs to cancer cells [20]. These growth factors are small polypeptide molecules which bind to transmembrane receptors and activate various signaling pathways like mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) pathways and transcription

factors like signal transducers and activators of transcription (STATs) [21]. TGF-β along with FGF helps in tumor cell invasion by upregulating the secretion of matrix metalloproteases MMP2 and MMP9 while downregulating the tissue inhibitors of matrix metalloproteases (TIMP1, TIMP2, TIMP3 and TIMP4) [21, 22]. TGF-β regulates physiological cell processes like apoptosis and differentiation under normal circumstances, however genetic and epigenetic events transform TGF-β into tumor promoter [21]. Various signaling pathways like mitogen-activated protein kinase-extracellular signal-regulated kinases (MAPK/ERK), wineglass-related integration site (WNT)/β-catenin and phosphatidylinositol 3-kinase/ protein kinase B/ mechanistic target of rapamycin (PI3K/AKT/mTOR) get activated by TGF-β signaling and promote tumor cell invasion and metastasis [23, 24]. TGF-β also mediates the process of angiogenesis by activating other growth factors like FGF and vascular endothelial growth factor (VEGF). VEGF and its receptor vascular endothelial growth factor receptor (VEGFR) are expressed on breast cancer cells and other solid tumors to support tumor cell growth, survival and angiogenesis [25]. Along with angiogenesis VEGF also induces bone marrow progenitor cells which results in organ-specific metastasis of solid tumors [25].

Additionally, growth factors also induce transformation of several cell surface molecules and promote the process of migration [26]. The homotypic cell-cell adhesion is mediated by molecules called cadherins. Disruption of tight-junctions and epithelial E-cadherin molecules initiates the epithelial to mesenchymal transition (EMT) [8]. Further mesenchymal N-cadherin and

cadherin-11 expression promotes metastasis. The N-cadherin molecule activates (MAPK-ERK) pathway which results in MMP9 transcription [27].

Simultaneously chemokines like C-X-C chemokine receptor type 4 (CXCR4) aid to the migration of breast cancer cells to bones. In MDA-MB-231 breast cancer cells CXCR4-CXCL12 (C-X-C motif chemokine 12) (receptor-ligand) binding has been shown to support the directional migration through pseudopodia formation [20, 28].

A set of heterodimeric glycoprotein molecules called integrins; supplement the binding of tumor cells to the bone matrix. Integrins are mediators of cell attachment to the extracellular matrix. Breast cancer cells that express αvβ3 integrin exhibit affinity to bone matrix [29, 30]. Association of integrins αvβ3 and α5β1to receptor tyrosine kinases like (VEGFR), platelet-derived growth factor receptor (PDGFR) and epithelial growth factor receptor (EGFR) play a major role in tumor growth and initiation of ECM degradation.

1.2b ECM degradation and uPA-uPAR system:

ECM is mainly composed of proteins and proteoglycans which undergo constant remodeling to regulate various physiological and cellular functions [31]. ECM remodeling and degradation is carried out predominantly by proteases like MMPs, a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTs) and serine proteases like plasmin [32]. These ECM remodeling enzymes are controlled by transcriptional and posttranslational

regulation [33]. MMPs and plasmin exist in enzymatically inactive forms in cellular microenvironment [33]. Various proteases and other MMPs activate the enzymatic form of MMPs. However, plasminogen, the inactive form of plasmin is processed by serine proteases like uPA and tissue plasminogen activator (tPA) [34]. While both plasminogen activators (tPA &uPA) act as thrombolytic agents, uPA has been identified and characterized as the main plasminogen activator involved in ECM degradtion to promote the multistep process of tumor cell invasion, growth and metastasis [35].

uPA promotes ECM degradation in coordination with the uPA receptor (uPAR) [6, 7]. The uPA system has three integral parts; uPA, uPAR and several serine protease inhibitors (SERPINS) [36]. The plasminogen activation induced by uPA-uPAR interaction is kept in check by the inhibitors plasminogen activator inhibitor 1 (PAI1) and PAI2. Several studies indicate that disrupting the uPA system has significant role in blocking tumor growth. Targeting the catalytic activity of uPA and small molecule inhibitors of uPA-uPAR interaction are two well documented methods to inhibit the tumor growth [36, 37].

In breast and prostate cancer uPAR is expressed not only by the cancer but also by its surrounding stroma [38]. This feature advocates the use of various xenograft mouse models to evaluate the therapeutic efficacy of uPA-uPAR targeted therapeutic agents. Towards these goals, recently chemical antagonists, peptide inhibitors of uPA-uPAR interaction and anti-uPAR

antibodies have been demonstrated to have a greater effect on tumor growth inhibition [38, 39].

1.2c uPA system and Epigenetics:

Recent research shows that the uPA system is epigenetically controlled. Epigenetics is the branch of science which studies the heritable alterations of DNA and chromatin structure [40]. Most notable epigenetic modifications include DNA methylation, histone modifications and chromatin remodelling. Previous studies from our laboratory have shown a significant relationship between DNA hypomethylation and breast cancer metastasis [41-44].

To further our understanding of DNA methylation and its role in breast cancer progression we utilised epigenome wide association (EWAS) studies to sequence the whole epigenome of breast cancer patients. These studies helped us to explore epigenetic regulation of cellular transformation and breast cancer progression. Our T-cell epigenome wide association study resulted in one of the manuscripts presented in this thesis.

1.3 Osteosarcoma:

Osteosarcoma is one of the most common primary bone tumors, falling under the category of non-haematological bone tumors. It is most prevalent in children and adolescents, but also occurs in people above 50 years of age. The primary sites for osteosarcoma tumors are metaphyseal growth plates of long bones like femur, tibia and humerus. Stratification of osteosarcoma subtypes is very difficult due to characteristics like genetic instability and complex karyotypes. Various mutations, deletions, translocations and amplifications aid to tumor development. The most common feature of osteosarcoma tumors is the presence of immature osteoblasts. Targeted treatment for osteosarcoma is difficult due to different biological behaviour and molecular diversity in tumors. The five year survival rate of patients without metastasis is around 65%, whereas it is 25% for patients with metastasis [45-48]. Osteosarcoma patients experience severe bone pain, spinal cord compression and osteolysis which increase the risk of fractures exponentially. Metastasis occurs to other bones and lungs in patients diagnosed with metastasis. The current standard of care for osteosarcoma is surgical resection combined with chemotherapy (doxorubicin and cisplastin with or without methotrexate) [49].

Bone development is a complex process comprising of constant bone formation and resorption. Osteoblasts and osteoclasts are the cells responsible for bone remodelling. Osteoblasts are derived from mesenchymal cells found in bone marrow stroma. They express many transcription factors and proteins like RUNX- related transcription factor 2 (RUNX2), Osterix (OSX or SP7),

osteopontin and osteocalcin which help in bone formation by mineralizing the organic matrix of bone [50]. Another receptor expressed by osteoblasts, RANKL binds to RANK and helps in osteoclast differentiation [51]. Osteoclasts are the cells responsible for bone resorption, and are derived from monocytes of hematopoietic origin. Most of the bone lesions in osteosarcoma are osteolytic in nature [49]. Disrupted bone homeostasis is one of the major contributing factors for osteosarcoma development [52].

Osteosarcomas are genetically complex pediatric tumors, where various genetic mutations play a key role in tumor progression [53]. Two major tumor suppressor genes tumor protein p53 (TP53) and retinoblastoma (RB1) have been associated with osteosarcoma progression. Recently whole genome sequencing analysis of osteosarcoma tumors also verified the role of these two genes in osteosarcoma progression [54]. There are various signaling pathways implicated in osteosarcoma progression. Hedgehog, Notch and WNT pathways could serve as better targets for osteosarcoma treatment [49]. However, these pathways are also important in bone development and could also affect the normal development of adolescent osteosarcoma patients.

Tumor promoting events could be inherited through the germ line or somatically acquired in the lifetime of a patient. These factors could be of genetic or epigenetic origin. Targeting epigenetic changes could enhance the treatment options and block the tumor progression in early stages. Another problem associated with osteosarcoma is the rarity and heterogeneity of the disease which makes sample collection very difficult. In our osteosarcoma

study, we utilised two cell line models to characterize osteosarcoma proliferation and migration. To investigate the epigenetic factors involved, EWAS analysis was performed and the results were further analysed on histological tumor samples.

1.4 Bone Remodelling:

Bones form the supporting structure of our body and are integral part of our bone system. The outer bone structure looks immobile and static but the inner bone is extremely dynamic and continuously undergoes remodelling. Along with hematopoiesis the bone is also responsible for rapid production of new osteocytes. The mineralized connective tissue is comprised of several types of cells. Osteoblast, osteocytes, osteoclasts and inner lining cells are the most prominent cells involved in bone remodelling.

1.4a Bone Cells:

I. Osteoblasts:

Osteoblasts are the prominent bone forming cells which help in synthesis of new bone matrix. Preosteoblasts require RUNX2 and collagen type1, alpha 1 (Col1 A1) for differentiation. Various bone markers like alkaline phosphatase (ALP), Osterix (Osx), osteocalcin (OCN), bone sialoprotein (BSP) and collagen type I are expressed during the process of maturation. Differentiated osteoblasts are cuboidal in shape and have the capacity to transform into osteocytes or bone lining cells.

II. Bone lining cells:

As the name goes these cells line the surface of bones. The functions of bone lining cells are not very clear yet. These cells help in osteoclast differentiation and guard the bone against unnecessary bone resorption.

III. Osteocytes:

Majority of bone cells are osteocytes which are formed upon osteoblast differentiation. They have a discrete morphology depending on their location [55]. All osteocytes are connected to osteoblasts, bone lining cells and to each other by cytoplasmic processes which help in the transport of small molecules in the bone. Osteocytes and osteoblasts both are known to express RANKL which aids in the activation of the bone resorbing cells the osteoclasts.

IV. Osteoclasts:

Osteoclasts are multinucleated cells of hematopoietic origin. They possess a large number of vesicles and vacuoles, which gives them a foamy appearance. Two major proteins macrophage colony stimulating factor (M-CSF) and RANKL, secreted by osteoblasts, osteocytes and stromal cells, are responsible for differentiation of osteoclasts. Receptor activator of nuclear factor kappa- B (RANK) is expressed by preosteoclasts [56]. Which upon binding to RANKL triggers osteoclast differentiation. Another well-known decoy receptor for RANKL is osteoprotegrin (OPG), which binds to RANKL and inhibits the process of osteoclast differentiation [56].

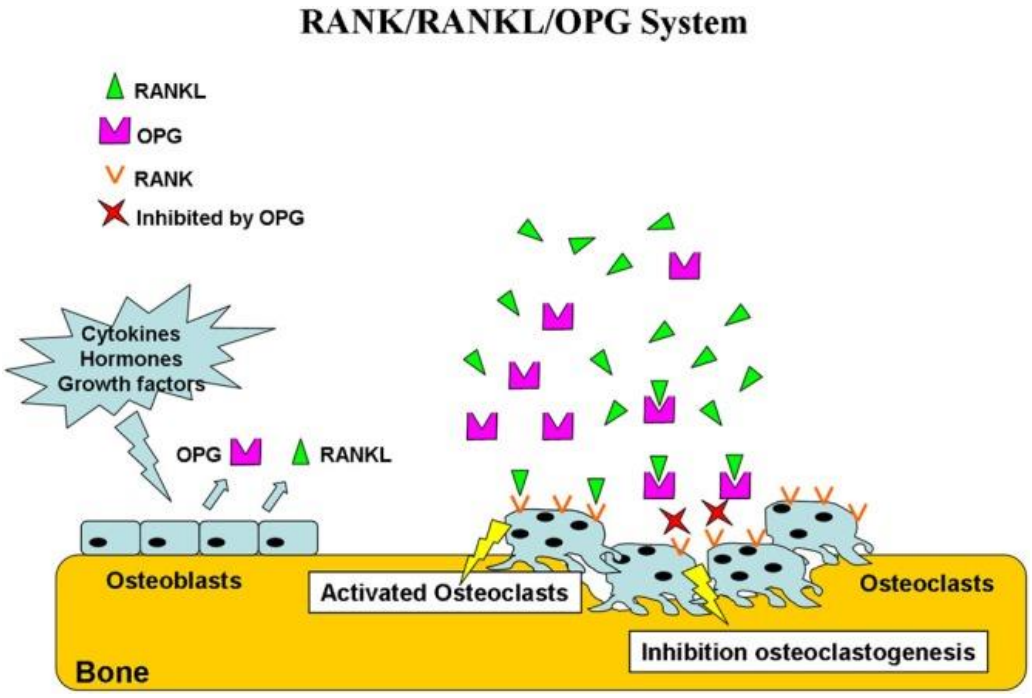

Figure 1: RANK/RANKL/OPG System: Osteoblasts produce RANKL and OPG under the control of various cytokines, hormones, and growth factors. OPG binds and inactivates RANKL, resulting in the inhibition of osteoclastogenesis. In the absence of OPG, RANKL activates its receptor, RANK, expressed on osteoclasts and preosteoclast precursors. The RANK-RANKL interaction leads to preosteoclast recruitment and fusion into multinucleated osteoclasts and to osteoclast activation and survival.

(Nardone V. et. al. 2014 Pharmacological management of osteogenesis. [57])

The bone cells orchestrate the process of bone remodelling. Osteoclasts initiate the process of bone resorption by removal of old bone cells; which is put to an end by formation of new bone by osteoblasts. Several bone diseases occur due to imbalance in the cycle of bone resorption and formation. Severe bone degradation by osteoclast results in osteoporosis, where bones become fragile and prone to fracture. Whereas, excessive bone formation that may

result from reduced osteoclast activity gives rise to a disease called osteopetrosis [58].

Reduced bone mineral density is one of the most common problems in the aging population. Postmenopausal women are at a greater risk of osteoporosis due to hormonal imbalance.

1.5 Epigenetics:

Epigenetics is the study of a stably heritable phenotype resulting from changes in a chromosome, without alterations in the DNA sequence. Three interlinked epigenetic processes regulate gene expression at the level of chromatin, namely DNA methylation, nucleosome remodeling and histone modifications. Our current understanding of epigenetics includes DNA methylation, histone post-translational modifications, functions of 5-methylcytosine and its oxidized derivatives. But a lot of basic questions remain unanswered. It is a well-known fact that epigenetic alterations contribute to carcinogenesis; but do these modifications cause cancer, or cancer is a consequence of these epigenetic changes is still a riddle. Various environmental factors, age and lifestyle changes influence the epigenetic mechanisms and the process of cancer progression. The epigenetic machinery controls the transcriptional regulation by silencing or altering the gene expression. Chromatin structure plays a major role in controlling gene expression. Heterochromatin is the highly condensed part of chromatin and contains most of the inactive genes. But the euchromatin is less condensed and contains active genes [59]. The building blocks of chromatin structure are nucleosomes, which consist of histone proteins and DNA. One unit of nucleosome consists of ~147 base pairs of DNA wrapped in 1.67 left-handed super helical turns around a histone octamer consisting of 2 copies of each of the core histones H2A, H2B, H3 and H4 [60]. Connected by a linker DNA up to 80 base pair long. The figure below shows the epigenetic modifications on histone tails and DNA [61].

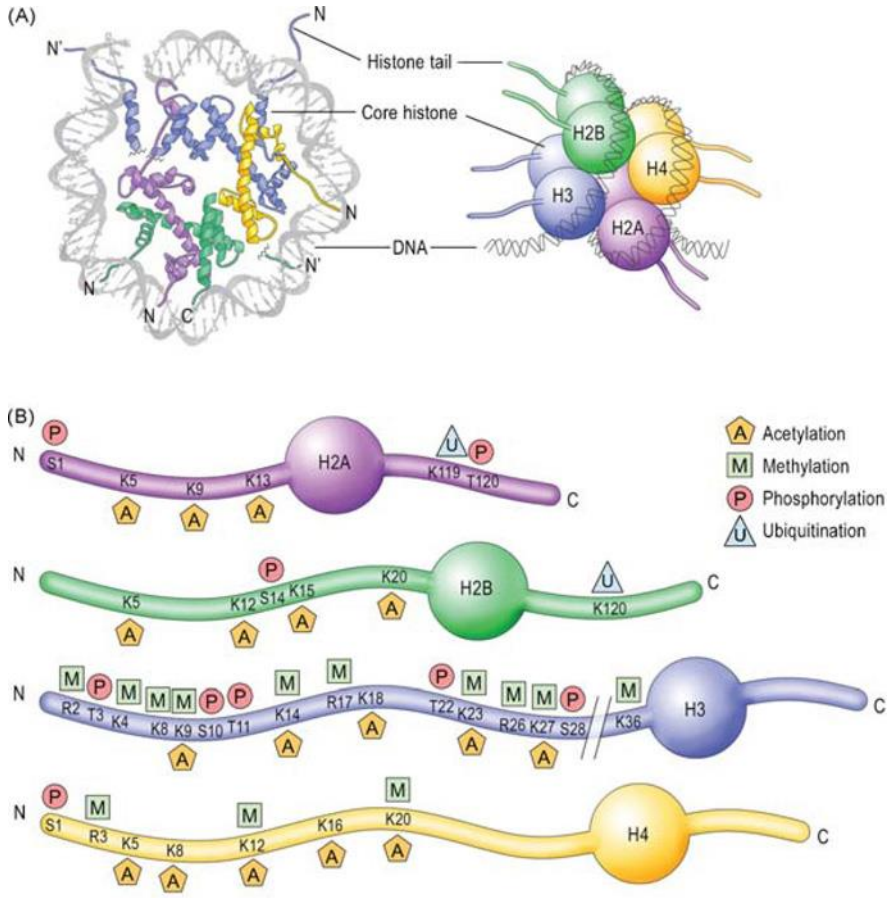

Figure 2: Epigenetic marks on histone tails and DNA: A, left) View of the nucleosome down the DNA super helix axis showing one half of the nucleosome structure. (Right) Schematic representation of the four-nucleosome core histones, H2A, H2B, H3 and H4. (B) Schematic representation of the N- and C-termini of the core histones and their residue-specific epigenetic modifications.

(**Alberini CM et. Al. Transcription factors in long-term memory and synaptic plasticity. [61]**)

1.5a Histone Modifications:

Although DNA methylation is the most extensively studied epigenetic modulation, it usually occurs with other epigenetic modifications like formation of nuclease resistant chromatin and histone modifications [62]. Histones were merely considered as "DNA-packaging" proteins, however recent studies have

shown their role as epigenetic regulators of gene expression as well. Histone deacetylases and histone methyltransferases are two prominent enzymes involved in histone modifications [63]. Various post-translational chemical modifications of histone proteins have been shown to regulate gene activity. Histone acetylation, methylation, phosphorylation, ubiquitination and sumoylation cause strategic change in chromatin structure and gene expression [64]. Histone modifications in combination with DNA methylation play an important role in nuclear organization and gene transcription. Hypermethylated DNA sequences are usually associated with histone hypoacetylation and hypermethylation which results in gene repression [65-67]. Histone acetylation and methylation occur at specific lysine residues present in the tails of nucleosomal histones. However, these histone modifications usually occur in specific combinations to regulate chromatin architecture and gene expression. A combination of deacetylation of histone H3 and H4, loss of histone H3K4 trimethylation, gain of H3K9 methylation and H3K27 trimethylation is commonly observed in hypermethylated CpG islands and promotors of the genes [65]. It has also been shown that hypoacetylation and hypermethylation of histone H3 and H4 can block the expression of certain tumor suppressor genes like p21 [68]. Global DNA hypomethylation and regional (CpG islands and promoters) hypermethylation is a well-known characteristic of cancer cells. However, a complete picture of all the epigenetic modifications is required to unravel the epigenetic process of cancer progression. Global loss of histone H4 monoacetylation and trimethylation has been observed in various cancers

including breast and liver cancers [69, 70]. Further analysis of these specific histone modifications is required to understand the global pattern of histone modifications in cancer cells.

The epigenetic profile of cancer cells provides an opportunity to devise prognostic, diagnostic and therapeutic strategies to control cancer progression. Histone modifications are posttranslational modification which can be studied with the help of techniques like mass- spectrometry [71]. However, genome wide application of mass-spectrometry presents several technical difficulties. Other possibilities are to analyse histone modifications at each specific amino acid residue by western blots and immunostaining technique [72]. While analysing histone modification, it is important to gather information about DNA sequences associated with histone modification along with type and the amount of modification. This can be accomplished by ChIP with antibodies against specific histone modifications. The DNA sequences associated with the histone modification gets immunoprecipitated which can be further analysed PCR with specific primers for candidate DNA sequence.

I. Histone acetyltransferase (HAT) & Histone deacetylases (HDAC):

The acetylation status of histones is regulated by two major enzymes HATs and HDACs [73]. The HAT enzymes are known for acetylation of histone group whereas, HDAC enzymes reverts the acetylated state of histones [74]. The nucleosome forming core histone proteins (H2A, H2B, H3 and H4)

contain a lysine rich amino-terminal tail, which harbours most of the post-translational modification sites [74]. The DNA molecules in the nucleosome core are enclosed by the amino-terminal tail of histone proteins [74]. This assembly of DNA molecules and amino-terminal tails determine the interaction of DNA and transcriptional regulators by acetylation/deacetylation of the lysine residues [74]. These lysine residues are usually acetylated by HATs, which results in the reduction of chromatin condensation and an increase in gene expression [75]. However, the chromatin condensation is restored by the HDACs by increasing the histone affinity to DNA [76]. The regulation of gene activity by HDACs was first shown in yeast HDAC mutants. HDAC inactivity induced gene silencing and resulted in the inhibition of transcriptional activity. Further studies utilising HDAC inhibitors showed a positive effect on gene expression of some mammalian genes with negligible effect on total transcriptional activity of the organism.[74] These studies exhibited selective regulation of transcriptional activity by HDACs [74].

The interaction of gene promoters and transcription factors depends largely on chromatin arrangement. HATs initiate chromatin rearrangement to expose gene promoter and thus provide access to transcription factors [75]. The lysine residues get a positive charge upon addition of acetyl group by HATs which results in reduced interaction between DNA and histone tails. This hyper-acetylation mediated by HATs results in increased transcriptional activation whereas, the HDAC mediated hypo-acetylation results in reduced transcriptional activity [74, 77].

1.5b Chromatin remodeling and the role of non-coding RNA (ncRNA):

Chromatin architecture regulates gene expression by controlling the accessibility of DNA to the transcriptional machinery [78]. Several factors like covalent histone modifications and chromatin binding proteins take part in chromatin remodeling and give rise to the highly condensed heterochromatin and an easily accessible, gene dense euchromatin [79]. The non-coding transcripts of RNA known as ncRNA take part in chromatin remodeling [78]. Various studies conducted in yeast *S. pombe,* the fruit fly *D. melanogaster* and the plant *A. thaliana* show the role of ncRNA in heterochromatin formation and silencing of genes [80-83]. While the role of ncRNA in the establishment of chromatin states in humans is still unknown, recent studies have established the role of ncRNA in the regulation of gene expression via epigenetic modifications [84, 85].

The ncRNA based on their size can be distinguished into long non-coding (lncRNA) and short non-coding RNA. The short non-coding RNA are more abundant in human genome and further classified into short interfering RNA (siRNA), micro RNA (miRNA) and piwi-interacting RNA (piRNA) [78]. The piRNAs are known maintain genomic integrity in the germ cells and are primarily active in the nucleus [86]. Whereas, cytoplasmic posttranscriptional gene silencing is regulated by siRNAs and miRNAs [87]. The activity of siRNAs and miRNAs depends on RNA-binding Argonaute (Ago) proteins [88]. Two prominent human Ago proteins, Ago1 and Ago2 are involved in small RNA induced gene regulation [89-92]. Recent studies have shown the role of

siRNAs in transcriptional gene silencing via increase in epigenetic marks like H3K27me3, H3K9me2 and DNA methylation [92-94]. Various histone modifications have been shown to be associated with transcriptional gene silencing mechanism [78]. Small RNA mediated transcriptional gene silencing in the promoter region of ubiquitin C gene requires HDAC1 and Ago1 protein [95].

Small RNAs are also known to induce transcriptional gene activation in the promoter and 3 prime regions of certain genes [96]. This phenomenon is called as RNA activation (RNAa) [97-99]. Promoter hypermethylation of E-cadherin gene was reversed by RNAa in prostate cancer and HeLa cells [98]. Several other studies have shown the role of RNAa mechanism in transcriptional gene activation via suppression of epigenetic marks like H3K9me2, H3K9Me3, H3K9 acetylation, H3K4 acetylation or gain of H3K4me2 and H3K4me3 [97, 98, 100]. However, the mechanism of siRNA and RNAa mediated chromatin remodeling is still unclear and further studies need to identify various key factors involved in this process.

1.5c DNA methylation:

One of the most extensively studied epigenetic modification is DNA methylation, it was first studied by Feinberg and Vogelstein in human tumors [101]. Methylation of DNA usually occurs at cytosine-guanine dinucleotides (CpG) on the 5^{th} carbon of the cytosine nucleotide. Upon methylation cytosine nucleotide turns into 5- methylcytosine and reduces the binding capacity of transcription factors to promoter/ enhancer regions of DNA. Various enzymes contribute to the process of DNA methylation and regulate the gene expression. The presence of methylated CpG dinucleotides at distinct places in genome results in the inactivation of genes.

I. Transcriptional regulation of gene expression via DNA methylation:

DNA methylation plays an important role at the time of embryonic development, cell proliferation and differentiation by controlling gene transcription and chromatin architecture. It can occur at cytosine and adenine nucleotides. But adenine methylation is only observed in prokaryotes. About 4-6% of all the cytosines are methylated in a normal human genome resulting in specific gene regulation [102]. The favourable sites for DNA methylation are cytosine nucleotides followed by guanine. These CpG sites are found throughout the genome at distinct places called as CpG islands and also in repetitive sequences and distal regulatory regions of the genes. Repetitive sequences are highly methylated in normal tissues and hypomethylated in

tumor tissues. It has also been observed that CpG islands located near promoter regions of certain genes are hypermethylated in tumors. Hypermethylation of tumor suppressor genes specifically at promoter regions and CpG islands causes gene silencing. Thus, hypermethylation and hypomethylation usually occur simultaneously in the same tumor tissue giving rise to distinct tumor subtypes.

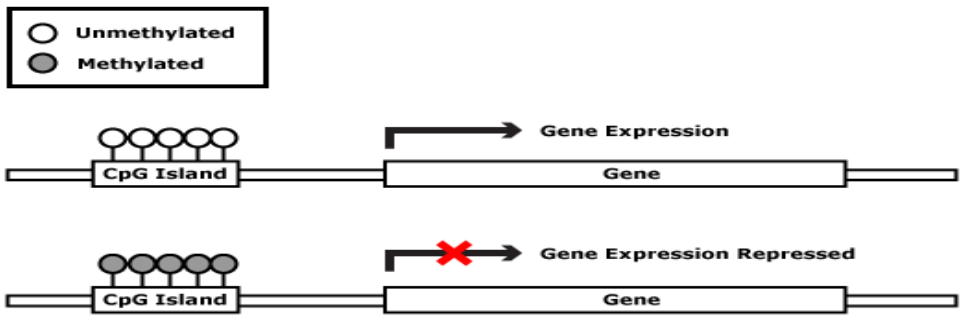

Figure 3: Regulation of gene expression by DNA methylation

II. DNA methyltransferases (DNMTs):

Global hypomethylation and regional hypermethylation is the hallmark characteristic of cancer. These aberrant changes in the cancer epigenome are attributed to a set of enzymes called as DNA methyl transferases (DNMT). DNMT overexpression has been observed in majority of cancer tissues. There are five known members of DNMT family, but only three of them, namely DNMT1, DNMT3A and DNMT3B are known to participate in the maintenance of and *de novo* methylation [103]. DNA methylation plays a crucial role at the time of embryogenesis and germ cell development. DNMT3A and DNMT3B are known to initiate de novo methylation. Whereas, DNMT1 is a maintenance enzyme which along with DNMT3B forms a complex with oncogenic transcription factors and induces methylation of promoter region of the genes [104].

Fortunately, epigenetic changes can be reversed to restore normal gene functions. The first FDA approved epigenetic drugs utilized the concept of DNMT inhibition to reverse hypermethylation in cancer tissues. There are two DNMT inhibitors which are currently used as standard treatment for Myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) [105, 106]. These two DNMT inhibitors are cytidine analogs namely, 5- azacytidine (Vidaza) and 5-aza- 2- deoxycytidine (Dacogen). Vidaza and Dacogen proved very efficient in blood borne malignancies and improved the quality of life and survival rate of these patients. Vidaza is currently under clinical trial for treatment of solid tumors [107]. Thus, hypermethylating agents offered

promising results for certain cancers. However, the downside of the treatment cannot be overlooked. There are toxicities associated with overdose and prolonged treatment with hypermethylating agents [108]. Global hypomethylation was one of the first characteristic observed in cancer tissues. Co-existence of hypomethylation and hypermethylation in the same tumor tissue makes it more difficult to treat. Identification and targeting specific regions of heterogeneous cancer tissues is really important. Use of DNA methylating agents either in combination or alone can provide new insights to cancer treatment.

III. Role of MBD2 enzyme in DNA hypomethylation:

DNA methylation is an active process which is regulated by a set of enzymes. The DNMT enzymes induce DNA methylation which is kept in check by methyl-CpG-binding domain protein 2 (MBD2) [109]. MBD2 belongs to a family of DNA binding nuclear proteins. There are five members in MBD family: methyl CpG binding protein 2 (MeCP2), MBD1, MBD2, MBD3 and MBD4 [110]. All the members of MBD family share a common methyl binding domain, containing 70 amino acid residues, which has a capacity to bind specifically to the methylated CpG sites [110]. The first few studies regarding MBD2 activity advocate its role in chromatin remodelling and silencing of methylated genes [111-113]. While the role of MBD2 in DNA methylation is disputed, it has been recently shown that MBD2 converts 5'-methylcytosine to 5'-hydroxymethylcytosine by releasing a formaldehyde molecule [114].

Various studies have shown that MBD2 reduces the methylation potential of cells *in vitro* [115-117]. Using MBD2 antisense oligonucleotide its role in breast and prostate cancer invasion and metastasis was shown *in vitro* and *in vivo* studies [42, 107, 118]. These studies have collectively identified global DNA hypomethylation as one of the major epigenetic mechanism regulating gene transcription in tumor tissue and identification of MBD2 as a novel therapeutic target to establish mutilation equilibrium in malignancy.

IV. S- adenosylmethionine (SAM):

Another candidate inhibitor of DNA hypomethylation is S-adenosylmethionine. SAM is a naturally occurring compound which is found in many tissues to play a role in several physiological functions including immune response, degradation of chemicals like serotonin, dopamine and melatonin in the brain [119].

Additionally SAM plays an important role in the maintenance of cellular growth, repair and metabolism of various neurotransmitters [119]. Vitamins like B12 (cobalamine) and B9 (folate) are required for SAM metabolism and production [120, 121] . Various studies have shown that SAM reduces joint pain from osteoarthritis, relieves depression and liver disorders

where it can be given via oral route to allow easy availability and accessibility to a larger population[120, 122-124] .

Mechanism of Action:

SAM is usually synthesized from the amino acid methionine and ATP and is a by-product of one carbon metabolism in two correlated metabolic cycles of folate and methionine [121]. Transfer of one methyl group from SAM results in the production of S-adenosylhomocysteine (SAH). SAH is then recycled and again utilised for SAM metabolism [125].

Dosage:

SAM is consumed as a dietary supplement and is readily available in the form of capsules and tablets which can be taken orally. The daily recommended dose as a dietary supplement is 200 mg for adults. The recommended dosage differs depending on the health condition.

Liver disorders: 600- 1200mg per day.

Osteoarthritis: 600- 1200mg per day.

Depression: 800- 1600mg per day [120].

SAM is a structurally unstable compound having a half-life of about 100 minutes. Considering these facts, stable salt forms of SAM are given as oral supplements. These oral supplements of SAM reach its peak plasma levels following four to five hours after consumption. Although, bioavailability of

intramuscularly injected SAM is much higher (96%) as compared to the oral forms (5%) as shown in various clinical studies and continues to be the preferred route of administration[120, 126]. With its increasing use several side effects like gastrointestinal disorders, dyspepsia and anxiety have been now identified as well. However its prolonged use and the associated risk of damage to the chromosomal DNA due to its DNA alkylating effects remains an potential area of concern which needs close monitoring and additional investigation [127-129].

1.6 Role of epigenetics in human physiology and diseases:

The epigenetic modifications provide a secondary level of control over the gene transcription machinery. Specifically, DNA methylation, histone modification and ncRNA mediated regulations are known to alter the gene expression patterns in human genome [130]. DNA methylation is also known to play an important role in various biological processes like aging and carcinogenesis [131]. However, certain environmental factors like diet, use of alcohol and tobacco, exposure to chemical carcinogens, physical activity and stress are also known to influence epigenetic modifications [132-135]. DNA methylation plays an important role in various stages of human development, starting with the embryonic development starting at the level of the male genome at the time of fertilisation which is followed by *de novo* methylation of the embryo [136]. It is observed that *de novo* methylation is absent at the time of normal physiological development, but present in complex disorders like cancer [131]. A better understanding of mechanisms governing methylation patterns at the time of development comes from studies utilising DNMT1 knockout mice, which results in loss of DNA methylation and embryonic lethality [137]. It has also been shown that conditional inactivation of DNMT1 results in activation of tissue-specific genes in fibroblast cells [138]. Another important role of DNA methylation mechanism is observed at the time of X-chromosome inactivation, where ncRNA induced genomic imprinting of parental alleles is maintained by DNMT1 activity [139-141].

In case of cancer, various epigenetic modifications lead to chromosomal instability, activation of oncogenes and silencing of tumor suppressor genes [142, 143]. Histone modifications, DNA methylation and various DNA-binding proteins regulate gene expression in cancer and have been described above.

I. Environmental factors, aging & diet:

Epigenetic modification are reversible changes and can be affected by various environmental factors, diet and aging which leads to abnormal phenotypes [131]. The process of aging has been associated with DNA hypomethylation in aging animals and cultured fibroblast cells [144-146]. However, the hypermethylation of certain genes like estrogen receptor, IGF2 and MYOD has also been observed in aging people [132, 147]. As age related predisposition to cancer is considered as one of the most important risk factor in carcinogenesis. The hypermethylation of CpG islands in aging people may lead to development of various types of cancers [148-150].

Diet is a well known factor which plays an important role in the onset of various diseases. Several vitamins like folic acid and dietary supplements of methyl group donors are well described to affect the cellular methylation patterns [151]. Decreased folic acid consumption has been associated with, neural tube defects and genomic instability [152-155]. Also, overexpression of oncogenes like c-ras, c-myc or c-fos with DNA hypomethylation has been observed in rats fed with methyl-deficient food [156, 157]. These findings

suggest a positive correlation between aging, environmental factors like diet and the predisposition to long term diseases like cancer. Future studies regarding reversal of these epigenetic modifications through diet and lifestyle changes and aging individuals will shed a new light in this field.

1.7 DNA Methylation Analysis:

The field of Epigenetics is developing at an exponential rate while adding substantial advances to our understanding of cancer research. Abnormal epigenetic alterations lead to impaired gene activity which in turn causes faulty cellular metabolism and initiates the process of tumorigenesis [158, 159]. Thus, there is a current need to analyse and sequence the epigenome to find novel biomarkers for diagnosis and better treatment options for patients. There are three major epigenetic modifications namely; DNA methylation of cytosine residues, post-translational modification of histones and microRNA gene expression regulation [160]. Around 45 years ago DNA methylation was proposed as primary factor controlling gene regulation and cellular proliferation [161, 162]. It is still the best known epigenetic modification in humans. DNA methylation analysis has been widely used in recent years in combination with next generation sequencing (NGS) techniques [163]. Various sequencing platforms are available which provide DNA analysing equipment and bioinformatics tools for data analysis. Some well-known DNA sequencing platforms for DNA methylation analysis are: Roche, illumina, Ion Torrent and PacBio Technology. All these DNA sequencing platforms utilise the NGS methods, which involve a series of steps for sequencing and analysing the epigenome. First, the genomic DNA is fragmented and utilised for library template preparation. The templates are then immobilised and sequenced in a DNA analyser. The resulting data is analysed by specific bioinformatics software provided by the sequencing platforms. One of the most important

feature of NGS technology is the immobilisation of DNA templates, which helps in execution of billions of sequencing reactions at the same time.

In human genome, the regions which are densely populated with CpGs are called “CpG islands”. These CpG islands and promoter regions of these genes are usually unmethylated. Whereas, the majority of CpG sites constituting the repetitive elements and regions with low CpG density are highly methylated [164]. Overall, the methylated and unmethylated CpG sites are scattered throughout the genome which requires whole genome sequencing with the precision of single base-pair resolution. Various methods of sequencing methylated DNA are available today, which help in locus-specific and genome wide analysis. Figure 4 shows a decision tree to select various DNA methylation analysis techniques [164].

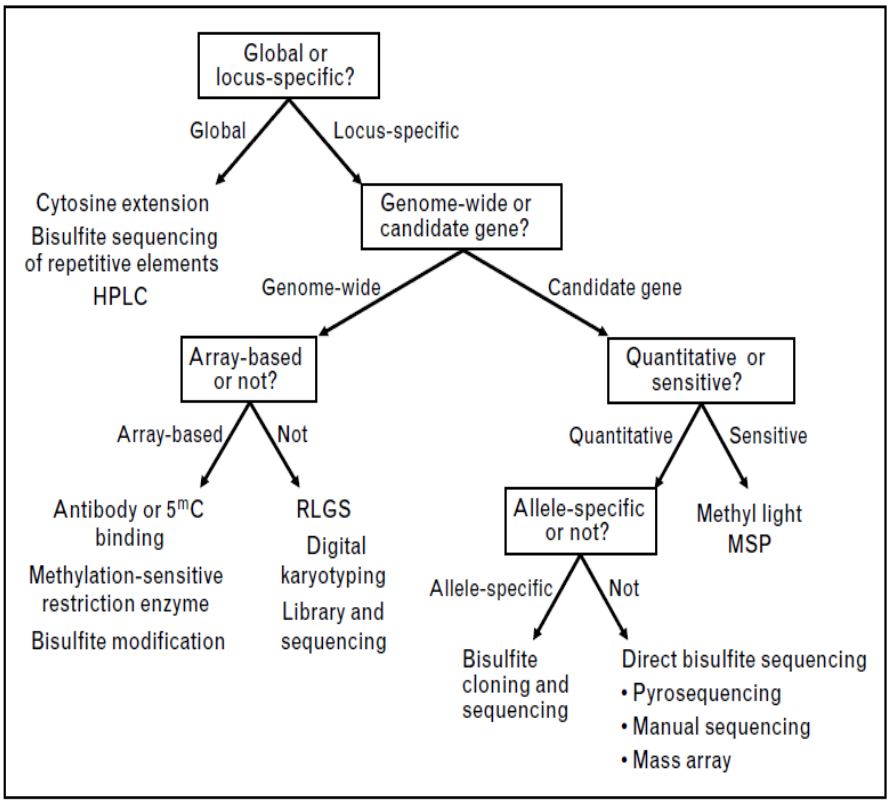

Figure 4: A flowchart showing selection of DNA methylation analysis methods (**Shen L. et al "Methods of DNA Methylation Analysis" [164]**).

Global DNA methylation analysis can be performed by high performance liquid chromatography (HPLC) [71]. However, it requires large amounts of high-quality genomic DNA and not suitable for studies with large number of samples. In most cases PCR amplification of DNA is required, but DNA methylation signature is usually lost in subsequent PCR reactions. To overcome these problems, alternative pre-treatment of DNA prior to analysis is performed. These pre-treatments include affinity enrichment, endonuclease digestion and bisulphite conversion. Affinity enrichment techniques rely on the affinity of methylated DNA towards specific agents. Two major affinity

enrichment techniques are Methyl Cap-seq and MeDIP-seq. Methyl Cap-seq technique is based on the capture of methylated DNA by methyl CpG binding protein 2. The captured DNA is eluted and sequenced for further analysis. Whereas, the MeDIP-seq technique utilises immunoprecipitation of methylated DNA with anti-5-methylcytosine antibodies [165]. Another way to analyse regional methylation patterns involve restriction endonuclease digestion. Two restriction endonucleases HpaII and MspI have been used extensively for this purpose. Both these enzymes recognise and cleave the site 5'-CCGG-3'. HpaII cleaves this recognition site only when it unmethylated. However, MspI cleaves its recognition site irrespective of its methylation state [166]. The specific cleavage criteria of these two restriction endonucleases enables us to analyse the entire genome by breaking it down in smaller fragments. The digested fragments are then used for library construction and subsequent sequencing analysis.

However, restriction endonuclease and affinity enrichment methods have a bias towards CpG islands and repetitive sequences [163]. To avoid problems associated with these techniques, bisulphite conversion method was adopted which requires the incubation of DNA with sodium bisulphite resulting in the conversion of unmethylated cytosine residues in uracil. The methylated cytosine residues remain unaffected by the bisulphite treatment which can be easily detected by DNA sequencing analysis [163]. This can be achieved by either partial sequencing or whole genome sequencing of bisulphite converted DNA.

The partial sequencing methods include reduced representation bisulphite sequencing (RRBS) and sure select method. RRBS involves MspI assisted restriction digestion of genomic DNA followed by electrophoretic separation, amplification and sequencing. MspI breaks DNA fragments into 50-250bp long sequences which represent majority of promoters and CpG islands [167]. Although RRBS is a cost effective method, the major limitation is the random splicing by MspI which may result in the loss of significant DNA sequences [163]. The second method of choice is sure select Methyl-seq. This platform is developed by Agilent technologies where predesigned RNA probes are utilised, which represent majority of regulatory regions like CpG islands, DNase I hypersensitive sites, gene promoters, Refseq genes [168]. This allows analysis of regions which cannot be detected by RRBS sequencing method.

1.7a Epigenome Wide Association Studies (EWAS) & Global analysis of DNA methylation:

The partial sequencing methods are cost effective and enable us to analyse large number of samples at the same time. However, the major drawback is the risk of loosing valuable DNA methylation sites which occur outside of the captured DNA. A complete analysis of DNA methylation sites is only possible with the epigenome wide sequencing of DNA. Illumina platforms provide array based whole genome bisulphite sequencing methods which allow DNA methylation analysis at single base resolution. Three different platforms have been developed by illumine namely Illumina 27K, Golden gate assays and

Illumina Human Methylation 450K bead chip. Illumina Human Methylation 450K bead chip assay is the most recent platform and has been used extensively in cancer translational analyses and databases such as The Cancer Genome Atlas (TCGA) [169].

1.7b Illumina Human Methylation 450K bead chip assay:

One of the most commonly used assays for methylation analysis uses the Illumina Human Methylation 450K bead chip. The bead chip array covers more than 485000 methylation sites, 99% Refseq gene regions, 96% of CpG islands and other CpG dinucleotides located outside CpG islands and promoter regions. This broad range of CpG sites represents approximately the whole epigenome and provides a platform to study various cohorts of disease associated or cancer patient samples. With Illumina 450K methylation technology any number of diseased cases and control cohorts can be compared and analysed to identify the epigenetically modified methylation patterns.

One of the major objective of the present study was to investigate distinct methylation patterns in cancer cases and control samples. I have utilised Illumina 450K methylation analysis for preparation of all the three manuscripts presented in this thesis.

1.7c Bioinformatics analysis:

Data analysis is an essential part of array based methylation techniques. The epigenome wide analysis of DNA methylation results in a large amount of valuable data which requires meaningful graphical representation. These large

datasets can be easily processed with the help of bioinformatic softwares. The illumina array platform provides “Genome Studio software” for bioinformatic analysis of various array and sequencing datasets [170]. These datasets can be easily exported to Genome studio for further analysis. Data processing, visualisation and results analysis can be performed with data analysis tools supported by Genome studio. A wide range of illumina assay platforms including gene expression, genotyping, DNA methylation, protein analysis and many more can be processed with Genome studio.

The graphical user interphase and visualisation tools help in integrating the data through multi-modal examination and provide a functional control of the data to the user. The methylation module can be utilised for analysis of DNA methylation arrays like illumina 450K bead chip. The methylation module calculates methylation levels of individual experimental groups, which are represented as beta values. The differential gene expression levels of two or more groups can be compared which are calculated as p-values and can be exported in tabular format for further analysis [171]. Illumina genome viewer and illumina chromosome browser help in the visualisation of CpG island methylation. The results can be graphically presented in a wide range of charts, graphs and histograms [172]. The data generated by gene expression, methylation modules and other can be combined together for an integrated analysis of large projects. Overall, Genome studio provides data analysis at single base resolution with high precision and easy workflow tools. Genome

studio software has been used for data analysis in two studies presented in this thesis.

I. CHAMP (Chip Analysis Methylation Pipeline) analysis:

CHAMP is statistical analysis software which utilises the computer programming language "R" for its operations. CHAMP software package was developed by Bioconductor software organisation for analysis of Illumina Human Methylation 450K and EPIC (a software used in healthcare systems) data analysis. Various analysis options like quality control assessment, data normalization, identification of differentially methylated genes and analysis of copy number alterations are provided by CHAMP [173].

Batch correction is an essential part of DNA methylation data analysis. In majority of clinical studies, the samples are analysed in several batches at different time points, which may affect the quality of resultant data. To avoid these problems and correct the technical errors, a statistical batch correction is performed. Champ software provides batch correction by using ComBat method [174]. The statistical data analysis of the breast cancer study presented in this thesis was performed with help of CHAMP software.

II. IPA (Ingenuity Pathway Analysis) software:

Array based sequencing platforms provide large amount of datasets which are simplified by statistical methods. However, these statistical methods only provide users with significant molecules or gene names. Further

integration and interpretation of these datasets is required for in-depth understanding of the biological systems. Qiagen's IPA software provides a complete network analysis of the molecules or genes generated by array results [175]. The relationships, mechanisms, functions and expression patterns of the key genes from array results are analysed by IPA software. Valuable information regarding upstream/downstream regulators and target genes and proteins can be extracted by IPA analysis. The network analysis also helps in identification of the most promising biomarker candidates within the experimental datasets [176]. Network analysis of illumina 450K methylation datasets was performed by IPA software in the studies presented in this thesis.

1.7d Validation of methylation status:

Pyrosequencing:

Pyrosequencing is a tool for DNA sequencing that detects pyrophosphate (PPi) molecules released during DNA synthesis. A series of enzymatic reactions are utilised for synthesis of a complementary strand of DNA to a single strand of DNA. DNA polymerase and a mixture of chemiluminescent enzymes are primarily involved in pyrophosphate (PPi) detection. This technique helps in mapping DNA methylation patterns up to single nucleotide resolution. Depending on the requirement, either single CpGs or a group of CpGs located in a specific region of the gene can be quantitatively analysed.

One of the most important application of pyrosequencing is the validation of EWAS signals. Pyrosequencing analysis of DNA samples requires bisulfite conversion of DNA. For this purpose, DNA is treated with sodium bisulphite which results in the conversion of all the unmethylated cytosine bases into uracil. Whereas, the methylated cytosine bases stay protected from this treatment. Upon bisulfite conversion the regions of interest are amplified with the help of specifically designed primers by PCR. The resulting PCR amplicons are then utilised for pyrosequencing analysis which reads every single nucleotide in the DNA strand and helps in quantification of methylated CpGs. Various CpGs located in a small region of a gene can have different levels of methylation which can be effectively quantified by pyrosequencing [177].

Cancer is a complex developmental disorder in which various growth factors, proteases and enzymes regulate the process of tumor growth and metastasis. These factors play an important role in regulating cellular process like gene transcription, cell-cycle, DNA repair mechanisms and apoptosis. Various mutations, deletions, translocations, and amplifications aid to tumor development [178]. Especially down regulation of tumor suppressor genes and up regulation of tumor promoting genes is observed in all known human cancers [179]. These transcriptional and post transcriptional regulations are governed by epigenetic mechanisms like DNA methylation, histone modifications and chromatin remodeling [180]. DNA methylation in particular is an important player in orchestrating changes in gene expression in cancer [181, 182]. In the present study, I have focused on evaluating the role of DNA methylation in bone remodeling, osteoporosis and cancer progression. The manuscripts presented in this study demonstrate the use of DNA methylation mechanism for early diagnosis and development of new and effective therapeutic strategies for diseases like osteosarcoma, breast cancer and osteoporosis.

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Chapter 2

S-adenosylmethionine blocks osteosarcoma cells proliferation and invasion *in vitro* and tumor metastasis *in vivo*: therapeutic and diagnostic clinical applications.

Preface

Previous studies in our lab have shown the effect of S-adenosylmethionine in blocking tumor growth and skeletal metastasis in various hormone dependent malignancies.

In this study, we investigate the methylation based therapeutic strategies for a primary bone tumor - osteosarcoma (OS). Epigenome-wide association studies (EWAS) were utilised to study differential methylation patterns in tumor cell lines. Results obtained from EWAS studies were further evaluated in biopsies of normal bone and OS patients.

Abstract

Osteosarcoma (OS) is an aggressive and highly metastatic form of primary bone cancer affecting young children and adults. Previous studies have shown that hypomethylation of critical genes is driving metastasis. Here we examine whether hypermethylation treatment can block OS growth and pulmonary metastasis. Human OS cells LM-7 and MG-63 were treated with the ubiquitous methyl donor S-adenosylmethionine (SAM) or its inactive analogue S-adenosylhomocysteine (SAH) as control. Treatment with SAM resulted in a dose dependent inhibition of tumor cell proliferation, invasion, cell migration, and cell cycle characteristics. Inoculation of cells treated with 150 μM SAM for six days into tibia or via intravenous (i.v.) route into Fox Chase SCID mice resulted in the development of significantly smaller skeletal lesions and a marked reduction in pulmonary metastasis as compared to control groups.

Epigenome-wide association studies (EWAS) showed differential methylation of several genes involved in OS progression and prominent signaling pathways implicated in bone formation, wound healing and tumor progression in SAM treated LM-7 cells. Real time PCR (qPCR) analysis confirmed that SAM treatment blocked the expression of several pro-metastatic genes and additional genes identified by EWAS analysis. Immunohistochemical analysis of normal human bone and tissue array from OS patients showed significantly high levels

of expression of one of the identified gene PDGFA. These studies provide a possible mechanism for the role of DNA demethylation in the development and metastasis of OS to provide a rationale for the use of hypermethylation therapy for OS patients and identify new targets for monitoring OS development and progression.

Introduction

OS is third most common child hood cancer affecting long bones accounting for 20% of all bone cancers[1,2]. Late stage OS tumors are known to cause lung metastasis resulting in the high morbidity and mortality. Late stage disease is highly aggressive with 5-year event free survival in 60-70% patients[3,4]. While recent advances in neo-adjuvant chemotherapy and surgery has improved the long-term survival rates of patients without metastatic disease, patients who exhibit metastasis continue to respond poorly to chemotherapy and have poor prognosis[5-8]. This poor response to therapy is also associated with a high incidence of drug toxicity and efforts to change chemotherapeutic regimen has yielded limited success with no improvement in outcome[9]. Therefore, it is crucial to understand the molecular mechanism of tumor metastasis for early diagnosis, predict prognosis and identify new targets for the development of more effective therapeutic strategies.

OS is a rare tumor which is often difficult to classify. The primary malignant tumor is characterized by genetic instability and complex karyotypes[10]. Various mutations, deletions, translocations and amplifications aid to tumor development[10]. Mostly alterations in two prominent tumor suppressor genes *TP53* and *RB1* are associated with tumorigenic activity[11]. The p53 and retinoblastoma protein pathways are known for controlling apoptosis, DNA repair and cell-cycle regulation. However, epigenetic mechanisms are also known to contribute to the tumor development process in various types of cancers including OS[12-14]. These epigenetic modifications mainly involve DNA

methylation, histone modifications and chromatin remodeling[15]. The epigenome can regulate the alterations of DNA and associated proteins without affecting the original DNA sequence[16]. One of the fundamental epigenetic modifications is the methylation of cytosine residues in CpG dinucleotides. Atypical methylation patterns have been observed in majority of cancers, which result in the inactivation of tumor suppressor pathways[17]. Additionally, extensive hypomethylation of tumor promoting genes is also described to enhance the overall process of oncogenesis. A recent delineation of the landscape of DNA methylation in liver cancer revealed wide spread hypomethylation of promoters of genes involved in migration and invasion including several classic prometastatic genes[18]. Hypermethylation of DNA caused by DNA methyltransferase enzymes (DNMTs) and histone acetylation by histone acetyltransferase (HAT) and histone deacetylase (HDAC) has been the prime focus of the epigenetic studies in the recent past[19]. Drugs that target DNMTs and HDAC are under clinical trials for treatment of solid tumors and have already been approved for hematological malignancies[19]. However, inhibition of DNA methylation could also result in activation of prometastatic genes and aggravate cancer metastasis[20,21]. We therefore proposed that inhibition of demethylation of prometastatic genes could serve as a strategy to block cancer metastasis[22].

SAM is a common co substrate involved in methyl group transfer reactions[23]. We have previously shown that SAM treatment causes hypermethylation of urokinase type plasminogen activator (uPA) in breast cancer cells and the knock down of methyl DNA binding protein 2 resulting in silencing

of the uPA gene by reverting the hypomethylated state of this gene in breast and prostate cancer cells[24,25]. We have also previously shown that SAM could inhibit the pro-invasive effects of the DNA methylation inhibitor Vidaza (5-azacytidine) on non-invasive breast cancer cells[25]. We therefore tested in the present study whether methylating agent SAM would be effective in suppressing metastasis in OS *in vitro* and *in vivo* using well established models of OS by effecting key signaling pathways involved in bone remodeling and tumor progression. Since methylation of tumor suppressor genes could stimulate cancer growth, we also determined whether SAM would not exhibit such an adverse effect. Our data show that SAM is effective in inhibiting both invasiveness and tumor growth. These data have important implications on therapy of metastatic OS.

Materials and Methods

Cell culture

Human OS cells LM-7 and MG-63 were obtained from the American Type Culture Collection and maintained in MEM with 10% fetal bovine serum, 2mmol/L L-glutamine and 100 unit/ml penicillin sulfate/streptomycin sulfate. Cells were incubated with different doses of SAM or SAH (New England Biolabs, Mississauga, Ontario) as previously described[25].

Cell proliferation invasion and wounding assay

LM-7 and MG-63 cells were plated in duplicates at a density of 9 x 10^5 and 5 x 10^5 cells, respectively in 10 mL of culture media in plates. The effect of two different doses of SAM (75.0 and 150.0 µM) was evaluated. The invasive capacity of LM-7 and MG-63 cells was examined using two-compartment Boyden chamber Matrigel invasion assay (Costar Transwell, Corning Corporation, Sigma-Aldrich, Oakville, ON) following treatment with SAH or SAM for six days as described previously[25].

For wound healing analysis, cells LM-7 and MG-63 cells were treated with SAH or SAM (75 and 150 µM) for six days in the presence of 10% FBS. Cells were then plated in six well plates to form a monolayer and then wounded manually with a sterile 1,000µL pipette tip in the center of each well. Cells were grown in the presence of 2% FBS and migrating cells where photographed at different time points. Analysis and quantification was carried out using Image Pro-Plus software and calculated as percentage wound healing using the

equation, % wound healing = [1 - (wound area at Tx h / wound area at T_0)], where Tx is the respective time point and T_0 is the time immediately after wounding. These experiments were repeated twice in duplicates.

Cell cycle analysis

For cell cycle analysis LM-7 cells were treated with SAH or SAM (75µM and 150µM**)** every 48 hours for six days and were fixed by adding 70% of ice-cold ethanol. Fixed cells were washed with PBS and then treated with 1 U of DNase-free RNase and stained with 0.05mg of propidium iodide for one hour. Cell cycle analysis was performed on a FACS Calibur machine. Results were analyzed further using the FlowJo Software.

Quantitative real-time PCR (qPCR)

For qPCR analysis total cellular RNA from SAH and SAM treated LM-7 cells was extracted using TRIzol (Invitrogen Life Technologies, Burlington, ON) according to the manufacturer's protocol. Two microgram of total RNA was used for reverse transcription (RT) reaction. 25ng of cDNA was used in a 20µl reaction with SYBR green mix, 0.8µM forward and reverse primers. PCR was performed in an ABI StepOne Plus with the following conditions: denaturation 95^{o}C 10 min; amplification 95^{o}C 15s, annealing temperature 1 min, for 40 cycles.

Illumina Methylation 450K Analysis

LM-7 cells were treated with vehicle or 150 μM of SAM for six days. Genomic DNA was quantified using PicoGreen protocol (Quant-iT^{TM} PicoGreen® dsDNA Products, Invitrogen, P-7589) and read on a SpctraMAX GeminiXS Spectrophotometer. Bisulfite conversion of 500ng of genomic DNA was performed using the EZ-96 DNA Methylation-GOLD Kit (Zymo Research, Irvine, California). The Illumina Methylation 450K kit was used for the microarray experiment as described by the manufacturer's protocol, except that 8□l of bisulfite converted template was utilized to initiate the amplification step. The Illumina Hybridization oven was used for incubating amplified DNA (37°C) and for BeadChips hybridization (48°C). A Hybex incubator was used for fragmentation (37°C) and denaturation (95°C) steps. The X-stain step was carried out in a Tecan Freedom evo robot with a Te-Flow module. Arrays were scanned in Illumina iScan Reader. Data analysis was performed with the Methylation module (version 1.9.0) of the Genome Studio software (Illumina; version 2011.1) using HumanMethylation450_15017482_v1.2.bpm manifest. Statistical threshold was set at a false discovery rate of >0.05, differential score (statistical power) of >0.13 and delta beta (differential methylation) between the groups was set at >0.15.

Tissue Microarray slides for osteosarcoma cases were obtained from US Biomax Inc. (Rockville, MD) whereas all normal cases were from iliac crest. Rabbit polyclonal antibodies for EXOC7 and PCGF3 (Abcam, Toronto, ON) were used at 1:10, 1:10 and 1:1000 dilution respectively. Rabbit polyclonal

antibody to PDGF AA (Abcam) was used as primary antibody at 1:1000 dilution. Heat-mediated antigen retrieval was performed by Tris/EDTA pH 9.0 buffer, EnvisionTM FLEX Target Retrieval Solution (Dako, Burlington, ON) at 1:50 dilution; and Phosphate buffer containing hydrogen peroxide, 15 mmol/L NaN3 and detergent, EnvisionTM FLEX Peroxidase-Blocking Reagent (Dako) was used as blocking reagent. Dextran coupled with peroxidase molecules and goat secondary antibody molecules against rabbit immunoglobulins in buffered solution containing stabilizing protein and preservative, EnvisionTM FLEX/HRP, (Dako) was used as secondary antibody for 30 minutes. 3,3'-diaminobenzidine tetrachloride, EnvisionTM FLEX DAB+ Chromogen (Dako) and buffered solution containing hydrogen peroxide and preservative, EnvisionTM FLEX Substrate buffer (Dako) were added. The slides were counterstained with hematoxylin (1a Harris hematoxylin solution by MERCK KGaA, Darmstadt, Germany). Sections were washed twice for 10 minutes in Tris buffered saline solution containing Tween 20, pH7.6 (EnvisionTM FLEX Wash Buffer (DAKO) at 1:20 dilution after every step during the procedure. Slides were mounted with DPX (MERCK, KGaA).

Stained slides were scored for proportion and intensity of staining in cells by two pathologists. Staining intensity was assessed as negative, mild, moderate or strong. Percentage of positive cells showing different intensity staining patterns were noted, and then rounded off to the nearest 10th percentage. Percentage of cells showing mild intensity were given 1 score, percentage of cells showing moderate intensity were given 2 score and those with strong intensity

staining were given 3 score[26,27]. A total score was obtained by adding the products of these different intensity scores as follows. Total Score= (percentage of cells with mild intensity staining x 1) + (percentage of cells with moderate intensity staining x 2) + (percentage of cells with strong intensity staining x 3)

Animal protocols

For in vivo studies, LM-7 cells were treated with SAH or SAM (150 μM) for six days in MEM+10% FBS. At the end of the treatment, cells were harvested in sterile saline. 6 week-old male Fox Chase severe combined immune deficient (SCID) mice, obtained from Charles River, St-Constant, Quebec, Canada, were anesthetized using a cocktail of ketamine (50mg/kg), xylazine (5mg/kg), and acepromazine (1mg/kg) intramuscularly. LM-7 cells viability was confirmed by Trypan blue assay and cells were inoculated at 2×10^5 cells per mouse in 40 μL saline with a 27-gauge needle into the left tibia using a drilling motion. The mice were monitored weekly for tumor burden. On week 4, a digital radiography of hind limbs of all animals was done using a Faxitron X-ray machine (Faxitron X-ray Corp., Lincolnshire, Illinois, USA) to monitor the development of skeletal lesions. The mice were then euthanized, and the left tibias were collected and fixed in 10% buffered formalin solution for 24 hours. The X-ray scoring method is described as follows: no lesions or minor changes, small lesions, significant lesions (minor peripheral margin breaks, 1% to 10% of bone surface disrupted), and significant lesions (major peripheral margin breaks, >10% of bone surface broken) rating 0 to 4, respectively[28-32].

In lung metastasis studies, LM-7 cells treated with 150 μM of SAH or SAM were inoculated in 6 weeks old female BALB/c nude mice, tumor formation and pulmonary metastasis was monitored for a period of 14 weeks[33,34]. Control and experimental animals were sacrificed at the end of this period and lungs were harvested and fixed. Metastatic nodules were counted on surfaces of all lung lobes and the number recorded as the number of lung metastases for each tumor-bearing animal. All the experimental animal protocols were in accordance with the McGill University Animal Care Committee guidelines.

Statistical analysis

Results were analyzed as the mean ± SEM, and comparisons of the experimental data were analyzed by an independent two-sample t test at $P < 0.05$ level of significance.

Results

Effect of SAM on OS cells proliferation, invasion and migration

Methylation of tumor suppressor genes could result in increased growth rate, which might counteract any anti-metastatic property of SAM. We therefore first determined whether SAM treatment would result in adverse increase in cancer cell growth rate. We examined the effect of SAH and SAM treatment on two invasive human OS cell lines LM-7 and MG-63. Treatment of LM-7 and MG-63 with 75μM and 150 μM dose of SAM for 6 days resulted in significant inhibition of LM-7 and MG-63 cell proliferation as compared to control cells treated with similar doses of SAH (Figure 1A).

We then determined whether SAM pre-treatment affects the invasive potential of OS cells using Boyden chamber Matrigel invasion assay. Pre-treatment of LM-7 and MG-63 cell lines with different doses (75 μM and 150 μM) of SAM reduced tumor cells invasion in a dose dependent manner (Figure 1B). In order to rule out the possible confounding anti-proliferative effects of SAM as shown in panel A, we counted the tumor cells in both upper and lower part of Boyden chamber. Results from this analysis showed similar number of tumor cells during this treatment demonstrating that the observed anti-invasive effects are not due to the ability of SAM to alter cell proliferation.

The effect of SAM on cell migration was analyzed by wound healing assay using LM-7 and MG-63 cell lines. A significant reduction in wound healing (%) was observed in SAM-treated (75 and 150 μM) LM-7 cells compared with SAH treated control cells at 48, 72 and 120 hours and MG-63 cells at 24, 48 and 72 hours after wounding (Figure 2). 150 μM of SAM was most effective in blocking cell migration in LM-7 and MG-63 at 120 hours and 72 hours respectively.

Effect of SAM on cell cycle

Tumor cell's ability to form colonies in soft agar is an index of their aggressive potential. We therefore examined the effect of SAM on the number of colonies formed by LM-7 and MG-63 cells. Following treatment of these cells with (75 and 150 μM) of SAM, a significant and dose-dependent decrease in the number of colonies formed was observed compared to control (SAH treated) group of cells (Figure 3A).

We then examined the effects of different doses (75 and 150 μM) of SAM on cell cycle kinetics to further confirm that SAM treatment wouldn't result in silencing of tumor suppressor mechanisms and enhancement of cell cycle progression. FACS analysis of cell cycle distribution on control and SAM treated cells showed a significant increase in the number of tumor cells in G_2/M phase with simultaneous decrease in S phase in the SAM treatment group as compared to control group of cells (Figure 3B). Thus, not only doesn't SAM accelerate the progression of the cell cycle as anticipated if it silenced tumor suppressor genes it rather inhibits progression through arresting cells at the G_2/M phase of the cell cycle.

Effect of SAM on OS metastasis in vivo

Next we examined the effect of SAM on development and progression of skeletal lesions in our xenograft model of OS by using highly invasive LM-7 cells. Control and SAM (150 μM) treated LM-7 cells were inoculated directly into the tibia of male Fox Chase mice as described in "Materials and Methods". Control animals developed skeletal lesions at week 8 which continued to increase in size and number of lesions over time. In contrast, animals treated with LM-7 cells treated with SAM exhibited reduced total skeletal lesion area (~34%) represented as X-ray score as

compared to the control group of animals inoculated with SAH at week 8 post tumor cell inoculation (Figure 4A).

Since lung metastasis is a common occurrence in OS, we next examined the effect of SAM treatment on the development of lung metastasis using our lung metastasis model as described in "Materials and Methods". Control animals inoculated with SAH treated LM-7 cells developed large lung metastasis detected at the end of these studies 14 weeks post tumor cell inoculation. In contrast, experimental animals inoculated with SAM treated LM-7 cells exhibited a marked decrease in number and size of lung metastasis (Figure 4B).

Effects of SAM on epigenome wide methylation in OS

SAM is a global hypermethylating agent raising the concern that it will indiscriminately affect DNA methylation particularly methylating tumor suppressor genes, which could result in enhancing cancer cell growth. Although our cellular studies described in Figures 1 and 3 demonstrated that SAM didn't block tumor suppressor mechanisms but rather enhanced tumor suppression it is nevertheless important to exclude the possibility that SAM increases methylation of tumor suppressor genes. We therefore performed an epigenome wide analysis of the changes in DNA methylation triggered by SAM using Illumina 450K bead arrays which provide a representative coverage of CGs at transcription start sites, 5' regulatory regions, CG shores as well as in the gene bodies. DNA was isolated from LM-7 cells treated with 150 μM of SAH and SAM for 6 days. This dose and time period of treatment was found to be most effective in inhibiting tumor

cell proliferation, invasion and migration (Fig. 1-2). Results from these studies presented in supplementary Table 1 (S-1) which lists the statistically significant CGs whose methylation was altered in response to SAM treatment reveal that SAM has remarkably a very specific and particularly limited effect on the methylome. None of the known tumor suppressor genes altered their state of methylation in response to SAM treatment while the sites that were hypermethylated were associated with genes that were known to play a key role in tumor growth and metastasis (S-1). Ingenuity pathway analysis (IPA) showed that the hypermethylated genes are members of key intracellular signaling pathways that are known to be involved in OS growth and metastasis but there were no genes in tumor suppressor pathways that seem to be affected (S-2).

Effect of SAM on the expression of OS associated genes

Due to the complex nature of OS progression several molecular pathways and genes are implicated in its growth and metastasis. In order to understand the anti-tumor effects of SAM we first analyzed the expression of well-established genes which are known to alter tumor cell proliferation, invasion and metastasis as well as genes that were hypermethylated by 150 μM 6d SAM treatment as determined by the Illumina bead array analyses (S-1, 2). The qPCR results presented in Figure 6 show the analysis of RNA from control and 150 μM SAM treated cells. SAM treatment reduced the expression of genes implicated in tumor cell invasion, metastasis and angiogenesis such as *matrix metalloproteinase (MMP) 2 and 9, vascular endothelial growth factor (VEGF), plasminogen*

activator inhibitor 1 (PAI-1) and *uPA*. Additionally, SAM treatment also markedly reduced the expression of transforming growth factor β *(TGF-β)* and *runt related transcription factor 2 (RUNX2)* [Figure 5 A].

We also selected three representative genes of Exocyst Complex Component 7 (EXOC7), Polycomb Group Ring Finger 3 (PCGF3) and Platelet-Derived Growth Factor Alpha (PDGFA) which were found to be hypermethylated following SAM treatment. These genes are involved with several intracellular signaling pathways that are known to affect tumor growth and metastasis[35,38]. qPCR analysis of control and experimental LM-7 cells show that expression of these genes was markedly reduced following SAM treatment supporting the hypothesis that SAM triggered hypermethylation leads to silencing of several genes critical for metastasis (Figure 5B).

Expression of new candidate genes in cancer and normal tissues

We determined the levels of expression of these genes (EXOC7, PCGF3, PDGFA) in normal bone and clinical biopsies from osteosarcoma patients in a tissue array using commercially available antibodies as described in "Material and Methods". Results from osteosarcoma array demonstrated a higher PDGFA expression in different stages of osteosarcoma as compared to normal bone ($p<0.05$). No significant differences in the level of expression of PDGFA were observed between different stages of osteosarcoma (Figure 6). Antibodies against EXOC7 and PCGF3 showed a high nonspecific staining at multiple dilutions resulting in inconclusive results for immunohistochemistry (data not shown).

Discussion

Aberrations in DNA methylation pattern is one of the hallmarks of cancer where by controlling the transcription of tumor suppressor and pro-metastatic genes it can regulate the multi-step process of tumor progression[39]. In the majority of studies to date focus has been on understanding the hypermethylation of tumor-suppressor genes and targeting these processes therapeutically whereas little attention was paid to the potential role of hypomethylation of pro metastatic genes. However, an increasing body of evidence suggests that hypomethylation of prometastatic genes could promote cancer metastasis. This points to the possibility that drugs that induce hypermethylation of prometastatic genes could serve as antimetastatic agents. We have previously shown that the ubiquitous methyl donor SAM can inhibit DNA demethylation *in vitro* and *in vivo*[24,25] and can lead to hypermethylation and silencing of pro-metastatic genes. SAM is a particularly attractive agent since it is a FDA approved nutritional supplement with little documented toxicity.

In this study we provide a proof of principle that SAM could act as an antimetastatic agent in osteosarcoma. Towards these goals we used two *in vivo* models of osteosarcoma which allowed the evaluation of the effect of SAM in bone and in blocking distant metastasis (lungs). Combined with these models we used several *in vitro* assays to determine the mechanism of these anti-tumor effects of SAM. The first concern with using a hypermethylating agent in cancer is that it will lead to silencing of tumor suppressor genes through increased DNA methylation and that such an effect will override its beneficial effects on

inhibition of metastasis. Our results show that SAM treatment had a significant effect on reducing tumor cell proliferation and altering cell cycle kinetics by reducing the number of cells in S phase and arresting them at G_2/M phase. This suggests that SAM does not inhibit tumor-suppressor mechanisms; on the contrary SAM triggers mechanisms that arrest cell growth and makes them susceptible to radio- and chemo-therapy. As hypothesized SAM inhibited invasion and migration and thus blocked basic mechanisms driving metastasis while avoiding silencing of tumor suppressor mechanisms. Although SAM reduced both proliferation and invasion the effects of SAM on cell invasion were found to be independent of cell death or inhibition of proliferation as similar number of control (SAH) and experimental (SAM) treated tumor cells were observed in upper parts of Boyden (Fig. 1). We then evaluated the effect of SAM on OS metastasis *in vivo*. Inoculation of SAM treated cells exhibited a significantly reduced number of lung metastasis when injected via tail vein *in vivo*. *In vivo* SAM treatment did not increase cell proliferation as anticipated if tumor suppressor genes were silenced by this hypermethylating agent but resulted in inhibition of cell proliferation. The fact that transient treatment *in vitro* was sufficient to block invasion and growth *in vivo* without further treatment with SAM is consistent with the hypothesis that the "*in vitro*" treatment epigenetically "reprogrammed" the OS cells to become less invasive and tumorigenic. The ability of epigenetic drugs to "reprogram" cancer cells carries important therapeutic advantage. The specificity of these SAM mediated effects was confirmed by simultaneous treatment with its inactive analogue SAH which lacks

the methyl group and doesn't cause hypermethylation and showed no effects on invasion and growth.

Although SAM is a global hypermethylating agent, the biological effects observed suggest specificity[40]. A plausible mechanism for SAM action is that it results in coordinate silencing of critical genes for OS metastasis but doesn't silence tumor suppressor genes. In order to understand the underlying molecular mechanism mediating these significant *in vitro* and *in vivo* affects, we first examined the change in the levels of expression of genes implicated in tumor metastasis in general and skeletal metastasis in particular. PCR analysis of control (SAH) and experimental (SAM) treated LM-7 cells showed a marked inhibition in the expression of tumor promoting genes (*MMP-2, MMP-9, VEGF, PAI-1, uPA*) and genes (*uPA, TFG-β, RUNX2*) which are known to promote the development and progression of skeletal metastasis. *MMP-2* and *MMP-9* are two key regulators of extracellular matrix (ECM) remodeling and play a crucial role in angiogenesis, migration of cancer cells and metastasis. *VEGF* is a major angiogenic growth factor[41]. uPA and PAI-1 are integral components of plasminogen activator system and play important roles in ECM degradation and invasion of cancer cells[43]. TGF-β and RUNX2 are involved in osteoblast differentiation and skeletal metastasis[43,44]. TGF-β arrests cell cycle at G1 phase and initiates differentiation or apoptosis of normal cells, however in metastatic cancer it is known to stimulate invasion and metastasis by up regulating the uPA mRNA and SMAD4 signaling[9,45]. *RUNX2* is a gene which has a well-established role in bone biology and skeletal metastasis[46]. Recently it has been shown that

increased residence of RUNX2 at mitotic chromosomes may reflect its epigenetic function in "bookmarking" of target genes in cancer cells[47]. The fact that SAM targeted these genes provides a plausible mechanism for its anti OS effects seen in our study.

The idea that SAM has a specific effect on OS that targets prometastatic genes for silencing but not tumor suppressor genes was supported by a methylome analysis of changes in DNA methylation in LM-7 triggered by SAM (S-1). Remarkable in spite of the fact that it is a general methyl donor only a small number of genes were affected by SAM (S-1) but they seem to particularly target critical pathways for metastasis and tumor growth (S-2). Ingenuity pathway analysis of these genes that became differentially methylated are involved in critical signaling pathways that were known to play a role in tumorigenesis but none of the known tumor suppressor genes that are hypermethylated in cancer. Following IPA analysis, we selected three genes that are hypermethylated by SAM treatment (*EXOC7, PCGF3, PDGFA*) which are implicated in several key intracellular signaling pathways, regulation of gene transcription and tumorigenesis as shown in S-1. We then determined the change in the levels of expression of these candidate genes (EXOC7, PCGF3 and PDGFA) in OS cells following treatment with SAM. Experimental cells treated with SAM showed a marked suppression in the expression of these genes as determined by qPCR analysis.

Using immunohistochemcial analysis we determined the significance of identified genes (EXOC7, PCGF3, PDGFA) in the OS development and

progression. Towards these goals we used commercially available OS tissue array and normal bone from our institution. Commercially available antibodies against EXOC7 and PCGF3 showed high nonspecific staining at multiple dilutions and results from these studies are not shown. However, antibody against PDGFA showed specific staining of bone cells. Results of this analysis as shown in Figure 6 show low levels of PDGFA expression normal bone samples. In contrast PDGFA expression was markedly high in bones of OS patients. While these results clearly showed the induction of PDGFA in OS, limited number of samples from early stages (Ia, Ib) restricted our ability to establish a correlation with disease progression. These results are particularly significant as PDGFA is up regulated in several cancers due to its ability to alter cell proliferation, differentiation, angiogenesis and metastasis[48-50].

Collectively, these results provide support that SAM can serve as a viable and attractive anti-cancer agent which blocks various tumor promoting genes and signaling pathways. Our studies identify OS "signature" candidate genes, which are hypomethylated in OS and may serve as efficient biomarkers for diagnosis and prognosis of OS patients. Since SAM is already being used in oral formulation, it can provide beneficial effects in both preventive and therapeutic settings using improved and stable forms of SAM. Results from these studies also provide new therapeutic opportunities where methylation therapy alone or in combination with various therapeutic strategies currently under development to target genes which we have identified like uPA and its receptor to elicit strong

synergistic effects to significantly reduce morbidity and mortality in cancer patients in general and those with OS in particular.

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Figures (Chapter 2)

Figure 1

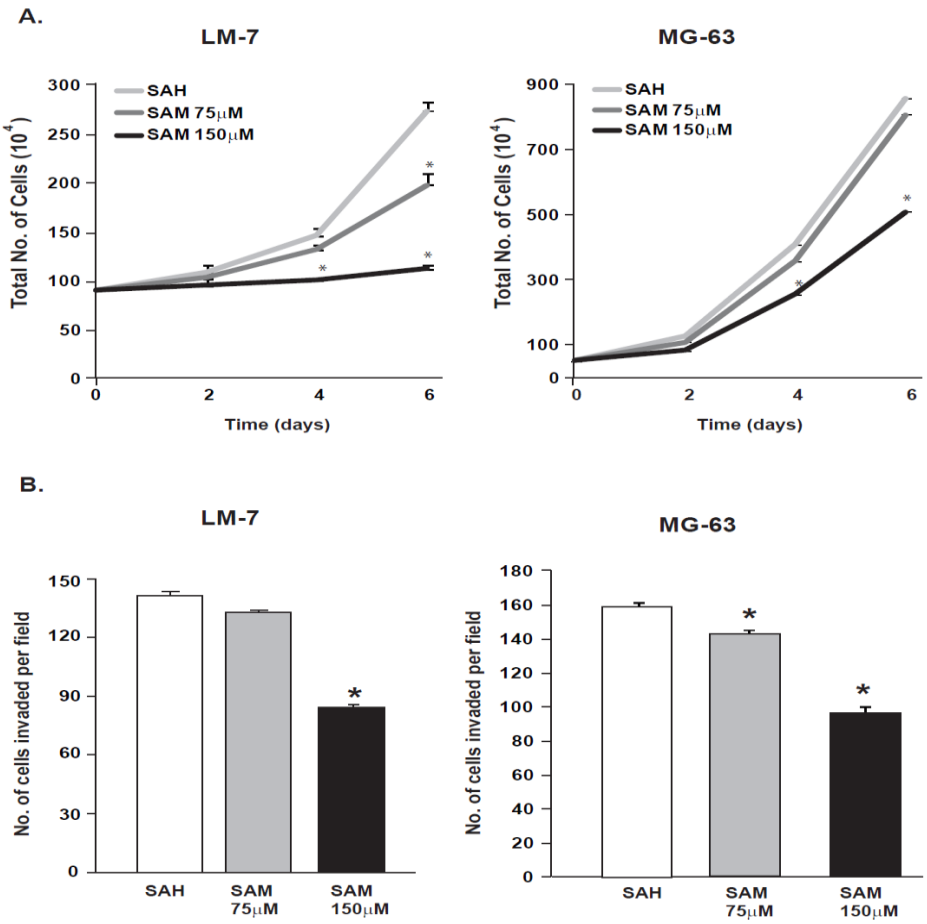

Figure 1. Effect of SAM on OS cells proliferation and invasion *in vitro*

Human OS cancer cells LM-7 and MG-63 were plated in 10ml plates and treated with 150 □□ of SAH as control (SAH) or two doses (75 and 150 μM) of SAM. Cell growth rate was determined in each group by trypsinization and counting the number of cells by Coulter counter as described in "Materials and Methods" (panel A). LM-7 and MG-63 cells invasive capacity was evaluated by using a Boyden chamber Matrigel invasion assay. After 18 h of SAM (75 and 150 μM) treatment, the invaded cells were fixed, stained and ten random fields were counted. Number of cells invading is shown as bar diagram ± SEM (panel B) as described in "Materials and Methods". Results are presented as the mean ± SEM of two different experiments in duplicate from control and experimental cells. Significant differences from the control (SAH) is represented by an asterisk ($p < 0.05$).

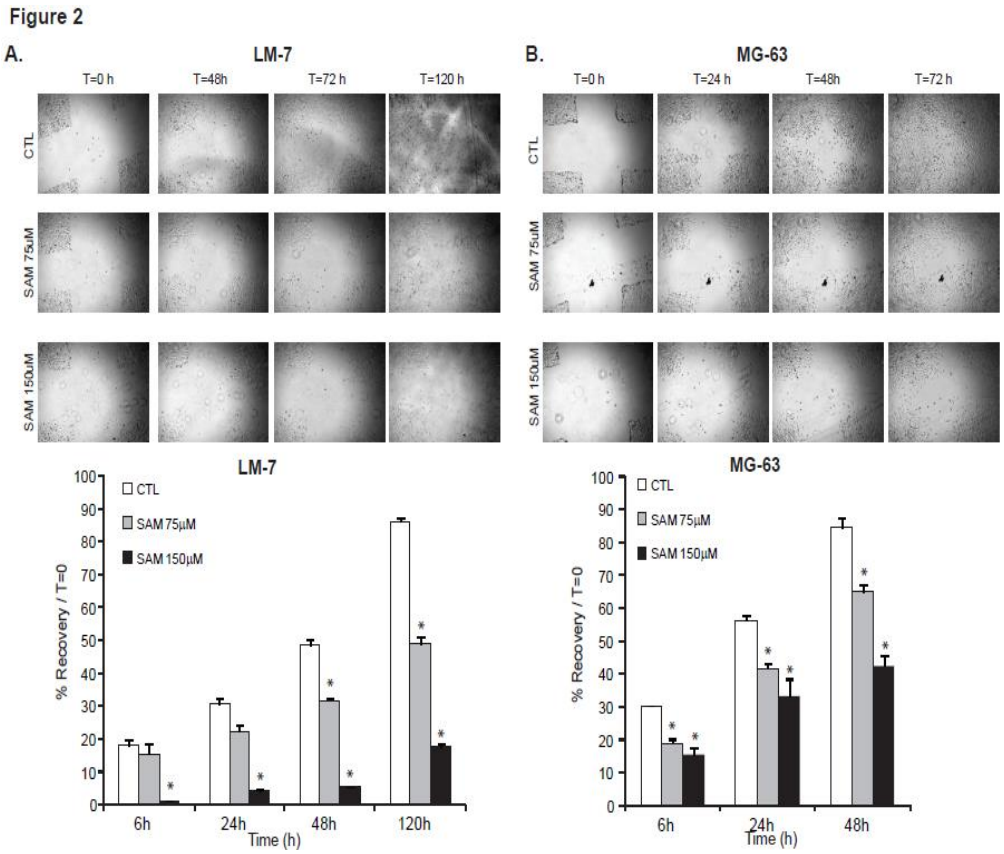

Figure 2. Effect of SAM on OS cells migration *in vitro*

Wound healing assay was carried out by seeding LM-7 and MG-63 cells in six well plates and allowing them to grow as a monolayer and making a wound as described in "Materials and Methods". These cells were treated with 150 µM SAH as control (SAH) or two different doses of SAM (75 and 150 µM) containing 2% fetal bovine serum and migrating cells were photographed at different time points. Percent wound healing was recorded at different time points, and percentage of wound healing with respect to T_0 was calculated using the equation described in "Materials and Methods". Results are presented as the mean ± SEM of two different experiments in duplicate from control and experimental cells. Significant differences from the control (SAH) is represented by an asterisk (p <0.05).

Figure 3

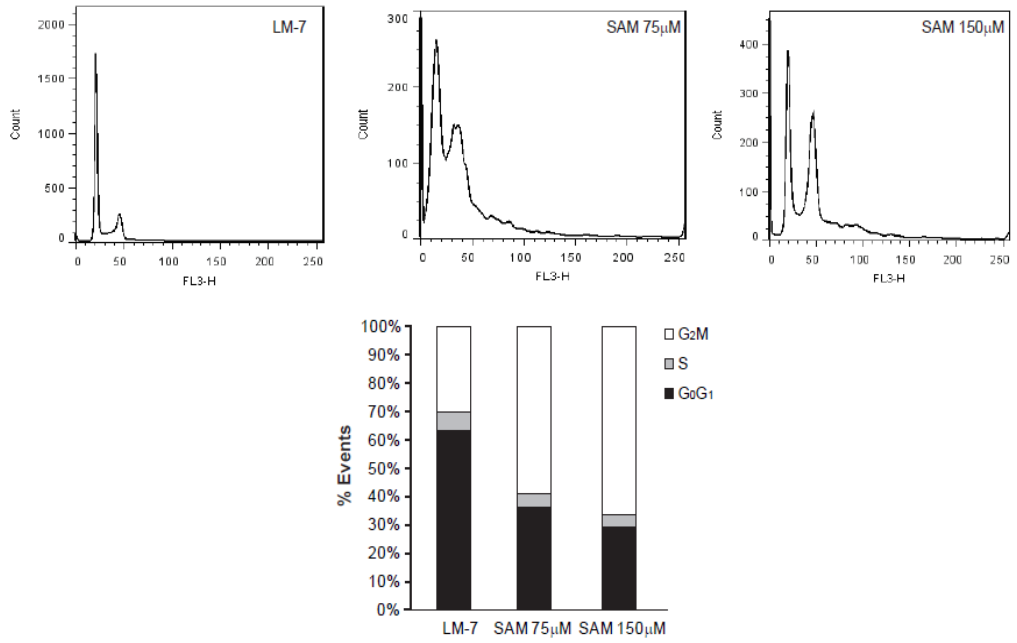

Figure 3. Effect of SAM on OS cellcycle kinetics *in vitro*

LM-7 and MG-63cells were treated with 150 □M of SAH as control (SAH) or SAM (75 and 150 □M). Treated cells were then fixed and stained with propidium iodide. FACS analysis was performed as described in "Materials and Methods" (Panel A). Results are presented as the mean ± SEM of two different experiments in duplicate from control and experimental cells. Significant differences from the control (SAH) is represented by an asterisk (p <0.05).

Figure 4

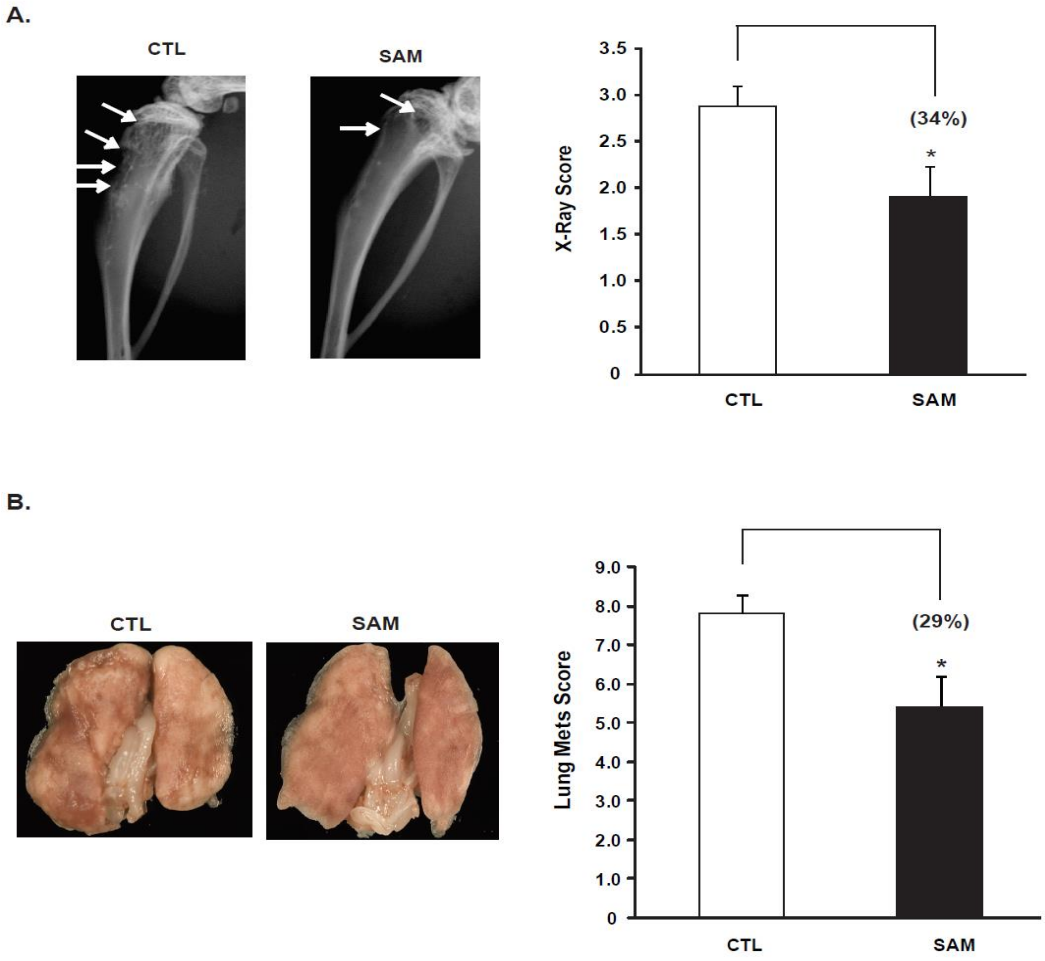

Figure 4. Effect of SAM on OS skeletal lesions and lung metastasis *in vivo*

Panel A: Male Fox Chase SCID mice were inoculated with ($2x10^5$) LM-7 cells treated with 150 µM of SAH as control (SAH) or 150 µM of SAM for seven days via i.t route. Development of skeletal lesions was determined at weekly intervals by X-ray using Faxitron and lesion area was determined as described "Materials and Methods". Representative X-ray and lesion score of control and experimental animals at week 4 post tumor cell inoculation is shown. Skeletal lesions are highlighted by arrows. Panel B: Male Balb/c nude mice were inoculated with ($2x10^5$) LM-7 cells treated with 150 µM of SAH as control (SAH) or 150 µM of SAM for seven days and injected via tail vein. At week post tumor cells inoculation control and experimental animals were sacrificed and number lung metastasis was determined as described in "Materials and Methods". Photomicrographs of representative lungs in each group are shown. Result represents the mean ± SEM of ten animals in each group. Significant differences from control are represented by asterisks (p <0.05).

Figure 5

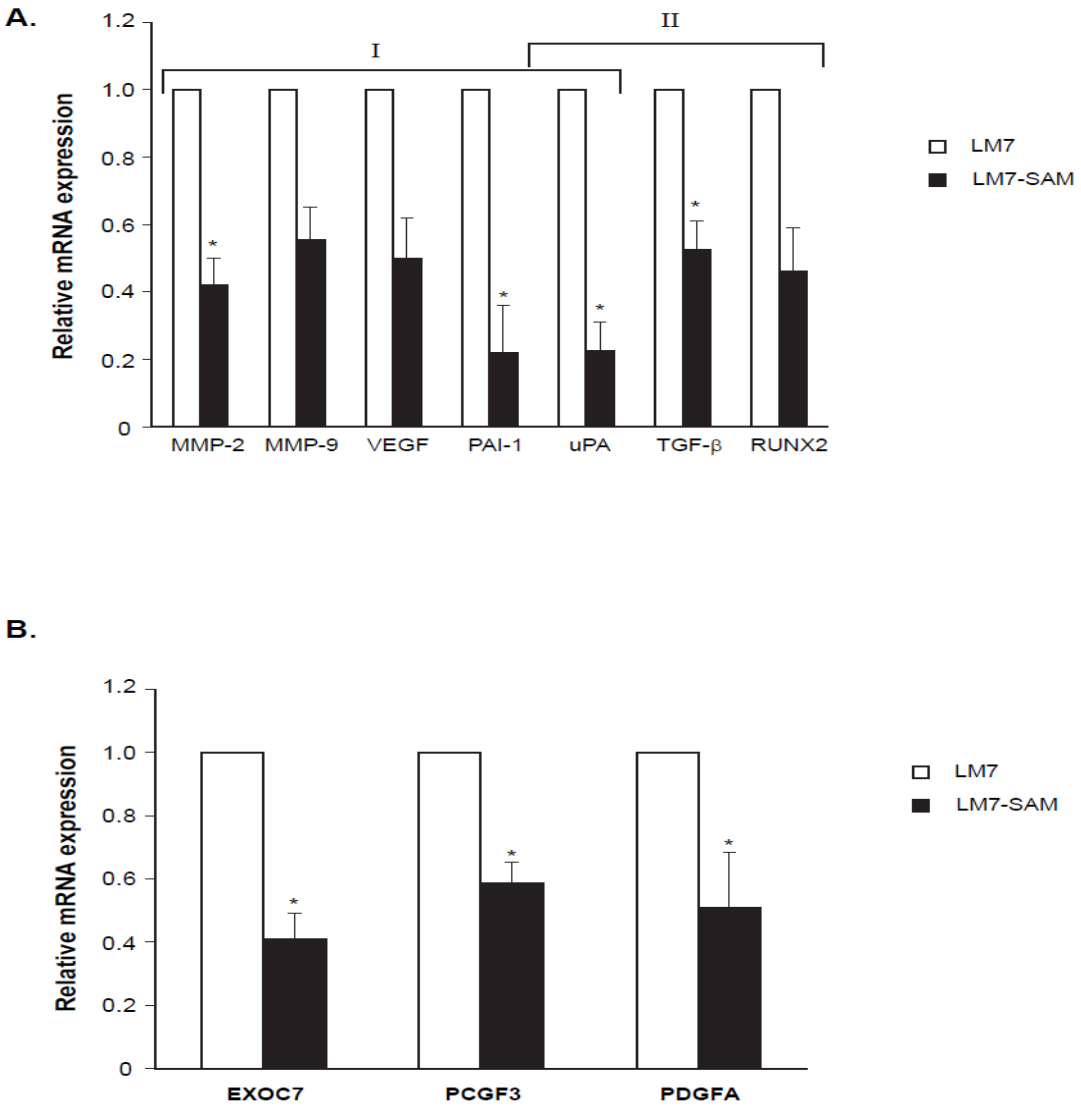

Figure 5. Effect of SAM on the expression of genes associated with OS metastasis

LM-7cells were treated with 250 μM of SAH as control (SAH) or with 250 μM of SAM for seven days, and total cellular RNA was isolated with TRIzol. RNA from control and treatment groups were analyzed for the expression of genes involved in tumor progression and skeletal metastasis (panel A) and hypomethylated genes identified by illumina analysis (panel B). Changes in the mRNA expression of the representative genes were determined by plotting the relative ratio against GAPDH which was used as loading control. Results are presented as the mean ± SEM of two different experiments in duplicate from control and experimental cells. Significant differences from the control (SAH) is represented by an asterisk (p <0.05).

Figure 6

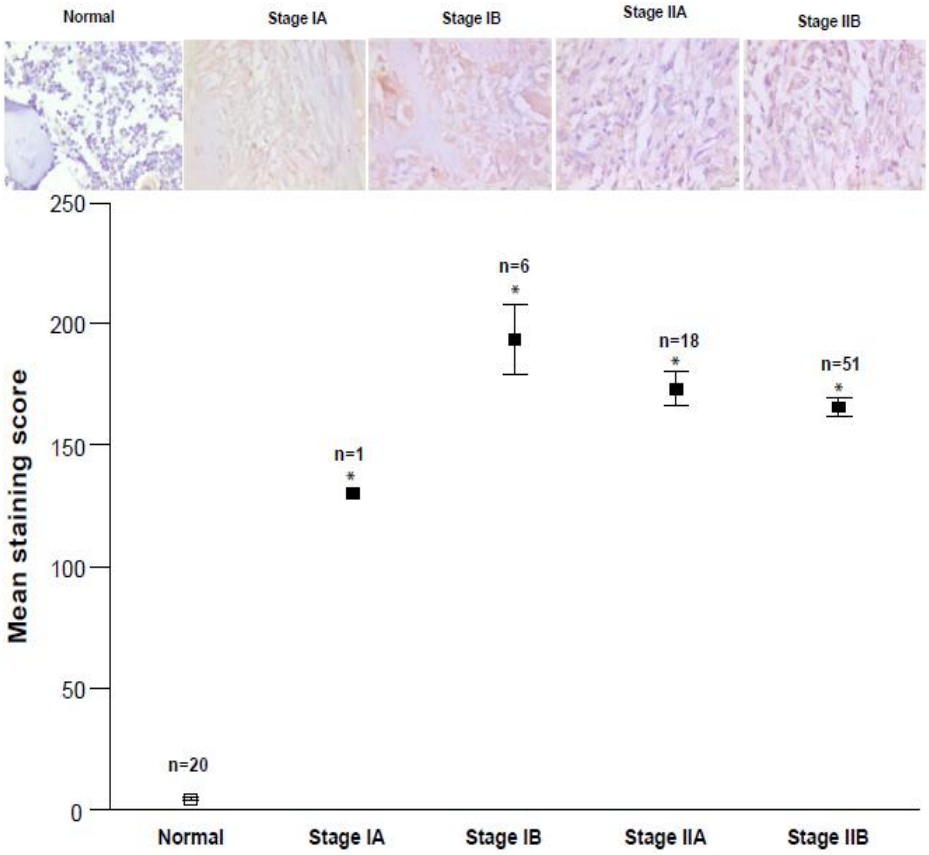

Figure 6. Immunohistochemical analysis for PDGFA expression in normal bone and osteosarcoma patients.

Tissue array obtained from of osteosarcoma patients of different stages (Ia, Ib, IIa, IIb) as defined by the American Cancer Society and normal bone (Normal) were stained with PDGFA specific antibody and staining intensity was quantitated as described in "Materials and Methods"). The staining intensity was calculated and a total mean staining score = (Percentage of cells with mild intensity staining x 1) + (Percentage of cells with moderate intensity staining x 2) + (Percentage of cells with strong intensity staining x 3) was calculated and represented in panel B. Representative images from normal bone and different stages of osteosarcoma are shown (upper panel).

Tables (Chapter 2)

Table 1. Differentially methylated genes and their functions

UCSC_REFGENE_NAME	Function	UCSC_REFGENE_ACCESSION	TargetID	UCSC_REFGENE_GROUP	LM7_SAM.Delta Beta	LM7_SAM.DiffScore
EXOC7	Member of exocyst complex, vesicular trafficking	NM_001145298;NM_001145297; NM_015219;NR_028133;NM_001145299; NM_001013839	cg26287080	Body	0.29	113.7
			cg09973371		0.17	83.6
			cg03655023		0.19	79.7
PCGF3	Member of polycomb group, PcGPRC1 complex-chromatin remodeling	NM_006315	cg03271827	5'UTR	0.19	74.6
NXN	Nucleoredoxin, negative regulator of Wnt signaling pathway	NM_022463	cg05176970	Body	0.16	57.8
PDGFA	Growth factor, embryonic development, cell proliferation, migration chemotaxis	NM_002607;NM_033023	cg14496282	Body	0.16	32.3
PRKAR1B	Regulatory subunit of the cAMP-dependent protein kinases	NM_001164759;NM_001164762; NM_001164758;NM_001164760; NM_001164761;NM_002735	cg13558627	Body	0.12	31.4
			cg01635128		0.15	29.6
PTPRN2	Development of nervous system and pancreatic endocrine cells	NM_002847;NM_130842;NM_130843	cg00859877	Body	0.14	25.5
			cg08284447		0.11	25.2
ERICH1	Glutamate-rich protein 1	NM_207332	cg11416102	Body	0.13	25.2
INS-IGF2;IGF2AS; IGF2;	Insulin-like growth factor, potent mitogen, exhibits osteogenic effects	NR_003512;NR_028044; NM_001127598;NM_001007139; NR_028043;NM_000612	cg04072545	Body;TSS1500;5'UTR;	0.09	21.9
			cg14317384		0.16	20.0
			cg05146307		0.10	17.7

1

PRKAR1B	Regulatory subunit of the cAMP-dependent protein kinases	NM_001164762;NM_002735; NM_001164759;NM_001164761; NM_001164760;NM_001164758	cg22433798	3'UTR	0.15	16.4
CTSH	Cathepsin H, degradation of proteins in lysosomes	NM_004390;NM_148979	cg12604181	1stExon;5'UTR	0.13	15.7
			cg05546241		-0.12	-15.5
ERICH1	Glutamate-rich protein 1	NM_207332	cg23474407	Body	-0.17	-19.6
			cg25247689		-0.17	-21.4
FRMD4B	Member of GRP1 signaling complex, recruited in response to insulin receptor signaling	NM_015123	cg10178228	TSS200	-0.16	-24.6
PTPN14	Protein tyrosine phosphatase, lymphangiogenesis, regulates TGF-β, tumor suppressor	NM_005401	cg10952190	TSS1500	-0.15	-37.7
HOXA11	Transcription factor, part of developmental regulatory system	NM_005523	cg27309564	Body	-0.14	-41.8
			cg18430555		-0.18	-72.4
LOC442459		NR_024608	cg03789606	Body	-0.21	-76.6
			cg16578226		-0.29	-163.9

2

Table 2 Ingenuity pathway analysis of hypermethylated genes in SAM treated LM7 cells

Ingenuity Canonical Pathways	p-value	Ratio	Molecules
Glioma Signaling	8.11E-04	1.77E-02	IGF2,PDGFA
Glioblastoma Multiforme Signaling	1.93E-03	1.19E-02	IGF2,PDGFA
Thioredoxin Pathway	2.76E-03	1.25E-01	NXN
Vitamin-C Transport	6.43E-03	4.55E-02	NXN
Sonic Hedgehog Signaling	1.37E-02	2.86E-02	PRKAR1B
Axonal Guidance Signaling	1.54E-02	4.14E-03	PDGFA,PRKAR1B
Netrin Signaling	1.78E-02	1.72E-02	PRKAR1B
Neuroprotective Role of THOP1 in Alzheimer's Disease	1.83E-02	1.82E-02	PRKAR1B
Amyloid Processing	2.33E-02	1.64E-02	PRKAR1B
Phototransduction Pathway	2.42E-02	1.49E-02	PRKAR1B
PXR/RXR Activation	3.05E-02	1.09E-02	PRKAR1B
Macropinocytosis Signaling	3.09E-02	1.3E-02	PDGFA
Growth Hormone Signaling	3.13E-02	1.28E-02	IGF2
Melatonin Signaling	3.18E-02	1.23E-02	PRKAR1B
Leptin Signaling in Obesity	3.40E-02	1.18E-02	PRKAR1B
BMP signaling pathway	3.40E-02	1.18E-02	PRKAR1B
Dopamine Receptor Signaling	3.45E-02	1.04E-02	PRKAR1B
PDGF Signaling	3.49E-02	1.16E-02	PDGFA
VDR/RXR Activation	3.54E-02	1.14E-02	PDGFA
Melanocyte Development and Pigmentation Signaling	3.89E-02	1.05E-02	PRKAR1B
α-Adrenergic Signaling	3.89E-02	9.17E-03	PRKAR1B
CDK5 Signaling	3.98E-02	1.03E-02	PRKAR1B
PAK Signaling	3.98E-02	9.09E-03	PDGFA
G Beta Gamma Signaling	4.03E-02	8.26E-03	PRKAR1B
IL-1 Signaling	4.20E-02	9.17E-03	PRKAR1B
PPAR Signaling	4.25E-02	9.35E-03	PDGFA
Antioxidant Action of Vitamin C	4.34E-02	9.09E-03	NXN
IGF-1 Signaling	4.38E-02	9.35E-03	PRKAR1B
Neuropathic Pain Signaling In Dorsal Horn Neurons	4.52E-02	9.17E-03	PRKAR1B
Nitric Oxide Signaling in the Cardiovascular System	4.56E-02	8E-03	PRKAR1B
Renin-Angiotensin Signaling	4.91E-02	7.94E-03	PRKAR1B
Sphingosine-1-phosphate Signaling	4.96E-02	8.13E-03	PDGFA
Androgen Signaling	5.04E-02	6.9E-03	PRKAR1B
Gαs Signaling	5.04E-02	8E-03	PRKAR1B
Corticotropin Releasing Hormone Signaling	5.09E-02	6.9E-03	PRKAR1B
Sperm Motility	5.31E-02	6.99E-03	PRKAR1B
Synaptic Long Term Potentiation	5.40E-02	7.69E-03	PRKAR1B
Atherosclerosis Signaling	5.44E-02	7.25E-03	PDGFA
P2Y Purigenic Receptor Signaling Pathway	5.48E-02	6.94E-03	PRKAR1B
Gαi Signaling	5.48E-02	7.41E-03	PRKAR1B
Cellular Effects of Sildenafil (Viagra)	5.79E-02	6.45E-03	PRKAR1B
GNRH Signaling	5.83E-02	6.54E-03	PRKAR1B
eNOS Signaling	5.88E-02	6.45E-03	PRKAR1B
Insulin Receptor Signaling	5.92E-02	6.71E-03	PRKAR1B
Ovarian Cancer Signaling	5.97E-02	6.58E-03	PRKAR1B
Human Embryonic Stem Cell Pluripotency	6.01E-02	6.21E-03	PDGFA
AMPK Signaling	6.01E-02	5.56E-03	PRKAR1B
Cardiac β-adrenergic Signaling	6.05E-02	6.33E-03	PRKAR1B
Relaxin Signaling	6.14E-02	6.1E-03	PRKAR1B
Hepatic Cholestasis	6.23E-02	5.46E-03	PRKAR1B
Hepatic Fibrosis / Hepatic Stellate Cell Activation	6.23E-02	6.45E-03	PDGFA

Hepatic Fibrosis / Hepatic Stellate Cell Activation	6.23E-02	6.45E-03	PDGFA
Tight Junction Signaling	6.83E-02	5.99E-03	PRKAR1B
Gap Junction Signaling	6.96E-02	5.52E-03	PRKAR1B
Cdc42 Signaling	7.18E-02	5.38E-03	EXOC7
Dopamine-DARPP32 Feedback in cAMP Signaling	7.27E-02	5.35E-03	PRKAR1B
RAR Activation	7.74E-02	5.24E-03	PRKAR1B
CREB Signaling in Neurons	7.78E-02	4.83E-03	PRKAR1B
Ephrin Receptor Signaling	7.78E-02	4.76E-03	PDGFA
PPARα/RXRα Activation	7.83E-02	5.03E-03	PRKAR1B
Sertoli Cell-Sertoli Cell Junction Signaling	7.87E-02	5.05E-03	PRKAR1B
Calcium Signaling	8.00E-02	4.61E-03	PRKAR1B
Role of NFAT in Cardiac Hypertrophy	8.08E-02	4.78E-03	PRKAR1B
Clathrin-mediated Endocytosis Signaling	8.21E-02	5.05E-03	PDGFA
ERK/MAPK Signaling	8.34E-02	4.74E-03	PRKAR1B
Breast Cancer Regulation by Stathmin1	8.55E-02	4.67E-03	PRKAR1B
Actin Cytoskeleton Signaling	9.44E-02	4.13E-03	PDGFA
cAMP-mediated signaling	9.70E-02	4.42E-03	PRKAR1B
Cardiac Hypertrophy Signaling	9.87E-02	4.02E-03	PRKAR1B
Colorectal Cancer Metastasis Signaling	1.06E-01	3.73E-03	PRKAR1B
G-Protein Coupled Receptor Signaling	1.13E-01	3.62E-03	PRKAR1B
Role of Macrophages, Fibroblasts and Endothelial Cells	1.32E-01	2.92E-03	PDGFA
Molecular Mechanisms of Cancer	1.47E-01	2.58E-03	PRKAR1B
Protein Kinase A Signaling	1.62E-01	2.46E-03	PRKAR1B

Chapter 3

DNA methylation signatures of breast cancer in peripheral T-cells

Preface

Genetic and epigenetic mechanisms play a key role in cancer development and progression. Previous studies from our laboratory have shown the role of DNA methylation in breast cancer progression. Immune system plays an important role in recognizing and eliminating transformed cancer cells. Cancer immunosurveillance is a well-known mechanism which can be utilised to delineate the mechanism of cancer growth.

In the current study, we investigate the DNA methylation signatures found in peripheral cells of the immune system and their role in breast cancer progression.

Abstract

Background: Breast cancer is the second most leading cause of death in women worldwide. The biggest challenge towards breast cancer control is a complete and precise diagnosis. Ability of the immune system to identify tumor cells has led to immune surveillance which can identify factors acting as defense mechanism in cancer. These changes include both genetic and epigenetic mechanism effecting breast cancer and its outcome.

Objective: To determine DNA methylation signatures found in peripheral blood T- cells in breast cancer patients and compare it with age matched normal control group of women.

Materials and Methods: Blood samples from breast cancer patients (19 early stage and 9 late stage patients) and 9 age matched normal females were collected. T-cells were isolated from peripheral blood cells using magnetic bead separation method. DNA was isolated from the T-cells samples and subjected to illumina 450 K analysis.

Results: Illumina 450K Raw data was analysed by CHAMP pipeline. The methylation data from early and late stage breast cancer patients was compared with age matched normal females separately which resulted in a list of 1590 and 12705 differentially methylated genes respectively. To elucidate the most differentially methylated genes in both early and late stage breast cancer patients, we overlapped the two gene lists. The resultant list of 1363 common probes was utilised for further validation. Pyrosequencing analysis for

the most significant genes was performed, to validate the results obtained from illumina 450 K methylation analysis.

Conclusion: The current study provides a proof of principal for the role of DNA methylation in breast cancer progression and presents the evidence of association between the immune cells and epigenetic regulation of cancer. The DNA methylation signatures present in T-cell DNA could be utilised for a better diagnosis and prognosis in breast cancer patients.

Introduction:

Breast cancer is one of the most prevalent malignancy in women affecting as many as one in nine women resulting in a high incidence of morbidity and mortality [1]. Selecting personalized therapeutic strategies for these patients remains a challenge due to variability in tissue morphology and cancer phenotype [2, 3] Towards these goals serval classifications criteria based on histopathology and genetic profiles of breast tumors have been developed[4, 5]. Depending on tissue or site of origin, breast cancers can be categorized into ductal and lobular carcinoma; whereas characteristics like hormone responsiveness and genetic signatures place breast cancers into specific subtypes like Estrogen/Progesterone receptor positive or human epidermal growth factor receptor 2 HER2/neu positive tumors [6, 7].

Current standard of care for breast cancer involves a combination of hormone therapy, chemotherapeutic agents, surgery and radiation. Hormone therapy comprising of selective estrogen receptor modulators (SERMs) and aromatase inhibitors are current treatment of choice for hormone (estrogen) receptor positive breast cancers [8]. Certain prognostic biomarkers like HER2 have shown promising outcome for a specific subgroup of HER2 positive patients. Favourable results have been obtained via treatment with monoclonal antibodies like trastuzumab and lapatinib.

Despite these advances the search for better predictive and prognostic biomarkers and identification of individualized molecular targets continues.

Recently molecularly targeted biomarker bases therapies like poly-ADP-ribose polymerase inhibitors (PARPi) and tyrosine kinase inhibitors have gained attention [9, 10]. However, the biggest hindrance towards breast cancer treatment remains the understanding of the molecular stratification of breast cancers. Involvement of various signaling pathways, genetic profiles and chromosomal aberrations create further difficulties in choosing treatment options for individual patients [11].

Unlike genetic mutations, DNA methylation occurs specifically in promoter regions and CpG islands of the genes and can be measured consistently [12]. The promoter regions and CpG islands are densely populated with CpG sites, however gene body methylation is also observed in many genes resulting in differential gene expression. Moreover, molecular alterations like DNA methylation found in tumor cells are reflected in peripheral blood cells and can be used as an efficient tool for non-invasive approach to determine tumor phenotype [13]. Recent studies have shown that peripheral cells of immune system can serve as promising candidates for early detection of breast cancer and serve as efficient biomarkers [14, 15]

The host immune system constantly monitors and eliminates any non-self-antigens or pathogens [16]. T-cells are the most prominent members of host-immuno-surveillance system, which control the tumor growth [17]. The cellular infrastructure of the human body including these peripheral immune cells is governed by epigenetic mechanisms which regulate transcriptional machinery [18]. The key role of these epigenetic changes in the detection and

monitoring of cancer has been demonstrated in the recent years [19, 20]. These epigenetic signatures vary in different individuals and result in specific gene regulation. DNA methylation is one of the most important epigenetic alteration accompanying tumorigenesis [21]. These DNA methylation signatures can be easily detected in blood samples obtained through non-invasive approach. Peripheral cells of immune system help in eliminating tumor cells and can be easily obtained from the blood. In the current study we hypothesized that the immune system plays an important role in eliminating and controlling cancer growth, invasion and metastasis and can therefore be associated with changes in DNA methylation signatures found in peripheral blood immune cells.

Materials and Methods:

Study Populations:

The study design was approved by ethics committee of McGill University Health Center (MUHC). Peripheral blood samples from healthy controls and breast cancer patients was obtained from the oncology clinic of MUHC following the approval by the institutional review board (IRB) and written consent was obtained from all control and breast cancer patients. Detailed information about the breast cancer cases and controls is shown in Table 1.

T-cell isolation:

All the peripheral whole blood samples were stored in EDTA tubes until Leukocyte isolation. Leukocytes were freshly isolated from whole blood by using ficoll gradient separation. The leukocyte cell pellets were immediately frozen at -80° C until further use. First, B cells were positively isolated using a Dynabeads CD19 positive isolation kit (Invitrogen). Subsequently these B cell-depleted leukocytes were used for T-cell purification with a Dynabeads CD3 positive isolation kit (Invitrogen). The B and T-cell pellets were immediately frozen at -80° C for further DNA/RNA isolation. DNA was isolated from T and B cells using AllPrep DNA/RNA/Protein Mini Kit from Qiagen.

Illumina 450K methylation analysis:

Genomic DNA from all the breast cancer cases and controls was quantified using Picogreen protocol (Quant-iTTM PicoGreen_ dsDNA Products, Invitrogen, P-7589) and read on a Spctra-MAX GeminiXS Spectrophotometer. Bisulfite conversion of 500 ng of genomic DNA was performed using the EZ-96 DNA Methylation-GOLD Kit (Zymo Research, Irvine, CA). The Illumina Methylation 450K kit (San Diego, California, USA) was used for the microarray experiment as described by the manufacturer's protocol, except that 8 lL of bisulfite converted template was utilized to initiate the amplification step. The Illumina hybridization oven was used for incubating amplified DNA (37°C) and for BeadChips hybridization (48°C).

A Hybex incubator was used for fragmentation (37°C) and denaturation (95°C) steps. The X-stain step was carried out in a Tecan Freedom evo robot with a Te-Flow module. Arrays were scanned in Illumina iScan Reader. Data analysis was performed with the Methylation module (version 1.8.0) of the GenomeStudio software (Illumina; version 2011.1) using Human Methylation450_15017482_v1.2. bpm manifest. Statistical threshold was set at a false discovery rate of >0.05, differential score (statistical power) of ±0.33, and delta beta (differential methylation) between the groups was set at >0.15.

Statistical analysis:

The raw data obtained from the 450 K arrays were processed from the IDAT files through to normalisation with BMIQ [22] using the ChAMP [23]

pipeline, batch correction for technical replication dataset using ComBat [24] and all subsequent analysis was performed with the R statistical software v3.2.1.

Quality control of the array data included removal of 2394 probes for which any sample did not pass a 0.01 detection P-value threshold, filtering probes with a beadcount less than 3 has removed 267 from the analysis. Filtering probes with SNPs as identified in Nordlund et al, has removed 28391 from the analysis. Filtering probes that align to multiple locations as identified in Nordlund et al, has removed 8482 from the analysis.

Pyrosequencing Analysis:

Genomic DNA (200-500ng) was used for bisulfite converted using the EZ-DNA methylation Gold Kit (Zymo Research). Pyrosequencing validation of selected genes as predicted by penalise software were performed (See Table xx for list of primers used). The number of genes validated by pyrosequencing was limited by the amount of DNA obtained from these clinical samples. Samples were prepared by performing PCR amplification of selected CGs. PCR reactions were conducted using Hot star enzyme in Biometra T Gradient and T3 thermocyclers. Pyrosequencing was performed using standard methods; briefly, biotinylated PCR products were incubated with streptavidin sepharose bead (GE Healthcare, Canada), followed by denaturation. Beads containing the biotinylated strand were released into 25µl annealing solution and 0.3mM sequencing primer per well. Pyrosequencing was performed using the

PyroMark Q24 machine and results were analyzed with PyroMark® Q24 Software (Qiagen). Collected data was expressed as mean ± standard error of the mean (SEM) and using Student's t-test, p-value< 0.05. The statistical analysis was performed using Prism (GraphPad Software Inc, San Diego, California).

Results:

Genome wide methylation analysis of T-cell DNA from breast cancer patients:

To delineate the DNA methylation differences appearing in peripheral blood T-cells of breast cancer patients compared to normal females, we compared methylation profile of DNA extracted from 9 age matched normal female samples to the group of 28 breast cancer patients (19 females with breast cancer stages 1 and 2, five with stage 3 and four with stage 4). For this purpose, $CD3^+$ T cells were immune-magnetically isolated from whole blood and processed with the Illumina Infinium HumanMethylation450 BeadChip assay [25]

Almost all breast cancer patients were estrogen and progesterone receptor positive and HER2 receptor negative (for clinical characteristics see Table 1[a and b]). To exclude confounding clinical factors involvement in DNA methylation we performed linear regression analysis for age or hormonal status (ER, PR and HER2). None of these confounding factors showed consistent correlation with average methylation values across the group.

The raw data for all samples were processed through the ChAMP analysis pipeline [23] (see Methods). After filtering single nucleotide polymorphisms (SNPs), and other bad probes, the analysis proceeded with 445978 probes. LIMMA R package revealed 10859 probe sets (Figure 1A) with statistically significant differential methylation between normal female controls

and female with breast cancer ($p<0.05$). Heatmap and hierarchical clustering analysis of the DNA methylation profiles of 9 normal and 26 cancer individuals grouped all cancer patients together, suggesting that the DNA methylation profile in T cells is similar in breast cancer (Figure 1A). Breast cancer is associated with overall DNA hypomethylation as seen on heatmap and boxplot (Figure 1A, B). We also compared the difference in methylation between normal controls and early (1, 2) and controls and late (3, 4) stages.

Remarkably, we found much less, 1902 (Figure 3) differentially methylated probes associated with 1590 genes in early stages compared to 30312 associated with 12705 genes in late stages of breast cancer. Overlap of early and late stages (Figure 4) resulted in 1363 probes (P=9.47e-321, hypergeometric).

Functional significance of differentially methylated CpGs in the early stage of breast cancer:

To assess which gene networks, functional categories and canonical pathways are affected in T cells of breast cancer patients we used Ingenuity Pathway Analysis (IPA) tool. Table 2 shows the detailed list of top cancer related canonical pathways.

Validation of DNA methylation obtained from Illumina 450K by Pyrosequencing:

We further validated DNA methylation levels obtained from illumina 450 K by pyrosequencing. Pyrosequencing analysis was limited by the

remaining amount of T-cell DNA. A few representative samples were used for validation purposes. 9 normal and 5 breast cancer T-cell DNA samples were subjected to bisulphite conversion and pyrosequencing analysis. The results showed similar differential methylation patterns as shown in illumina 450K methylation analysis. We found significant correlation between illumina analysis and pyrosequencing validation. Most of the significant genes were hypomethylated in breast cancer patients compared to normal females. 7 probes/CGs were found to be significantly differentially methylated in the representative breast cancer and normal female samples (Figure not shown).

Discussion:

Aberrant DNA methylation is one of the hallmarks of cancer tissue. However, less is known, about the aberration occurring in DNA methylation in non-cancer tissues of cancer patients. Peripheral blood can serve as a promising source that can be used also as a diagnostic tool.

We hypothesize, that this approach can also be applicable in the detection of early stage breast cancer. Though, various biomarkers for breast cancer have been proposed, none of them are helpful in complete and precise diagnosis of breast cancer [26]. The current imaging methods are also restricted by the size and volume of growing tumor tissue. Mostly the current methods of breast cancer detection depend on invasive methods like biopsy of tumor tissue or collection of circulating tumor cells. Screening methods like mammograms, X-rays and other breast imaging tests are limited by the size of tumor [27, 28]. Early detection of breast cancer before the appearance of tumors, could improve breast cancer diagnosis and prognosis.

Early detection of breast cancer and better prognosis is the need of the hour. The mechanisms governing cancer development and metastasis need to be unraveled. Cancer cells are known to exploit various gene regulation mechanisms to their benefit and transform into invasive and metastatic phenotype. Contrary to the predominant current understanding, cancer cells not only alter by genetic but also via epigenetic programs to maintain growth and survival [18]. Cancer cells frequently escape the immune surveillance

mechanisms and disseminate to newer sites for metastasis. These metastatic cancer cells are epigenetically programmed to alter the genetic machinery and establish themselves in the favorable environment. The peripheral cells of immune system constantly patrol the body to protect it from pathogens, exogenous antigens and are able to identify the transformed cells [29]. Role of T-cells in cancer immune surveillance has been documented recently [30, 31]. This led us to our hypothesis that progression of cancer is associated with DNA methylation signatures in T-cells. In the present study we demonstrate a significant role of T-cell DNA methylation in breast cancer patients, indicating the presence of peripheral molecular markers of breast cancer in T-cells.

Our epigenome wide methylation analysis showed 1363 significantly differentially methylated genes in early and late stage breast cancer patients as compared to age matched normal females. For this purpose, we utilized 28 breast cancer patients (19 females with breast cancer stages 1 and 2, five with stage 3 and four with stage 4) and compared them to 9 age matched normal female samples. T-cell DNA from both groups of normal females and breast cancer patients was analyzed for significant methylation differences. Each group of early and late stage breast cancer patients was first compared separately to the control group of normal females. This resulted in two lists of significant probes which were distinctly different in the breast cancer patients as compared to normal females. To avoid technical issues and confounding effects we compared the differentially methylated probes in early and late stage breast cancer cohorts and overlapped the resultant lists.

This list of significant probes was further utilized for validation by pyrosequencing analysis. Validation of significant probes was limited by the small amount of leftover T-cell DNA after epigenome wide analysis. 7 probes were analyzed by pyrosequencing analysis which showed similar correlation to illumina methylation analysis even in the small number of representative samples. All these probes had at least one significant CG which was differentially methylated in T-cells in breast cancer patients. The genes belong to key signaling pathways known to play an important role in DNA repair, cell differentiation as shown in Table 2 and serve as good predictor of breast cancer diagnosis.

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Figures (Chapter 3)

Figure 1

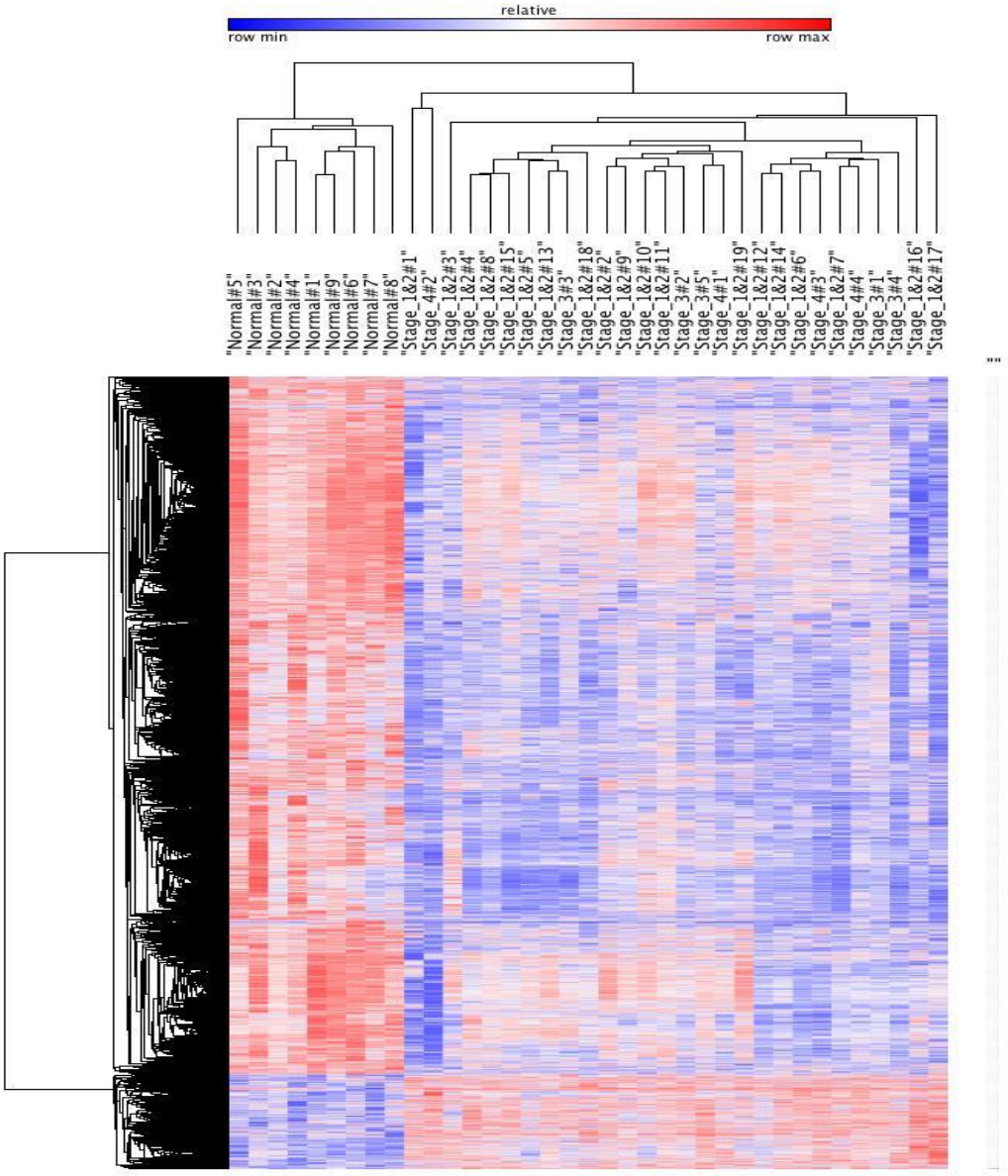

Figure 1: Differential methylation pattern of breast cancer patients and age matched controls**:** Heat map and hierarchical clustering analysis of the DNA methylation profiles of 9 normal and 28 breast cancer patients. Breast cancer is associated with overall DNA hypomethylation as seen on heat map. The analysis was performed using the Hmisc R package. 10772 CpG sites (8283 hypomethylated and 2489 hypermethylated CpG sites) are shown with FDR adjusted p value <0.05.

Figure 2

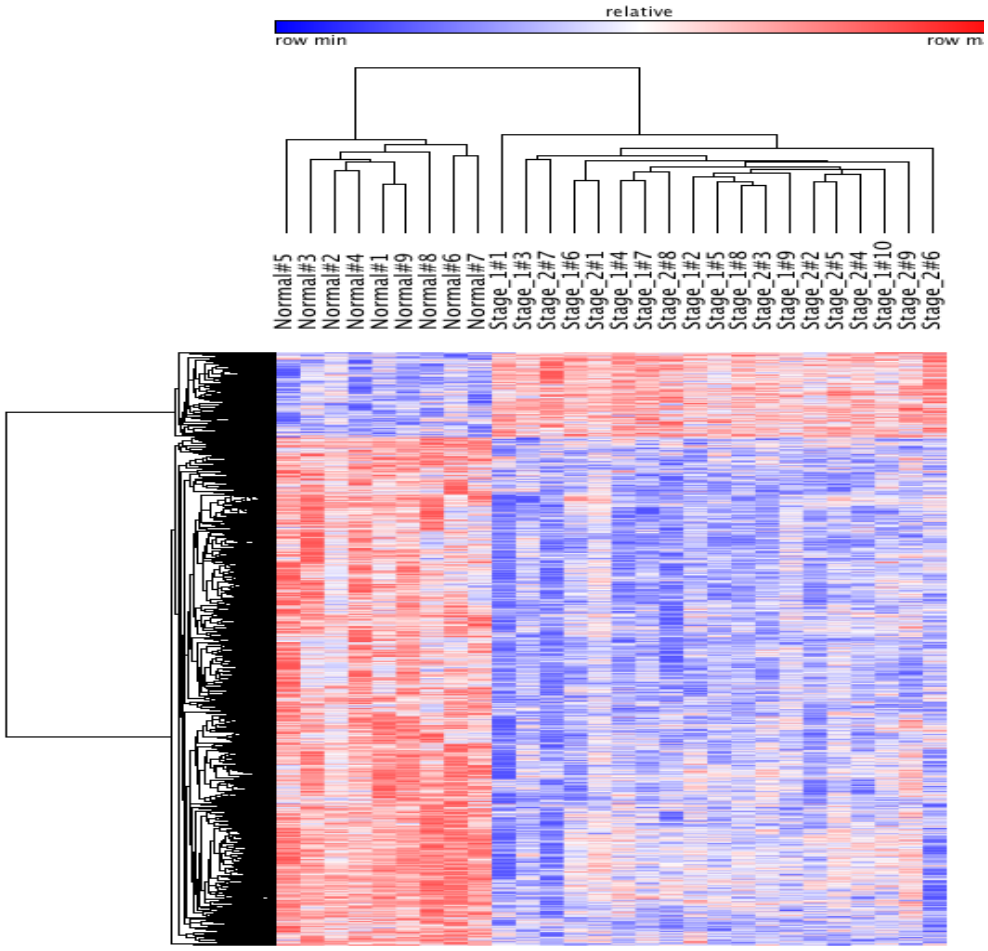

Figure 2: Differential methylation pattern of early stage breast cancer patients and age-matched controls**:** Heat map and hierarchical clustering analysis of the DNA methylation profiles of 9 normal and 19 early stage (stage 1& 2) breast cancer patients. Relationship between early cancer stages and methylation profiles was performed by linear regression analysis using the Hmisc R package. 10772 CpG sites (8283 hypomethylated and 2489 hypermethylated) are shown with FDR adjusted p value <0.05.

Figure 3

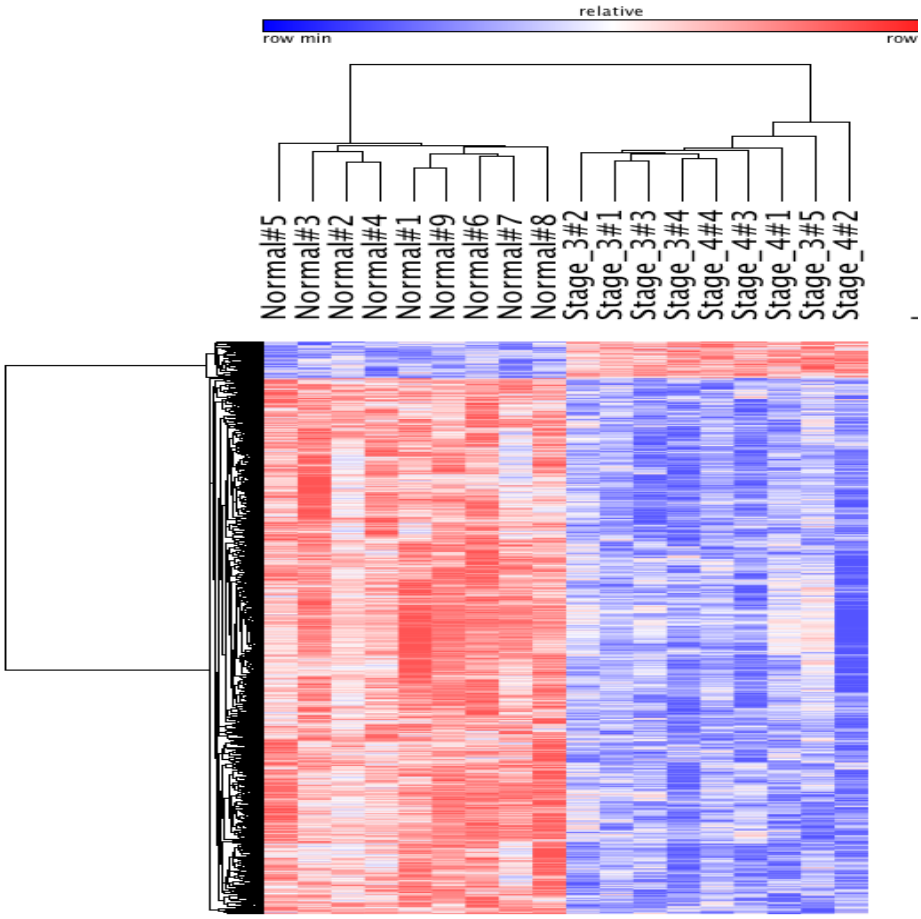

Figure 3: Differential methylation pattern of late stage breast cancer patients and age-matched controls**:** Comparison of late stage breast cancer (stage 3 & 4) with normal females showing 30312 CpGs probes (27049 hypomethylated and 3263 hypermethylated). These differentially methylated probes were associated with 12705 genes in late stages of breast cancer. The 30312 CpG probes were annotated to 12705 genes.

Figure 4

Figure 4: Common differential methylated sites in early and late stage breast cancer patients: Overlap of early and late stages resulted in 1363 probes (P=9.47e-321, hypergeometric). These results demonstrate strong correlation of breast cancer stages with DNA methylation profile in T cells of breast cancer patients.

Tables (Chapter 3)

Table 1(a): Clinical Characteristics of patients

Normal Controls

Patient #	Age	Stage	ER	PR	HER2
Normal1	54	N/A	N/A	N/A	N/A
Normal2	69	N/A	N/A	N/A	N/A
Normal3	67	N/A	N/A	N/A	N/A
Normal4	61	N/A	N/A	N/A	N/A
Normal5	50	N/A	N/A	N/A	N/A
Normal6	58	N/A	N/A	N/A	N/A
Normal7	57	N/A	N/A	N/A	N/A
Normal8	54	N/A	N/A	N/A	N/A
Normal9	83	N/A	N/A	N/A	N/A

Breast Cancer Patients - Stage 1

Patient #	Age	Stage	ER	PR	HER2
Stage1_1	71y	1 &2	+	+	-
Stage1_2	57y	1 &2	+	+	-
Stage1_3	56y	1 &2	+	-	-
Stage1_4	75y	1 &2	+	+	-
Stage1_5	56y	1 &2	+	+	+
Stage1_6	58y	1 &2	+	+	-
Stage1_7	66y	1 &2	+	+	-
Stage1_8	85y	1 &2	+	+	-
Stage1_9	69y	1 &2	+	+	-
Stage1_10	67y	1 &2	+	+	+

Table 1(b): Clinical Characteristics of patients

Breast Cancer Patients - Stage 2

Patient #	Age	Stage	ER	PR	HER2
Stage2_1	55y	1 &2	+	+	-
Stage2_2	68y	1 &2	+	+	-
Stage2_3	54y	1 &2	+	+	-
Stage2_4	43y	1 &2	+	+	-
Stage2_5	66y	1 &2	+	+	-
Stage2_6	59y	1 &2	+	+	-
Stage2_7	61y	1 &2	+	+	-
Stage2_8	55y	1 &2	+	+	-

Breast Cancer Patients - Late Stage

Patient #	Age	Stage	ER	PR	HER2
Stage3_1	67y	3	-	-	+
Stage3_2	77y	3	+	+	-
Stage3_3	85y	3	+	Weakly+	-
Stage3_4	68y	3	+	+	-
Stage3_5	83y	3	?	?	?
Stage4_1	64y	4	+	+	-
Stage4_2	62y	N/A	N/A	N/A	N/A
Stage4_3	55y	4	-	-	-
Stage4_4	57y	4	+	+	-
Stage4_5	55y	1 &2	+	+	-

Table 2: Top cancer related canonical pathways

Ingenuity Canonical Pathways	**p-value**	**Ratio**
PTEN Signaling	9.69E-05	1.61E-01
p53 Signaling	8.78E-04	1.53E-01
RAR Activation	1.37E-04	1.21E-01
Salvage Pathways of Pyrimidine Ribonucleotides	1.52E-03	1.51E-01
Cell Cycle: G1/S Checkpoint Regulation	1.60E-03	1.72E-01
Cyclins and Cell Cycle Regulation	2.66E-03	1.54E-01
STAT3 Pathway	4.64E-03	1.51E-01
ERK5 Signaling	4.67E-03	1.59E-01
Role of CHK Proteins in Cell Cycle Checkpoint Control	5.80E-03	1.64E-01
HIPPO signaling	6.00E-03	1.4E-01
CDK5 Signaling	7.26E-03	1.31E-01
Telomerase Signaling	7.26E-03	1.31E-01
Wnt/β-catenin Signaling	7.26E-03	1.12E-01
Non-Small Cell Lung Cancer Signaling	1.70E-02	1.38E-01
Regulation of the Epithelial-Mesenchymal Transition Pathway	1.78E-02	1.03E-01
PDGF Signaling	1.86E-02	1.3E-01
VDR/RXR Activation	2.02E-02	1.28E-01
GADD45 Signaling	2.58E-02	2.11E-01
Glioma Signaling	3.03E-02	1.16E-01
DNA Methylation and Transcriptional Repression Signaling	3.08E-02	2E-01
Thyroid Cancer Signaling	3.34E-02	1.5E-01
Bladder Cancer Signaling	3.95E-02	1.15E-01

Chapter 4

Identification and Validation of an Epigenetic Signature of Osteoporosis in Post-menopausal Women

Preface

Osteoporosis is one of the most common bone related disorder affecting elderly people. Low bone mass and deteriorating bone tissues are the most prominent characteristics of osteoporosis.

Previous studies from our lab have established the significant role of epigenetics in gene transcription and disease progression. In the current study we investigate the osteoporosis associated DNA methylation patterns in postmenopausal osteoporotic patients. We utilised Epigenome-wide association studies (EWAS) to delineate epigenetic signatures found in whole blood DNA of osteoporotic patients.

Abstract

Background: Osteoporosis (OP) is one of the most common age-related progressive bone disease in the elderly people. Approximately every one in three women and one in five men are predisposed to the risk of OP. Postmenopausal OP is one of the major concerns as it leads to reduced bone mineral density and an increased risk of fractures.

Objective: To delineate DNA methylation signatures found in whole blood samples in osteoporotic patients.

Materials and Methods: Total 60 women aged 51-89 were enrolled in the study. Blood samples from 30 osteoporotic and 30 age matched normal females were collected and DNA was isolated from these whole blood samples. Two different cohorts of 22 osteoporotic and 22 normal control samples were sent for illumina 450K methylation analysis.

Results: Illumina 450K Raw data was analysed by Genome studio software. Two different cohorts of osteoporotic female patients were compared with age matched normal females which resulted in a list of 1234 differentially methylated CpG sites in osteoporotic patients. T-test, Anova and post-hoc statistical analysis were performed and 77 significantly differentially methylated CpG sites were obtained. Pyrosequencing analysis for the most significant genes was performed, to validate the results obtained from illumina 450 K methylation analysis.

Conclusion: The current study provides a proof of principal for the role of DNA methylation in the development and progression of OP. The DNA methylation signatures present in osteoporotic blood samples favor the use of DNA methylation analysis for better diagnosis and prognosis in osteoporotic patients.

Introduction

Osteoporosis is a disease characterized by low bone mass and deterioration of bone tissue affecting elderly people. The incidence of osteoporosis is relatively high in postmenopausal women compared to men [1, 2] . Major advances have been made in the treatment of osteoporosis in the recent years, but no single cause has been identified yet. Genetic background, lifestyle and environmental factors contribute to the complex character of the disease [3]. The current treatment for osteoporosis includes bisphosphonates, denosumab, calcitonin, estrogen, estrogen antagonists and parathyroid hormone analogs and peptides, calcium and vitamin D which have reduced the risk of fractures extensively [4, 5]. However, the underlying cause of osteoporosis has not been unraveled yet.

In spite of its static appearance bone is a highly dynamic connective tissue, which constantly undergoes remodelling. Various bone cells like osteoclasts and osteoblast take part in bone resorption and formation [6]. Excessive bone resorption or formation results in bone related disorders like osteoporosis and osteopetrosis [7]. Various factors are responsible for maintenance of proper bone structure and remodelling. Bone resorption is initiated with the recruitment of various endocrine hormones, signaling molecules in the proximity of the bone lining cells including osteoclasts and osteoblasts [6]. The current need is to find an appropriate diagnostic tool for early detection and monitor prognosis of osteoporosis. Human blood is the most readily available tissue, which can serve as a promising candidate for this

purpose [8]. Peripheral blood cells (lymphocytes and monocytes) can serve as efficient biomarkers and dictate the disease outcome precisely. The epigenetic and genetic machinery regulates the cellular interactions and transcriptional regulation in the human body [9]. The peripheral blood cells could be utilised to identify these epigenetic signatures responsible for diseased state [10]. The epigenetic signatures vary in different individuals and result in specific genetic regulation. DNA methylation is one of the most important epigenetic alteration studied extensively in recent years [11]. We hypothesized that osteoporosis progression can be tracked with changes in DNA methylation signatures found in peripheral blood cells.

In this study we carried out epigenome wide methylation analysis to determine the DNA methylation signatures found in peripheral blood cells. We observed significantly different DNA methylation patterns in postmenopausal osteoporotic patients compared to age matched normal females. These studies demonstrate an intricate relationship between peripheral blood cells and osteoporosis associated DNA methylation patterns. We explore this hypothesis in two different cohorts of postmenopausal osteoporotic patients.

Materials and Methods

Study Populations:

The study design was approved by ethics committee of Canadian Multicenter Osteoporosis Study (CaMoS). Whole blood DNA samples from healthy controls and osteoporotic patients was obtained from Royal Victoria Hospital with the consent from all the subjects. Detailed information about the osteoporotic patients and controls is shown in Table 1.

Illumina 450K methylation analysis:

Genomic DNA from all the osteoporotic patients and controls was quantified using Picogreen protocol (Quant-iTTM PicoGreen_ dsDNA Products, Invitrogen, P-7589) and read on a Spctra-MAX GeminiXS Spectrophotometer. Bisulfite conversion of 500 ng of genomic DNA was performed using the EZ-96 DNA Methylation-GOLD Kit (Zymo Research, Irvine, CA). The Illumina Methylation 450K kit (San Diego, California, USA) was used for the microarray experiment as described by the manufacturer's protocol, except that 8 lL of bisulfite converted template was utilized to initiate the amplification step. The Illumina hybridization oven was used for incubating amplified DNA (37°C) and for BeadChips hybridization (48°C).

A Hybex incubator was used for fragmentation (37°C) and denaturation (95°C) steps. The X-stain step was carried out in a Tecan Freedom evo robot with a Te-Flow module. Arrays were scanned in Illumina iScan Reader. Data analysis was performed with the Methylation module (version 1.8.0) of the

GenomeStudio software (Illumina; version 2011.1) using Human Methylation450_15017482_v1.2. bpm manifest. Statistical threshold was set at a false discovery rate of >0.05, differential score (statistical power) of ±0.33, and delta beta (differential methylation) between the groups was set at >0.15.

Illumina 450K methylation data analysis:

Two different groups were used for methylation analysis, the reference group consisted of 22 age matched normal female samples and two different study group of 10 and 12 osteoporotic patient samples. The genome studio analysis of the normal females and 1st cohort of osteoporotic patients resulted in a list of 13293 differentially methylated CGs in the two different groups.

To further validate our findings a second cohort of 12 osteoporotic patient DNA samples were sent for illumina 450K methylation analysis and compared with the reference group of 22 normal females. This comparison resulted in a list of 4454 differentially methylated CGs. These two lists of differentially methylated CGs were used to find common differentially methylated CGs. For this purpose, the two lists were overlapped which resulted in 1233 differentially methylated CGs common in both the cohorts of osteoporotic patients. The list of 1233 CGs was used for t-test statistical analysis. A t-test was performed on the average beta values of individual patients for both the cohorts separately. This resulted in a list of 77 CGs significantly differentially methylated in two different cohorts. Clustering of CGs was performed using one minus pearson correlation.

For group comparisons between the reference group, 1st cohort and 2nd cohort ANOVA and post-hoc statistical analysis were used. 13 top significant differentially methylated CGs were then used for further validation by pyrosequencing analysis.

Pyrosequencing Analysis:

Genomic DNA (200-500ng) was used for bisulfite converted using the EZ-DNA methylation Gold Kit (Zymo Research). Pyrosequencing validation of selected genes as predicted by penalise software were performed. The number of genes validated by pyrosequencing was limited by the amount of DNA obtained from these clinical samples. Samples were prepared by performing PCR amplification of selected CGs. PCR reactions were conducted using Hot star enzyme in Biometra T Gradient and T3 thermocyclers. Pyrosequencing was performed using standard methods; briefly, biotinylated PCR products were incubated with streptavidin sepharose bead (GE healthcare, Canada), followed by denaturation. Beads containing the biotinylated strand were released into 25µl annealing solution and 0.3mM sequencing primer per well. Pyrosequencing was performed using the PyroMark Q24 machine and results were analyzed with PyroMark® Q24 Software (Qiagen). Collected data was expressed as mean ± standard error of the mean (SEM) and using Student's t-test, p-value< 0.05. The statistical analysis was performed using Prism (GraphPad Software Inc, San Diego, California).

Statistical Analysis:

Results were analyzed as the mean ± SEM, and comparisons of the experimental data were analyzed by an independent two-sample t test at $P < 0.05$ level of significance.

Results

Genome wide methylation analysis of whole blood DNA from osteoporotic patients:

To elucidate the distinct methylation patterns appearing in peripheral blood DNA samples, we compared whole blood DNA samples from osteoporotic patients to normal age matched females. For this purpose, the methylation profile of whole blood DNA samples from two different cohorts of osteoporotic females consisting of 10 and 12 patients respectively was compared to 22 normal age matched females. The epigenome wide methylation analysis was performed by Illumina Infinium HumanMethylation450 BeadChip assay [12]

Most of the osteoporotic patients had fractures at the time of blood withdrawal (for clinical characteristics see Table 1). To exclude confounding clinical factors involvement in DNA methylation we performed linear regression analysis for age or fracture status. None of these confounding factors showed consistent correlation with average methylation values across the group (Data not shown).

The genome studio analysis of the normal females and 1st cohort of osteoporotic patients resulted in a list of 13293 differentially methylated CGs. To further validate our findings a second cohort of 12 osteoporotic patient samples were sent for illumina 450K methylation analysis and compared with the reference group of 22 normal females. This comparison resulted in a list of

4454 differentially methylated CGs. These two lists of differentially methylated CGs were used to find common differentially methylated CGs in both the cohorts. For this purpose, the two lists were overlapped which resulted in 1233 differentially methylated CGs common in both the cohorts of osteoporotic patients (Figure 1). The list of 1233 CGs was used for t-test statistical analysis. A t-test was performed on the average beta values of individual patients for both the cohorts separately. This resulted in a list of 77 CGs significantly differentially methylated in two different cohorts as shown in the heatmap (Figure 2). Average beta value corresponding to each significant CG per individual was used to construct a heatmap using GENE-E software (Broadinstitute.org). Clustering of CGs was performed using one minus pearson correlation.

For group comparisons between the reference group, 1st cohort and 2nd cohort ANOVA and post-hoc statistical analysis were used. 13 top significant differentially methylated CGs were then used for further validation and biomarker prediction.

Pyrosequencing Validation:

To verify the results obtained from illumina 450 K methylation analysis and biomarker prediction by penalise, 13 differentially methylated CG sites were selected based on ANOVA and group comparisons for validation by pyrosequencing. Pyrosequencing analysis was limited by the remaining amount of DNA. All the individual DNA samples were utilised for bisulphite

conversion and pyrosequencing validation of 4 most significant genes. The results showed similar differential methylation patterns as shown in illumina 450K methylation analysis. Most of the significant genes were hypermethylated in osteoporotic patients compared to normal females. 4 probes/CGs were found to be significantly differentially methylated in the representative osteoporotic patients and normal female samples (Figure 3).

Discussion

Recently several effective options have been employed for the treatment of osteoporosis which reduced the risk of fractures greatly. For further expansion of these treatment options and early diagnosis of osteoporosis a proper diagnostic tool needs to be constructed [13]. The peripheral blood cells constantly patrol the body to protect it from pathogens, exogenous antigens and get transformed in the event of disease. DNA methylation signatures present in peripheral blood cells could predict osteoporosis outcome. In the present study we demonstrate a significant role of DNA methylation in diagnosis and prognosis of osteoporotic patients, indicating the presence of molecular markers in peripheral blood cells.

Our epigenome wide methylation analysis showed 1233 significantly differentially methylated genes in osteoporotic patients as compared to age matched normal females. For this purpose, we utilized two different cohorts of osteoporotic patients comprising of 10 and 12 patients respectively and compared them to 22 age matched normal females. Whole blood DNA from both groups of normal females and osteoporotic patients was analyzed for significant methylation differences. Each group of osteoporotic patients was first compared separately to the control group of normal females. This resulted in two lists of significant probes which were distinctly different in the osteoporotic patients as compared to normal females. To avoid technical issues and confounding effects we compared the differentially methylated probes in two osteoporotic cohorts and overlapped the resultant lists, to obtain common

differentially methylated probes in both the cohorts. This bigger list of common probes was further scrutinized by group comparisons (post-hoc analysis), ANOVA and T-test statistical analyses. The shorter list resulting from statistical analysis served as our starting point for biomarker prediction. We used the methylation data from normal females which were age matched and were evaluated for our ongoing studies in women with breast cancer. The penalize software predicted 13 probes which could serve as molecular biomarkers in the second cohort (Table 2).

The predicted probes were present in the regulatory regions of significant genes involved in cellular metabolism, transcriptional regulation and bone formation. This suggests that epigenetic mechanisms play an important role in the development and progression of osteoporosis. This list of significant probes was further utilized for validation by pyrosequencing analysis. Table 2 shows the functions of the genes employed for validation by pyrosequencing. Validation of significant probes was limited by the small amount of leftover DNA after epigenome wide analysis. 4 probes were analyzed by pyrosequencing analysis which was significantly differentially methylated. The following 4 probes/ CGs were found to be significantly different by pyrosequencing: ZNF267, RHOJ, CDKL5 and PDCD1.

ZNF267 is involved in transcriptional regulation and unlike other genes was found to be hypomethylated in osteoporotic patients. Hypomethylation of ZNF267 may result in its overexpression and further alteration of expression of other downstream genes. RHOJ is the member of RHO family of proteins and

is involved in growth and survival via AKT signaling pathway. The protein encoded by RHOJ gene is also associated with angiogenesis. CDKL5 protein is involved in kinase activity and was found to be hypermethylated in all the osteoporotic patients. CDKL5 is also known to be involved in phosphorylation and gene regulation. Another gene which was hypermethylated in osteoporotic patients was PDCD1, which is involved in T and B- cell activity and may act as cell death inducer. Hypermethylation of PDCD1 may result in reduced expression of this gene and further affect T-cell functions. Recent studies suggest that PDCD1 is involved in autoimmune prevention. Experimental mice lacking PDCD1 had compromised cardiovascular functions [14].

The genes identified by EWAS analysis indicate that epigenetic modifications may be the underlying cause of osteoporosis development. Most of the differentially methylated genes in osteoporotic patients were involved in transcriptional regulation, cellular metabolism, angiogenesis and apoptosis. The epigenetic alterations result from environmental changes, lifestyle and aging. These alterations can be stably reversed and utilised to reprogram the gene expression mechanisms. The panel of epigenetically modified genes presented in this study may serve as an early diagnostic tool and help in the betterment of current treatment options for osteoporosis. Follow up studies in a larger cohort of normal and osteoporotic women will validate these finding. Identification of the osteoporosis methylation profile will allow us to the early identification of at risk women and potentially identify new therapeutic targets for osteoporosis.

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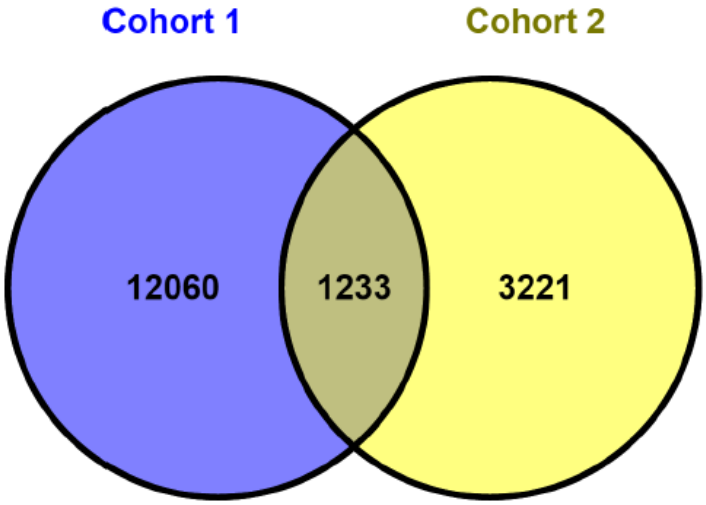
Figures (Chapter 4)

Figure 1



Common differentially methylated genes in two cohorts of osteoporosis patients.

Figure 1: Common differentially methylated sites in 2 cohorts of osteoporotic patients. Overlap of two different cohorts of osteoporotic patients compared with age-matched normal females resulted in 1233 significant differentially methylated probes.

Figure 2

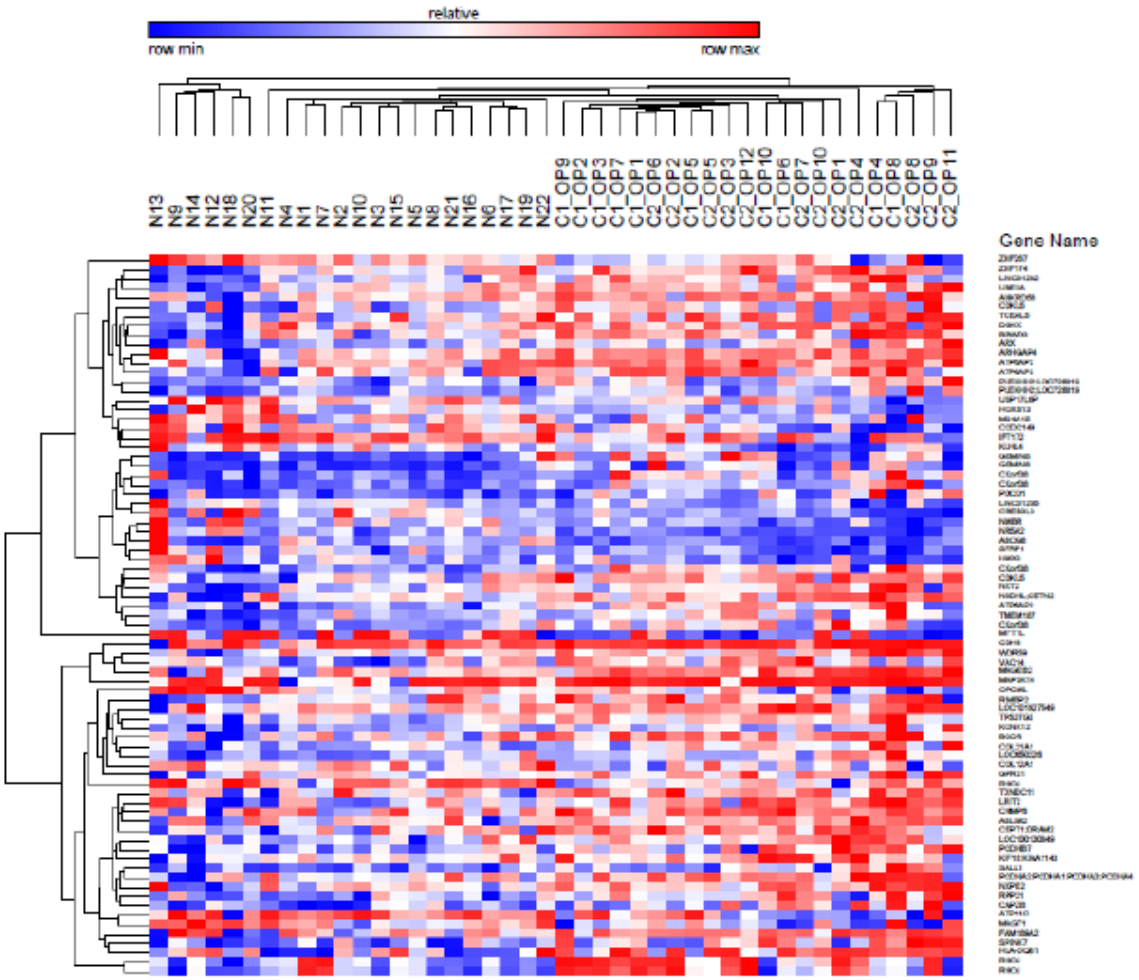


Figure 2: Differential methylation pattern of osteoporosis patients and age-matched controls: Heat map and hierarchical clustering analysis of the DNA methylation profiles of 22 normal and 22 osteoporotic patients was performed using one minus pearson correlation. Average beta value corresponding to 77 significant CG per individual was used to construct a heatmap using GENE-E software (Broadinstitute.org).

Figure 3

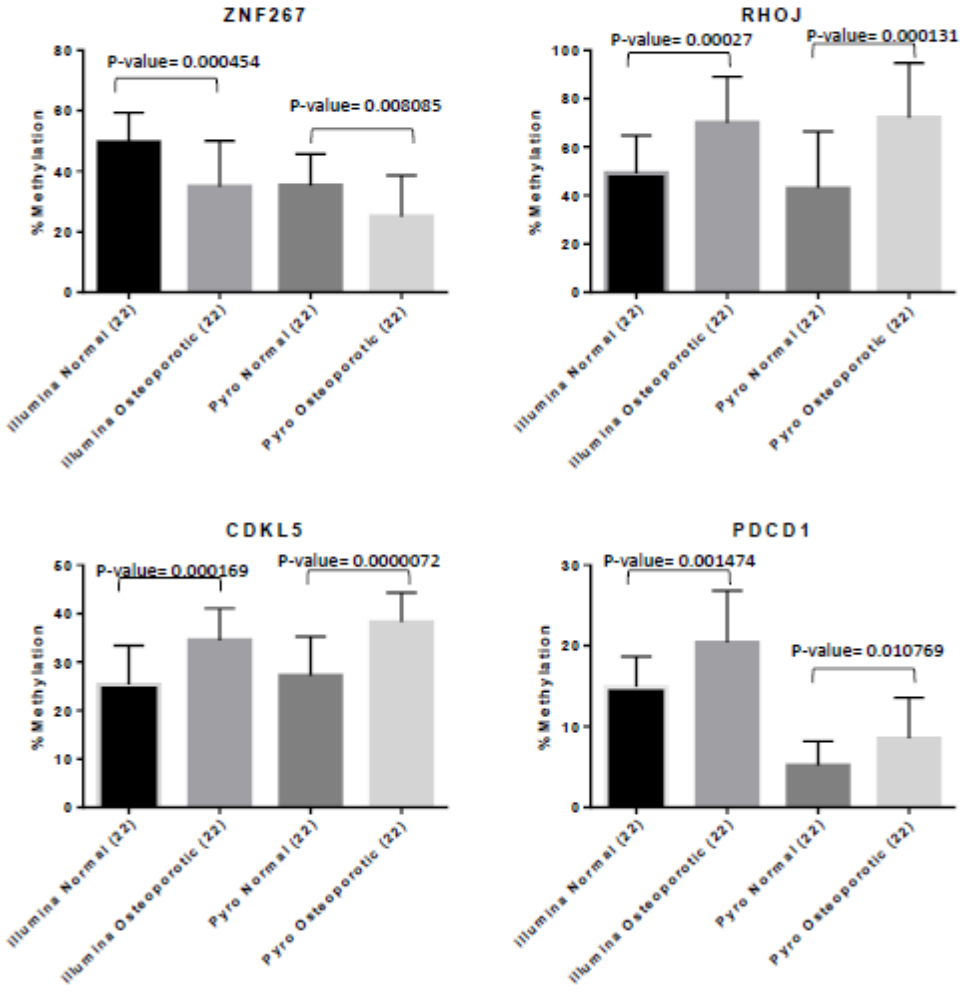


Figure 3: Pyrosequencing analysis of significant differentially methylated genes**:** Pyrosequencing was performed using the PyroMark Q24 machine and results were analyzed with PyroMark® Q24 Software (Qiagen). Collected data was expressed as mean ± standard error of the mean (SEM) and using using Student's t-test, p-value< 0.05. The statistical analysis was performed using Prism (GraphPad Software Inc, San Diego, California).

Tables (Chapter 4)

Table 1(a): Clinical Characteristics of Patients

Patient #	Patient ID	Gender	Age	Fracture
N1	HA02042	Female	85	No
N2	HA04704	Female	76	No
N3	KN02554	Female	73	No
N4	HX02987	Female	77	No
N5	SK02951	Female	72	No
N6	SK04589	Female	85	No
N7	CA05972	Female	80	No
N8	KN05004	Female	79	No
N9	CA08220	Female	79	No
N10	CA08464	Female	83	No
N11	HA06592	Female	75	No
N12	SK02104	Female	77	No
N13	TO03062	Female	52	No
N14	KN03030	Female	75	No
N15	KN03548	Female	71	No
N16	TO03162	Female	75	No
N17	KN03631	Female	79	No
N18	TO05618	Female	71	No
N19	SK06497	Female	77	No
N20	QC08071	Female	84	No
N21	CA09942	Female	79	No
N22	QC04373	Female	67	No

Table 1(b): Clinical characteristics of patients

Patient #	Patient ID	Gender	Age	Fracture
OP1_C1	SK01897	Female	71	No
OP2_C1	KN02331	Female	71	No
OP3_C1	KN01769	Female	81	No
OP4_C1	KN03289	Female	89	No
OP5_C1	SK04126	Female	88	Yes
OP6_C1	SK07074	Female	75	Yes
OP7_C1	KN07538	Female	82	No
OP8_C1	KN06786	Female	84	No
OP9_C1	QC02332	Female	82	Yes
OP10_C1	CA08593	Female	79	No
OP11_C2	HA06150	Female	71	Yes
OP12_C2	HA04713	Female	73	No
OP13_C2	SK02072	Female	74	Yes
OP14_C2	SK01601	Female	77	Yes
OP15_C2	SK01829	Female	51	No
OP16_C2	CA03180	Female	69	No
OP17_C2	SK06096	Female	87	Yes
OP18_C2	SK06830	Female	72	No
OP19_C2	KN05405	Female	75	Yes
OP20_C2	TO08284	Female	77	No
OP21_C2	CA08443	Female	87	Yes
OP22_C2	CA09766	Female	77	Yes

Table 2: Differentially methylated genes and their functions

Gene_Name	TargetID	UCSC_Accesion	Function
ZNF267	cg01879726	NM_003414	Transcriptional regulation
RHOJ	cg07157030	NM_020663	GTP-binding proteins in the Rho family, may regulate angiogenesis
CDKL5	cg07519908	NM_003159	member of Ser/Thr protein kinase family with protein kinase activity
PDCD1	cg08457169	NM_005018	regulation of T-cell function, possible cell death inducer
COL21A1	cg12569497	NM_030820	XXI collagen protein, maintains integrity of the ECM
MAP3K14	cg13133387	NM_003954	serine/threonine protein-kinase
RHOJ	cg13199429	NM_020663	GTP-binding proteins in the Rho family, may regulate angiogenesis
MAGEB2	cg15715746	NM_002364	Melanoma Antigen Family B2, associated with melanoma
RPP21	cg16058990	NM_024839	protein subunit of nuclear ribonuclease P
PCDHB7	cg16583552	NM_018940	Potential calcium-dependent cell-adhesion protein
ABLIM2	cg19651341	NM_032432	Actin Binding LIM Protein Family, Member 2
IFT172	cg21604741	NM_015662	ciliary assembly and maintenance
CAPZB	cg24593138	NM_017765	regulation of cell morphology and cytoskeletal organization

Chapter 5: General Discussion

Carcinogenesis is a long term process that starts much before the symptoms arise. Favourable advances have been made in cancer research within the past few decades which reinstate the role of genetic mutations in cancer development and progression. Various mutations, translocations and faulty mechanisms of DNA repair and duplication contribute to the process of carcinogenesis. Identification of carcinogenic events is one of the most important goal in cancer research right now. Two signaling pathways namely, PTEN/PI3K/AKT and FGFR/FGF pathways were found to be most commonly altered in a recent phase I trial, conducted to identify molecular alterations in various advanced cancers [1].

Another challenge posed by many solid tumors is intra-tumor heterogeneity. A recent study regarding breast tumors shows different set of genetic alterations in metastatic sites of breast cancer which were absent in the primary tumor [2]. Breast cancer is one of the most extensively studied cancer with highest degree of heterogeneity [2]. Almost all cancers develop resistance to treatment over time. Clonal selection and genetic diversity of tumor cells are the main reasons for acquired resistance [3]. In a recent study regarding treatment resistant breast tumors, TP53, PIK3CA and GATA3 were found to be genetically altered [3]. Despite these advances in cancer research, the goal to minimize cancer related deaths seems elusive.

The current standard of care for cancer treatment involves a combination of surgery, chemotherapy and radiation therapy [4]. Targeted therapy is the choice of treatment for a small subset of cancer patients. For example, inhibitors of molecules like mTOR and CDK4/6 have been approved and found to be effective in a small subset of population with specific tumor subtypes [5, 6]. Given the diverse range of tumor subtypes, personalized medicine seems to be an attractive treatment option for breast cancer patients. However, only two targetable molecules, ER and HER2 have been used successfully for diagnosis and treatment of breast cancer patients [7].

Cancer outcome completely relies on early detection and prevention of disease progression. There is a dire need for clinical trials and observational studies on the new treatment options [8]. For development of better diagnostic tools and therapeutic targets, following four facts need to be determined. 1) Identification of oncogenic drivers. 2) Identification of the genes responsible for tumor resistance in late stages. 3) Faulty DNA repair mechanisms and mutations need to be elucidated, and 4) Interpretation of immune escape mechanisms of tumors [7]. The oncogenic drivers or triggers which start the process of cancer development need to be identified and distinguished from downstream events that help in cancer progression. Current literature cites a vast range of regulatory molecules involved in cancer progression [9, 10]. However, the suitability of these molecules as therapeutic targets and effectiveness is still questionable.

Tumor suppressor genes and oncogenes are major key players in the process of carcinogenesis. Silencing of tumor suppressor genes and onset of oncogenes is responsible for cancer progression [11]. Epigenetic modifications like DNA methylation are at the core of these gene silencing effects [12]. The crosstalk between genetic and epigenetic factors is responsible for proper maintenance of cellular mechanisms, and any imbalance results in cancer progression. It has been observed that both global hypomethylation and regional hypermethylation coexist in the same tumor subtype. The current need is to unravel the contrasting role of DNA methylation in carcinogenesis and target selective signature molecules to control cancer progression [11]. A combination of current therapeutic approaches with epigenetic therapy could bring great changes in cancer outcome [13]. The three manuscripts presented in this thesis emphasise on the role of epigenetics in cancer development and progression.

5.1 S-adenosylmethionine blocks osteosarcoma cells proliferation and invasion *in vitro* and tumor metastasis *in vivo*: therapeutic and diagnostic clinical applications

Cancer is one of the diseases where DNA methylation has been proposed as a target for development of novel therapeutics. DNA methylation regulates gene expression programs which monitor cell proliferation, invasion, migration, metastasis and promote tumor growth. Although, DNA hypermethylation and hypomethylation both are observed in cancer, they affect different DNA sequences [14]. Global hypomethylation has been observed frequently in case of cancer which, occurs as an active replication independent mechanism [15, 16]. Analysis of methylation patterns in various cancers revealed hypomethylated genes involved in cancer progression [17, 18]. Various signaling pathways involved in EMT transition, invasion and migration are also shown to be altered by DNA hypomethylation [19]. Despite these findings the major focus of attention remained in development of DNA methylation inhibitors. Very few DNA methylating agents have been utilised for cancer treatment so far. Previous studies from our laboratory have shown the role of DNA methylating agent SAM in blocking cancer progression. A genome wide analysis of SAM treated prostate cancer cells showed involvement of JAK/STAT pathway in cancer metastasis [20]. The mechanism of DNA methylation by SAM appears to be very selective with negligible adverse effects. SAM abrogates DNA hypomethylation by methylating and silencing MBD2 gene promoter and activates DNMT-associated DNA

methylation of various prometastatic genes [21]. Thus, treating cancer-associated DNA hypomethylation with SAM is an attractive approach to control cancer progression and metastasis.

In the first manuscript presented in this thesis, we examined the role of DNA methylation patterns on development and metastasis of one of the most common primary bone tumor, OS. The ubiquitous methyl donor SAM was employed to induce DNA hypermethylation in two human OS cell lines LM-7 and MG- 63. Treatment with DNA methylating agent SAM, reduced tumor cell proliferation and invasion. DNA methylation of tumor cells also caused their arrest in G2M phase and altered cell cycle kinetics.

We also show reduced lung metastasis in our OS mouse model. These results clearly exhibit that SAM caused reversal of unwanted epigenetic modifications and reprogrammed tumor cells to become less invasive and tumorigenic. Another concern regarding DNA methylating agents is hypermethylation and silencing of regulatory genes and tumor suppressor genes. The results in our study resolve this issue as none of the tumor suppressor genes were silenced by SAM treatment. We also some significant genes *EXOC7, PCGF3, PDGFA* which were altered upon SAM treatment and could serve as prognostic indicators of OS onset and development.

The results from our study show that SAM is a potent anticancer agent which can serve as preventative as well as anti-metastatic agent. Future studies involving a combination of SAM and other DNA methylation inhibitors will elucidate the scope of new therapeutics, targeting epigenetic modifications.

Exploiting the methylation potential of SAM appears to be a promising approach as it is a FDA approved and commercially available natural molecule with negligible toxicity.

5.1a Limitations:

Human OS cell lines were utilised for in vivo studies which do not exhibit complete interaction between host and tumor cells due to species differences. Also, cell lines get extensively manipulated during cell culture which may result in altered characteristics. A transgenic OS mouse model could be employed for future studies which will help in analysing the effects of SAM treatment on development of OS progression. A transgenic mouse model that mimics human OS [22] will also help in studying the effects of SAM on early and late stages of OS development.

SAM is prescribed as an anti-depressant and dietary supplement. However, it has been reported that SAM administration can induce anxiety and mania in certain individuals [23]. Bioavailability and toxic effects of the administered doses should be determined in future studies. Another concern regarding SAM administration is the genomic stability. Cancerous cells are prone to mutations, chromosomal instability and aneuploidy [24]. Effect of SAM treatment on genomic stability of cancer cells should be evaluated in future studies.

5.2 DNA methylation signatures of breast cancer in peripheral T-cells:

Early diagnosis remains one of the major challenge in breast cancer treatment. Almost all of the diagnostic techniques available today are limited by the size of tumor [25, 26]. Various blood-based biomarkers have been utilised in past for breast cancer detection, but none of them could effectively predict the breast cancer outcome [27]. Prognosis of breast cancer relies extensively on molecular classification [28]. However, the determination of the molecular subtype of breast cancer requires biopsy samples or collection of circulating tumor cells from the patients. Circulating tumor cells are only available in late stages of cancer and the surgical removal of biopsy tissue samples puts the patients at a higher risk of developing metastasis. Development of a non-invasive diagnostic tool for breast cancer detection and follow-up for relapse is critically important.

Various molecular targets have been utilised for breast cancer treatment, considering the intrinsic properties of tumors. However, the systemic changes occurring in the body extensively affect the cancer growth. One of the prominent mechanism that accompanies cancer development is immune recognition of cancer cells [29]. Recent evidence from various animal studies and clinical trials suggest that cancer cells evade immune system in three steps: elimination, equilibrium and escape [28, 30, 31]. It has also been shown that tumor infiltrating cytotoxic $CD8^+$ T cells improve the outcome of human regressive melanoma, esophageal, ovarian and colorectal cancer [32-38]. Recently, tumor specific immune signature reflected by tumor infiltrating

lymphocytes were identified in DNA methylation and transcriptome analysis of breast cancers [39, 40].

Progression of cancer is associated with DNA methylation changes which can be predicted in peripheral immune cells. Various tissue and cell-type specific DNA methylation patterns are observed in human body [41, 42]. It has been shown previously that DNA methylation at 5' region of the gene results in silencing whereas, recent studies indicate the role of gene body methylation in altered gene expression mechanisms [43-48]. Thus, two different mechanisms control gene expression in tissues, the hereditary genetic information and cell-type specific methylation patterns. Several other factors like environmental exposures and early life social experiences can also influence the epigenetic machinery [49, 50]. The altered epigenetic status further transforms functional gene expression networks and gives rise to a cancer-specific DNA methylation signature [51]. Previous studies from our laboratory have identified specific DNA methylation signatures associated with breast cancer cells *in vitro* and *in vivo* [52-56]. These specific DNA methylation patterns of breast cancer cells create distinct signatures which allow stratification of various sub-types of breast cancer [57-59]. Recent studies utilised a set of few genes or representative CG sites to map DNA methylation patterns in breast cancer [60].

The breast cancer study presented in this thesis employs epigenome wide analysis of T-cells derived from breast cancer patient samples. This allows us to explore the genome wide picture of DNA methylation exhibited by peripheral T-cells in breast cancer patients. Previous studies have shown

specific DNA methylation patterns associated with breast and ovarian cancer in peripheral blood cells [61-65]. However, these conventional approaches were confounded by multiple cell types in blood. In the present breast cancer study we have utilised the most prominent cell type involved in immune recognition of cancer cells [66, 67]. The crosstalk between immune system and cancer cells forms the underlying objective of this study. Immune system is constantly in a tug of war with developing cancer cells and T-cells being the most important players in immunosurveillance help in this mechanism [32, 34]. In this whole process T-cells adapt and transform themselves to better counteract the cancer cells [33, 37].

We utilised circulating T-cells from blood samples of breast cancer patients for epigenome wide analysis. Epigenetic modifications in T-cells of breast cancer patients were compared to an age matched cohort of normal females. The results from our EWAS analysis show epigenetic alterations in prominent genes involved in PTEN signaling, p53 signaling and RAR activation pathways which are well-known and implicated in breast cancer progression in various studies. Future studies will be performed to explore the functional impact of significant differentially methylated genes in PTEN and p53 pathways.

In this study we also attempt to resolve the issue of non-invasive method of sample collection from cancer patients. Tumor biopsies often put patients to a higher risk and location of tumor sometimes makes it more

difficult. Small quantities of blood samples can be easily drawn from patients non-invasively and can be utilised for diagnostic and prognostic purposes. We isolated T-cell DNA from patient samples which were further utilised for EWAS analysis and validation by pyrosequencing. We show that T-cells possess epigenetic signatures of breast cancer which can be employed for prediction of early diagnostic markers of breast cancer.

5.2a Limitations:

A. DNA methylation signatures detected in circulating T-cells might be different from tumor infiltrating T-cells. However, tumor infiltrating T-cells share a common T-cell antigen CD3 with circulating T-cells. This feature makes our approach more feasible and less complex than others.

B. The present study focuses on one particular cell type, when there are several other important cell types present in blood. This approach reduces cellular complexity and minimizes confounding heterogeneity exhibited by cell-type specific DNA methylation patterns. However, another prominent cell type that takes part in immune surveillance is the B-cells. We have also analyzed the DNA methylation patterns of top most significant genes from EWAS analysis in B-cells (Data not shown). These results show similar DNA methylation profiles in B-cells as well, which further supports the findings of the present study. T and B-cells are the most important regulators of immune functions. Future studies employing a validation cohort of breast cancer patients will illustrate the DNA methylation patterns in T and B-cells both. These

methylation patterns of T and B-cells from EWAS analysis will help in constructing highly specific noninvasive epigenetic based diagnostic biomarkers for breast cancer patients.

5.3 Identification and Validation of Epigenetic Signature of Osteoporosis in Post-Menopausal Women:

The third manuscript presented in this thesis investigates the role of epigenetic alterations in osteoporosis. Various factors like aging and environmental exposures affect epigenetic mechanisms [68]. Thus, it is reasonable to expect the involvement of a strong epigenetic component in the onset of a complex disorder like osteoporosis [69]. Osteoporosis is one of the most common bone related disorder and post-menopausal women are at highest risk for it. We procured whole blood DNA samples from healthy controls and osteoporotic patients from the DNA bank of Canadian Multicenter osteoporosis study (CaMos).

EWAS analysis was performed on two different cohorts of osteoporotic patients and an age matched cohort of normal females. This unprecedented approach of epigenome wide analysis of osteoporotic patients further emphasises on the role of DNA methylation in Osteoporosis. The results from the study show significant alterations in genes involved in transcriptional regulation, cell cycle progression, and apoptosis. DNA hypomethylation was observed in most of the genes in osteoporotic females.

This indicates the role of DNA methylation in osteoporosis onset and disease progression. The present study The results of the study could be further

utilised for biomarker prediction and early diagnosis of osteoporosis in post-menopausal females.

5.4 Shortcomings:

5.4a Illumina 450 K analysis:

Illumina 450K analysis technique was employed for all the three manuscripts presented in the thesis as it is one of the best epigenome wide analysis technique available today. It is an array based analysis technique which covers 485,000 CpG sites, which cover 96% of the CpG islands and 99% of Refseq genes. However, the idea that certain important epigenetic sites may remain undetected cannot be overruled. The probes utilized for building illumina microarray are preselected genetic probes available in public databases like RefSeq [70]. Results obtained from such analyses could lead to a biased understanding of epigenetic mechanisms in disease progression and outcome. Future techniques providing complete coverage of epigenetically significant sites are required which will enhance our understanding of epigenetic mechanisms.

5.4b Bioinformatics analysis:

Genome studio software and 'R' package was employed for bioinformatics analysis of EWAS data. These softwares are based on standard statistical tools that give number based analysis of data. Biological significance of certain important epigenetic alterations may remain unexplored due to the

statistical inclination of these softwares. A robust bioinformatics pipeline should be designed in future for impartial analysis of EWAS data.

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Appendix

Contribution to Original Knowledge:

1. We have demonstrated that hypermethylation treatment with SAM reduces OS tumor cell proliferation, invasion and migration by altering the cell cycle characteristics.
2. We have demonstrated that inoculation of SAM treated LM7 cells resulted in smaller skeletal lesions and reduced pulmonary metastasis *in vivo*.
3. EWAS studies have shown that SAM alters the methylation pattern of genes EXOC7, PCGF3 and PDGFA which are involved in OS progression, bone formation and wound healing.
4. Immunohistochemical analysis of normal bone and OS patient tumor samples showed elevated expression of PDGFA in OS patient samples.
5. In the breast cancer study, we provide a proof of principle for the role of DNA methylation in breast cancer progression. We present the evidence of association between the peripheral immune T-cells and epigenetic regulation of cancer.
6. We demonstrate epigenetic regulation of several genes involved in PTEN pathway, p53 pathway and RAR activation in breast cancer patient samples.
7. In the osteoporosis study, we provide a proof of principal for the role of DNA methylation in the development and progression of OP.

8. We demonstrate the adequacy of whole blood DNA samples as better diagnostic and prognostic tool to study DNA methylation signatures in osteoporotic patients.
9. EWAS studies have shown a crucial role of epigenetic regulation in significant genes involved in transcriptional regulation, angiogenesis, growth and cell survival in osteoporotic patients.