Osteosarcoma (OS) progression is associated with increased nuclear levels and transcriptional activity of Active-β-catenin (ABC)

by

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Abstract

Osteosarcoma (OS) is an aggressive primary bone malignancy having peak incidence in children and young adults <25 years of age. Outcome remains poor for most patients with metastatic disease which develops in 1 of every 5 cases. However, despite the use of multimodal chemotherapeutic and surgical treatments, survival rates have remained relatively constant over the past two decades. Presently, there is no reliable marker available for predicting invasive/metastatic disease. This highlights the need to understand the underlying biology of OS to design improved therapeutic interventions and identify prognostic and/or predictive markers which will facilitate stratification of patients to administer treatment options relevant to each patient group, thus allowing for better management of OS.

The canonical Wnt/β-catenin pathway is important for a number of cellular processes and is also known to be a critical pathway in OS development and progression. Thus, we investigate Wnt/β-catenin signaling, specifically, the roles of its effector molecules β-catenin and the transcriptionally active form of β-catenin, Activated-β-Catenin (ABC), in OS progression.

We used an *in vitro* model comprising of two pairs of OS cell lines that model OS progression. Our results show significantly upregulated nuclear ABC levels and increased activity of ABC, but not β-catenin, in metastatic OS cell lines compared to parental ones. Furthermore, our immunohistochemical studies showed presence of positive nuclear ABC staining in 85% of the OS tissue cores tested and a significant association between nuclear ABC staining and age. Hence, our findings propose ABC as a prognostic marker for survival in OS.

Preface

This thesis is an original work by Noureen Ali. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, "Osteosarcoma (OS) progression: a role for β-catenin/Active-β-Catenin." No. pro0065705, 9th June 2016.

A dedication to my loving parents Ramzan Ali and Naseem Ramzan

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List of abbreviations

ABC – Activated-β-Catenin

AJCC – American Joint Committee on Cancer

ALP – Alkaline phosphatase

AP-1 – Activator protein 1

APC – Adenomatous polyposis coli

AYA – Adolescents and young adults

β-TrCP – β-Transducin repeat-containing protein

BAD – Bcl-2-associated death promoter

BIM – Bcl-2-interacting mediator of cell death

circRNA – Circular RNA

CK1 – Caesin kinase 1

CSL – CBF-1/Suppressor of Hairless/Lag-1

CT – Computed Tomography

CTGF – Connective tissue growth factor

DFS – Disease free survival

DKK – Dickkopf

DLG2 – Disks large homolog 2

Dvl – Dishevelled

ECM – Extracellular matrix

ERM – Ezrin/Radixin/Moesin

FFPE – Formalin-fixed, paraffin-embedded

FGF – Fibroblast growth factor

Fz – Frizzled

GRM4 – Glutamate receptor metabotropic 4

GSK3β – Glycogen synthase kinase 3β

HER2 – Human epidermal growth factor receptor 2

HES – Hairy/Enhancer of Split

HIF-1α – Hypoxia-inducible factor-1α

IF – Immunofluorescence

IGF – Insulin-like growth factor

IHC – Immunohistochemical/ immunohistochemistry

KRAS – Kristen RAt Sarcoma

LDH – Lactate dehydrogenase

LFS – Li-Fraumeni syndrome

LRP-5/6 – Low density lipoprotein receptor-related protein 5/6

MAPK – Mitogen-activated protein kinases

MDM2 – Mouse double minute 2 homolog

miRNA – Micro RNA

MMP – Matrix metalloproteinase

MRI – Magnetic Resonance Imaging

MSCs – Mesenchymal stem cells

MTP-PE – Muramyl tripeptide phosphatidylethanolamine

OPN – Osteopontin

OS – Osteosarcoma or Osteogenic sarcoma

OSX – Osterix

PEDF – Pigment-epithelial derived factor

PET – Positron Emission Tomography

PI3K – Phosphoinositide-3-kinase

PKB – Protein kinase B

pRB – Retinoblastoma protein

PTEN – Phosphatase and tensin homolog

PTH – Parathyroid hormone

RANK – Receptor activator of nuclear factor κ-B

RANKL – Receptor activator of nuclear factor κ-B ligand

RB1 – Retinoblastoma 1

RECK – Reversion-inducing cysteine rich protein with Kazal motifs

RFS – Relapse-free survival

RUNX2 – RUNX-related transcription factor 2

sFRP – Secreted frizzled-related protein

SNP – Single nucleotide polymorphism

TCF/LEF – T-cell factor/ Lymphoid enhancer-binding factor

TGF-α – Transforming growth factor alpha

TGF-β – Transforming growth factor beta

THBS1 – Thrombospondin 1

TP53 – Tumor protein p53

VEGF – Vascular endothelial growth factor

Wif-1 – Wnt inhibitory factor 1

Chapter 1

Introduction

1.0. Introduction

1.1. Bone structure

Bone is a specialized form of connective tissue which serves as a tissue and an organ system in higher vertebrates (Downey & Siegel, 2006; Florencio-Silva, Sasso, Sasso-Cerri, Simões, & Cerri, 2015). Its primary functions are locomotion, protection of soft-tissue structures, in deposition and homeostasis of minerals such as calcium and phosphate and in providing an environment for hematopoiesis within bone marrow spaces (Datta, Ng, Walker, Tuck, & Varanasi, 2008; Robling, Castillo, & Turner, 2006). Bone comprises of four kinds of cells including osteoblasts, osteoclasts, osteocytes and bone lining cells (Downey & Siegel, 2006; Marks & Popoff, 1988). Osteoblasts originate from mesenchymal stem cells (MSCs) and are the primary bone-forming cells. They secrete osteoid or the bone matrix, which then solidifies and becomes the compact part of the bone (Damoulis & Hauschka, 1997; Florencio-Silva *et al*., 2015). On the other hand, there are osteoclasts, which originate from hematopoietic stem cells and are the bone-resorbing cells. Their activity is regulated by osteoblasts and they play an important role in bone remodelling (Downey & Siegel, 2006; Florencio-Silva *et al*., 2015). The third type of bone cells, known as osteocytes, are the most abundant cells in the bone (Franz-Odendaal, Hall, & Witten, 2006). These cells are trapped within osteoid and mainly act as sensors of mechanical changes in the bone (Knothe Tate, 2003). The bone lining cells are thin, elongated cells covering most of the bone's surface and are known to be metabolically inactive (Downey & Siegel, 2006; Miller, de Saint-Georges, Bowman, & Jee, 1989). The structure of a long bone can be broadly divided into three segments including the diaphysis, the metaphysis and the epiphysis. As can be seen from the figure 1, the diaphysis is the hollow shaft in the middle of the bone and consists of bone marrow in its central core. At each end of the long bone are bulbous ends, also known as the epiphysis, which consist of the cancellous or the spongy bone. Connecting the epiphysis to the diaphysis is the metaphysis which consists of the growth plate from where cells initially start to divide and continue bone formation (Clarke, 2008; Downey & Siegel, 2006). Importantly, metaphysis is most common site for occurrence of osteosarcoma (OS) and thus is an important region for study of OS (Kundu, 2014; C. S. Wang *et al*., 2012).

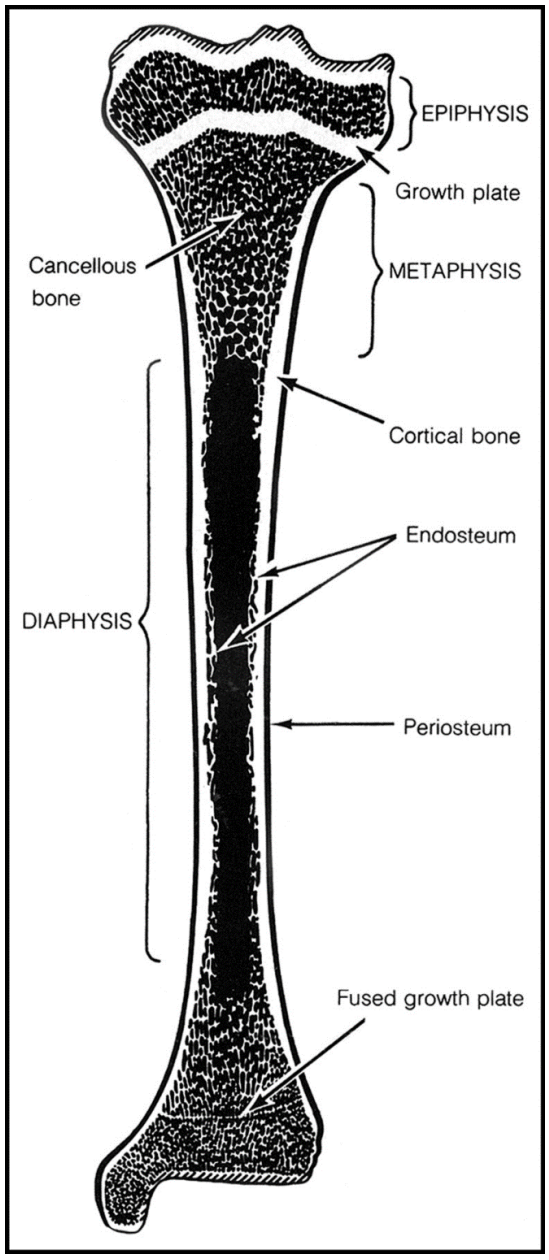


Diagram reprinted with permission from Clarke *et al.,* 2008

Figure 1. Schematic diagram of a tibia showing important bone structures

Most of the bone is covered with a tough layer of cells known as periosteum. Beneath the periosteum is the cortical bone, which is the compact part of the bone. The diaphysis is the middle long shaft and epiphysis is present at both ends. Within the epiphysis is the cancellous bone. Between epiphysis and diaphysis is the metaphysis, which also has growth plate at the end. The figure was reprinted with permission from the publisher.

1.2. Overview of OS

OS is rare but the most common primary bone malignancy, its incidence is bimodal, affecting children and young adults who are less than 25 years of age or people older than 60 years (Ottaviani & Jaffe, 2009a). It is characterized by production of osteoid or immature bone by malignant osteoblasts (Yu Cai, Cai, & Chen, 2014). With the current combination of multi-agent chemotherapy and surgery, 5-year disease-free survival (DFS) rate for patients with localized OS is about 60-70% (Durfee, Mohammed, & Luu, 2016). However, the overall survival rates for patients with OS have remained relatively constant over past two decades and more advances in therapy are needed to improve them (Lewis *et al*., 2007). OS also has a high tendency to become aggressive and invade locally as well as cause early distant metastasis, with pulmonary metastasis being the most common (Yu Cai *et al*., 2014; C. H. Lin, Ji, Chen, & Hoang, 2014). Approximately 20% of patients present with metastasis at the time of diagnosis and the 5-year overall survival rate for patients with metastatic disease is about 20-30% (Durfee *et al*., 2016; Kaste, Pratt, Cain, Jones-Wallace, & Rao, 1999; Mialou *et al*., 2005). These statistics about OS highlight the need to design better treatment strategies which will restrict OS growth, limit metastasis and also improve survival rates and overall quality of life of OS patients (Yu Cai *et al*., 2014). One approach to designing effective therapeutic strategies is to understand both the dysregulated signaling pathways in OS and the underlying mechanisms that result in OS initiation and development. Hence, our study aims to discern the role of dysregulated Wnt/β-catenin signaling, mainly its effector molecules β-catenin and Activated-β-Catenin (ABC), in OS tumor development and proposes the use of ABC as a marker of OS invasion and metastasis.

1.2.Initiation and formation of OS

(i) *Cell of origin*

The cell of origin for OS has been a topic of much debate. Some earlier studies believed OS to originate from osteoblasts and preosteoblasts, cells that are committed to differentiate into bone cells (Berman *et al*., 2008; Mutsaers *et al*., 2013; Walkley *et al*., 2008). In contrast, recent findings have shown malignant transformation to occur first in primitive multi-potent MSCs which then undergo a pattern of osteoblastic differentiation, resulting in the formation of malignant osteoblasts

(Dorfman & Czerniak, 1995; P. P. Lin *et al*., 2009; Shimizu *et al*., 2010). The ability of MSCs to differentiate into bone, cartilage, fat, muscle and stroma, and the formation of different histologic subtypes of bone tumor including chondroblastic, fibroblastic and osteoblastic, provide further evidence that this tumor likely arises from multi-potent stem cells than differentiated osteoblasts (Dorfman & Czerniak, 1995). Thus, with these contrasting findings, the crucial question of cell of origin for OS remains to be answered.

(ii) *Formation of OS*

OS usually starts to develop in the metaphysis of long bones of the body including the distal femur, proximal tibia and proximal humerus. Figure 2 shows the biologic growth pattern of OS. In healthy individuals, the MSCs differentiate into preosteoblasts, cells which are committed to become osteoblasts. These preosteoblasts express markers of early osteoblastic differentiation including RUNX2 and OSX. Following this, the preosteoblasts differentiate into mature osteoblasts which express additional markers of osteoblastic differentiation including osteocalcin and osteopontin (OPN). These mature osteoblasts continue to secrete bone matrix or osteoid, and as osteoid is being secreted, some of these osteoblasts become trapped in the bone matrix, eventually becoming osteocytes. However, this normal process of osteogenesis can be disrupted if genetic mutations occur in MSCs, preosteoblasts or mature osteoblasts. Due to these genetic alterations, there is incomplete differentiation of MSCs into osteoblasts, resulting in the formation of immature osteoblasts. These immature osteoblasts become OS cells, secreting malignant osteoid and constituting the formation of OS tumor (Adamopoulos, Gargalionis, Basdra, & Papavassiliou, 2016a). OS has a higher tendency to form in long bones which have large growth plates with increased cellular division and growth activity (Clark, Dass, & Choong, 2008). The growth pattern of this bone malignancy occurs in a radial manner, resulting in the formation of a bone-like mass. When this mass grows and applies pressure on adjacent muscles, it compresses them into a layer known as the 'reactive zone.' Small nodules of the tumor, called 'satellites' invade into the reactive zone. Based on the anatomical site of OS occurrence, the survival rates for patients vary, implicating the role of site of OS occurrence in its prognosis. For instance, the 5-year survival rate for OS in proximal tibia is 77.5% when compared to 5-year survival rate of 27-47% for OS occurring in the pelvic region (Clark, Dass, & Choong, 2008) . Typically, OS consists of immature

osteoid formation by spindle-shaped cells. Bone production by these cells occurs in a rather disorganized fashion with layers of malignant cells pushed against malignant bone (Spuy & Vlok, 2009). In addition to this, it may also consist of areas of benign-looking giant cells with enlarged blood-filled spaces and necrotic regions (Klein & Siegal, 2006).

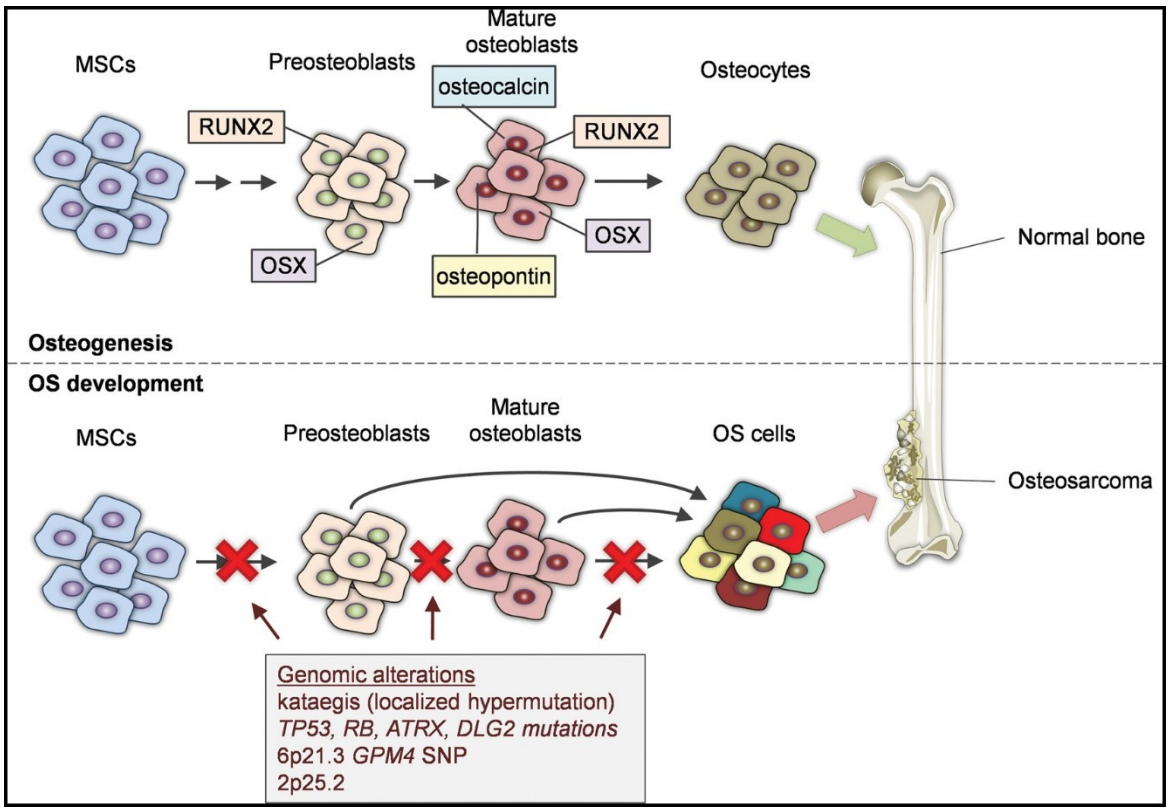


Diagram reprinted with permission from Adamopoulos *et al.,* 2016

Figure 2. Key steps in formation of OS

The upper panel of figure depicts the normal process of bone formation, from MSCs to mature osteoblasts and later osteocytes. Important markers of osteoblastic differentiation such as RUN-X2 and OSX are expressed to ensure complete differentiation of osteoblasts. The lower panel shows OS development which occurs due to critical genomic alterations in MSCs, preosteoblasts and mature osteoblasts, resulting in formation of OS cells. These OS cells secrete malignant osteoid and form the tumor. ATRX: transcriptional regulator ATRX; DLG2: disks large homolog 2; GRM4: glutamate receptor metabotropic 4; MSCs: mesenchymal stem cells; OS: osteosarcoma; RB1: retinoblastoma 1; RUNX2: RUNX-related transcription factor 2; SNP: single nucleotide polymorphism; TP53: tumor protein p53. The figure was reprinted with permission from the publisher.

1.4. Development and progression of OS

(i) *Tumor growth and cell proliferation*

Proliferation of malignant cells and the overall growth of tumor can occur via different mechanisms. However, two of the most prevalent processes include dysregulation of transcription factors and dysregulated expression of growth factors produced by cancer cells (Broadhead, Clark, Myers, Dass & Choong, 2011). Indeed, transcription factor complexes and individual transcription factors such as activator protein 1 complex (AP-1) and Myc are significantly upregulated in OS. The AP-1 complex is comprised of Fos and Jun proteins, which together regulate cell proliferation, differentiation and bone metabolism (Broadhead *et al*., 2011). An increased expression of Fos and Jun is seen in high-grade OSs compared to low-grade OSs and non-malignant osteoblastic lesions (Franchi, Calzolari, & Zampi, 1998; J. X. Wu *et al*., 1990). A study has shown that silencing of AP-1 regulated transcription resulted in decreased abilities for migration, invasion and metastasis in a murine model of OS (Leaner *et al*., 2009). Another important transcription factor which regulates cell division and growth and is significantly amplified in chemoresistant variant of OS cell lines, is Myc (Broadhead *et al*., 2011; Hattinger *et al*., 2009). A study by Scionti *et al*. showed that decreased Myc levels resulted in increased therapeutic effectiveness of methotrexate against OS cells (Scionti *et al*., 2008). Similar to this, another study has demonstrated loss of Myc activity to be correlated with inhibition of proliferation and promotion of differentiation, both of which will restrict tumor growth (Arvanitis, Bendapudi, Tseng, Gambhir, & Felsher, 2008).

Growth factors are critical for growth and proliferation of any cell but can also become a driver of uncontrolled cell growth if their expression is dysregulated. In cancer, the driving forces of tumor growth include overexpression of growth factors, increased and constitutive presence of their receptors and even amplification of the signals transmitted by these factors. The effects of growth factors are exerted through autocrine or paracrine or both mechanisms. Different studies have reported dysregulated expression of several growth factors in OS including transforming growth factor beta (TGF-β), insulin-like growth factor (IGF), connective tissue growth factor (CTGF) and parathyroid hormone (PTH) (Broadhead *et al*., 2011). In normal bone matrix, a significant portion of bone morphogenic proteins is comprised of TGF-β which regulates cell differentiation, proliferation, apoptosis and matrix formation (Broadhead *et al*., 2011). Expression of TGF-β is

significantly upregulated in high-grade OSs compared to low-grade OSs (Franchi, Arganini, *et al*., 1998). In addition, one study has shown between 30-50% reduction in cell growth when OS cell lines were cultured in growth medium with suppressed TGF-β activity, indicating the growth-inhibitory effects of TGF-β suppression on OS (Navid, Letterio, Yeung, Pegtel, & Helman, 2000). Similar to TGF-β, IGF growth factors have also been shown to be over expressed in OS (Broadhead *et al*., 2011). IGF factors act by interacting with their receptors and leading to activation of downstream signaling cascades such as PI3K (Phosphoinositide-3-kinase) and MAPK (Mitogen-activated protein kinases) for increased proliferative and anti-apoptotic effects (Rikhof, De Jong, Suurmeijer, Meijer, & Van Der Graaf, 2009). Studies have shown increased chemosensitivity and anti-tumor response as a result of suppressing IGF activity in OS cells *in vivo* and *in vitro* (Dong *et al*., 2010; Kolb *et al*., 2010; Y. H. Wang *et al*., 2010). Overall, the dysregulation of transcription and growth factors enable tumor cells to proliferate faster than other surrounding cells, resulting in rapid tumor growth. Hence, if these factors or their downstream signaling pathways are targeted, they can potentially reduce tumor growth and metastasis.

(ii) *Anchorage-independent growth*

OS cells have an increased tendency of becoming detached from the surrounding matrix and metastasize to other regions of the body. Under normal conditions, when cells become dissociated from basement membrane or matrix, a form of apoptosis, known as anoikis, is induced in the cells, eventually leading to cell death. However, OS cells gain the ability to evade anoikis and proliferate, even while having minimal or no cell-cell and cell-matrix contacts. This form of growth is also known as anchorage-independent growth and is an important feature of malignant cells. OS cells obtain this growth advantage by dysregulating signaling pathways that play a potential role in apoptosis. These include integrin signaling, Rho GTPase, PI3 kinase, PKB/Akt (Protein kinase B) and some components of apoptotic pathways (Broadhead *et al*., 2011). One possible way is by production of abnormal integrin subunits such as $\alpha_v\beta_6$ which cause activation of the PI3k pathway. Downstream of this signaling cascade is PKB/Akt, which then becomes activated, resulting in consequential inhibition of proapoptotic factor Bad (Bcl-2-associated death promoter) and survival of cancer cells (Janes & Watt, 2004; Nicholson & Anderson, 2002). Other studies have shown upregulation of Rho GTPases and MAPK signaling to lead to inhibition of Bad and Bim (Bcl-2

interacting mediator of cell death) respectively (Coniglio, Jou, & Symons, 2001; Ley *et al*., 2004). Thus, with the dysregulation of these otherwise tightly regulated apoptotic signals, malignant cells are able to thrive and proliferate without cell-cell and cell-matrix contacts.

(iii) *Angiogenesis*

For OS to grow and be sustained, there is an increased need for supply of nutrients and oxygen through blood vessels. This need is further aggravated by hypoxic conditions in tumor microenvironment which results in the release of hypoxia-inducible factor 1α (HIF-1α) (Duan & Xie, 2010). Following this, HIF-1α upregulates expression of vascular endothelial growth factor (VEGF) which in turn initiates endothelial cell proliferation, migration, vasodilation and formation and maturation of blood vessels (Duan & Xie, 2010; Matsumoto & Mugishima, 2006; Nagy, Dvorak, & Dvorak, 2007; Shibuya & Claesson-Welsh, 2006). VEGF expression can also be activated by growth factors upstream of it such as transforming growth factor alpha (TGF-α) and fibroblast growth factor (FGF) (Dvorak, 2005). Activation of VEGF results in the formation of a loosely arranged and leaky vascular network in the tumor and because it is not a well-formed network of blood vessels, it acts as a stimulus for release of more HIF-1α and VEGF. This creates a feed forward type of feedback loop by tumor cells, allowing them to expand the vascular network and support their growth (Broadhead *et al*., 2011; Liao & Johnson, 2007). OS is a relatively vascular tumor but studies showing association of VEGF with OS progression and metastasis have been controversial (Broadhead *et al*., 2011). Two findings show a positive correlation between VEGF expression and OS whereas one study reports no association between VEGF and the OS-associated microvascular network and metastasis at diagnosis (Hara *et al*., 2006; Kaya *et al*., 2000; Mantadakis *et al*., 2001). It is also important to note that in OS, the increased activation of proangiogenic factors such as VEGF is accompanied by downregulation of antiangiogenic factors including Thrombospondin 1 (THBS1), pigment epithelial-derived factor (PEDF) and reversion-inducing cysteine rich protein with Kazal motifs (RECK) (J. Cai, Parr, Watkins, Jiang, & Boulton, 2006; Clark, Thomas, Choong, & Dass, 2007; B. Ren, Yee, Lawler, & Khosravi-Far, 2006). In fact, a study by Ek *et al*. has demonstrated OS cell lines undergoing apoptosis when treated with PEDF, indicating the potential role of this and other antiangiogenic factors in therapy against OS (Ek, Dass, Contreras, & Choong, 2007).

1.5. OS classification

The most common type of OS, which accounts for approximately 75% of the cases, is the conventional central high-grade primary OS (Spina, Montanari, & Romagnoli, 1998). It is characterized by a high-grade mass of rapidly-dividing and transformed malignant cells with osteoid production and localized tissue invasion (Durfee *et al*., 2016). Based on the most prominent subtype of bone cells present within the conventional OS, it can be further classified into osteoblastic, chondroblastic, fibroblastic, small cell, telangiectatic, giant-cell rich and epithelioid forms (Klein & Siegal, 2006).

Apart from the conventional high-grade OS, a rare type of low-grade central OS also occurs which is locally invasive but has lower chances for metastasis and thus has overall higher survival rates (Fox & Trotta, 2013; Klein & Siegal, 2006; Puri, Jaffe, & Gelderblom, 2013). Particularly, if the tumor is not dedifferentiated, the 5 years survival rate is greater than 80% (Dujardin *et al*., 2011; Klein & Siegal, 2006). Prominent features of low-grade OS include fibrous areas with minimal number of atypical cells and small bony trabeculae with the presence or absence of chondroid regions. Due to the lack of typical features of OS, low-grade OS can sometimes be mistaken for fibrous dysplasia in radiographic images (Wadhwa, 2014). Another important defining feature of low-grade OS is the amplification of 12q13-15 chromosomal region which contains mouse double minute 2 homolog (MDM2) and cyclin-dependent kinase 4 (CDK4) genes, resulting in their protein over-expression. Hence, the expression of MDM2 and CDK4 can play a crucial role in diagnosis of low-grade OS (Dujardin *et al*., 2011).

Another broad classification of OS is surface OS, in which the epicenter of tumor is along or outside the bone cortex (Klein & Siegal, 2006). The different types of surface OS include parosteal OS, periosteal OS and high-grade surface OS. Amongst all three types of surface OS, parosteal OS is the most common, comprising of about 1-6% of all OS cases (Fox & Trotta, 2013). Parosteal OS appears in the form of sclerotic lesions which are attached to the underlying bone and typically have a ‘stuck on’ appearance. Their main features include slow growth, appearance of well-differentiated fibrous stroma with osseous components in histological examination and genetic mutations in MDM2 and CDK4 genes (Durfee *et al*., 2016; Yoshida *et al*., 2010). The prognosis is mainly worse for dedifferentiated form, which occurs in 25% of parosteal OS cases (Klein &

Siegal, 2006). The other type of surface OS, also known as periosteal OS, is located closer to the diaphysis than metaphysis and is more aggressive than parosteal OS (Inwards & Wenger, 2015; Klein & Siegal, 2006). These tumors consist of grade 2 cartilaginous matrix with some regions of osteoid deposition as well (Inwards & Wenger, 2015). For surgical treatment of periosteal OS, wide excision is sufficient and chances of metastasis are minimal when treated appropriately (Robert J. Grimer *et al*., 2005). The most aggressive and high-grade form of surface OS is known as high-grade surface OS and this form of OS typically has minimal or no medullary involvement. It is the least common type of surface OS, with less than 1% of all OS cases being high-grade surface OS. Pathologically, it appears similar to conventional high-grade OS and also has high chances of metastasis to lungs and other bones (James C. Wittig, n.d.).

1.6. Etiology of OS

(i) *Genomic instability*

One of the causes of OS can be mapped to changes in genetic composition, including inherited and acquired mutations. There are inherited genetic conditions which predispose individuals to OS. One such rare condition is Li-Fraumeni syndrome (LFS) which has germline mutation in tumor protein p53 (TP53) gene and approximately 30% of patients with LFS are also diagnosed with OS during their lifetime (Bougeard *et al*., 2015; Jinghui Zhang *et al*., 2015). Retinoblastoma is another such condition where germline mutation in retinoblastoma 1 (RB1) gene results in loss of Retinoblastoma protein (pRB), resulting in unregulated cell cycle progression (Kleinerman, Schonfeld, & Tucker, 2012; Wong, 1997). Studies have shown that in 60% of retinoblastoma cases, sarcomas develop as secondary tumors and this could be partly due to exposure to radiation during radiation therapy treatment of retinoblastoma (Hawkins, Draper, & Kingston, 1987; Wong, 1997). However, the majority of OS cases result from sporadic mutations, with loss of tumor suppressor genes being a common event (Kruzelock, Murphy, Strong, Naylor, & Hansen, 1997; Nellissery *et al*., 1998; Toguchida *et al*., 1989). Overall, with such randomly acquired mutations and some inherited predispositions, the etiology of OS remains complex to understand.

(ii) *Patient characteristics*

Apart from genetic predisposition, there are several characteristics which make individuals more susceptible to OS. One such factor is age, where adolescents between the ages of 10 and 25 and adults older than 65 years are at a higher risk for this disease (Durfee *et al*., 2016; Ries *et al*., 1999). According to one study, approximately 400 new cases of pediatric OS are diagnosed each year in the U.S. for children and adolescents younger than 20 years of age (Ottaviani & Jaffe, 2009b). This age brack*et al*so represents a period of rapid bone growth, where proliferating cells are more prone to mutations and vulnerable to oncogenic agents, resulting in increased chances for tumor formation (The American Cancer Society Medical and Editorial Content Team, 2017). Gender is also a risk factor for OS, with incidence of OS being higher in males than females (Linabery & Ross, 2008; Ottaviani & Jaffe, 2009b). It is also known that OS is more prevalent in African Americans and Hispanics compared to the white population, indicating that ethnicity could also be a potential risk factor for OS (Jemal *et al*., 2010). Hence, these individual characteristics increase the likelihood for occurrence of OS in the population.

(iii) *Ionizing Radiation*

Exposure to ionizing radiation is another important environmental risk factor for OS development. Patients who are given high doses of radiation for treatment of other malignancies are more likely to develop OS at a later age (Huvos, 1987). Specifically, patients with Ewing sarcoma are at a higher risk of subsequent development of OS due to their exposure to high doses of radiation during treatment (Supramaniam *et al*., 2008). Other elements which are implicated to be causative agents for OS include radium-224 and thorium-232. The latter was used as a radiocontrast agent in the past (Harrist, Schiller, Trelstad, Mankin, & Mays, 1979; Loutit, 1970). Thus, overall, ionizing radiations are thought to play a role in causing OS.

1.7. Signs and symptoms of OS

(i) *Pain and swelling*

Pain is one of the most common symptoms for OS and can initially be activity-related but later becomes pain at rest or night pain (Geller & Gorlick, 2010). Since pain is such a generic symptom and can also be mistaken for growing pains in young children, this symptom can often be ignored in the beginning. Such pain is also associated with trauma or vigorous physical exercise, both of which are common in the target population of children and young adolescents (Meyers & Gorlick, 1997). One review reported that the median time from presentation of symptoms to the time of diagnosis is about 4 months, but there was significant variation in the dataset (Geller & Gorlick, 2010). In some cases, a palpable mass or painful swelling can also be a symptom for OS (Durfee *et al*., 2016).

(ii) *Bone fractures*

Bone fracture is a rare symptom for OS but is still reported in some cases. They are more likely in telangiectatic OSs in which the bones become weaker compared to other forms of OS and thus have an increased tendency to cause fractures at the site of the tumor. One study also reported that approximately 5-10% of patients show pathological fracture as a first sign of illness (Scully, Ghert, Zurakowski, Thompson, & Gebhardt, 2002).

1.8. Diagnosis of OS

(i) *Imaging techniques*

When patients present with the above-discussed symptoms, they are further examined via imaging. Usually, the most basic and primary imaging test performed is radiographic imaging or x-ray of the affected area. The tumor presents itself as a lytic, sclerotic or a combination of lytic-sclerotic lesion in the radiograph. Commonly, penetration of mass into adjacent tissue is also seen. A radial or sunburst pattern of ossification in soft tissue is a usual feature of OS. For accurate determination of extent of tumor penetration into bone and soft-tissue, cross-sectional imaging techniques such as Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) are used (Gillespy *et*

al., 1988). Usually, MRI of the entire bone is performed to assess the extent of bone marrow invasion, to evaluate the presence of any soft-tissue mass and determine its size, to study tumor relationship with surrounding structures such as muscle, subcutaneous fat, etc. and to look for presence of skip metastasis (Durfee *et al*., 2016; Geller & Gorlick, 2010). Although considered inferior to MRI in diagnosis, CT scan of the thorax plays an important role in detecting pulmonary metastasis and is considered to be the preferred method of detection (Fox & Trotta, 2013; Neifeld, Michaelis, & Doppman, 1977). However, the accurate detection of small metastatic nodules (<5 mm in size) through CT scan is challenging and the diagnosis should be confirmed histologically as well. (Geller & Gorlick, 2010; Marina, 2004). In addition to this, the role of positron emission tomography (PET) scan in OS diagnosis and staging continues to emerge. Specifically, there have been findings which support success of PET imaging in evaluating histologic response to chemotherapy and in predicting progression-free survival (Geller & Gorlick, 2010). For instance, one study has reported that total lesion glycolysis prior to chemotherapy shows positive correlation with poor overall survival and that an increase in total lesion glycolysis post-chemotherapy is associated with worse progression-free survival (Costelloe *et al*., 2009). Other reports have also shown that PET scan can be useful in identifying tumors with increased metabolic activity and thus identify high-grade tumors (J F Eary & Mankoff, 1998; Janet F. Eary *et al*., 2002). Overall, these imaging techniques form an essential component of OS diagnosis.

(ii) *Biopsy*

In addition to diagnostic radiography, research studies have placed significant importance on use of needle biopsies for confirmation of tumor diagnosis (Davies, Livesley, & Cannon, 1993). Biopsy can either be accomplished through an open incisional or via core needle biopsy. Generally, open incisional biopsy is preferred over core needle procedure as the former yields sufficient amount of tissue for examination and is also reported to have approximately 96% diagnostic success (Geller & Gorlick, 2010). It is imperative that the biopsy is performed by the surgeon who will later perform the definitive resection to ensure that the biopsy tract is removed with the final surgical resection (H J Mankin, Mankin, & Simon, 1996). Additionally, the tissue sample obtained in open biopsy can also be used for research studies to facilitate the understanding of OS pathogenesis (Meyers & Gorlick, 1997). On the other hand, core biopsy technique is minimally

invasive, can be performed under local anesthesia, preserves tissue architecture and usually provides sufficient specimen for diagnosis (Pramesh, Deshpande, Pardiwala, Agarwal, & Puri, 2001). This procedure is also reported to have a positive predictive value of more than 98%, indicating its efficacy in the diagnostic process (Altuntas *et al*., 2005). Thus, based on the type of case, the appropriate method of biopsy can be employed for diagnosis and staging of OS.

1.9. Staging of OS

In order to determine appropriate chemotherapeutic and surgical treatment and the prognosis of the disease, it is important to categorize it via tumor staging (Isakoff, Bielack, Meltzer, & Gorlick, 2015; Spina *et al*., 1998; Vasquez *et al*., 2016). Staging uses different parameters to classify tumors based on their grade and presence of metastasis, etc. Currently there are two staging systems which are used for OS, including the Enneking and the American Joint Committee on Cancer (AJCC) staging systems (Tables 1 and 2 respectively). The Enneking staging defines tumors to be either low or high grade, intracompartmental or extracompartmental and have presence or absence of metastasis (Geller & Gorlick, 2010). The AJCC system was developed after Enneking and uses size of the tumor to distinguish small-sized tumors from larger ones (<8cm or >8cm) (Durfee *et al*., 2016). Even though there are some differences between the two staging systems, most tumors are classified similarly in both systems as the major predictor of prognosis is metastasis and that is defined in the same way by both systems (Moore & Luu, 2014). Hence, staging is an essential part of OS diagnosis and treatment and therefore should be done accurately and carefully to appropriately guide patient care and recruitment to clinical trials.

Table 1. Enneking Staging System for malignant musculoskeletal tumors (The table is reproduced from Jawad *et al*., 2010 with their permission).

Stage	Grade	Size	Metastasis
IA	Low	T1 - intracompartmental	M0 – none
IB	Low	T2 – extracompartmental	M0 – none
IIA	High	T1 – intracompartmental	M0 – none
IIB	High	T2 – extracompartmental	M0 – none
III	Any	Any	M1 – regional or distant

Table 2. AJCC Staging System for bone sarcoma. (The table is reproduced from Durfee *et al.,* 2016 with their permission.

Stage	Grade (G)	Size (T)	Lymph Node (N)	Metastasis (M)
IA	G1-low	T1<8 cm	N0-none	M0-none
IB	G1-low	T2>8 cm	N0-none	M0-none
IIA	G2-high	T1<8 cm	N0-none	M0-none
IIB	G2-high	T2>8 cm	N0-none	M0-none
III	Any G	Any T	Skip metastasis	Skip metastasis
IVA	Any G	Any T	N0-none	M1-lung metastasis
IVB	Any G	Any T	N1-lymph node metastasis or N0	M1-non-lung metastasis

1.10. Treatment strategies for OS

(i) *Chemotherapeutic treatment*

Before the advancement of chemotherapy, OS had poor survival rates. When patients were treated with either surgery and/or radiotherapy, the 2-year overall survival rates were approximately 15-20% (Friedman & Carter, 1972; Marcove, Miké, Hajek, Levin, & Hutter, 1970; Marina, 2004). In fact, the majority of patients had metastasis at the time of diagnosis and those with localized disease would soon develop the metastatic form of this disease, resulting in poor prognosis for patients (Durfee *et al*., 2016). A study by Jaffe et. al in the 1970s published about the efficacy of methotrexate in managing metastasis in advanced stage OS (Jaffe, 1972). Later on, multiagent chemotherapy proved to be more effective in improving survival compared to the use of single agent, with one study showing an increase in the 6-year survival rate from 11 to 61% with use of multiagent chemotherapy (Link *et al*., 1986). Doxorubicin and methotrexate were commonly used in combination and resulted in relapse-free survival (RFS) rates of up to 60% (Geller & Gorlick, 2010). In addition to this, several randomized clinical trials have demonstrated the use of chemotherapy in improving survival rates for OS (Link *et al*., 1986).

Current administration of chemotherapeutic treatment in North America and Europe includes methotrexate, adriamycin and cisplatin (MAP) (S. Ferrari & Serra, 2015; Isakoff *et al*., 2015). Typically, patients are given neoadjuvant chemotherapy for a period of about 6-10 weeks before surgical resection (S. Bielack *et al*., 2009; Geller & Gorlick, 2010). Following surgical removal of tumor and a short period for wound healing, patients are given maintenance chemotherapy for an additional period of 29 weeks. This entire treatment schedule has shown cure rates in approximately 70% of patients with localized disease. However, in patients with metastasis, the same treatment regimen results in long-term survival rates of <20% (Geller & Gorlick, 2010). On the other hand, the use of ifosfamide with or without etoposide has been under debate. Some studies have reported response rates of 30-40% for patients with recurrent or metastatic OS but others have shown increase in toxicity and no improvement in survival with its use (Carli, Passone, Perilongo, & Bisogno, 2003; Carrle & Bielack, 2006; Chou *et al*., 2005; S. Ferrari *et al*., 2005). In terms of toxicity, recent findings have shown no increase in survival rates for patients who are given high dose chemotherapy when compared to low doses of the same drugs (W.-G. Wang, Wan,

& Liao, 2015). There is also the issue of resistance to chemotherapy, which often depends on a multitude of factors and is becoming an important area of investigation in recent studies (He, Ni, & Huang, 2014).

(ii) *Surgical treatment*

For OS treatment, chemotherapy is complemented with surgical removal of the tumor and affected adjacent tissue. Patients can undergo either amputation of the limb in which tumor is present or limb salvage surgery. Previously, amputation was the optimal method for removing the tumor and preventing its recurrence. However, with the advent of chemotherapy, advanced imaging techniques and reconstruction strategies, limb salvage has become the predominant form of surgical treatment for OS patients (Robert J. Grimer, 2005; Marulanda, Henderson, Johnson, Letson, & Cheong, 2008). In limb salvage surgery, the tumor is removed while keeping surrounding tissue and important neurovascular structures intact. If dissected carefully, most of limb's function can be preserved (Durfee *et al*., 2016). In fact, according to studies, 85% of high-grade appendicular OS tumors can be removed carefully and successfully, while maintaining limb function (Robert J. Grimer, 2005; Marulanda *et al*., 2008). Even though it is important to preserve limb function, surgical removal should be performed to minimize chances of local recurrence (Gaetano Bacci *et al*., 2006).

Another important consideration during surgical resection is the reconstruction of affected bone and surrounding area. A number of options are available for reconstruction and they include bone allografts, endoprosthetic devices, biological constructs or even a combination of these elements (Durfee *et al*., 2016; Geller & Gorlick, 2010). Bone allografts of comparable size and anatomy can be obtained from donors and their successful integration depends on biological union and remodeling between host and implanted bone. Since the bone allografts are harvested with ligamentous soft-tissue attachments, it results in improved soft-tissue integration as well as osseous and vascular integration (Henry J. Mankin, Gebhardt, Jennings, Springfield, & Tomford, 1996). However, these allografts are unsuccessful in 17-20% of cases due to non-union, infection and fractures. Another method of reconstruction is the use of endoprosthetic replacements which has

become the preferred choice of reconstruction in past few years. Their modular form allows them to be adjusted intraoperatively to suit anatomical requirements of each patient. Importantly, they offer improved reconstruction for metaphyseal tumors and since the majority of OS cases in children occur in the metaphysis, this form of reconstruction is crucial for pediatric patients (Durfee *et al*., 2016). Despite these advantages, endoprosthetic replacements are accompanied with the risk of infections and eventual mechanical failure, with most long-term OS survivors requiring one or more revision surgeries later during their lives (R. J. Grimer *et al*., 2016).

In addition to the existing chemotherapeutic and surgical forms of treatment for OS, other alternative methods are also emerging which show potential for improving survival rates for OS patients. These include tyrosine kinase inhibitors, immunomodulatory agents such as muramyl tripeptide phosphatidylethanolamine (MTP-PE), human epidermal growth factor receptor 2 (HER2) targeted therapy and signal transduction pathway inhibitors (Durfee *et al*., 2016; Fleuren, Versleijen-Jonkers, Boerman, & van der Graaf, 2014; Shaikh *et al*., 2016).

1.11. Biomarkers in OS

At present, much of the diagnostic and prognostic information available for OS patients is derived through clinical markers. These markers include tumor size, disease stage, presence of metastasis at diagnosis and histologic response to preoperative chemotherapy. (C. Kong & Hansen, 2009; Gaetano Bacci *et al*., 2006; Bieling *et al*., 1996; Foukas *et al*., 2002; Yonemoto *et al*., 1998). However, clinical markers provide physicians with limited information and have some disadvantages as well. For instance, to observe the histologic response to neoadjuvant chemotherapy, patients have to undergo chemotherapeutic treatment, which can be intense and can result in short and long-term side effects for patients (Haddy, Mosher, Dinndorf, & Reaman, 2004). Therefore, in addition to clinical markers, a number of molecular and circulating molecules have been proposed as biomarkers for OS. However, no single reliable marker has been identified to date, which can predict OS biological behavior and metastasis. Table 3 summarizes a list of molecular markers and their association with OS prognosis.

(i) *Ezrin*

Ezrin belongs to the ezrin/radixin/moesin (ERM) family of proteins and is encoded by the VIL2 gene. Its primary functions include serving as a linker between plasma membrane proteins and the actin cytoskeleton and in the functional expression of membrane proteins at the cell surface (Hunter, 2004; C. Kong & Hansen, 2009; Sauvanet, Wayt, Pelaseyed, & Bretscher, 2015). A comparison study between conventional low-grade and high-grade OS has shown absence of ezrin expression in all low-grade OSs with significant immunoreactivity in many high-grade samples (Park *et al*., 2006). Additionally, previous studies have shown high ezrin expression in both metastatic and non-metastatic OS and have proposed it to be a prognostic marker for outcome in OS patients (S. Ferrari *et al*., 2008; Kim, Song, Cho, Lee, & Jeon, 2007; Y. F. Wang, Shen, Xie, Wang, & Huang, 2011). However, it is important to note that high ezrin expression was seen in both, metastatic and non-metastatic OS, suggesting that it might not be a suitable marker for differentiating between metastatic and non-metastatic OS. Hence, there is a need for discovering such markers for OS which are able to predict metastases along with other factors.

(ii) *Survivin*

Survivin belongs to the Inhibitor of Apoptosis family of proteins and acts via reducing the activity of procaspases (procaspase 3 and 7) which in turn results in inhibition of cell death for those with apoptotic signals (C. Kong & Hansen, 2009). Different studies have reported expression of surviving in OS patients. For instance, one study has shown nuclear and cytoplasmic staining of surviving to be correlated with increased malignancy (W. Wang, Luo, & Wang, 2006). In addition to this, another study has reported significant differences in survivin levels in patients with metastatic OS compared to those with non-metastatic OS (E Osaka *et al*., 2006; Eiji Osaka *et al*., 2007). Thus, the inhibitory role of survivin may contribute to OS progression and can be studied further to delineate its use as a biomarker.

(iii) *Alkaline Phosphatase*

Different studies have shown upregulated levels of alkaline phosphatase (ALP) in OS patients when compared to control samples (Limmahakhun *et al*., 2011; Ouyang *et al*., 2013; Tian *et al*., 2014). These increased levels of ALP are seen to be correlated with poor prognosis of OS and are also suggested to reflect osteoblastic activity of OS cells (Gaetano Bacci, Picci, Ferrari, Orlandi, *et al*., 1993; Bramer, van Linge, Grimer, & Scholten, 2009). A study by Han *et al* evaluated pre-chemotherapy serum ALP levels in 177 OS patients and showed a positive correlation between increased prechemotherapy ALP levels and reduced disease-free and overall survival (Han *et al*., 2012). Similar to this, results of a meta-analysis of published data showed significant association between high serum ALP levels and overall poor survival ($p<0.001$), disease-free survival ($p<0.001$) and occurrence of metastasis at the time of diagnosis ($p=0.006$) (H. Y. Ren, Sun, Li, & Ye, 2015). However, serum ALP levels are known to be upregulated in children and adolescents, making it challenging to differentiate the increased ALP levels between healthy children and adolescents and OS patients (Rauchenzauner *et al*., 2007; Szulc, Seeman, & Delmas, 2000; Turan *et al*., 2011). Moreover, studies that have measured and correlated ALP levels in preadult OS patients, have used inconsistent normal cut-off values for ALP levels in their analyses, making their findings possibly confounding (H. Y. Ren *et al*., 2015). Thus, even though serum ALP has shown to be associated with poor OS prognosis, the limitations discussed above should be considered while using it as a biomarker for OS.

(iv) *Lactate dehydrogenase*

The enzyme lactate dehydrogenase (LDH) plays a role in the interconversion of pyruvate and lactate, providing NAD+ for ongoing glycolysis (McClelland, Khanna, González, Butz, & Brooks, 2003; Tan, Gerrand, & Rankin, 2018). This enzyme is known to reflect tumor burden in many different types of tumor and is also of prognostic value in some tumors including pancreatic, lung, prostate as well as Ewing's sarcoma (Albain, Crowley, LeBlanc, & Livingston, 1990; G. Bacci *et al*., 1999; Smaletz *et al*., 2002; Tas *et al*., 2001; Walenta & Mueller-Klieser, 2004). One study investigated levels of LDH in patients with OS of the extremities and concluded that patients who presented with metastasis at the time of diagnosis had significantly higher LDH levels compared to those with localized disease ($p<0.0001$). Interestingly, pre-treatment patients with localized

disease who showed increased levels of LDH had a significantly greater chance of disease relapse compared to those who presented with normal levels. This indicates that pre-treatment serum LDH levels can be of prognostic significance for patients with OS of extremities and can be useful while planning therapeutic treatment plan (G. Bacci *et al*., 1994). Another meta-analysis by Chan *et al*. determined high serum LDH levels to be correlated with poor prognosis for OS patients (J. Chen, Sun, Hua, & Cai, 2014). Despite these promising studies, there are contradictory results for association of LDH levels with prognosis of OS and further validation for its use as a biomarker is required (J. Chen *et al*., 2014).

(v) *Matrix Metalloproteinases*

The matrix metalloproteinase (MMP) family of proteins encompasses more than twenty extracellular zinc-dependent endopeptidases, which are involved in digesting different components of the extracellular matrix (ECM) (Husmann *et al*., 2013). The ability of MMPs to degrade ECM makes them important for cancer cells as they assist in processes such as tumor growth, invasion and metastasis. For instance, when activated, MMPs can degrade basement membrane, allowing cancer cells to pass through the membrane and enter the circulatory system to metastasize to other organs/sites of the body (Liotta & Kohn, 2001). Another way in which MMPs promote tumor growth is by enhancing the bioavailability of certain growth factors, which are residing in ECM via degradation of ECM (Chambers & Matrisian, 1997). Hence, different MMPs have been known to be enhanced in various malignancies including lung, colon, breast, prostate, pancreatic, bladder and bone cancers (Bjørnland *et al*., 2005; Chambers & Matrisian, 1997; Eissa *et al*., 2007; Roy *et al*., 2008). For OS, several MMPs, including MMP2 and MMP9 have been described to be upregulated and have also been correlated with poor OS prognosis (Bjørnland *et al*., 2005; C. Ferrari *et al*., 2004; Uchibori *et al*., 2006). Thus, it is essential to study the expression of MMPs in OS malignancy and understand how it affects patient outcomes' and prognosis.

(vi) *Circular RNAs*

Circular RNAs (circRNAs) are classified as non-coding RNAs which are generated by back splicing of exons and have covalent bonds between the 3' and 5' ends, resulting in circular structures. These RNAs can potentially regulate gene expression at the transcriptional or post-transcriptional level by acting as sponges for micro RNAs (miRNA) (Greene *et al*., 2017). Each circRNA can interact with singular, multiple or whole families of miRNAs, indicating that even a small number of circRNAs can be very potent in their role as miRNA sponges (Kristensen, Hansen, Venø, & Kjems, 2018). Interestingly, some studies have shown expression of specific circRNAs in OS. For example, a study by Huang *et al*. identified circNASP to have significantly higher expression in OS cells compared to normal cells and implicated a potential role of circNASP in OS cell proliferation and tumor invasion (Huang, Chen, Pan, & Yu, 2018). Another study has demonstrated circPVT1, a circRNA, to be upregulated in OS and that its increased levels correlate with poor prognosis in OS patients (Kun-Peng, Xiao-Long, & Chun-Lin, 2018). Apart from overexpression of these RNAs in OS, there are also circRNAs which are downregulated and can even act as potential tumor suppressors. One such example would be of circHIPK3, where OS patients with reduced circHIPK3 expression were shown to have overall shorter survival times, and that lower circHIPK3 expression was correlated with lung metastasis and more advanced tumor stage (Xiao-Long, Kun-Peng, & Chun-Lin, 2018). Similar to this, another circRNA, hsa_circ_0002052, has been shown to be significantly reduced in OS cell lines and have negative association with survival and prognosis of OS patients (Z. Wu, Shi, & Jiang, 2018). In fact, this study has suggested that hsa_circ_0002052 suppresses OS growth and progression by exerting inhibitory effects on miR-1205/APC2 components of the Wnt/β-catenin pathway, which is also the signaling pathway of focus in our study (Dokanehiifard & Soltani, 2018; Z. Wu *et al*., 2018; Yang *et al*., 2018). Thus, by acting as miRNA sponges and regulating gene expression at the transcriptional level, circRNAs affect proliferation, invasion and metastasis of OS cells (C. Wang, Ren, Zhao, Wang, & Wang, 2018). Current studies have also indicated dysregulation of circRNAs is correlated with clinical progress and prognosis of OS patients (Liu *et al*., 2017). Overall, research on use of circRNAs as prognostic biomarkers and therapeutic targets for OS is in its early stages but shows potential for their use in future.

(vii) *Micro RNAs*

Micro RNAs (miRNAs) are short non-coding RNAs, about 20-25 base pairs in length, which regulate gene expression by affecting stability and translation of their target mRNA molecules (Raimondi *et al*., 2016; Rossi *et al*., 2014). Aberrant expression of miRNAs has been seen in different tumor types, where they can act as tumor suppressors or oncogenes (Y. W. Kong, Ferland-McCollough, Jackson, & Bushell, 2012). Several studies have investigated their diagnostic and prognostic potential and have proposed them to be promising blood cancer biomarkers (Rossi *et al*., 2013). Specifically, they have also been of interest in the diagnosis and prognosis of OS (Kobayashi, Hornicek, & Duan, 2012). For instance, a study by Cao et. al, showed significant downregulation of miR-326 in OS patients' serum in comparison to normal healthy controls and that reduced levels of miR-326 were correlated with distant metastasis, advanced tumor stage and shorter overall survival rates (Cao *et al*., 2016). In addition to this, there are miRNAs which act as oncogenes, contributing to initiation and progression of OS. Correlational analysis of miRNAs with clinicopathological features of OS patients show that the majority of miRNAs had a positive association with advanced clinical stage, increased tumor size, distant metastasis and in some cases, with resistance to chemotherapeutic treatment (Larrea *et al*., 2016; X. Wang *et al*., 2017). Also, a study has highlighted significantly reduced blood levels of miR-199a-5p in post-operative OS patients compared to pre-operative ones, indicating its potential to be used as a biomarker for monitoring disease recurrence in post-surgery follow-up patients (G. Zhou *et al*., 2015). To conclude, miRNAs show potential to be used as OS biomarkers.

Table 3. Summary of molecular markers associated with OS prognosis (Table reprinted with permission from (Clark *et al*., 2008).

Factor	General role in cancer	Levels in OS	Prognostic bearing in OS	Potential for therapy in OS
VEGF	Angiogenesis	Up	Controversial (Mohammed *et al*. 2007; Kaya *et al*. 2000; Hara *et al*. 2006; Mantadakis *et al*. 2001)	Yes (Folkman 2004; Tjin Tham Sjin *et al*. 2006)
PEDF	Anti-angiogenesis	Down	Undetermined	Yes, awaiting further preclinical trials (Ek *et al*. 2007)
MMP-2, MMP-9	Extra-cellular matrix invasion	Up	Correlation (Foukas *et al*. 2002)	Yes, good results in other cancers (Nemunaitis *et al*. 1998)
uPA/uPAR	Increases plasmin and MMPs. Pro-invasion	Up	Correlation (Choong *et al*. 1996)	Yes, reduced invasion if down regulated (Dass *et al*. 2005)
RECK	Anti-invasion, anti-angiogenesis	Down	Undetermined	Undetermined
P-glycoprotein	Drug resistance. Other unidentified pathways	Up	Correlation, specific to doxorubicin therapy (Baldini *et al*. 1999; Park *et al*. 2001)	Undetermined
CXCR4	Chemotaxis, organ-specific metastasis. Pro-invasion	Up	Correlation (Laverdiere *et al*. 2005)	Yes, good evidence in mice (Perissinotto *et al*. 2005)
p53	Cell cycle control	Down/mutated	Correlation (Park *et al*. 2001)	Undetermined
ErbB-2	Cell signalling, proliferation	Mixed results	Controversial (Onda *et al*. 1996; Somers *et al*. 2005; Zhou *et al*. 2003)	Undetermined
Survivin	Inhibits apoptosis	Up	Correlation (Osaka *et al*. 2006)	Undetermined

HLA class I	Absence allows immune system evasion	Down	Correlation (Tsukahara *et al*. 2006)	Undetermined
Ezrin	Cell signalling, cell interaction, metastasis	Up	Correlation (Khanna *et al*. 2004; Park *et al*. 2006)	Yes, potentially using Rapamycin (Wan *et al*. 2005)
Rb	Tumor suppressor, transcription control	Down/mutated	Correlation (Wadayama *et al*. 1994; Benassi *et al*. 1999; Feugeas *et al*. 1996)	Undetermined
PTHrP/PTHR1	Proliferation, invasion, hypercalcaemia	Up	Undetermined	Undetermined
c-Fos	Transcription	Up	Indirect correlation (Gamberi *et al*. 1998)	Undetermined

1.12. Dysregulated signaling pathways in OS

(i) *RANKL/RANK pathway*

The receptor activator of nuclear factor κ-B (ligand) or RANKL/RANK pathway is crucial for maintaining bone homeostasis by regulating differentiation, activation and function of bone-resorbing osteoclasts. The RANK receptor is present on osteoclasts and becomes activated when bound by its ligand RANKL, which in turn is produced by cells of the osteoblast lineage. The uncontrolled activation of this pathway in malignant and metastatic bone results in initiation of an ongoing cycle of osteoclastogenesis and tumor growth. A number of different studies have discussed the role of this pathway in OS, with one study even correlating the increased expression of RANKL with poor response to post-operative chemotherapy and decreased cancer-free survival (Lamoureux *et al*., 2007; Lee *et al*., 2011; Molyneux *et al*., 2010; Mori *et al*., 2007; Wittrant *et al*., 2006). Also, Bransletter *et al*. assessed the expression of RANK and its ligand RANKL in human OS samples and showed expression of RANKL in 68% of samples with no staining for RANK seen in OS tumor cells. In contrast, another study shows positive expression of RANK in 76.9% of human OS cases, with presence of RANK being negatively associated with disease-free survival and significant resistance to chemotherapy (Zhu *et al*., 2017). Overall, more research is required to elucidate the role of the RANKL/RANK pathway in OS tumor development and progression.

(ii) *PI3K/Akt pathway*

The PI3K/Akt/mTOR pathway consists of a series of protein kinases and is a crucial signaling pathway for many physiological and pathological processes including cell proliferation, autophagy, and cell growth (Adamopoulos, Gargalionis, Basdra, & Papavassiliou, 2016b; Jian Zhang, Yu, Yan, Wang, & Wang, 2015). A plethora of evidence confirms the dysregulation of this pathway in almost every type of human cancer, with amplifications and mutations occurring in approximately every member of this pathway (Porta, Paglino, & Mosca, 2014; Yuan & Cantley, 2008). In fact, PI3K/Akt signaling is dysregulated in majority of localized OS and in 100% of advanced-stage OS, indicating its potential role in progression of this malignancy (W. Zhou *et al*., 2014). The pathway can contribute to OS development via tumorigenesis, angiogenesis, chemoresistance, proliferation, invasion, metastasis and apoptosis (Jian Zhang *et al*., 2015). One

of the negative regulators of this pathway is phosphatase and tensin homolog (PTEN) and loss or inactivity of PTEN is a common event in OS, resulting in dysregulation of the PI3K cascade and constitutive activation of its downstream effector molecule Akt which then maintains the proliferative and anti-apoptotic state in malignant cells (Persad *et al*., 2016; Sansal & Sellers, 2004). A study on high-grade OS cell lines showed active Akt signaling among 20 of those cell lines and that inhibition of Akt activity restricted proliferation in cell lines having wild-type Kristen RAt Sarcoma (KRAS) function (Kuijjer *et al*., 2014). Given the involvement of PI3k/Akt pathway in OS development, this pathway has become an important target for drug therapy to improve OS prognosis and long-term survival rates for patients. Development and use of inhibitory molecules for this pathway is underway and includes several molecules such as PTEN activators, Dual PI3k/mTOR inhibitors, Akt inhibitors as well as some natural compounds which target different components of this pathway (Jian Zhang *et al*., 2015). Overall, the downregulation of the PI3k/Akt signaling pathway may be an important tool in therapeutic advances against OS.

(iii) *Notch pathway*

Notch signaling has been conserved evolutionarily and contributes to normal bone development by regulating both osteoblasts and osteoclasts (Bai *et al*., 2008; Hughes, 2009; Sciaudone, Gazzerro, Priest, Delany, & Canalis, 2003; Tezuka *et al*., 2002; Yamada *et al*., 2003). On a broader level, this pathway is crucial for different cellular processes such as cell fate determination, stem cell renewal, differentiation, survival and cell proliferation (Artavanis-Tsakonas, Rand, & Lake, 1999). The Notch signaling pathway comprises of Notch receptors, Jagged and Delta-like ligands and transcription factors belonging to Hairy/Enhancer of Split (HES) and HES-related repressor families of proteins. Once bound by ligand, the Notch receptor undergoes proteolytic cleavage and translocates to the nucleus to act as a co-activator in transcriptional complexes of CSL (CBF-1/Suppressor of Hairless/Lag-1), which then regulate transcription of downstream target genes (Hughes, 2009). Interestingly, the Notch pathway has been implicated to be oncogenic in tumors such as lung, colorectal and breast etc. as well as act as a tumor suppressor in B cell malignancies, neural crest and skin tumors (Allenspach, Maillard, Aster, & Pear, 2002; Leong & Karsan, 2006; Nickoloff, Osborne, & Miele, 2003; Radtke & Raj, 2003; Zweidler-McKay *et al*., 2005). In OS, studies support the role of Notch pathway in promoting tumor invasion and metastasis. A study by

Hughes *et al*. has shown increased levels of the components of the Notch pathway in metastatic OS cell lines compared to normal human osteoblasts and non-metastatic OS cells (Hughes, 2009). Inhibition of Notch signaling by γ-secretase inhibitors resulted in reduced cell proliferation and decreased invasiveness in OS cells and restricted tumor development in nude mouse xenografts *in vivo* (Hughes, 2009; Tanaka *et al*., 2009). Another study demonstrates that the majority of Notch pathway activation can potentially be a result of surrounding endothelial cells and pericytes than the OS cells themselves, since the latter express relatively low levels of Notch ligands. This indicates that targeting the Notch pathway for therapy could be challenging as its activation is non-uniformly distributed across a tumor (McManus, Weiss, & Hughes, 2014). Thus, there is still more to understand about the role of the complex Notch pathway in OS to use it as an effective therapeutic target to treat the malignancy.

1.13. Wnt/β-catenin signaling in OS

(i) *Canonical Wnt/β-catenin pathway*

The Wnt pathway is a highly conserved pathway which is a critical regulator of important cellular processes including cell fate determination, proliferation, differentiation and homeostasis (Jamieson, Sharma, & Henderson, 2012; Logan & Nusse, 2004; Luu *et al*., 2004; MacDonald, Tamai, & He, 2009). The mammalian genome consists of 19 conserved Wnt genes (Clevers & Nusse, 2012). The Wnt pathway can be divided into canonical (β-catenin dependent) and non-canonical (β-catenin independent) signaling. The canonical pathway functions by regulating the amount of transcriptional co-activator β-catenin which is critical in regulating expression of downstream target genes of this pathway. On the other hand, the non-canonical pathway works independent of β-catenin and can be further divided into planar cell polarity pathway and Wnt/Ca^{+2} pathway. The planar cell polarity pathway plays a role in asymmetric organization of cell cytoskeleton to cause polarized organization of structures. The Wnt/ Ca^{+2} pathway plays an important in several processes during embryogenesis including dorsal axis formation and gastrulation via causing release of intracellular Ca^{+2} from endoplasmic reticulum (Komiya & Habas, 2008; Sheldahl *et al*., 2003). Since our study focuses on canonical Wnt/β-catenin signaling in OS, we will look at the mechanism of canonical Wnt/β-catenin signaling in detail in a cell.

In the canonical Wnt/β-catenin pathway, when Wnt ligands are absent, β-catenin is constitutively phosphorylated and targeted for degradation by the degradation complex. The degradation complex consists of the scaffolding protein axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β) and caesin kinase 1 (CK1). Thus, in the absence of Wnt binding, specific amino acid residues of β-catenin are phosphorylated by CK1 (Ser45) and GSK3β (Thr41, Ser37 and Ser33) and ubiquitinated by β-Transducin repeat-containing (β-TrCP), resulting in subsequent proteasomal degradation of β-catenin. However, when Wnt signaling is on, the Wnt ligands bind to its cell-surface receptor Frizzled (Fz) and co-receptor Low-density lipoprotein receptor-related protein 5/6 (LRP5/6). This binding leads to the recruitment of another protein called Dishevelled (Dvl) to the plasma membrane, which in turn causes disintegration of the degradation complex. With the degradation complex disintegrated, it can no longer target β-catenin for degradation. Hence, this leads to stabilization and accumulation of β-catenin, such that it can now translocate to the nucleus and bind to the TCF/LEF family of transcription factors to cause transcription of its downstream target genes (MacDonald *et al*., 2009; Zhan, Rindtorff, & Boutros, 2017). This mechanism is also shown diagrammatically in Figure 3.

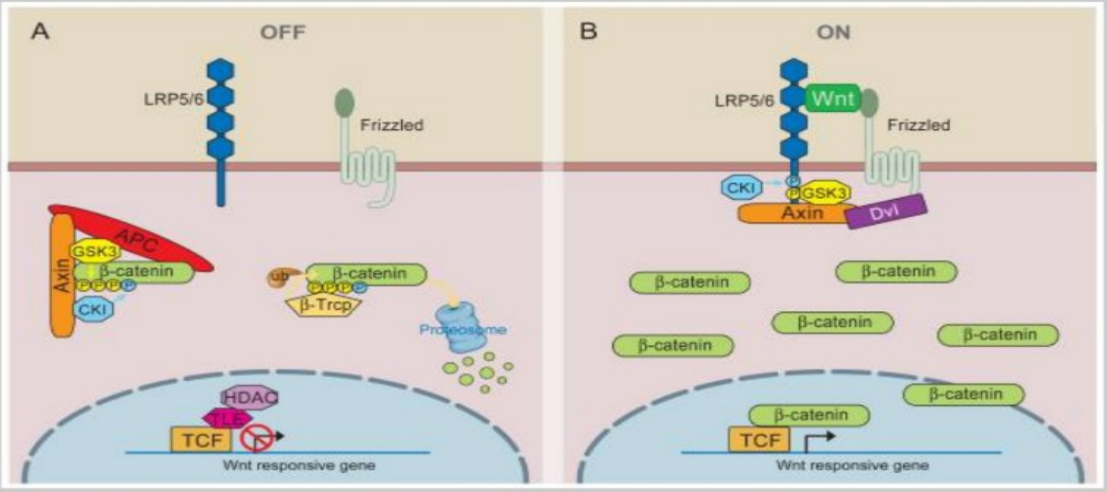


Diagram reprinted from Macdonald B *et al.,* 2009

Figure 3. Wnt/β-catenin signaling pathway

In the absence of Wnt ligand binding to Fz and LRP5/6, β-catenin is targeted by a degradation complex comprising of Axin, AP1, GSK3β and CK1, for proteasomal degradation via phosphorylation and ubiquitylation. However, when Wnt ligand binds to its receptor Fz and co-receptor LRP5/6, a protein Dvl is recruited to cell membrane which then recruits Axin, GSK3β and CK1 to cell membrane, resulting in disintegration of degradation complex. Thus, with degradation complex no longer intact, β-catenin is not degraded and is free to enter the nucleus and act as a transactivation factor to regulate transcription of downstream target genes. This figure was reprinted with permission from the publisher.

(ii) *Overview of canonical Wnt/β-catenin pathway in OS*

In bone, Wnt signaling is important for bone modeling during embryogenesis and development as well as bone remodeling which continues to occur throughout the life. Particularly, signaling from Wnt ligands is known to regulate differentiation of osteoblasts (Regard, Zhong, Williams, & Yang, 2012). Dysregulated Wnt/β-catenin signaling is known to play a role in different types of cancers including colon, gastric, lung, prostate and bone cancers (McQueen *et al*., 2011; Mohinta, 2007; Najdi, Holcombe, & Waterman, 2011; Tomita *et al*., 2007; Zi *et al*., 2005). In addition to dysregulation of Wnt/β-catenin in different cancers, studies have also reported activity of this pathway in OS. For instance, primary human OS samples and OS cell lines show both increased activation of the canonical Wnt pathway and an upregulated expression of Wnt ligands and receptors, indicating the potential role of this pathway in OS development (Yu Cai *et al*., 2014; C. Chen *et al*., 2015). These observations can further be supported by two studies by Chen *et al*. and Ma *et al*. which show immunohistochemical expression of the Wnt-10b ligand in 75% of human OS samples and a significant upregulation of Wnt-3a in the OS cell line SaOS-2 when compared to a human fetal osteoblast (hFOB) cell line, respectively (K. Chen *et al*., 2008; Ma *et al*., 2013). In addition, a study by Hoang *et al*. investigated the expression of LRP-5 in four OS cell lines (U2OS, SaOS2, HOS and 143B) and not only showed its overexpression in all cell lines but also demonstrated an association between increased expression of LRP-5 and tumor metastasis and worse overall disease-free survival rate in patients (B. H. Hoang, Kubo, Healey, Sowers, *et al*., 2004). Thus, the increased expression of Wnt ligands and receptors suggest an autocrine or paracrine mechanism for upregulating Wnt signaling in OS development (Yu Cai *et al*., 2014).

(iii) *β-catenin in OS*

β-catenin, which is the main effector molecule of the Wnt/β-catenin pathway, is a 92 kDa protein and belongs to the armadillo family of proteins. It consists of three domains, including the N-terminal domain, the armadillo domain which has 12 consecutive armadillo repeats and the C-terminal domain (Xu & Kimelman, 2007). Interestingly, β-catenin exists in different pools in the cell: at the cell membrane as part of the adherens junction, in the cytoplasm where its levels are tightly regulated and in the nucleus where it plays a role in transcriptional regulation of its downstream target genes (Voronkov & Krauss, 2013). Levels of intracellular β-catenin are mainly

regulated by Wnt signaling, such that with the dysregulation of oncogenic Wnt signaling, the amount of β-catenin existing in the cell also varies. Various studies have investigated the expression of β-catenin in OS and have obtained contradictory findings. For murine OS models, a study by Iwaya *et al*., proposed that there was increased levels of nuclear β-catenin associated with OS progression, while Kidani *et al.,* in contrast, showed decreased metastatic potential with increased cytoplasmic β-catenin (Iwaya *et al*., 2003; Kidani *et al*., 2014). Similarly, for studies targeting human OS cell lines and human OS samples, conflicting results have been seen for cytoplasmic and nuclear staining of β-catenin. For instance, one study by Haydon *et al*. showed cytoplasmic and/or nuclear accumulation of β-catenin in 70% of OS patients (Haydon *et al*., 2002). However, it was later confirmed that only 3 of the 47 patients evaluated in this study showed positive nuclear β-catenin staining (Ng *et al*., 2005). In addition, another study by Du *et al*., showed no nuclear β-catenin staining in 46 OS samples tested, but cytoplasmic and membranous β-catenin staining was present in 32 of 46 samples (Du, Yang, Yang, Tian, & Zhu, 2014). There is also another finding by Cai *et al*. which concludes no positive correlation between nuclear β-catenin levels and OS progression (Yongping Cai *et al*., 2010). Another study by Lu *et al*. has reported positive cytoplasmic β-catenin staining in 66 out of 96 OS cases, with correlational analysis indicating that aberrant β-catenin expression was significantly associated with metastasis and decreased patient survival (Lu *et al*., 2015). In support of these findings, accumulation of β-catenin is shown to be a common event in OS, but correlation of its accumulation with OS progression have been conflicting. Overall, these findings make it more important to understand how expression of β-catenin changes with OS progression and its correlation with the development of the disease.

(iv) *Role of Wnt antagonists in OS*

There are two types of secreted Wnt pathway antagonists which function to suppress increased tumorigenesis and metastatic potential of OS. One group of antagonists consists of those which bind directly to Wnt ligands, inhibiting a stimulatory response to Wnt signaling. Examples of this category of antagonists include Wnt inhibitory factor-1 (WIF-1), secreted frizzled-related protein (sFRP) family and cerebrus. The other group of antagonists inhibits the Wnt pathway by binding to the receptors, thereby preventing the interaction between Wnt ligands and its receptors.

Molecules belonging to the Dickkopf (DKK) family of proteins and sclerostin constitute the other group of Wnt antagonists (C. H. Lin *et al*., 2014). These antagonists are increasingly becoming an important target for therapy against OS via suppression of the Wnt/β-catenin pathway.

Different studies have investigated the role of the Dickkopf family of antagonists in OS and all conclude that it results in suppression of OS development. Expression of Dkk-3 in OS cells resulted in decreased translocation of β-catenin to the nucleus, preventing its binding to TCF/LEF for transcription of target genes (B. H. Hoang, Kubo, Healey, Yang, *et al*., 2004). Similarly, another study co-expressed Dkk-3 with a dominant-negative LRP-5 mutant in OS cells and demonstrated significantly reduced cell motility and invasion. In the same study, when Dkk-3 transfected OS cells were injected in nude mice, it decreased both tumor growth and lung metastasis in the mice (C. H. Lin *et al*., 2013). Thus, based on the understanding about Dkk's impact on Wnt/β-catenin signaling in OS, these antagonists could be a potential target for therapy. Similar to Dickkopf family, sclerostin, which is the other extracellular Wnt antagonist, acts by binding to LRP-5/6 to inhibit Wnt/β-catenin signaling (Holdsworth *et al*., 2012; X. Li *et al*., 2005). One study investigated the expression of sclerostin in OS cell lines and found significant difference in its expression among the OS cell lines. It also showed a positive association between sclerostin expression and ALP, which generally correlates with poor overall survival in OS patients (Shen *et al*., 2016). Hence, the role of sclerostin in OS is still unclear and there is a need to study its role in this malignancy in more detail.

In addition, the Wnt antagonists which bind to the Wnt ligands themselves have also been studied in great detail. One such ubiquitous antagonist for this pathway is WIF-1, which is known to be deregulated in a range of malignancies including lung, breast, prostate, bladder and OS (Rubin *et al*., 2010; Wissman *et al*., 2003). This secreted protein consists of a WIF domain that enables it to interact directly with Wnt ligands (Hsieh *et al*., 1999). Research has shown epigenetic silencing of WIF-1 via hypermethylation in its promoter region in OS tissue and cell lines (Kansara *et al*., 2009). Silencing of this protein in primary OS correlates with increased tumor growth and development in mice (Kansara *et al*., 2009). In another study, injection of WIF-1-transfected OS cells in nude mice resulted in decreased tumorigenesis and lung metastasis (Rubin *et al*., 2010).

Important properties of cancer cells including anchorage-independent growth, upregulated activity of MMPs for degrading extracellular matrix and increased motility – all were suppressed by re-expression of WIF-1 in OS cells (C. H. Lin *et al*., 2014). Along with the WIF-1 antagonist, the role of Frzb, which belongs to the sFRP family, has also been studied in OS and other tumors (C. H. Lin *et al*., 2014). Frzb consists of an amino-terminal cysteine-rich domain which is structurally similar to the ligand-binding domain of Frizzled. This similarity allows Frzb to act as an inhibitor of Wnt signaling by binding competitively to Wnt ligands, thus preventing the interaction of these ligands with Frizzled receptors (B. Hoang, Moos, Vukicevic, & Luyten, 1996; Leyns, Bouwmeester, Kim, Piccolo, & De Robertis, 1997). Decreased expression of Frzb is observed in OS tissue and cell lines, indicating its downregulation in this tumor (Mandal *et al*., 2007). Also, c-Met, which is a downstream target of the Wnt/β-catenin pathway, can be inhibited *in vitro* by Frzb (Guo, Xie, *et al*., 2008; Patanè *et al*., 2006; Zi *et al*., 2005). Thus, this class of Wnt-ligand binding antagonists, can also prove to be critical targets in OS treatment.

Apart from these antagonists, a group of molecules, also known as small molecule Wnt inhibitors, also show potential for delaying and reducing tumorigenesis and metastasis (Yu Cai *et al*., 2014). In fact, studies have identified two molecules, curcumin and PKIF118-310, which can significantly reduce nuclear β-catenin and inhibit invasion and migration in OS cell lines (Leow, Tian, Ong, Yang, & Ee, 2010). Another study has identified a small molecule inhibitor, 3289-8625, which reduces tumorigenesis in prostate cancer cells and can also have promising effects in OS cell lines (Grandy *et al*., 2009). Overall, with the current body of knowledge regarding antagonists and small molecule inhibitors of the Wnt pathway, there is a high potential for effective treatment therapies against OS.

1.14. Activated β-catenin (ABC) in OS

In most studies, nuclear β-catenin has been reported as the sole effector molecule of the Wnt/β-catenin pathway, where it transmits the signals by acting as a transcriptional/trans-activator. However, a study by Staal *et al*. in 2002 showed that stability of β-catenin was insufficient for promoting β-catenin/TCF transcriptional activity. The study used two different systems that

prevented β-catenin from being degraded: (i) a pharmacological proteasomal inhibitor ALLN (N-acetyl-Leu-Leu-Norleu-al) and (ii) CHO cells having a temperature sensitive mutation in E1 ubiquitin conjugation enzyme. In both cases, the amount of accumulated β-catenin was increased but no change could be seen in downstream β-catenin/TCF transcriptional activity. Importantly, a form of β-catenin, that is unphosphorylated at Ser37 and Thr41 at its N-terminal domain, was elevated with Wnt1 stimulation and promoted TCF transcriptional activity (Staal, van Noort, Strous, & Clevers, 2002). This partially phosphorylated form of β-catenin, also known as **A**ctivated **β**-**C**atenin or ABC, constitutes a unique pool of β-catenin. The figure below shows the structure of β-catenin and ABC with their respective phosphorylation sites (Figure 4). Another study by van Noort *et al*. further supported this model by designing an antibody (Anti-Active-β-catenin) which recognizes amino acids 36-44 of β-catenin, specifically when Ser37 and Thr 41 are non-phosphorylated (Van Noort, Meeldijk, Van Der Zee, Destree, & Clevers, 2002). Thus, the study by Staal *et al*. provided strong evidence of the mediation of Wnt signaling by ABC, making it important to investigate how ABC levels change in an environment of dysregulated Wnt signaling. ABC is largely monomeric, localizes mainly to the nucleus and is also found to be intrinsically more transcriptionally active than the non-phosphorylated form of β-catenin (Maher, Mo, Flozak, Peled, & Gottardi, 2010). One finding has also shown high levels of nuclear ABC compared to total unphosphorylated cellular β-catenin to be associated with a significantly worse overall survival in melanoma (Lopez-Bergami, Fitchman, & Ronai, 2008). Hence, the role of ABC needs to be studied in more detail to have a better understanding of how it is dysregulated in OS and its possible contribution in OS progression.

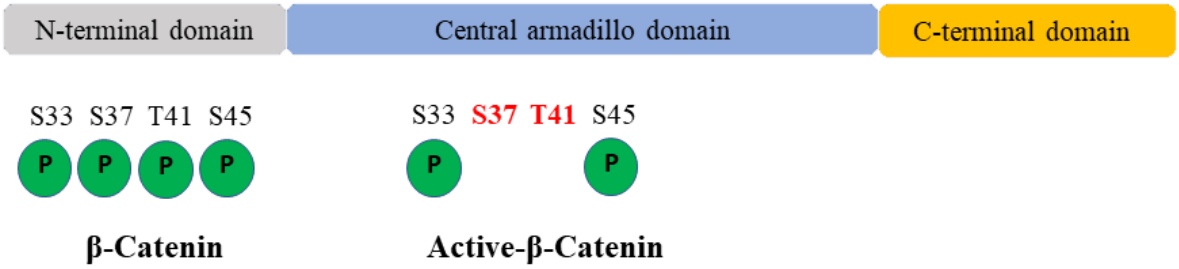


Figure 4. Diagrammatic representation of β-catenin and ABC.

The figure shows structure of β-catenin/ABC which consists of three domains including the N-terminal domain, the central armadillo domain and the C-terminal domain. The four putative amino acid residues present in the N-terminal domain are serine 33, serine 37, threonine 41 and serine 45. When all four residues are phosphorylated, β-catenin is targeted for degradation. However, when serine 37 and threonine 41 are not phosphorylated, a transcriptionally more active form of β-catenin, also known as Activated-β-Catenin (ABC) is produced.

1.15. Why is a reliable marker for OS needed?

Prior to advancement in chemotherapy and surgical treatments, amputation of the limb was the standard form of treatment for OS and survival rates were low (G. Bacci *et al*., 2001; S. S. Bielack *et al*., 2002; Provisor *et al*., 1997). Later, around the 1980s, with the advent of adjuvant and multi-agent chemotherapy and limb-sparing surgery, survival rates greatly improved for OS patients to approximately 70% (C. Kong & Hansen, 2009). However, these survival rates have remained relatively constant over the past few decades, pressing on the need for improvement in current treatment strategies as well as advancement in existing prognostic and diagnostic measures. Moreover, about 1 in 5 cases of localized OS eventually develop metastasis despite intensive chemotherapeutic treatments (Gaetano Bacci *et al*., 2006; S. S. Bielack *et al*., 2002; Durfee *et al*., 2016; Meyers *et al*., 1992; Mialou *et al*., 2005; Rodriguez-Galindo *et al*., 2004; Tabone *et al*., 1994; Wunder, Nielsen, Maki, O'Sullivan, & Alman, 2007). Currently, 80% of patients who do not present with distant metastasis at the time of diagnosis will become long-term survivors of this malignancy (C. Kong & Hansen, 2009). This also means that the remaining 20% of patients who do not show distant metastasis at diagnosis will not be long-term survivors. The same group of patients also seem to be resistant to chemotherapeutic treatment as their survival rates do not improve even after intensification of chemotherapy post-surgery (Gaetano Bacci, Picci, Ferrari, Ruggieri, *et al*., 1993). In addition to this, multi-drug chemotherapy administered to OS patients remains as one of the most intense and exhausting chemotherapeutic treatments given for solid tumors (C. Kong & Hansen, 2009). In fact, as a result of these treatments, there have been reports on development of renal, blood and cardiac disorders as well as the occurrence of secondary tumors (G. Bacci *et al*., 2000; S. Ferrari *et al*., 2005; Gaffney *et al*., 2006; Ta, Dass, Choong, & Dunstan, 2009). Moreover, studies have also demonstrated that despite intensification of chemotherapy dosage, further improvement is not seen and that the increased drug toxicity has also resulted in complications in certain cases (S. Ferrari & Palmerini, 2007; Haddy *et al*., 2004; Lewis *et al*., 2007; Meyers *et al*., 1998). All these factors emphasize the need to stratify patients based on the potential for aggressive biological behavior of OS and to administer the most optimal chemotherapeutic and surgical treatment relevant to each group of patients. Such a stratification would need more relevant markers than the ones already present in the clinic to help predict response to therapy, progression of disease and chemoresistance, etc. (C. Kong & Hansen, 2009; Raimondi *et al*., 2017). Hence, based on our findings about ABC in metastatic OS cell lines

compared to parental ones and its expression detected by IHC in OS Tumor Tissue Array (TMA), our study proposes the use of ABC as a potential biomarker for OS. We hypothesize that expression of ABC would help predict OS invasion and/or metastasis and aid physicians in planning the most appropriate treatment regimen for patients.

1.16. Hypothesis

We hypothesize that ABC drives osteosarcoma progression.

1.17. Objectives

1. Evaluation of ABC levels and its transcriptional activity in *in vitro* model of OS progression.

2. Evaluation of ABC as a marker of OS progression.

Chapter 2

Materials and Methods

2.0.Materials and Methods

2.1. Cell lines and culture conditions

The SaOS2-LM7 and its parent cell line SaOS2 were kind gifts from Dr. Eugenie Kleinerman, The University of Texas, M.D. Anderson Cancer Center, USA. HOS (Catalog no. CRL-1543™), HOS-143B (Catalog no. CRL-8303™), MCF-7 (Catalag no. HTB-22™) and HT1080 (Catalog no. CCL-121™) were purchased from ATCC. The SaOS2 cell line was originally derived from the primary OS of an 11-year old girl. Its metastatic counterpart was established by injecting SaOS2 cells in nude mice and allowing the tumor to grow. The first lung metastases were detected after 6 months, following which the cells from metastatic lungs were isolated and reinjected into another nude mice. This process was repeated 6 additional times to obtain the more metastatic SaOS2-LM7 cell line (Jia, Worth, & Kleinerman, 1999). The other metastatic cell line HOS-143B was derived from transformation of the HOS cell line with the ki-ras oncogene (Ek, Dass, & Choong, 2006). The metastatic potential of HOS-143B can also be confirmed by Luu *et al*.'s study where orthotopic injection of HOS-143B cells in mice resulted in increased tumorigenicity and spontaneous pulmonary metastases (Luu *et al*., 2005). Another study by Mohseny *et al*. showed development of multiple lung metastases after subcutaneous and intramuscular injection of HOS-143B cells in mice (Mohseny *et al*., 2011). Hence, both these studies confirm the high metastatic potential of HOS-143B cell line. All cell lines were cultured in Minimal Essential Medium (MEM) (Catalog no. 10320-021, Gibco), supplemented with 10% fetal bovine serum (FBS) (Catalog no. 12483-020, Gibco), 1x penicillin-streptomycin (Catalog no. 15140-122, Gibco), 1mM sodium pyruvate (Catalog no. 11360-070, Gibco) and 2mM L-Glutamine (Catalog no. 25030-081, Gibco) at 37ºC and 20% O_2 and 5% CO_2. For passaging, cells were incubated in 0.25% Trypsin for 4 minutes at 37ºC and 20% O_2 and 5% CO_2. Following incubation, trypsin activity was stopped by addition of supplemented MEM medium. To remove trypsin from cells in suspension, they were centrifuged at 800 rpm for 5 minutes. Supernatant was removed, and the cell pellet resuspended in supplemented MEM medium and seeded in a new flask.

2.2. Conditioned media concentration and gelatin zymography

All OS cell lines including SaOS2, SaOS2-LM7, HOS and HOS-143B were seeded in 6-well plates. When the cells were 80% confluent, FBS supplemented medium was replaced with 500 µl serum-free medium and incubated for 24 hours. Following incubation, conditioned medium was collected and transferred to an Eppendorf tube and centrifuged at maximum speed for 1 minute. The supernatant was then transferred to a Centricon filter (Catalog no. UFC501024, Millipore) and concentrated according to the manufacturer's protocol. Simultaneously, all cells from which conditioned media were collected, were counted for each cell line. This was important for normalizing results of MMP activity with number of cells

10ul of the concentrated conditioned medium in 6X loading buffer was loaded onto an 8% SDS-PAGE containing 2 mg/ml gelatin substrate (Catalog no. G8150, Sigma Aldrich). Conditioned medium from HT1080 fibrosarcoma cells was used as a positive control. It is ideal to use conditioned medium from HT1080 cells as it contains both MMP2 and MMP9 (Toth, Sohail, & Fridman, 2012). At the completion of electrophoresis, gels were washed in 2.5% Triton X-100 *v/v* in water for 3 times, 20 minutes each. The washed gels were incubated overnight in incubation buffer (composed of NaCl, $CaCl_2$, Tris and NaN3) at 37ºC and then stained with 0.05% Coomassie Brilliant Blue G-250 (Catalog no. B 1131, Sigma) for 2 hours. The gels were then de-stained using aqueous 4% methanol: 8% acetic acid and imaged using the Bio-Rad Gel Doc apparatus and Quantity One software.

2.3. Transwell® Invasion Assay

For cell invasion assay, a Transwell® unit (8 µM) coated with BD Matrigel Basement Matrix was used. The chamber was placed in a 24-well plate. SaOS2, SaOS2-LM7, HOS, HOS-143B and MCF-7 cells were counted (40000-50000) and seeded in the upper compartment of the chamber. MCF-7 was used as a negative control and an empty chamber without cells was used as a blank. The cells were incubated in 0.1% FBS DMEM for 24 hours at 37ºC and 5% CO_2. DMEM supplemented with 10% FBS was added to the lower compartment of the well to act as a chemoattractant. After completion of the incubation period, the chamber was removed from each

well and cells present on the upper face of the membrane were removed using a cotton swab. For cells which were able to invade to the lower face of the membrane, they were washed with 1X PBS and fixed with ice-cold 100% methanol (-20ºC) at room temperature for 20 minutes. The cells were then stained with 0.5% crystal violet for 15-20 minutes. For counting the cells, the lower face of the membrane was analysed using a 10X High Content Microscope and MetaExpress software.

2.4. Preparation of whole cell lysate

Cells were grown to 90% confluence in a 100 mm dish, washed twice with ice-cold 1X PBS (Catalog no. SH3025601, Thermo Scientific) and trypsinized in 0.25% Trypsin-EDTA 1X (Catalog no. 25200-056, Gibco) for 4 minutes at 37ºC. Trypsinized cells were pelleted down via centrifugation (800 rpm for 5 minutes) and resuspended in 1X PBS. The cell suspension was diluted 1 in 10 and $1x10^6$ cells were counted using a hemocytometer. These cells were pelleted at 500 rpm for 4 minutes. For lysis, approximately 100 µl of ice-cold 1X RIPA lysis buffer (Catalog no. 20-188, Millipore) supplemented with 1X phosphatase inhibitor cocktail (Catalog no. 524629, Millipore) and 1X protease inhibitor (Catalog no. P8340, Sigma Aldrich), was added to the cells. The cell pellet was resuspended in lysis buffer by vortexing at maximum speed for 15 seconds followed by incubation on ice for 10 minutes. After this, the lysate was sonicated twice for 3 seconds with 2 minutes incubation in between. Finally, the cell resuspension was centrifuged at maximum speed (13,200 rpm) for 10 minutes at 4ºC. The supernatant was immediately collected in a pre-chilled eppendorf tube and stored at -20ºC until further use. Throughout the procedure, resuspended cells were kept on ice to avoid any denaturation of proteins.

2.5. Cytoplasmic and nuclear fraction isolation

For isolation of cytoplasmic and nuclear fractions, the NE-PER nuclear/cytoplasmic extraction kit (Catalog no. 78833, Thermo Scientific) was used according to the manufacturer's protocol. The following volumes of reagents were used for both pairs of OS cell lines (Table 3). To reduce cytoplasmic contamination in our nuclear fractions, an additional wash step was introduced after collection of each cytoplasmic fraction. The pellet was first centrifuged at maximum speed for 2 minutes and any additional cytoplasmic fraction was removed by vacuum. It was then resuspended

(tap mixing) in 500 µl of ice-cold 1X PBS followed by centrifugation at maximum speed at 4ºC for 3 minutes. The supernatant was carefully removed by vacuum and the pellet was used for nuclear fraction isolation according to the manufacturer's protocol.

Table 4. Reagent volumes for the cytoplasmic and nuclear extraction kit

	Number of cells/pellet size	Cytoplasmic Extraction Reagent I (CER I)	Cytoplasmic Extraction Reagent II (CER II)	Nuclear Extraction Reagent (NER)
SaOS2/SaOS2-LM7	$1x10^6$ cells	200 µl	11 µl	50 µl
HOS/HOS-143B	$2x10^6$ cells	250 µl	20 µl	80 µl

2.6. Western Blot

Protein quantification of samples were carried out using a Pierce™ BCA (Bicinchoninic acid) protein assay kit (Catalog no. 23227, Thermo Scientific). The whole cell lysate samples were prepared by boiling 40 µg of protein in 1X loading buffer for 5 minutes. For the nuclear and cytoplasmic fractions, 36 µl of each fraction was boiled with 1X loading buffer for 5 minutes and then placed on ice for 5 minutes. The samples were run on a 7.5% SDS-PAGE and proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Catalog no. 1620177, Bio-Rad) at 110V for 70 minutes at 4ºC. Blocking of membrane was carried out for 1 hour using 5% non-fat dry milk powder in 1x TBS (Tris buffered saline) containing 0.1% Tween-20 (TBST), followed by incubation of the membrane in primary antibody overnight at 4ºC. The membranes were washed 3 times in TBST and then incubated in corresponding Horseradish peroxidase (HRP) linked secondary antibody for 1 hour at room temperature. After secondary antibody incubation, the blots were washed 3 times in TBST for 10 minutes each and visualized using SuperSignal West Femto (Catalog no. 34095, Thermo Fisher) or Western Lighting Plus ECL (Catalog no. NEL104001, Perkin Elmer).

The positive control used in Western blot experiments was non-stimulated A431 cell lysate (Catalog no. 12-301, Millipore Sigma). 2.5µg/100µl of β-merceptoethanol was added to A431 cell lysate and boiled for 5 minutes. The following antibodies were purchased commercially and used at the indicated dilutions: anti-β-catenin (Catalog no. 9587S, Cell Signaling) 1:1000; anti-Active-β-catenin (Catalog no. 05-665, Millipore) 1:500; anti-β-actin (Catalog no. sc69879, Santa Cruz) 1:10000; anti-α/β-tubulin (Catalog no. 2148; Cell signaling) 1:1000; anti-Lamin-B1 (Catalog no. MABE622) 1:1000; anti-GFP (Catalog no. 2555, Cell Signaling) anti-mouse IgG (Catalog no. NA934V, GE Healthcare); anti-rabbit IgG (Catalog no. NA931V, GE Healthcare) 1:10000. The figure below is a diagrammatic representation showing where anti-β-catenin and anti-Active-β-catenin antibodies bind to β-catenin and ABC proteins (Figure 5).

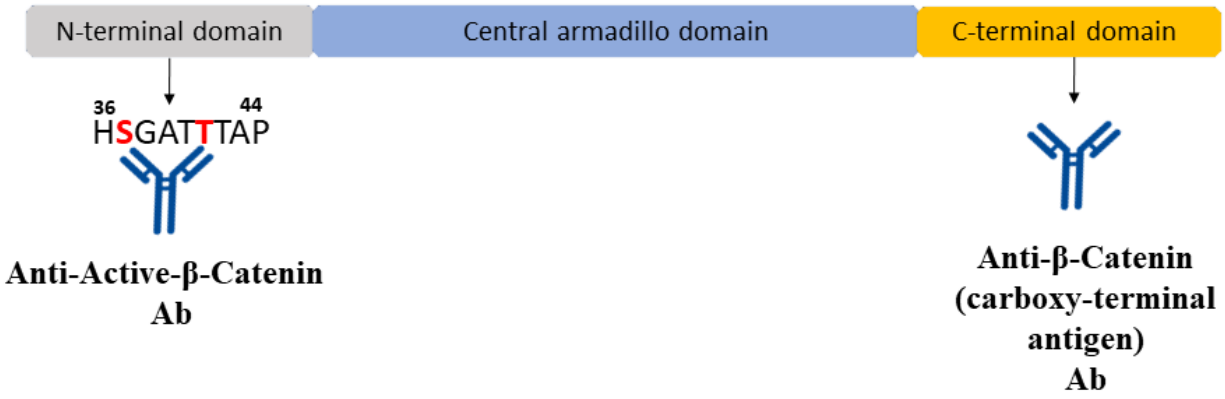


Figure 5. Binding sites for Anti-β-catenin and Anti-Active-β-catenin antibodies

The figure shows simplified structure of β-catenin/ABC and where the anti-β-catenin and anti-ABC antibodies bind to this protein. The anti-β-catenin antibody has its epitope in the C-terminal domain, such that it recognizes total β-catenin. The anti-ABC binds to N-terminal domain and its minimal epitope ranges from amino acid residue 36 to amino acid residue 44 of the protein and only recognizes ABC when serine 37 and threonine 41 are non-phosphorylated.

2.7. Immunofluorescence

Cells were grown to 30-40% confluence on coverslips. Cells were briefly washed with 1x PBS twice and fixed with 4% formaldehyde for 15 minutes at room temperature. Subsequently, cells were permeabilized with 100% methanol at -20°C for 10 minutes and blocked for 1 hour with 5% goat serum (Catalog no. 9023, Sigma Aldrich) in 1x PBS-Triton (0.3%). Cells were then incubated with 1:200 anti-β-catenin antibody (Catalog no. 2677, Cell Signaling) or 1:200 Anti-Active-β-catenin (ABC) diluted in blocking buffer overnight at 4°C. This was followed by incubation with AlexaFluor ® 555 goat anti-mouse antibody (Catalog no. A21422, Invitrogen) for visualization. At the completion of secondary antibody incubation, cell nuclei were stained with 300 nM 4', 6-diamidino-2-phenylindole (DAPI) for 7 minutes (Catalog no. D1360, Invitrogen). Coverslips were briefly rinsed with PBS and mounted on glass slides using Prolong antifade (Catalog no. P7481, Invitrogen). Washes were carried out 3 times, 5 minutes each with PBS after fixation, permeabilization and primary antibody incubation. Imaging was carried out at 40X magnification (oil immersion) using a Carl Zeiss Laser Scanning Microscope and image processing was carried out using LSM image browser software.

For co-localization study, cells transfected with ABC-pEGFP-C2, βcat-pEGFP-C2 and pEGFP-C2 plasmids were then used for immunofluorescence experiment according to the procedure described above.

2.8. High content microscopy

Cells were cultured in a 96-well plate (Catalog no. 655090, Greiner Bio one) to 70% confluence. Cell staining was carried out using the immunofluorescence protocol as described above. Images were taken at 10X (NA 0.3) magnification using an automated, high content screening system, ImageXpress Micro XLS, Molecular Devices (USA). The images were analyzed via cell scoring analysis which was performed in MetaXpress software, Molecular Devices. The cell scoring algorithm identifies a subpopulation of cells based on their fluorescence intensities. For our experiment, the software detected two fluorescence signals including Alexafluor-555 and DAPI via Cy3 and DAPI channels, respectively. The total number of cells were detected with DAPI

fluorescence which stained nuclei of the cells and cells positive for ABC were detected by signal from the Cy3 channel. To prevent inclusion of background staining, boundaries of cells and nuclei were demarcated. This was done based on the minimum and maximum size and minimum signal intensity from cells and nuclei. By providing these values to the software, the settings were configured to measure fluorescence intensity from cellular and nuclear areas of the cell. Once run, the software gave output values of cell/nuclear average intensity, cell/nuclear integrated intensity, etc.

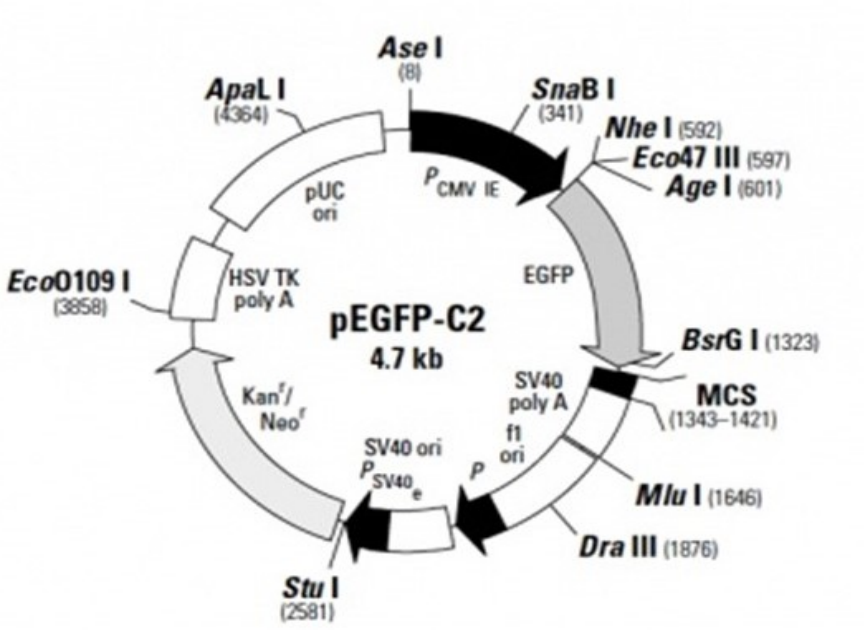
2.9. Quantitative Real-Time PCR

Total RNA was isolated using the RNeasy® Mini Kit (Catalog no. 74104, QIAGEN) as per the manufacturer's protocol. 1µg of total RNA was used for reverse transcription of Oligo (dT) (Invitrogen) and Superscript III reverse transcription (Vilnius, LT-02241, Applied Biosystems). Real time quantification of Cyclin D1, VEGF-A, MMP-2 and MMP-9 were assessed using power SYBR™ Green PCR Master Mix (Catalog no. 4309155, Applied Biosystems). GAPDH was used as the endogenous control. Samples were amplified with a precycling hold at 95˚C for 15 seconds, 30 cycles of annealing and extension at 60 ˚C for 1 minute. The following primers were used: Cyclin D1: sense (5`-CAT CTA CAC CGA CAA CTC CAT C-3`); Cyclin D1: anti-sense (5`-TCT GGC ATT TTG GAG AGG AAG -3`); VEGF-A: sense (5`-AGT CCA ACA TCA CCA TGC AG-3`); VEGF-A: anti-sense (5`-TTC CCT TTC CTC GAA CTG ATT T-3`); MMP-2: sense (5`-GGC CCT GTC ACT CCT GAG AT -3`); MMP-2: anti-sense (5`-GGC ATC CAG GTT ATG GGG GA-3`); MMP-9: sense (5'-CGA ACT TTG ACA GCG ACA AG-3'); MMP-9: anti-sense (5'-CAC TGA GGA ATG ATC TAA GCC C-3'); GAPDH: sense (5`-TCA ACG ACC ACT TTG TCA AGC TCA-3`); GAPDH: anti-sense (5`-GCT GGT GGT CCA GGG GTC TTA CT-3`). Each measurement was performed in triplicate with the Rotor-Gene-3000 instrument (Montreal Biotech Inc.) and analyzed using Rotor-Gene-6 Software. Gene expression was determined using the relative standard curve method normalized to GAPDH-binding protein expression. Histograms are reported as fold change of control which was set at 1.

2.10. ABC-pEGFP-C2 construct design

To design an ABC over-expression construct, we used pEGFP-C2 (Catalog no. 6083-1, Clontech) as the vector backbone. This backbone consists of several important features including: a CMV promoter, a GFP reporter gene and Neo/Kan resistance gene for bacterial and mammalian selection (Figure 6A). To mimic the partially phosphorylated state of ABC, modifications were made to amino acid sequence of wild-type β-catenin gene. As shown in Figure 6B, the N-terminal amino acids serine 33 and Serine 45, which are normally phosphorylated in ABC, were substituted by aspartic acid (D) to simulate constitutively phosphorylated serines. The N-terminal amino acids serine 37 and threonine 41, which are dephosphorylated in ABC, were represented by mutation of these amino acids to Alanine (A). The final pEGFP-ABC construct was created by GeneArt, Thermo Scientific and was verified by DNA sequencing at the Molecular Biology Facility, Department of Biological Sciences, University of Alberta.

A.



The plasmid map is reprinted from ©1997, CLONTECH Laboratories, Inc.

B.

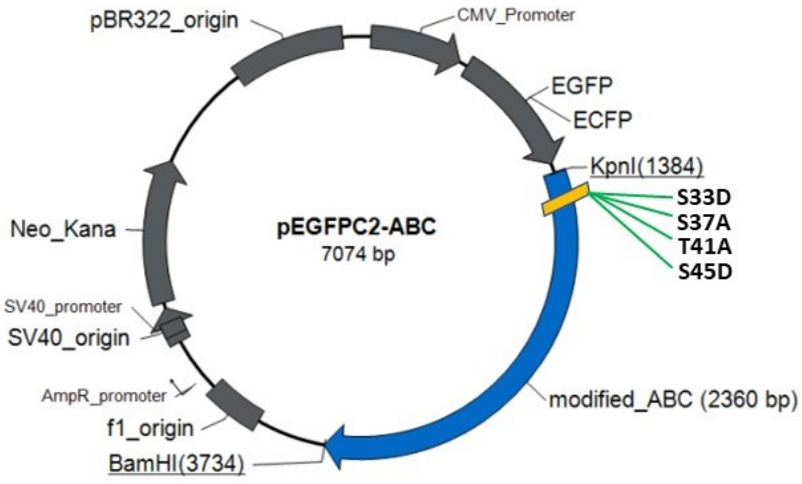


Figure 6. Detailed map of pEGFP-C2 and ABC-pEGFP-C2 plasmid constructs

A) pEGFP-C2 vector backbone. The map identifies unique restriction enzyme sites present in the sequence. All coding regions are indicated by arrows. **B)** ABC-pEGFP-C2 construct. The map identifies the unique restriction enzyme sites including KpnI and BamHI which were cut to insert the ABC gene into the plasmid. The amino acid substitutions made in β-catenin gene to mimic ABC are also shown in the diagram, using one-letter amino acid code. The arrow shows coding region of the plasmid. There is an arrow present at the end of EGFP but there is no stop codon such that transcription continues after EGFP, producing a fusion protein with ABC. Antibiotic resistance gene present in the plasmid include Kanamycin for bacterial selection and Neomycin for mammalian selection. EGFP: Enhanced green fluorescent protein; CMV promoter: Cytomegalovirus promoter; SV40: Simian virus 40.

2.11. Transient transfection of plasmids in SaOS2 and HOS cells

All transfection experiments carried out were transient transfections. For transfecting ABC-pEGFP-C2, βcat-pEGFP-C2 and pEGFP-C2 plasmids in SaOS2 and HOS cells, we compared the relative transfection efficiency of three different transfection reagents: Lipofectamine® LTX (Catalog no. 15338100, Thermo Fisher Scientific), Lipofectamine® 3000 (Catalog no. L3000008, Thermo Fisher Scientific) and FuGENE® 6 (Catalog no. E2691, Promega). Cells were seeded in 6-well plates and were transiently transfected when they were at 70-80% confluence. Quantities of reagents/well for the various transfection reagent cocktails are shown in Table 4 below. Transfections with each reagent was done according to the manufacturer's protocol. However, for Lipofectamine® LTX and Lipofectamine® 3000, the manufacturer's protocol was modified to reduce cell death post-transfection. For this, cells were incubated in transfection reagent + DNA complexes for only 6 hours at 37ºC and 20% O_2 and 5% CO_2. After this, the transfection reagent + DNA complexes were removed, and the cells were washed once with complete medium. They were then incubated overnight in complete medium at 37ºC and 20% O_2 and 5% CO_2. 24 hours post-transfection, the cells were visualized under a fluorescence microscope to determine transfection efficiency.

For co-localization study, SaOS2 cells in 6-well plate were transfected with Lipofectamine® LTX reagent using the protocol mentioned above.

Table 5. Optimal quantities of transfection reagents used

	Lipofectamine® LTX	Lipofectamine® 3000	FuGENE® 6
DNA	3.5 µg	3.5 µg	3 µg
OptiMEM (31985-070, Gibco)	150 µl x 2	150 µl x 2	90 µl
Transfection reagent	9 µl	7.5 µl	9 µl
Plus reagent (P3000™/PLUS™)	6 µl	6 µl	-

2.12. Tissue Immunohistochemistry

For immunohistochemical staining, formalin-fixed, paraffin embedded (FFPE) EOE tissue slides were obtained from Dr. Consolato Sergi, Department of Lab Medicine and Pathology, University of Alberta. We used EOE with different number of eosinophils for our experiments. Samples with no eosinophils were categorized as zero level, 5-15 eosinophils as intermediate and >25 as high level EOE stage. FFPE osteosarcoma tissue slides were obtained from Dr. Atilano Lacson, Department of Laboratory Medicine and Pathology, University of Alberta. FFPE colon cancer and spleen tissue blocks were obtained from Dr. Judith Hugh, Department of Laboratory Medicine and Pathology, University of Alberta. Colon cancer and spleen tissue were used as positive and negative controls respectively. The Osteosarcoma Tumor Tissue Array (TMA) was purchased from Folio Biosciences (Catalog no. ARY-HH0085) and consisted of 40 unique cores in duplicates. Information about each patients' age, sex, disease pathology and tumor stage was provided. The ethics approval for use of human tissue samples is in place for University of Alberta Hospital/ University of Alberta site.

Antibodies used for staining included: Anti-Active-β-catenin (Catalog no. 05-665, Millipore) and Anti-β-Catenin Clone 14/Beta-Catenin (Catalog no. 610154, BD Biosciences).

For deparaffinization and rehydration, TMA or tissue slides were baked at 60 degrees for 2 hours, immersed in xylene (2X) for 2 minutes followed by graded EtOH including 100% (2X), 95%, 85%, 75% and 50% respectively for 2 minutes each and then ddH2O for 5 minutes. Antigen presentation was carried out by immersing slides in boiling sodium citrate (10mM) for 30 minutes. The slides were washed 3X with wash solution (1X PBS with 0.05% Triton X-100) for 5 minutes each after each step starting from antigen presentation till after ABC solution incubation. Thereafter, slides were blocked for 2 hours in blocking buffer (1X PBS, 5% goat serum, 0.2% Triton X-100, 0.1% BSA and ddH_2O) followed by overnight incubation with primary antibody (1:200 dilution) at 4^0C. Following primary antibody incubation, slides were incubated with 0.3% H_2O_2 for 30 minutes and then incubated with HRP-labelled secondary antibody (Catalog no. NEF822001EA, Perkin Elmer) for 2 hours. For signal amplification, slides were treated with Tyramide Signal Amplification (Catalog no. NEL700A001KT, Perkin Elmer) reagent and Avidin-

Biotin Complex solution (Catalog no. PK-6100, Vector Laboratories) for 7 and 30 minutes respectively. After this, they were immersed in DAB chromogenic substrate (Catalog no. SK-4105, Vector Laboratories) for 1-10 minutes until a brown stain was detected and then washed under running tap water for 5 minutes. This was followed by hematoxylin (Catalog no. SH26-500D, Fisher Scientific) staining for 30 seconds, brief tap water washing, Scott's Top water (3.5g sodium bicarbonate, 20g magnesium sulphate and ddH_2O) incubation and washing again. Consequently, the slides were dehydrated in graded EtOH including 50%, 75%, 85%, 95% and 100% respectively for 2 minutes each, followed by xylene for 2 minutes as well. Coverslips were then mounted on slides using Permount (Catalog no. SP15-100, Fisher Scientific).

The TMA was scored by two pathologists who were blinded to all patient information. The cores in the TMA were scored based on the percentage of positive nuclear ABC staining cells in each core. The scoring was assigned as follows: 0 (no nuclear staining); 1+ (≤30% of positive nuclear staining cells); 2+ (31-60% of positive nuclear staining cells) and 3+ (>60% of positive nuclear staining cells. Since the cores were in duplicates, a difference in percentage of >10% for positive nuclear ABC cells was considered discrepant, and hence the higher score was selected. For any difference in percentage of <10%, average staining percentage was calculated for duplicate cores. The patients in TMA were divided into two age groups: (i) Children and Adolescents and Young Adults (AYA) for patients aged ≤ 25 years and (ii) Adults for patients aged > 25 years.

2.13. TCF/LEF Transcriptional Activity

The M50 Super 8X TOPFlash (Catalog no. 12456, Addgene) and M50 Super 8X FOPFlash (Catalog no. 12457, Addgene) plasmids were purchased from Addgene (they were a kind gift by Randall Moon to Addgene, (Veeman, Slusarski, Kaykas, Louie, & Moon, 2003)). HOS and HOS-143B cell lines were cultured in 100 mm plate to 80% confluence. TCF/LEF induced transcriptional activity was determined by using a TCF/LEF promoter-luciferase reporter construct, pTOPFlash, by techniques reported previously (Kidani *et al*., 2014) and according to the manufacturer's instructions (Promega E1500). Briefly, a luciferase reporter assay was performed whereby cells (HOS and HOS-143B) were transfected using Lipofectamine® 3000 (Catalog no. L3000008, Invitrogen) with the TCF promoter/luciferase reporter gene (pTOPFlash)

and subsequently treated with 10mM LiCl (for the activation of Wnt/β-catenin signaling). The TOPFlash-luciferase reporter construct specifically measures β-catenin-ABC/TCF regulated transcriptional function and is comprised of a multimeric synthetic β-catenin/TCF-4 binding site upstream of a Thymidine Kinase (TK) minimal promoter and a Luciferase open reading frame. A mutated TCF-Luciferase reporter construct (pFOPFlash) served as a negative control for TOPFlash activity. The reporter activity ratio was measured using a luminometer (Fluor Star OMEGA: BMG Labtech).

The plasmid map was reprinted from Addgene website.

Figure 7. M50 Super 8X TOPFlash plasmid construct map.

The M50 Super 8X TOPFlash plasmid map shows important features of this construct. All unique restriction enzyme sites are identified. Other important features such as the origin of replication and antibiotic resistance gene are also marked in purple color. All coding regions of the plasmid are shown with arrows. The luciferase gene is identified with a yellow arrow on the map.

2.14. Statistics

One and two-sided Student's t-test (GraphPad PRISM Software; GraphPad Software, Inc., CA, USA) were used to compare differences between groups. Results are presented as Mean ± SEM and values with $p<0.05$ were considered statistically significant. To check for correlation between patients' gender and nuclear ABC staining, the Fisher Exact test was used. Statistical analysis to compare tumors with positive/negative nuclear ABC staining with patient age was done using the non-parametric Mann-Whitney test. Association between stage of tumor and positive nuclear ABC staining was analyzed using the Kruskal Wallis Trend test.

Chapter 3

Results

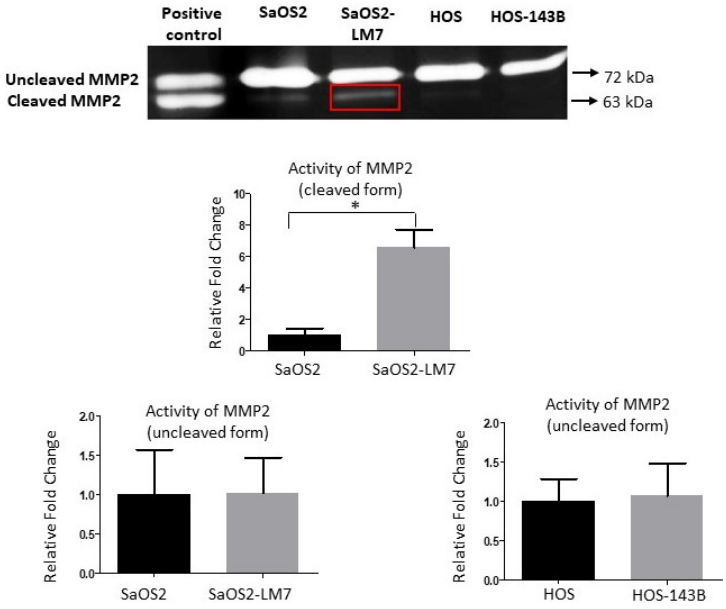
3.0.Results

3.1. Metastatic potential of OS cell lines increases with OS progression

Before using the OS paired cell lines, it was important to establish that SaOS2-LM7 and HOS-143B cell lines have higher metastatic potential than the SaOS2 and HOS cell lines, respectively. To confirm metastatic potential, MMP activity was measured via gelatin zymography. MMP2 and MMP9 are matrix metalloproteinases which are secreted as proenzymes into the extracellular space and activated by proteolytic cleavage (Kiczak *et al*., 2013). They are active in digestion of most extracellular matrix proteins, specifically the basement membrane, allowing tumor cells to invade through the matrix and metastasize to other sites in the body (Aimes & Quigley, 1995, Esparza *et al*., 1999). Since they help in metastasis, cancer cells with higher metastatic and invasive potential are considered to have higher activity of MMPs (Yu *et al*, 1996). In fact, a number of studies have investigated MMP2 and MMP9 expression in osteosarcoma. For instance, one study compared MMP2 and MMP9 activity with invasive properties of OS cell lines and demonstrated that the cell line with highest invasion also secreted highest amounts of MMP2 and MMP9 (Bjørnland *et al*., 2005). In another study, expression of MMP9 was shown to be present in 90% of OS patient samples tested, demonstrating that MMP9 activation is a likely event to occur in osteosarcoma (Yoo *et al*., 2005). In addition to this, two other studies have reported MMP2 and MMP9 expression to be associated with pulmonary metastasis and lower overall survival in osteosarcoma respectively (H. Li *et al*., 2014; M. Zhang & Zhang, 2015). With known expression of MMP2 and MMP9 in osteosarcoma and its potential association with invasion and metastasis, we measured and compared the activity of both MMPs in all four OS cell lines.

Our results show that the gelatinase activity of the pro-enzyme form or inactive form of MMP2 (~72 kDa) was similar in each pair of the two paired cell lines (Figure 8A). However, the gelatinase activity of the active form of MMP2 (~63 kDa) was significantly greater in SaOS2-LM7 compared to its parental cell line SaOS2 (Figure 8A). In contrast, the gelatinase activity of MMP9 (~82 kDa) was significantly greater in HOS-143B compared to the parental cell line HOS (Figure 8B). There was no measurable MMP9 activity in SaOS2/SaOS2-LM7 cells and we did not observe any measurable MMP2 activity in the HOS/HOS 143B cell pair.

A.



B.

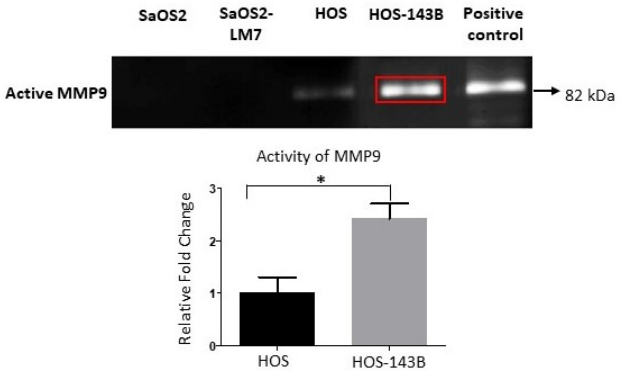


Figure 8. Gelatin zymography shows metastatic potential of OS cell lines increases with OS progression

Gelatin zymography for A) MMP2 B) MMP9, using concentrated conditioned media from cells grown in serum free media for 24 h, conditioned media from HT1080 cell line were used as positive control. **A)** No significant change was observed in the uncleaved form of MMP2 in both pairs of cell lines. However, a significant increase was observed in the activity of cleaved (active) MMP2 in SaOS2-LM7 compared to SaOS2 n=5, *p<0.05. **B)** For MMP9, there was a significant increase in its activity in HOS-143B compared to HOS, n=3, *p<0.05. n=2 repeats of this experiment were carried out by Geetha Vankateswaran.

3.2. Invasive potential of OS cell lines increases with OS progression

When cancer cells learn to survive without cell-cell adhesion, they can also dissociate from primary tumor and can invade through surrounding matrix to metastasize to other parts of the body. The ability of malignant cells to invade through the extracellular matrix is an essential component of metastasis and is supported by secretion of enzymes such as MMPs to digest the matrix and also by regulation of those proteins which are involved in cell migration and motility (Tracey A. Martin, Lin Ye, Andrew J. Sanders, Jane Lane, 2000). Thus, in addition to confirmation of metastatic potential for OS cell lines, it was important to determine their invasive abilities and whether the invasiveness increased with OS progression or not. The Transwell® Invasion Assay kit was used for measuring invasion of all four OS cell lines including SaOS2, SaOS2-LM7, HOS and HOS-143B. The non-invasive breast cancer cell line MCF-7 was used as a negative control and only medium was used as a blank. There was no invasion seen in both our negative control (MCF-7 cells) and in blank medium.

(i) *SaOS2/SaOS2-LM7*:

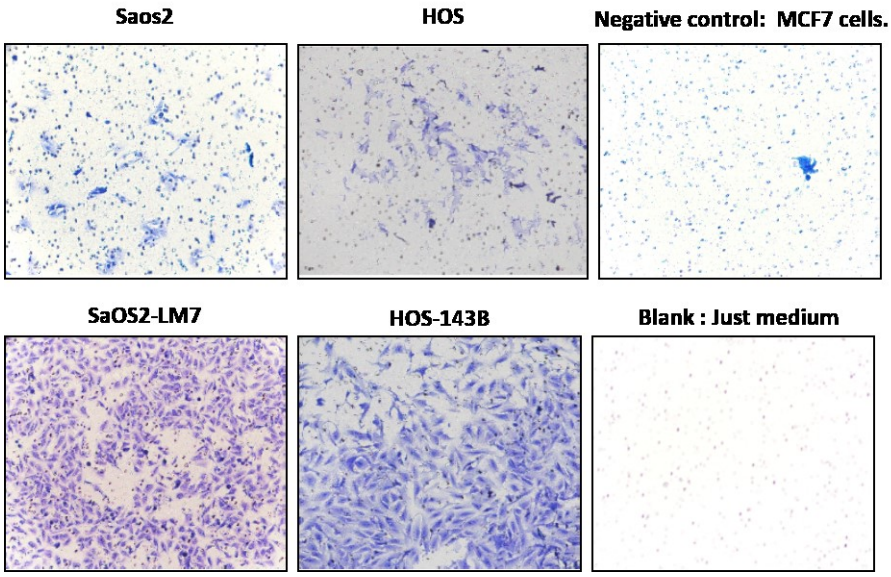
Our results show that for SaOS2-LM7, a significantly higher number of cells were able to invade through the matrix when compared to SaOS2 (*$p<0.05$). This corroborates our previous result of higher active MMP2 activity in SaOS2-LM7 compared to SaOS2 (Figure 8A). Thus, our results support increased invasive-metastatic ability for SaOS2-LM7 than SaOS2.

(ii) *HOS/HOS-143B*

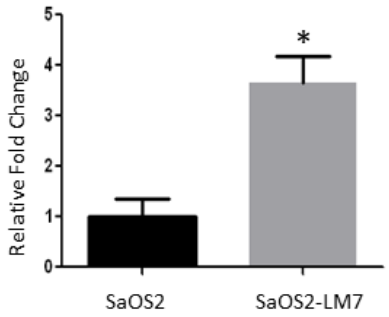
For the HOS/HOS-143B pair, there was approximately 3-fold higher number of invaded cells for the more metastatic HOS-143B compared to parental HOS cells (**$p<0.01$). This is also in agreement with significantly higher activity of active MMP9 seen in HOS-143B when compared to HOS cells (Figure 8B).

With increased invasion and metastatic ability, SaOS2-LM7 and HOS-143B can be determined as more invasive-metastatic than their parental counterparts SaOS2 and HOS, respectively and hence can be used as an effective model to show OS progression (from less invasive to more invasive-metastatic).

A.



B.



C.

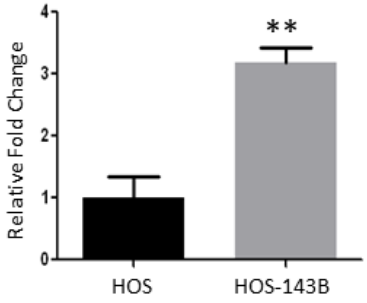


Figure 9. Cell Invasion Assay shows higher invasive potential of OS cell lines with OS progression

A) The upper panel shows images taken for invasive cells. **B)** There is a significant increase in number of invaded cells for SaOS2-LM7 compared to SaOS2; n=3, *$p<0.05$. **C)** Similarly, the number of cells that invaded to the lower face of the membrane was significantly higher for HOS-143B compared to HOS; n=3, **$p<0.01$.

3.3. Higher total cellular protein levels of ABC in metastatic OS cells

Since Wnt/β-catenin pathway is known to be dysregulated in Osteosarcoma and β-catenin/ABC are key effector molecules of this pathway, we investigated the protein levels of ABC and β-catenin in whole cell lysates of all four OS cell lines using Western blot analysis (MacDonald *et al*., 2009). β-actin was used as a loading control and showed equal loading for all samples.

(i) *SaOS2/SaOS2-LM7*

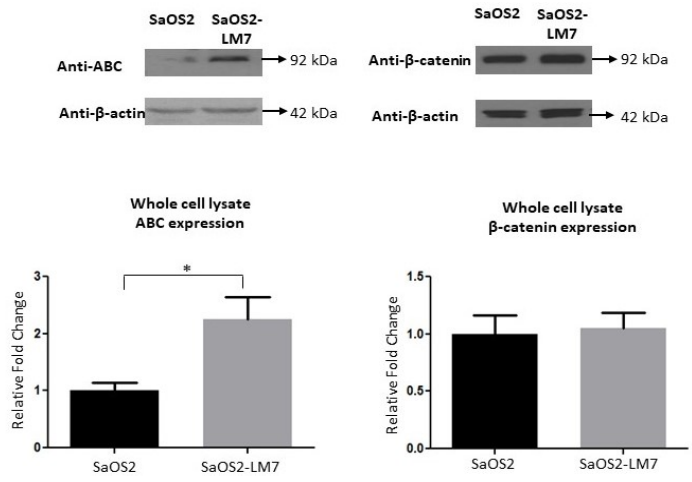
As shown in Figure10, cellular ABC protein levels (92 kDa) are greater in SaOS2-LM7 when compared to SaOS2 (*$p<0.05$). However, there was no significant difference seen in β-catenin levels between SaOS2 and SaOS2-LM7.

(ii) *HOS/HOS-143B*

In this pair, total cellular ABC protein levels for HOS-143B were approximately 2-fold higher in comparison to its parental cell line HOS (*$p<0.05$, Figure 10B). Similar to SaOS2/SaOS2-LM7 pair, β-catenin levels were not significantly different between HOS and HOS-143B.

Hence, our Western blot results showed greater total ABC levels in invasive-metastatic OS cell lines compared to parental ones, with no change in β-catenin levels.

A.



B.

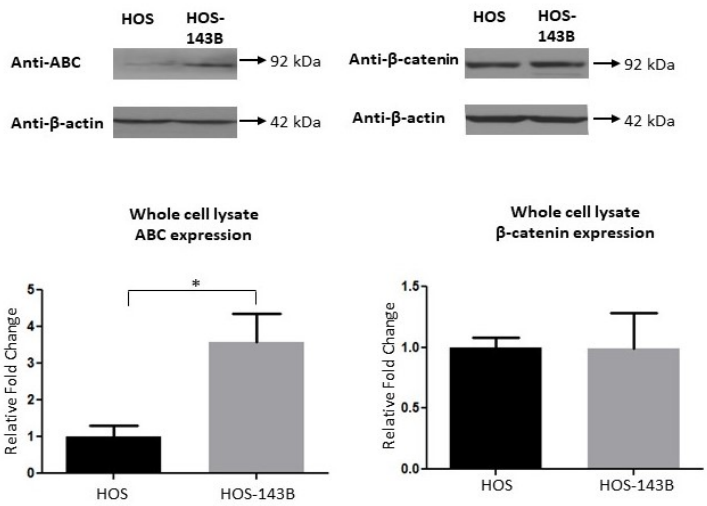


Figure 10. Western Blot analysis of ABC and β-catenin in whole cell lysate of OS cell lines

A) **SaoS2/SaOS2-LM7**. The cellular levels of ABC were increased significantly in the metastatic cell line SaOS2-LM7 compared to SaOS2 cells; n=3, *p<0.05. No significant difference in cellular levels of β-catenin was observed. **B) HOS/HOS-143B**. The cellular levels of ABC were higher in HOS-143B compared to HOS n=3, *p<0.05. However, no differences in the cellular levels of β-catenin was observed with OS progression. Blots represented are from one experiment and the graphs are representative of three experiments. All repeats of this experiment were carried out by Geetha Venkateswaran (Venkateswaran, 2016).

3.4. ABC levels are higher in nuclear fractions of SaOS2-LM7 & HOS-143B

With upregulated levels of total ABC in SaOS2-LM7 and HOS-143B, we next determined if ABC levels increase in the nuclear and/or cytoplasmic fractions of the cells. Since ABC is proposed to transduce Wnt/β-catenin signals by binding to TCF/LEF family of transcription factors, it would be pertinent to evaluate its levels in the nucleus (Staal *et al*., 2002). A higher presence of ABC in the nucleus could be potentially correlated to its increased activity in cells. Hence, we investigated ABC and β-catenin levels in cytoplasmic and nuclear fractions of OS cell lines via Western blot analysis. α/β Tubulin and Lamin B1 were mainly seen in cytoplasmic and nuclear fractions, respectively. This was to indicate the relative enrichment of these two subcellular fractions prior to immunoblotting and to ensure that there was minimal cross-contamination in our cytoplasmic and nuclear fractions.

(i) *SaOS2/SaOS2-LM7*

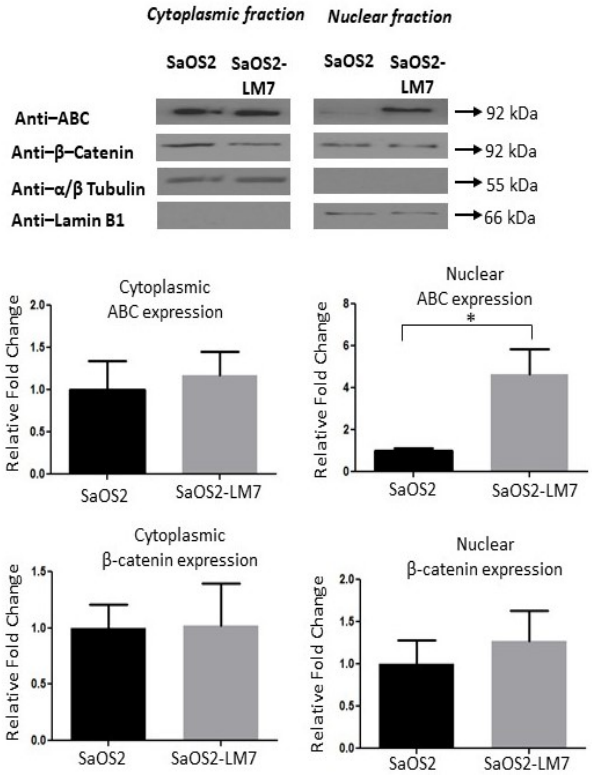
There is a significant, approximately greater than 3-fold increase, in ABC levels in nuclear fraction for SaOS2-LM7 compared to SaOS2 (*$p<0.05$, Figure 10A). However, there was no difference seen for ABC levels in the cytoplasmic fractions for this pair of cell lines. β-catenin levels were similar and comparable in the cytoplasmic and nuclear fractions of both cell lines in this pair.

(ii) *HOS/HOS-143B*

Similarly, for HOS/HOS-143B, ABC levels were significantly higher in nuclear fraction for the more metastatic HOS-143B compared to HOS cells (*$p<0.05$, Figure 10B). There was no difference seen for ABC levels in the cytoplasmic fraction and for β-catenin levels in cytoplasmic and nuclear fractions.

Taken together, our Western blot results show that high protein levels of total and nuclear ABC are present in SaOS2-LM7 and HOS-143B than in SaOS2 and HOS respectively.

A.


Cytoplasmic fraction
Nuclear fraction
SaOS2
SaOS2-LM7
SaOS2
SaOS2-LM7
Anti–ABC
Anti–β–Catenin
Anti–α/β Tubulin
Anti–Lamin B1
92 kDa
92 kDa
55 kDa
66 kDa
Cytoplasmic ABC expression
Nuclear ABC expression
*
Relative Fold Change
SaOS2
SaOS2-LM7
Cytoplasmic β-catenin expression
Nuclear β-catenin expression

B.

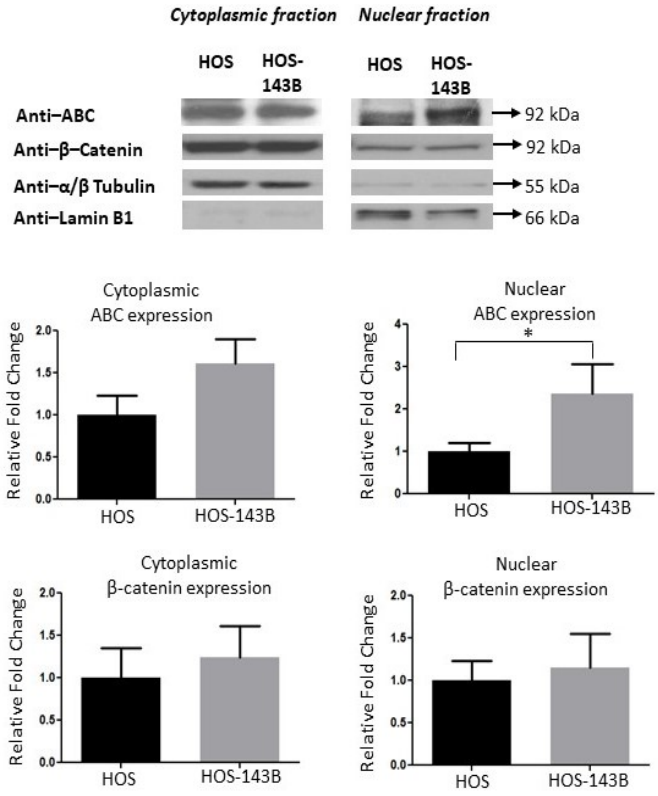


Figure 11. Western Blot analysis for ABC and β-catenin in cytoplasmic and nuclear fractions **A)** SaOS2/SaOS2-LM7. Nuclear ABC levels were significantly higher in SaOS2-LM7 compared to SaOS2; n=3, *$p<0.05$ (one-tailed t-test). However, no differences in the levels of cytoplasmic ABC, cytoplasmic β-catenin and nuclear β-catenin were observed. Blots represented are from one experiment and the graphs are representative of three experiments. All repeats of this experiment were carried out by Geetha Venkateswaran (Venkateswaran, 2016). **B) HOS/HOS-143B**. Nuclear ABC levels were significantly higher in HOS-143B compared to HOS; n=5, *$p<0.05$ (one-tailed t-test). However, no differences in the levels of cytoplasmic ABC, cytoplasmic β-catenin and nuclear β-catenin were observed. Blots represented are from one experiment and the graphs are representative of five experiments.

3.5. Differential localization pattern for ABC in OS cell lines

While cellular and sub-cellular levels of ABC and β-catenin were determined through Western blot analysis, the cellular localization of ABC and β-catenin was investigated via immunofluorescence (IF) analysis in the two paired cell lines.

(i) SaOS2/SaOS2-LM7

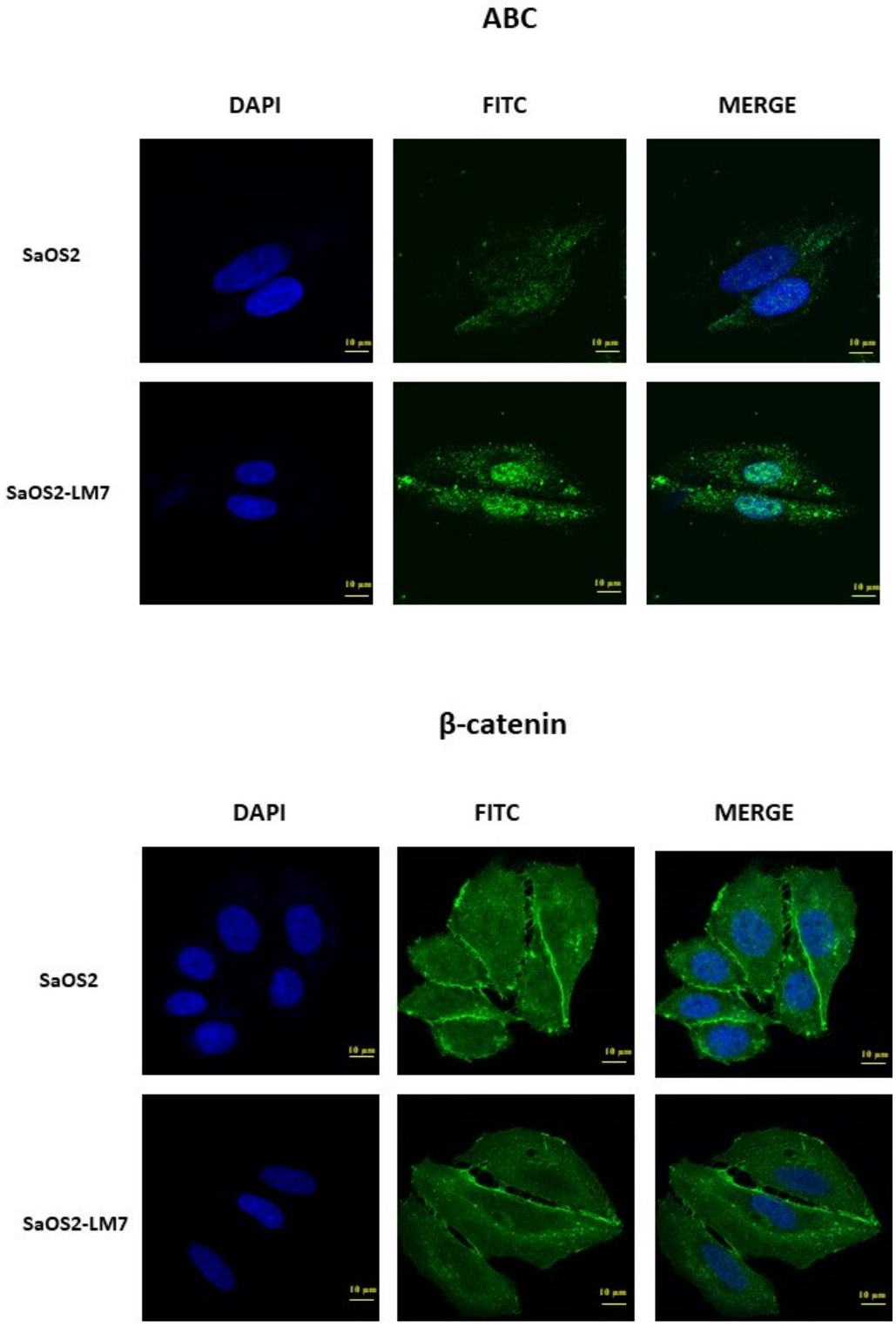
Our immunofluorescence results (Figure 12A) show a predominant nuclear localization of ABC. To be specific, the fluorescence intensity for ABC was higher in SaOS2-LM7 when compared to SaOS2. However, β-catenin localized mainly to the cytoplasm of the cells, with no detectable presence in the nucleus. Moreover, β-catenin's staining intensity was similar in both SaOS2 and SaOS2-LM7 cells.

(ii) HOS/HOS-143B

For this pair (Figure 12B), fluorescence for ABC could be seen predominantly in the nuclear and peri-nuclear region for both HOS and HOS-143B. However, greater fluorescence intensity for ABC was seen in HOS-143B when compared to HOS. Similar to SaOS2/SaOS2-LM7, β-catenin showed no difference in its localization and fluorescence intensity for both HOS and HOS-143B.

The observed patterns of localization for ABC and β-catenin in OS cell lines and higher fluorescence intensity for ABC in nuclei of more metastatic OS cell lines, suggest a putative role of ABC in OS progression.

A.


ABC
DAPI
FITC
MERGE
SaOS2
SaOS2-LM7
β-catenin
DAPI
FITC
MERGE
SaOS2
SaOS2-LM7

B.

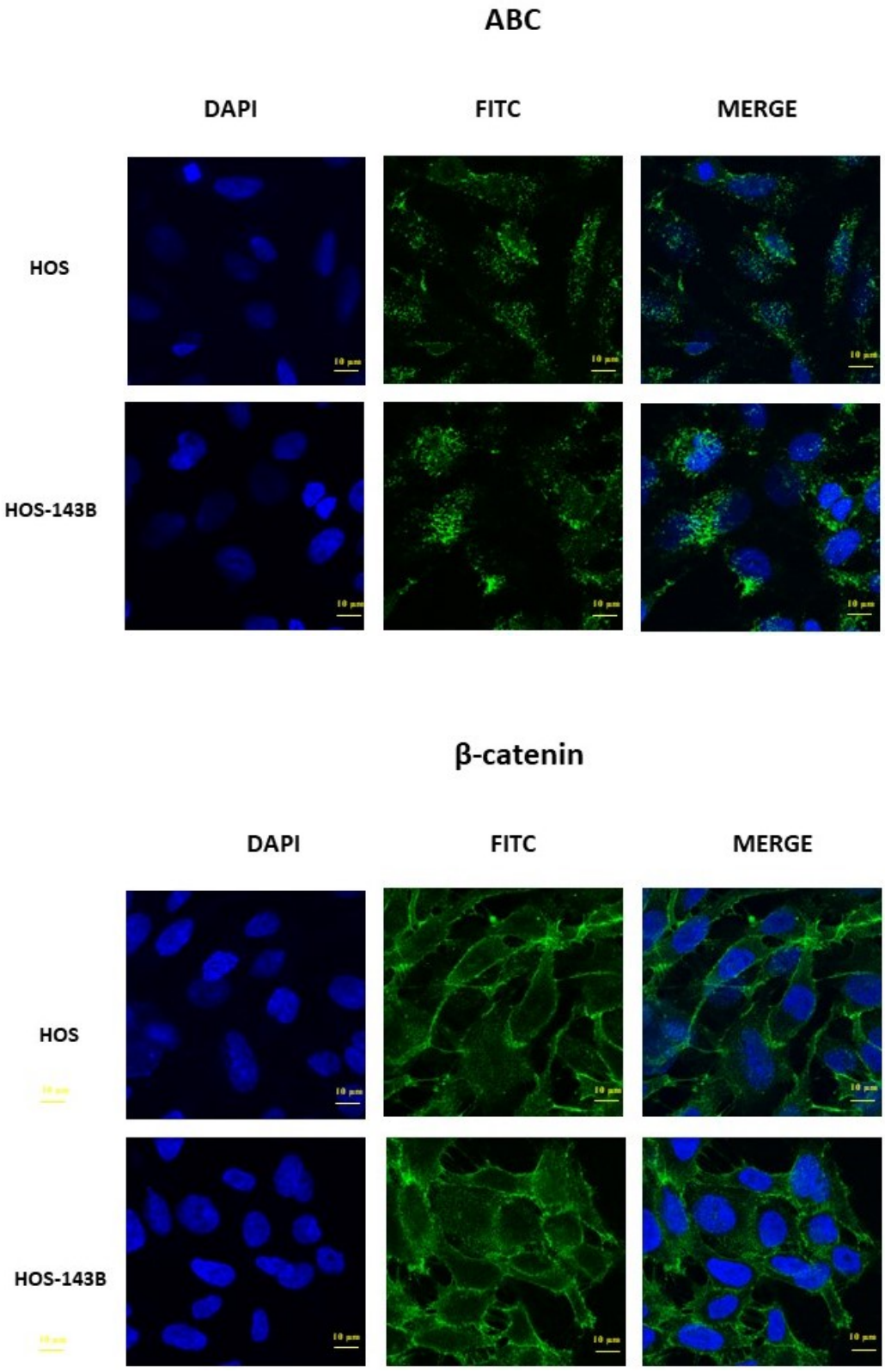

ABC
DAPI
FITC
MERGE
HOS
HOS-143B
β-catenin
DAPI
FITC
MERGE
HOS
HOS-143B

Figure 12. IF analysis for localization of ABC and β-catenin in OS cell lines.

A) SaOS2/SaOS2-LM7. ABC shows an increased localization in the nuclear region for SaOS2-LM7 compared to SaOS2 with β-catenin localized mainly to the cytoplasm for both SaOS2 and SaOS2/LM7; n=3. All repeats of this experiment were carried out by Geetha Venkateswaran (Venkateswaran, 2016). **B) HOS/HOS-143B** Immunofluorescence analysis shows increased nuclear localization of ABC in HOS-143B cell line compared to HOS with β-catenin localized mainly to the cytoplasm for both HOS and HOS-143B; n=3. n=1 repeat of this experiment was carried out by Geetha Venkateswaran (Venkateswaran, 2016).

3.6. Higher total cellular and nuclear ABC fluorescence intensity in metastatic OS cell lines compared to parental cell lines.

After observing ABC localization pattern and fluorescence intensity via immunofluorescence, we then quantified ABC fluorescence intensity through High Content analysis (HCS). Through HCS, we could not only quantify intensity from a large number of cells but could also measure fluorescence intensity from specific regions of the cell such as the nucleus.

(i) *SaOS2/SaOS2-LM7*

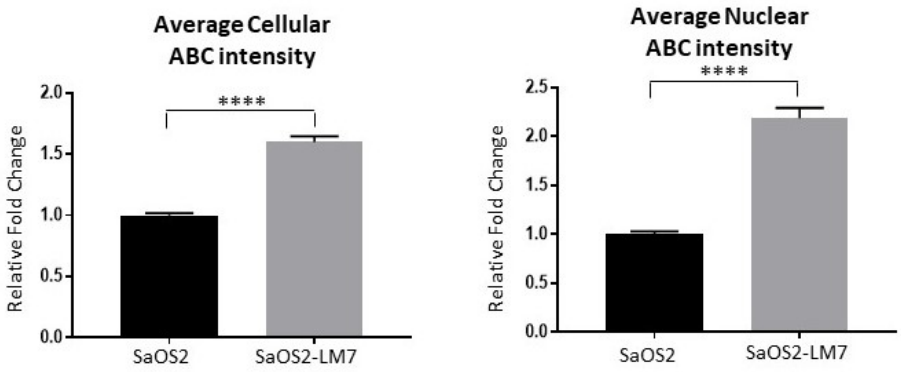
ABC intensity was significantly greater in total cellular and nuclear regions for SaOS2-LM7 when compared to SaOS2 (****$p<0.0001$, Figure 13A). This is in agreement with our results for Western blot analysis where we see a significant increase in ABC protein in whole cell lysate and nuclear fraction of SaOS2-LM7 compared to SaOS2.

(ii) *HOS/HOS-143B*

Similarly, for the HOS/HOS-143B pair, we see significantly higher ABC intensity from total cellular and nuclear regions for HOS-143B when compared to HOS (****$p<0.0001$, Figure 13B).

Thus, our high content analysis results supported our results of higher ABC presence in more metastatic OS cell lines compared to their parental cell lines.

A.



B.

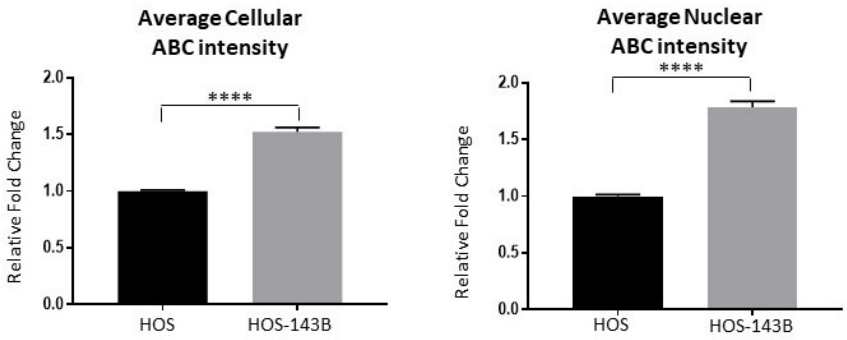


Figure 13. High Content Analysis for quantification of total cellular and nuclear ABC fluorescence intensity in OS cell lines.

Immunofluorescence images from high content microscopy were quantified using MetaXpress software for average cellular and average nuclear intensities. **A) SaOS2/SaOS2-LM7**. Analysis shows a very significant increase in the cellular and nuclear intensities of ABC with OS progression in SaOS2-LM7 compared to SaOS2; n=3, ****$p<0.0001$. **B) HOS/HOS-143B.** Similar significant increase is observed in cellular and nuclear ABC intensity in the more metastatic HOS-143B compared to HOS; n=3, ****$p<0.0001$. All repeats for HOS/HOS-143B pair were carried out by Geetha Venkateswaran.

3.7. Increased gene expression of downstream target genes of ABC in metastatic OS cell lines

Since ABC is a transactivation factor and binds to TCF/LEF to induce transcription of its downstream target genes, one way to measure ABC activity is by quantifying transcriptional expression of its downstream target genes in the two paired cell lines. We carried out this measurement via quantitative real-time PCR analysis. Four target genes were selected, and included MMP2, MMP9, Cyclin D1 and VEGFA. GAPDH was used as a housekeeping gene for endogenous control.

(i) SaOS2/SaOS2-LM7

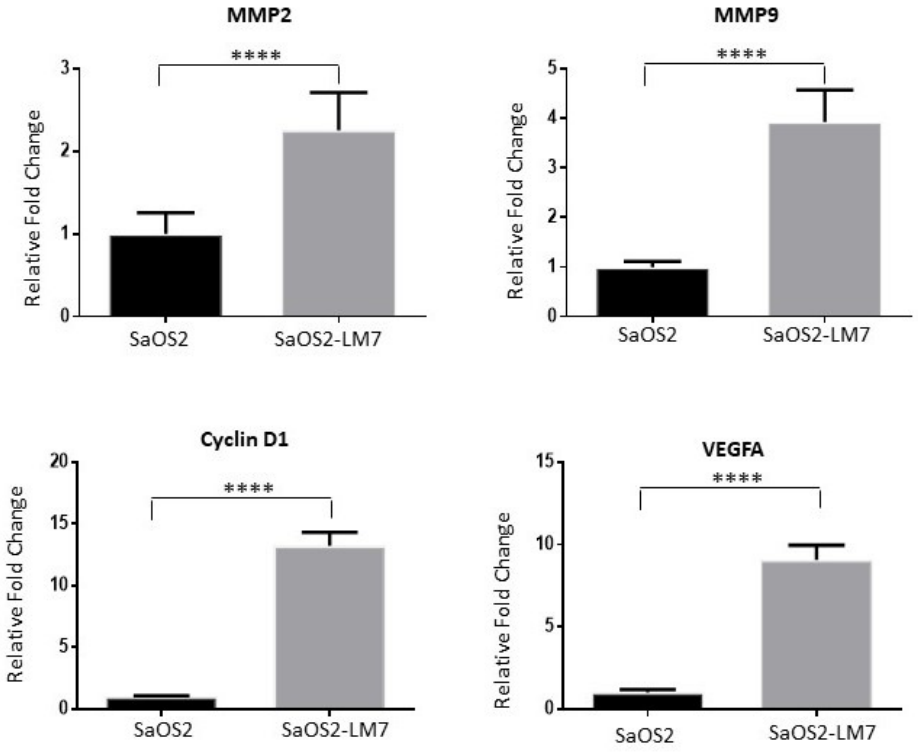
All four genes showed a significantly higher transcriptional expression in the more metastatic SaOS2-LM7 compared to SaOS2 (****$p<0.0001$, Figure 14A). Specifically, for Cyclin D1 and VEGFA, the gene expression was approximately 14-fold and 8-fold higher respectively for SaOS2-LM7 than SaOS2. The increased gene expression for MMP2 in SaOS2-LM7 also aligns with our previous results for higher MMP2 activity (active form) in SaOS2-LM7 compared to SaOS2. However, overall, the qRT-PCR results reflect significantly greater ABC activity in the more metastatic OS cell line for this pair.

(ii) HOS/HOS-143B

For the HOS/HOS-143B pair, there was increased gene expression for MMP2, Cyclin D1 and VEGFA in HOS-143B compared to HOS (*$p<0.05$, ****$p<0.0001$, Figure 14B). Similar to SaOS2/SaOS2-LM7, gene expression for Cyclin D1 was significantly higher (approximately 13-fold change) in HOS-143B when compared to HOS. Interestingly, there was no significant difference seen in MMP9 gene expression between HOS and HOS-143B which contradicts our previous finding of higher MMP9 activity (active form) in HOS-143B than in HOS.

Thus, the significantly upregulated transcriptional expression of these downstream target genes is consistent with higher ABC levels in more metastatic OS cell lines.

A.


MMP2
****
Relative Fold Change
3
2
1
0
SaOS2
SaOS2-LM7
MMP9
****
Relative Fold Change
5
4
3
2
1
0
SaOS2
SaOS2-LM7
Cyclin D1
****
Relative Fold Change
20
15
10
5
0
SaOS2
SaOS2-LM7
VEGFA
****
Relative Fold Change
15
10
5
0
SaOS2
SaOS2-LM7

B.

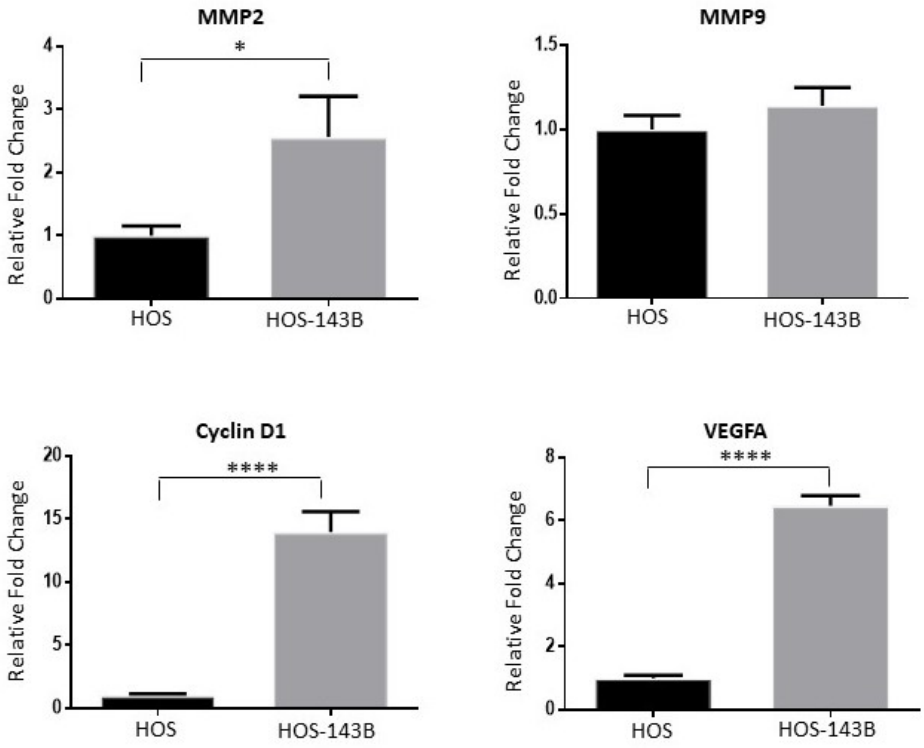


Figure 14. RT-qPCR analysis for quantifying expression of ABC target genes in OS cell lines. A) SaOS2/SaOS2-LM7. mRNA expression of ABC target genes (MMP2, MMP9, Cyclin D1 and VEGF-A). Target gene expression was quantified by RT-qPCR and shows that mRNA expression of all target genes is higher in the SaOS2-LM7 cell line compared to the parental SaOS2 cells. **B) HOS/HOS-143B**. mRNA expression of ABC target genes (MMP2, MMP9, Cyclin D1 and VEGF-A). Target gene expression was quantified by RT-qPCR and shows that mRNA expression of MMP2, Cyclin D1 and VEGF-A is significantly higher in HOS-143B compared to parental HOS cells. However, no significant difference was seen for MMP9 expression between HOS and HOS-143B cells. Each group of data is representative of 4 or more experiments. *$p<0.05$, ****$p<0.0001$.

3.8. TOPFlash activity significantly higher in HOS-143B compared to HOS cells

In addition to measuring ABC activity by qRT-PCR, we also used the TOPFlash reporter assay to determine if there were any significant differences for ABC-induced transcriptional activity in the more metastatic HOS-143B cells in comparison to parental HOS cells. FOPFlash plasmid was used as a negative control and showed background activity. As can be seen in Figure 15, HOS-143B cells showed approximately 3-fold higher activity compared to HOS cells (*$p<0.05$). This is also compatible with increased transcriptional expression of downstream targets genes of ABC in HOS-143B compared to HOS cells. Thus, our findings support that the higher ABC levels in HOS-143B cells compared to HOS cells result in greater ABC transcriptional activity in more metastatic OS cell lines compared to parental cells

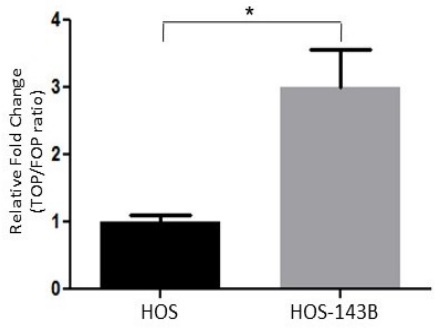


Figure 15. TOPFlash Luciferase assay shows higher TCF-mediated transcriptional activity in HOS-143B cell lines.

HOS and HOS-143B cells were transfected separately with TOPFlash and FOPFlash plasmids and 24 hours post-transfection, the cells were stimulated for approximately 10 hours with LiCl to activate Wnt signaling. Following this, the cells were lysed, and equal amounts of protein was added to each well of 96-well plate. Luciferin, the substrate of luciferase enzyme was also added to each well containing lysate and the bioluminescence produced as a result of luciferase's activity on luciferin was quantified using a bio-luminometer. TOPFlash Luciferase activity was significantly higher (approximately 3-fold) in HOS-143B cells compared to HOS cells. FOPFlash activity was measured as a control; n=3, *$p<0.05$. Ratio of TOP/Fop was calculated and then the ratio for HOS cells was normalized to 1 and the fold change for HOS-143B was calculated accordingly.

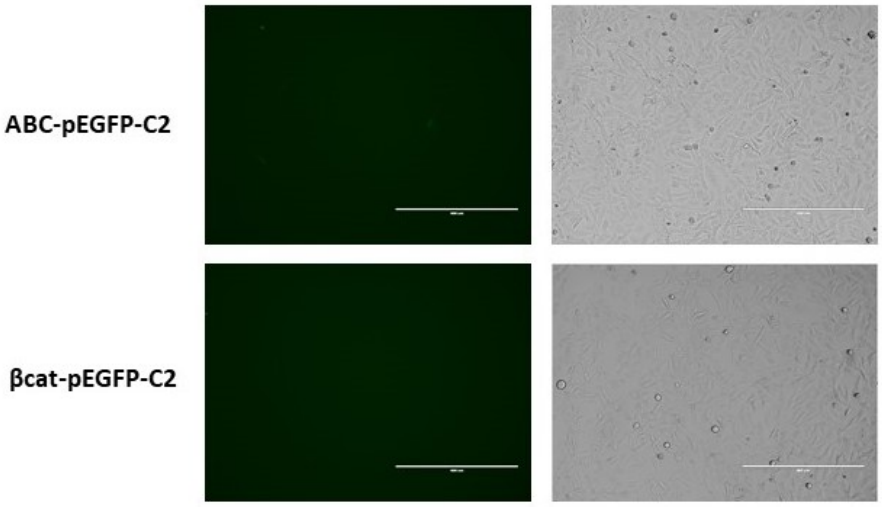
3.9. ABC-pEGFP-C2 construct

Our results have shown that in comparison to more metastatic OS cell lines (SaOS2-LM7 and HOS-143B), levels of endogenous ABC are relatively lower in parental SaOS2 and HOS cell lines. We wanted to study the effects of overexpressing ABC in the SaOS2 or HOS cell lines. Such an overexpression study would allow us to investigate how ABC is potentially contributing to OS progression. Our laboratory has previously generated an ABC overexpression construct with GFP fused to its N-terminus (ABC-pEGFP-C2). We also have a wild-type β-catenin construct with GFP fused to its N-terminus (βcat-pEGFP-C2). SaOS2 or HOS cells were transfected with ABC-pEGFP-C2 or βcat-pEGFP-C2 and their transfection efficiency was determined. An empty vector pEGFP-C2 was used as a control plasmid.

(i) Transient transfection efficiency of ABC-pEGFP-C2 and βcat-pEGFP-C2 in HOS cells

For optimum transfection of HOS cells with ABC-pEGFP-C2 and βcat-pEGFP-C2 plasmids, two different transfection reagents were tested according to manufacturers' protocols. These included Lipofectamine® 3000 and Fugene® 6 reagents. pEGFP-C2 plasmid was used as a control. After 24 hours of transfection, cells were checked for green fluorescence. Figure 16A shows that for HOS cells transfected transiently with ABC-pEGFP-C2 using Lipofectamine® 3000, there was very low transfection efficiency (5%) and 15-20% cell death. For ABC-pEGFP-C2 plasmid using Fugene® 6, there was almost no transfection that could be seen (Figure 16B). Similar results were observed for βcat-pEGFP-C2 with both transfection reagents. Compared to this, the transfection efficiency for pEGFP-C2 control plasmid was approximately 70% (results not shown). Thus, with such low transient transfection efficiencies, we decided to use SaOS2 instead for overexpressing our plasmids of interest.

A.



B.

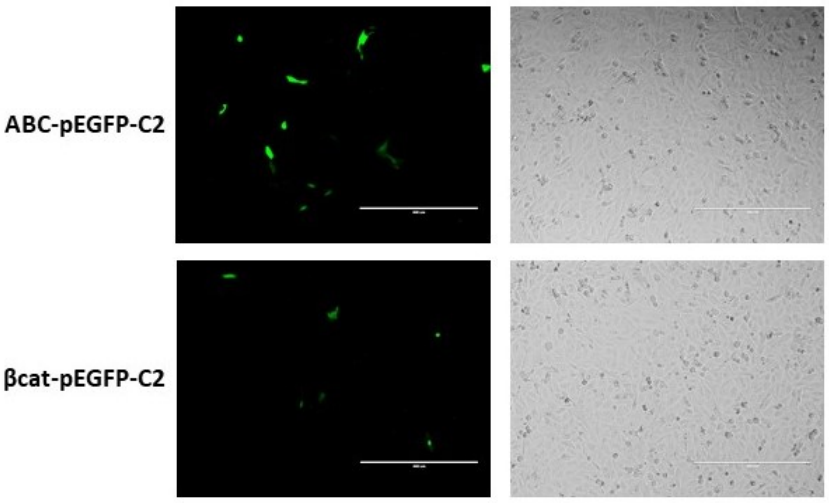


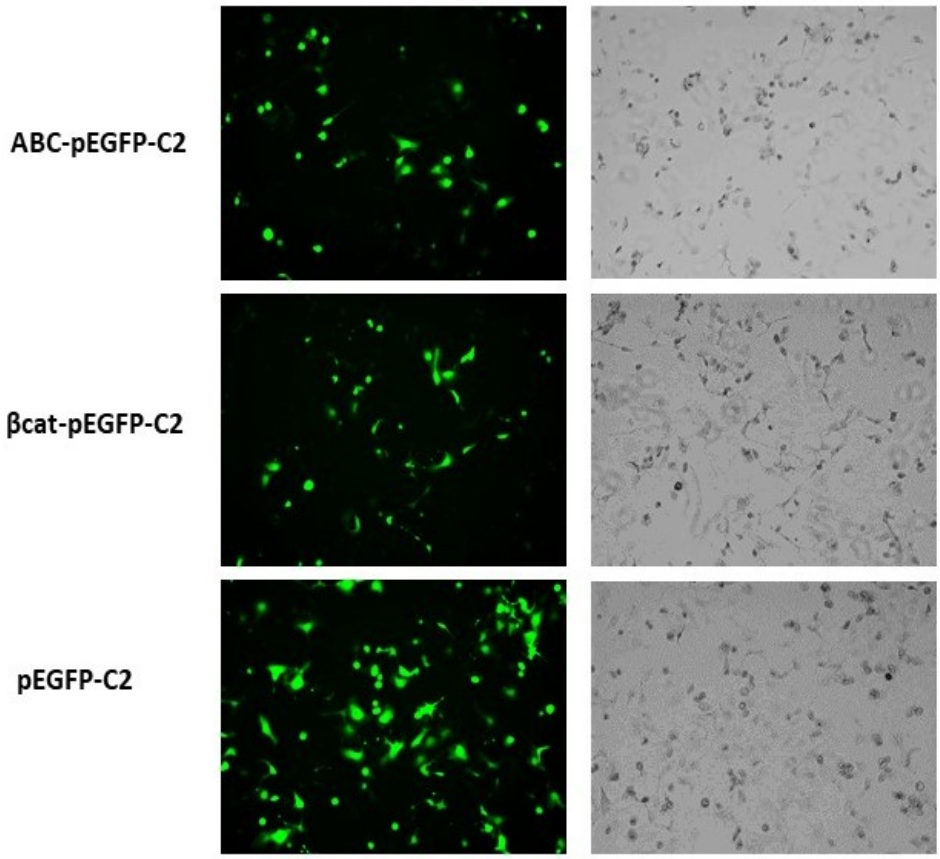
Figure 16. Comparing transient transfection efficiencies of plasmids using Fugene® 6 and Lipofectamine® 3000 in HOS cells.

Transient transfection efficiency for ABC-pEGFP-C2 & βcat-pEGFP-C2 in HOS cells. Using different transfection reagents, the cells were transfected with plasmids and incubated overnight at 37ºC and 20% O_2 and 5% CO_2. The cells were checked for transfection efficiency 24 hours post-transfection. **A) Fugene® 6**. Results showed that almost no cells were GFP-positive for both ABC-pEGFP-C2 & βcat-pEGFP-C2. **B) Lipofectamine® 3000**. Results showed approximately 5% transfection efficiency for both ABC-pEGFP-C2 & βcat-pEGFP-C2. pEGFP-C2 was used as control and showed approximately 70% transfection efficiency (results not shown).

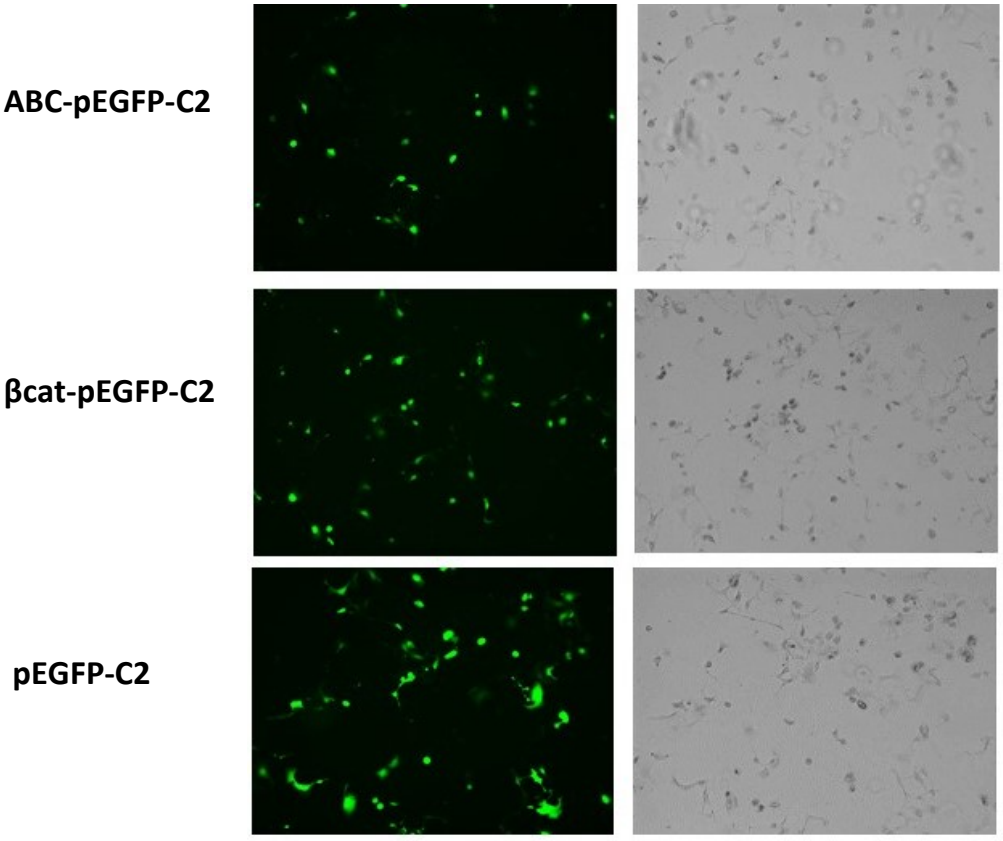
(ii) *Higher transient transfection efficiency of ABC-pEGFP-C2 with Lipofectamine® LTX - SaOS2 cells*

For optimum transfection of SaOS2 cells with ABC-pEGFP-C2 and βcat-pEGFP-C2 plasmids, three different transfection reagents were tested. These included Lipofectamine® LTX with Plus™ Reagent, Lipofectamine® 3000 and Fugene® 6 reagents. The pEGFP-C2 plasmid was used as a control. When checked for transient transfection efficiency after 24 hours, Fugene® 6 showed the least transfection for both plasmids and was not optimized further. For Lipofectamine® LTX and Lipofectamine® 3000, both showed at least 20-25% transfection efficiency and were optimized further. However, both these reagents also had approximately 15-20% cell death. Hence, to improve efficiency and reduce cell death, different quantities of transfection reagents and DNA and different incubation times of cells with transfection reagents were tested. Though improved transient transfection efficiency for ABC-pEGFP-C2 could be seen with both reagents, Lipofectamine® LTX showed higher transient transfection efficiency (~40-50%) compared to Lipofectamine® 3000 (~30-35%). The former also resulted in relatively healthier cells post-transfection compared to the latter reagent. Hence, Lipofectamine® LTX was chosen as the suitable reagent for delivering our plasmids to SaOS2 cells.

A. **B.B**



B.



C.

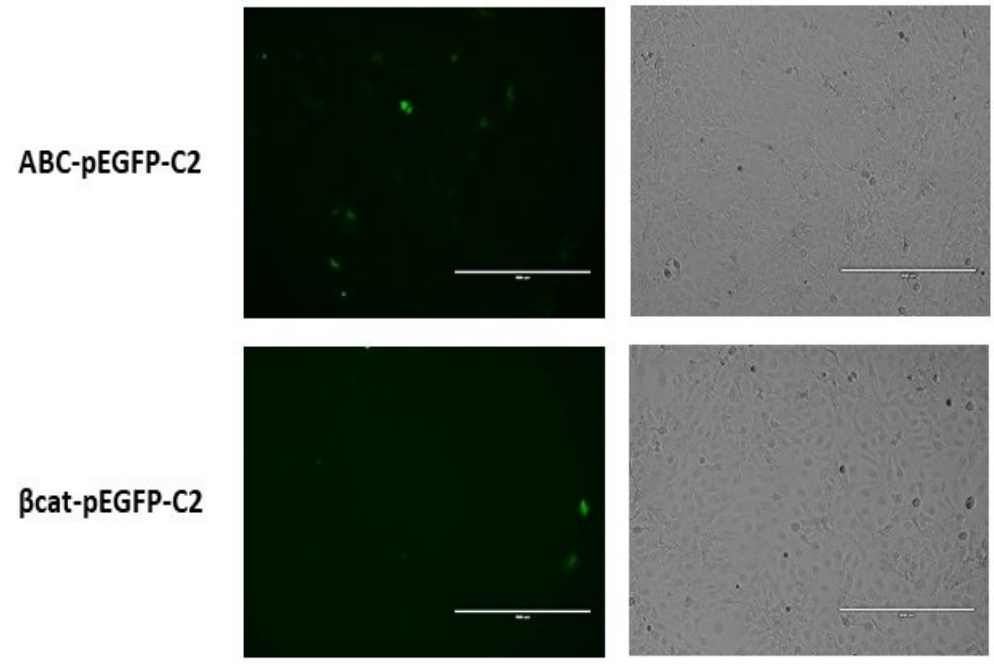


Figure 17. Comparing efficiencies of transient transfection of plasmids using Lipofectamine® LTX with Plus™, Lipofectamine® 3000 and Fugene® 6 in SaOS2 cells.

A) Lipofectamine® LTX with Plus™ reagent. Transient transfection efficiency for ABC-pEGFP-C2 & βcat-pEGFP-C2 in SaOS2 cells using Lipofectamine® LTX with Plus™ reagent. After 24 hours of transfection, approximately 45-50% cells were GFP-positive for both ABC-pEGFP-C2 and βcat-pEGFP-C2. pEGFP-C2 was used as control and showed approximately 70% transient transfection efficiency. **B) Lipofectamine® 3000 reagent.** Transient transfection efficiency for ABC-pEGFP-C2 and βcat-pEGFP-C2 in SaOS2 cells using Lipofectamine 3000 reagent. After 24 hours of transfection, approximately 25-30% cells were GFP-positive for both ABC-pEGFP-C2 and βcat-pEGFP-C2. pEGFP-C2 was used as control and showed approximately 70% transient transfection efficiency. **C) Fugene® 6 reagent.** Transient transfection efficiency for ABC-pEGFP-C2 and βcat-pEGFP-C2 in SaOS2 cells using Fugene® 6 reagent. After 24 hours of transfection, approximately 10% cells were GFP-positive for ABC-pEGFP-C2 and <5% for βcat-pEGFP-C2.

(iii) *Co-localization of endogenous and transiently transfected ABC and β-catenin in SaOS2 cells*

Prior to examining the effects of overexpressing ABC in SaOS2 cells, it was important to determine if the ABC-pEGFP-C2 and βcat-pEGFP-C2 constructs have similar localization as endogenous ABC and β-catenin. Our previous immunofluorescence results show a distinct cellular localization pattern for ABC and β-catenin, with ABC present mainly in the nucleus and β-catenin largely in cytoplasmic region of cells. For co-localization of transfected plasmids and endogenous ABC and β-catenin, transiently transfected cells were stained for immunofluorescence. SaOS2 cells transiently transfected with ABC-pEGFP-C2 and βcat-pEGFP-C2 plasmids were stained with anti-Active-β-catenin and anti-β-catenin primary antibodies respectively. To distinguish transfected from endogenous, different fluorescent tags were used i.e. GFP-fused protein for transfected proteins and CY3-tagged secondary antibody for endogenous proteins. However, it is important to note that anti-β-catenin antibody will bind to endogenous as well as overexpressed β-catenin, such that CY3-tagged secondary antibody will recognize both forms of β-catenin in the cells. SaOS2 cells transiently transfected with pEGFP-C2 plasmid were not stained with any primary antibody but only with CY3-tagged secondary antibody. As can be seen, both transiently transfected ABC-pEGFP-C2 and endogenous ABC localize mainly to the nucleus and the peri-nuclear region. The co-localization is shown by the yellow color which results due to merging of green and red for transfected and endogenous proteins respectively. However, for β-catenin, the endogenous protein is mainly cytoplasmic whereas the overexpressed form appears to be present in both, cytoplasm as well as the nucleus. Thus, it shows co-localization for transiently transfected βcat-GFP and endogenous β-catenin in cytoplasm as well as presence of βcat-GFP in the nucleus. Empty vector pEGFP-C2 was used as control and shows presence of GFP protein throughout the cell, with no expression seen for Cy3 fluorescence as no primary antibody was present to which CY3-tagged secondary antibody would bind. Hence, our results confirmed that the engineered plasmids for ABC and β-catenin have similar localization patterns to endogenous ABC and β-catenin, respectively. However, more repeats need to be done to confirm these results.

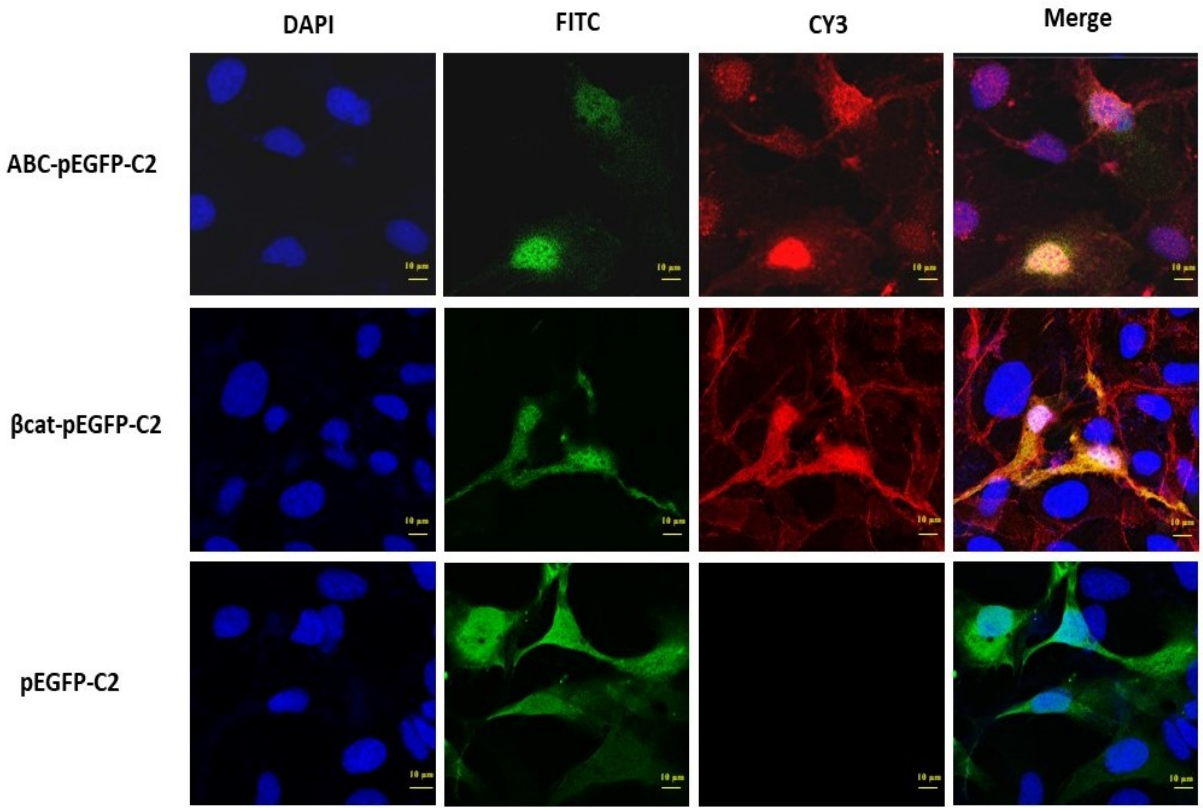


Figure 18. IF analysis for observing co-localization of overexpressed ABC-GFP and βcat-GFP with endogenous ABC and β-Catenin respectively.

Co-localization of transiently transfected constructs and endogenous ABC and β-catenin in SaOS2 cells. GFP signal in transiently transfected SaOS2 cells with ABC-pEGFP-C2 and βcat-pEGFP-C2. Both ABC-GFP and βcat-GFP co-localize with endogenous ABC and β-catenin (Alexa 555), respectively. The co-localization can be seen as yellow color in merge pictures. All images are taken at x40 magnification. Dr. Elizabeth Garcia helped in taking confocal images for this experiment.

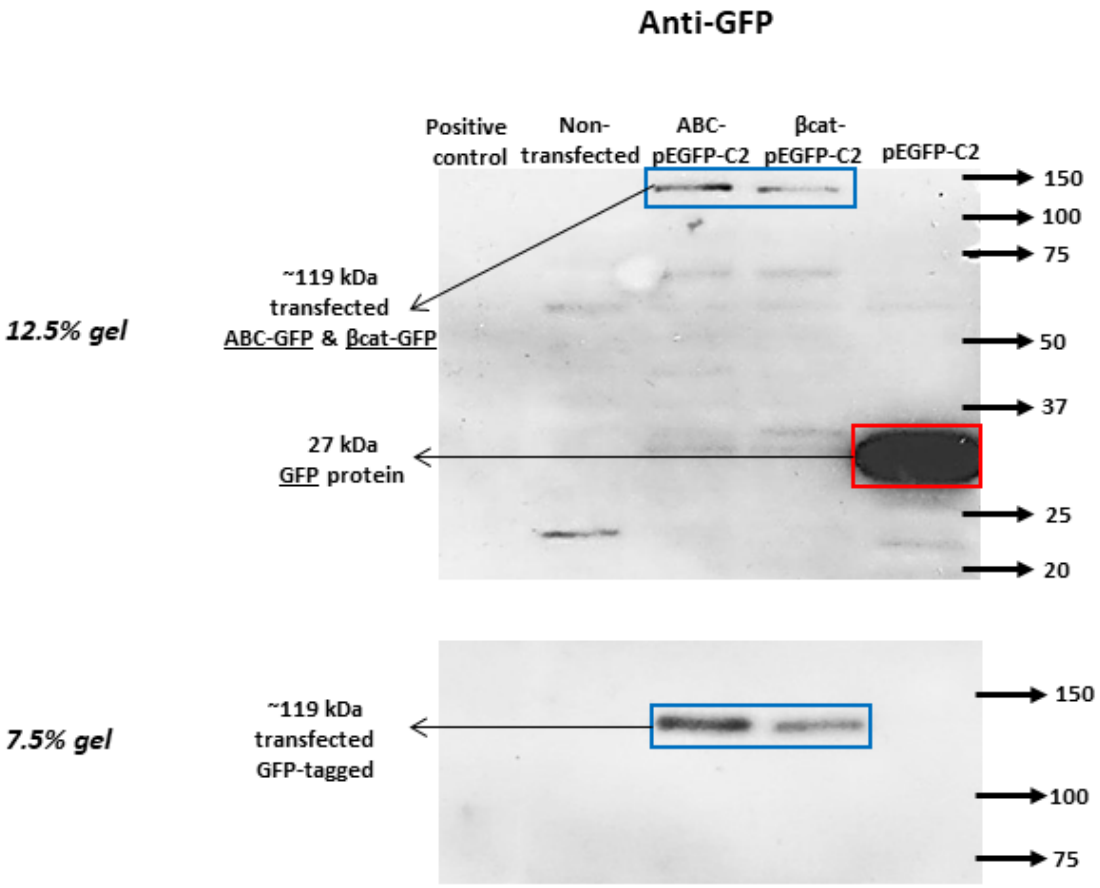
(iv) Western blot analysis of ABC-GFP, β-catenin-GFP and GFP proteins in SaOS2

In addition to visualizing over-expressed proteins through fluorescence microscopy, we determined their protein levels by Western blot. Lysates of SaOS2 cells transiently transfected with ABC-pEGFP-C2, βcat-pEGFP-C2 and pEGFP-C2 plasmids were collected and run on the gel. Lysate from non-transfected SaOS2 cells was also collected and analyzed on gel with other samples. Non-stimulated A431 cell lysate was used as positive control for endogenous ABC and β-catenin. Low molecular weight proteins give a better resolution on high percentage acrylamide gels whereas the reverse is true for high molecular weight proteins. Therefore, we prepared all samples in duplicates and ran them on 7.5% and 12.5% gels. This allowed us to visualize larger proteins such as ABC-GFP and Bcat-GFP = ~119kDa at a good resolution on 7.5% gel and low molecular weight GFP protein (~25 kDa) on 12.5% gel.

When blotted against anti-GFP antibody, we saw bands for ABC-GFP and βcat-GFP proteins at around 119 kDa, on both 12.5% and 7.5% gels. The total weight for fusion proteins includes weight for ABC/β-catenin (92 kDa) and GFP (27 kDa). A strong signal for GFP was seen for cells transfected with pEGFP-C2 only on the12.5% gel. No bands for GFP were seen in non-transfected or A431 positive control lysate. When stripped and blotted against anti-β-catenin antibody, we saw strong bands for endogenous β-catenin (92 kDa) in all samples including the non-transfected and the positive control. Since β-catenin antibody is C-terminal and the differences between ABC and β-catenin are in the N-terminal, such that both forms of ABC and β-catenin (transfected and endogenous) have the same amino acid sequence in the C-terminal, the antibody also detected bands for transfected ABC-GFP and βcat-GFP at approximately 119 kDa.

Hence, our Western blot results confirm the presence of transfected ABC-GFP and βcat-GFP proteins in SaOS2 cells.

A.


Anti-GFP
Positive control
Non-transfected
ABC-pEGFP-C2
βcat-pEGFP-C2
pEGFP-C2
150
100
75
50
37
25
20
12.5% gel
~119 kDa transfected ABC-GFP & βcat-GFP
27 kDa GFP protein
7.5% gel
~119 kDa transfected GFP-tagged
150
100
75

B.

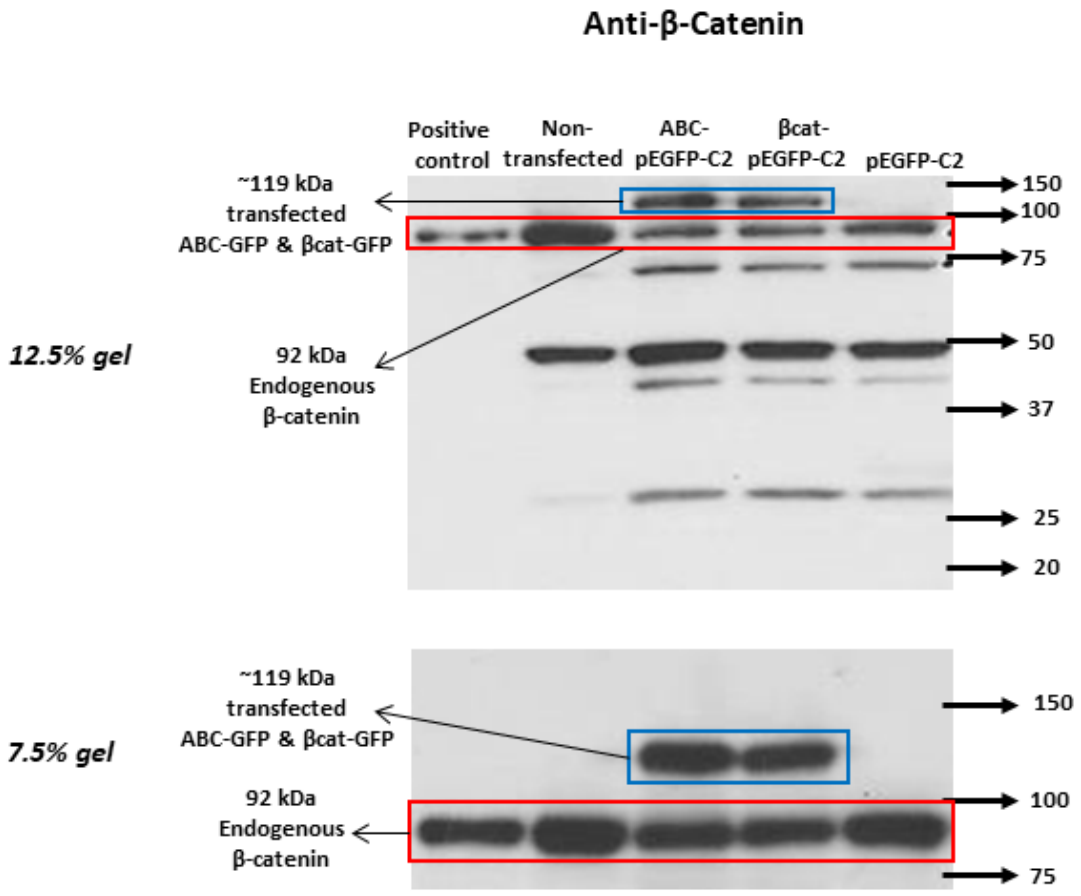


Figure 19. Western Blot analysis for overexpressed ABC-GFP, βcat-GFP and GFP proteins in SaOS2 cells.

The upper panel shows 12.5% gel and lower panel shows results from 7.5% gels. The same lysates were run on both gels. **A)** Blotting against anti-GFP antibody shows bands for ABC-GFP and βcat-GFP at around 119 kDa (92 kDa for ABC/βcat + 27 kDa for GFP) and a band for GFP at 27 kDa. **B)** The anti-β-catenin antibody, which is C-terminal, recognizes endogenous β-catenin (around 92 kDa) in all samples and transfected ABC-GFP and βcat-GFP proteins (around 119 kDa) in transfected lysates, respectively.

3.10. ABC and β-catenin staining in different levels of EOE tissue

Eosinophilic Esophagitis (EOE) is an allergic inflammatory disease which is characterized by dense oesophageal eosinophilia present in the tissue. As the number of eosinophils increase, the disease becomes more severe. Since β-catenin is a critical component of adhesion complexes, we expected to see its expression in the EOE tissue. However, presence of ABC was undetermined. Hence, to learn immunohistochemistry technique and visualize the immunostaining pattern for ABC and β-catenin, EOE tissue was used. For ABC staining, our results in figure 20 show nuclear and peri-nuclear staining for zero and intermediate level EOE tissue. The high level EOE shows more cytoplasmic staining for ABC. Contrary to this, β-catenin staining was strictly cell membranous and quite strong in zero level and high level EOE tissues. Weak β-catenin staining could also be seen in intermediate level EOE tissue, but this could possibly be due to experimental error. Overall, β-catenin staining was distinctly cell membranous in all three levels of EOE tissue whereas ABC staining was more nuclear and peri-nuclear, with some cytoplasmic staining present in intermediate level EOE tissue as well.

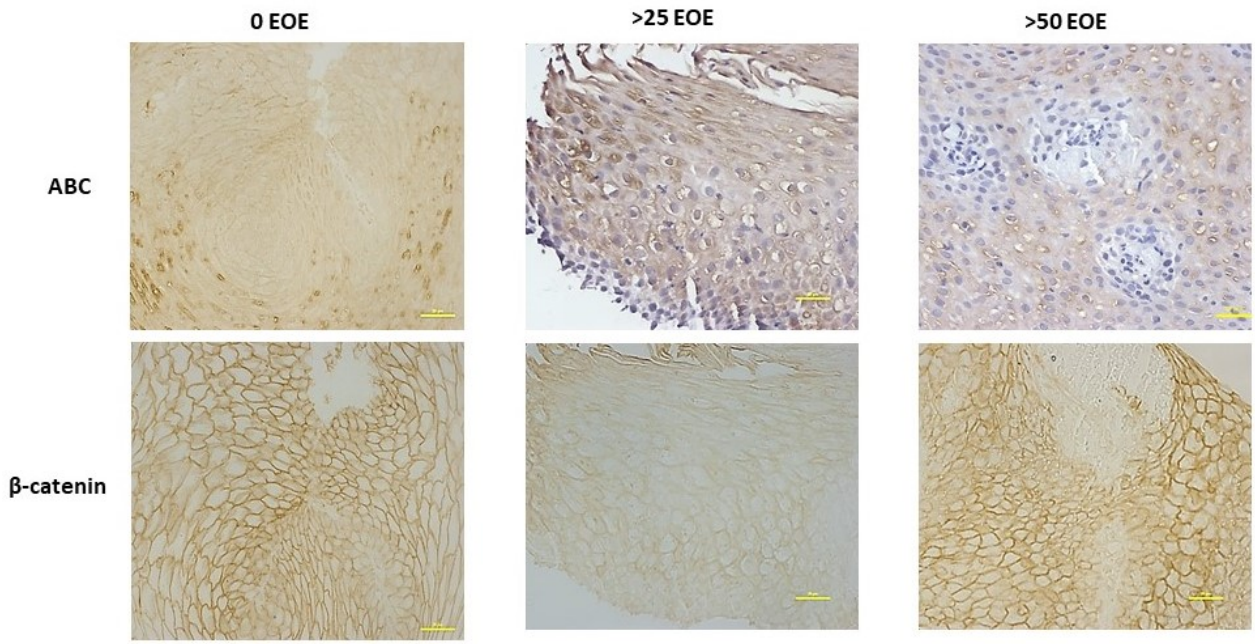


Figure 20. Comparison of IHC staining of ABC and β-catenin in 0, >25 and >50 EOE tissue.

ABC staining is mainly localized to the nuclear and peri-nuclear region of cells in EOE tissue. ABC staining is higher in >25 and >50 EOE compared to tissue with no EOE. However, no difference could be seen for ABC staining intensity between >25 and >50 EOE. β-catenin staining was predominantly cell-membranous for all three levels of EOE tissue. Staining intensity for β-catenin in >25 EOE was very weak. Dr. Hunter McColl helped in taking images for this experiment.

3.11. Optimization of ABC staining in normal bone, colon cancer and spleen tissue

ABC forms one part of the total β-catenin pool in the cells and to visualize this sub-pool of ABC in normal bone tissue, several optimizations were performed. These included testing different secondary antibody dilutions as well as using an additional amplification reagent and optimizing its dilution. Along with this, the antigen retrieval method used was modified to become less rigorous to prevent normal bone tissue from being lifted off the surface of slides. For controls, colon cancer tissue was used as a positive control and spleen tissue as a negative control. With these optimizations and modifications, ABC staining could be seen in colon cancer and in normal bone tissue with no staining in spleen tissue.

(i) Secondary antibody dilution optimization

To reduce non-specific staining and optimize ABC signal, different secondary antibody dilutions were tested.

a. 1:150

For this dilution, strong ABC staining could be seen in the colonic crypts of the colon cancer tissue. However, there were patches of brown stain present in different regions of colon cancer tissue, which indicated high background staining. For normal bone tissue, brown staining could be seen throughout the tissue and appeared to be quite non-specific to a particular region of cells. Even though the negative control spleen was expected to not have any staining, there was some brown staining that could be seen overlapping with some nuclei in the tissue. Thus, with high background staining, it was determined that 1:150 dilution was not optimal for visualizing ABC staining in tissues of our interest.

b. 1:200

For secondary antibody dilution of 1:200, strong ABC staining could be seen in colonic crypts of colon cancer tissue with minimal non-specific staining. The ABC staining in bone had a similar pattern and staining intensity to what was seen for 1:150 dilution.

Most of the spleen tissue had no staining except some faint background staining present in some regions of the tissue.

c. *1:500*

The dilution of 1:500 for secondary antibody showed very weak staining for ABC in colon cancer tissue, indicating that it was too diluted to detect the pool of ABC in this tissue. Contrary to what was seen in colon cancer tissue, normal bone had ABC staining throughout the tissue. However, this staining was relatively weaker than what was seen for 1:150 and 1:200 dilution. No ABC staining could be seen in spleen tissue.

Hence, after investigating the different dilutions, 1:200 was determined to be the optimal secondary antibody dilution for ABC staining in colon cancer, normal bone and spleen tissue.

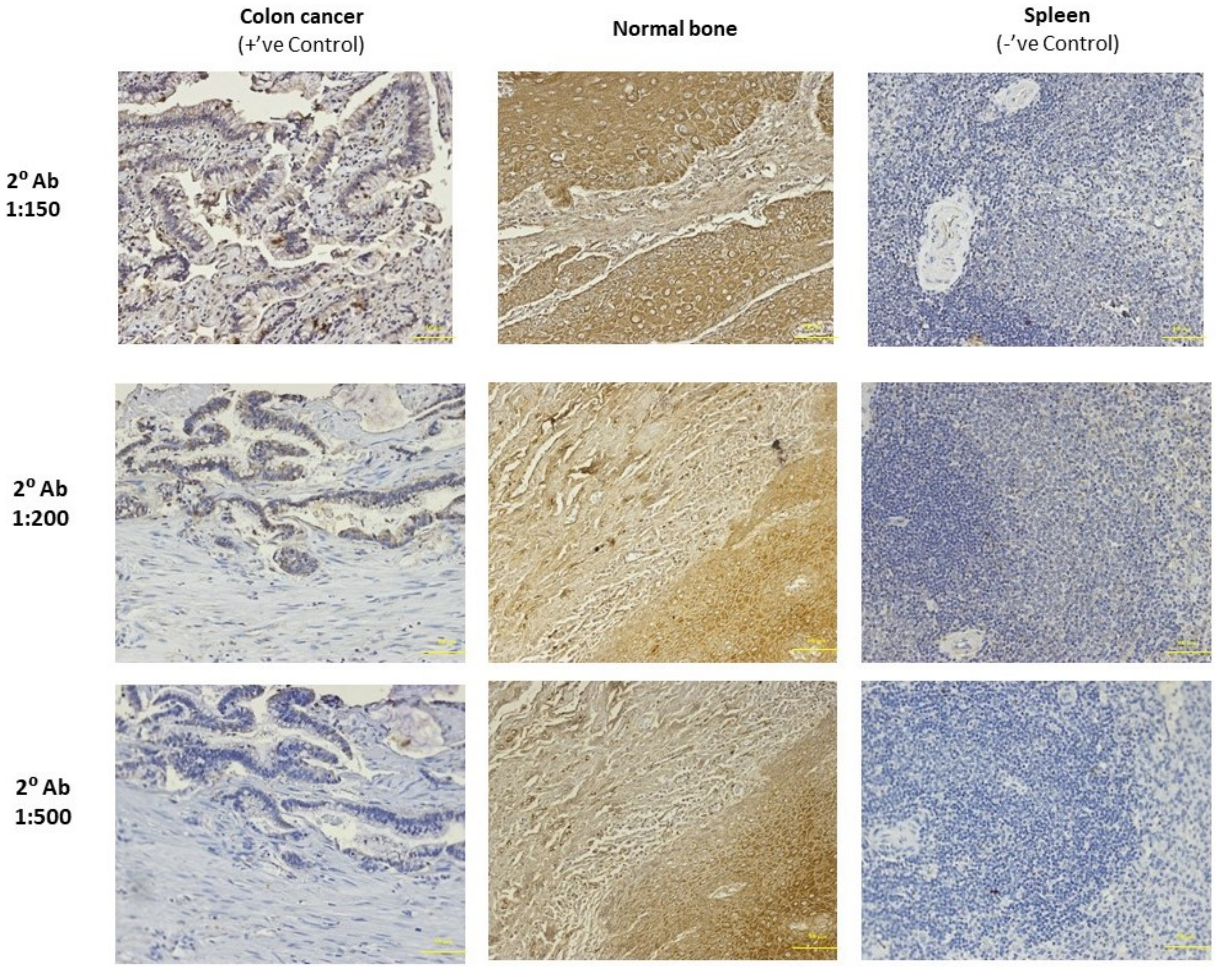


Figure 21. Optimization of 2°Ab dilution for IHC staining of ABC in colon cancer, normal bone and spleen tissue.

A) 1:150 shows strong staining for ABC in colonic crypts for colon cancer and no staining in spleen tissue. For normal bone, there was strong staining, but it was not localized to a specific region of cells/tissue. All images taken at x20. **B) 1:200**, high staining for ABC in colonic crypts for colon cancer and no staining in spleen tissue. Normal bone shows ABC staining but not localized to specific region of cells/tissue. **C) 1:500** shows relatively weaker staining for ABC in colon cancer and no staining in spleen tissue. For normal bone, there was some staining, but it was not localized to a specific region of cells/tissue. All images were taken at x20.

(ii) *Tyramide Signal Amplification (TSA) dilution optimization*

TSA is an amplification technique which uses biotinylated tyramine to detect low-abundance proteins in immunohistochemistry, immunocytochemistry, etc. This technique can also be used to reduce the amount of primary and secondary antibodies used since it is highly sensitive and can result in up to 1,000-fold reduction in the amount of antibody used (Perkin Elmer protocol and other websites). Hence, we tested for different TSA dilutions from using no TSA to 1:50 dilution for this reagent.

a. 1:25

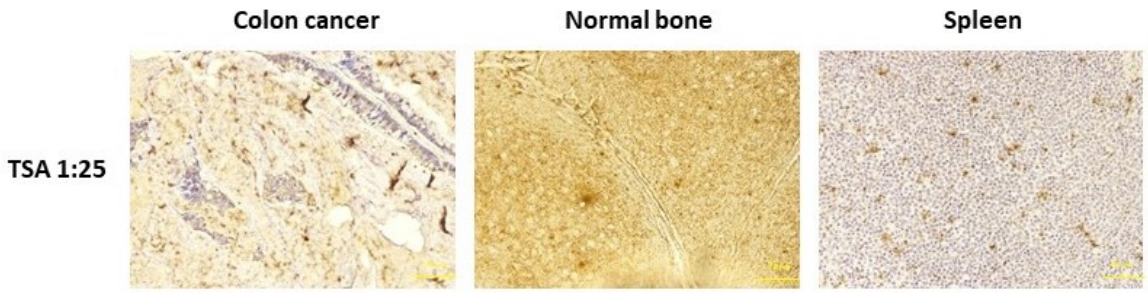
Our results for 1:25 dilution for TSA show strong background staining for ABC in all three tissues stained. In addition to ABC staining seen in colonic crypts for colon cancer tissue, widespread non-specific brown staining was apparent in other regions of the tissue as well. For normal bone tissue, high intensity ABC staining was visible throughout the tissue specimen. Spleen tissue also showed non-specific background staining in different regions of the tissue. Hence, our staining results determined that 1:25 TSA results in high background signal and doesn't allow specific ABC staining to be seen.

b. 1:50

Contrary to our above-mentioned results, 1:50 TSA dilution resulted in more specific ABC staining and much reduced non-specific background signal. In colon cancer tissue, strong ABC signal localized to the colonic crypts could be seen. There was minimal background staining in spleen tissue. For normal bone tissue, strong ABC staining was present in some regions with weak staining in others.

Overall, our results for different optimizations concluded the use of 1:200 secondary antibody dilution and 1:50 TSA dilution for colon cancer, normal bone and spleen tissues. Once optimized on these tissue samples, the same dilutions were used for ABC staining in osteosarcoma tissue.

A.



B.

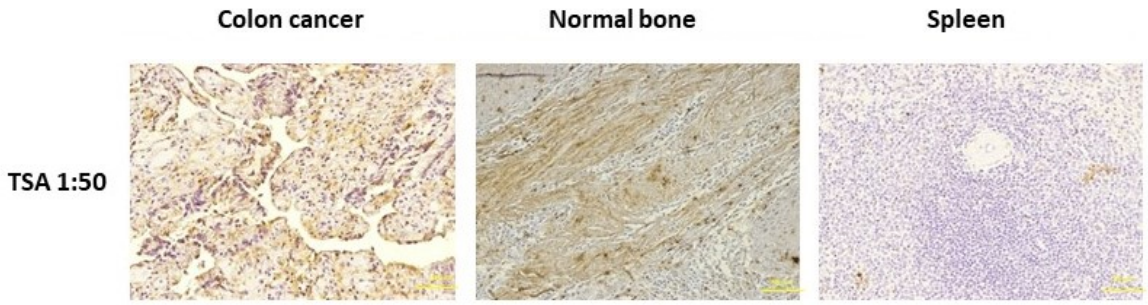


Figure 22. Optimization of TSA dilution for IHC staining of ABC in colon cancer, normal bone and spleen tissue.

A) TSA 1:25, strong staining for ABC in colon cancer and normal bone tissue. However, all tissues showed high non-specific background staining. **B) TSA 1:50** shows strong staining for ABC in colonic crypts of colon cancer and some parts of normal bone tissue. Almost no background staining could be seen in spleen tissue. All tissue sections are counter-stained with hematoxylin and imaged at x20.

3.12. Optimization of β-catenin staining in normal bone, colon cancer and spleen tissue

Along with ABC optimization, it was also important to determine suitable conditions for β-catenin staining in colon cancer, normal bone and spleen tissue samples. Specifically, it was essential to investigate whether TSA amplification reagent should be used for β-catenin staining or not. This is because β-catenin is more abundant than ABC and use of additional amplification reagents could result in saturated or high background signal. Hence, we tested for β-catenin staining with no TSA and with 1:50 dilution of TSA.

(i) No TSA

Since β-catenin is known to have high expression in colon cancer, we could see strong staining of β-catenin in colonic crypts even in the absence of TSA. However, no staining could be seen for β-catenin in normal bone tissue. Negative control spleen also did not show any staining for β-catenin.

(ii) 1:50 TSA

In the presence of TSA 1:50, strong staining for β-catenin was present in colonic crypts of colon cancer tissue with some non-specific staining as well. Unlike our results for no TSA in normal bone tissue, we could see a relatively stronger staining for β-catenin with this dilution. However, like ABC, β-catenin staining was present throughout the bone tissue making it difficult to determine a specific localization pattern for these proteins in bone tissue. As expected, spleen tissue showed no β-catenin staining.

Since bone is our main tissue of interest and no β-catenin staining could be seen in bone without TSA, we decided to use 1:50 TSA dilution for β-catenin staining in our tissues of interest.

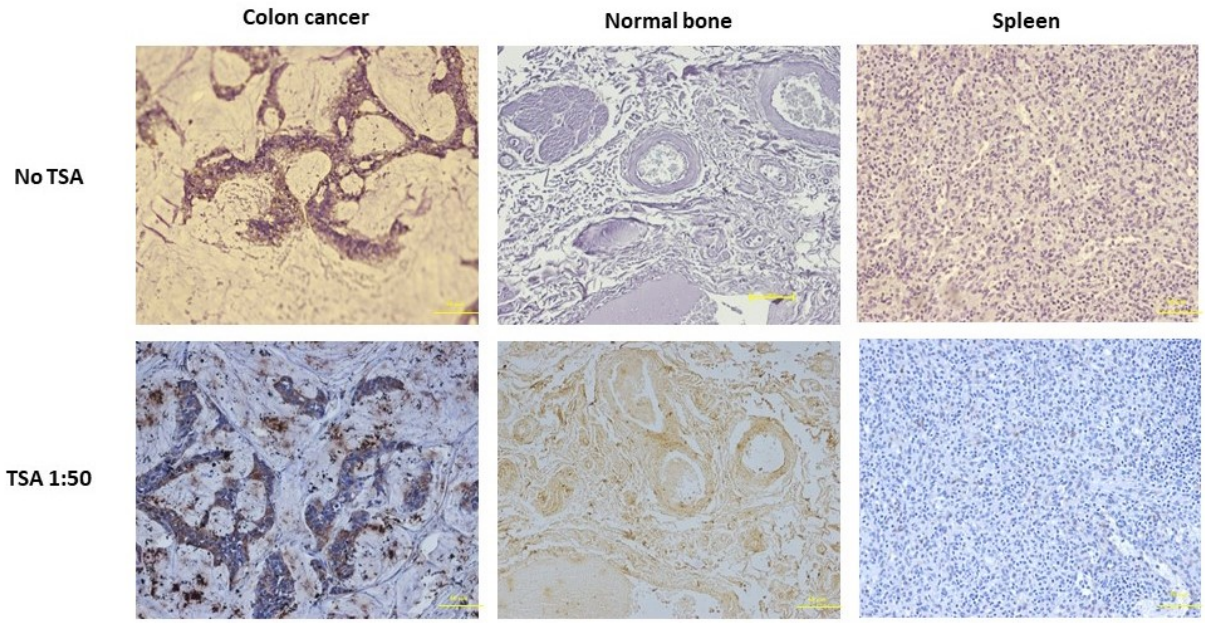


Figure 23. Optimization of TSA dilution for IHC staining of β-catenin in colon cancer, normal bone and spleen tissue.

For β-catenin staining in colon cancer tissue, no apparent difference could be seen in β-catenin intensity with and without TSA. However, TSA 1:50 showed staining of β-catenin in normal bone tissue in comparison to no staining without TSA. Spleen did not show any staining for both conditions i.e. with and without TSA. All images are taken at x20.

3.13. Presence of nuclear staining for ABC in Osteosarcoma tissue

Results from our *in vitro* cell culture-based analysis suggest that ABC levels and transcriptional activity are directly associated with OS progression. To explore the clinical relevance of our finding, we carried out immunohistochemical analysis (IHC) of ABC and β-catenin in OS tissue samples. Colon cancer tissue was used as a positive control and exhibited strong IHC staining for both β-catenin and ABC in the colonic crypts. Spleen was used as a negative tissue control and showed no staining for β-catenin or ABC. All OS tissue showed extensive staining for both ABC and β-catenin (Figure 24). However, a different staining pattern could be seen between ABC and β-catenin, with ABC staining being predominantly nuclear. β-catenin staining could be mainly seen in peri-nuclear and cytoplasmic regions.

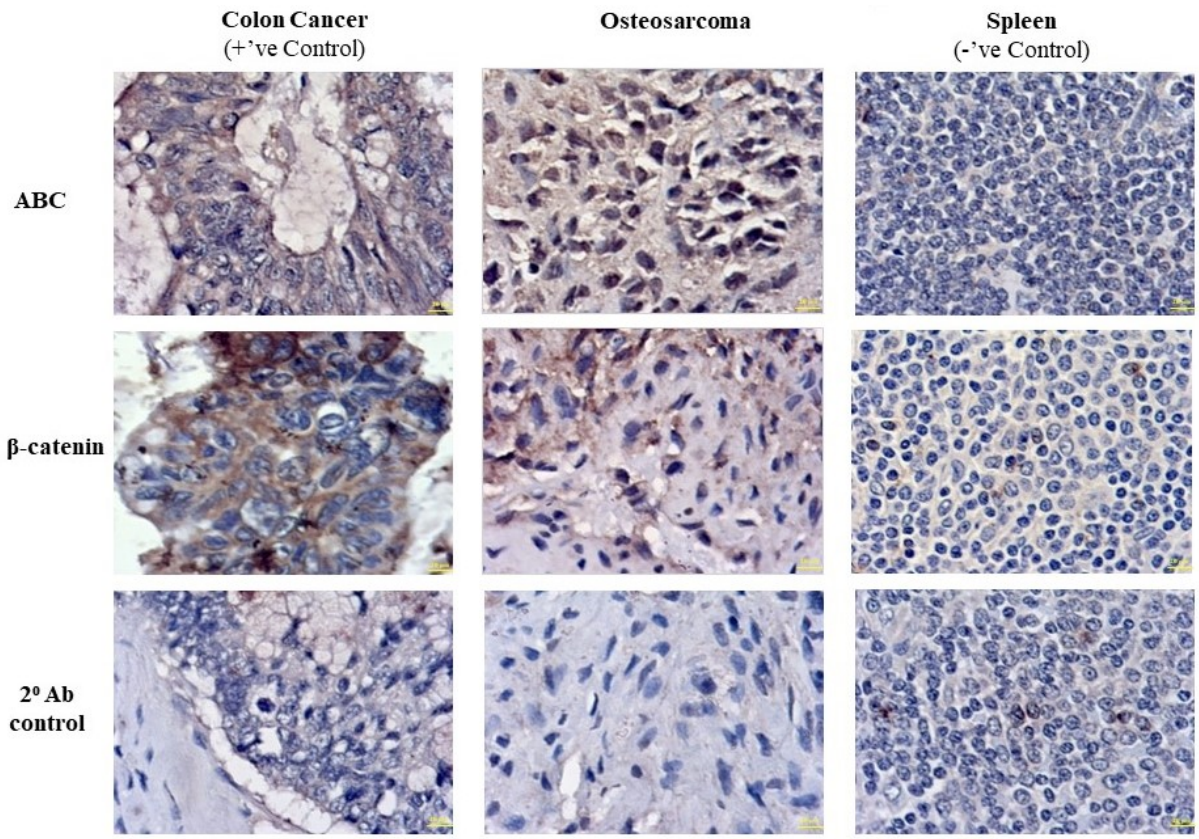


Figure 24. IHC analysis for cellular localization and intensity of ABC and β-catenin staining in osteosarcoma tissue.

Figure shows prominent nuclear staining of ABC in OS tissue. For β-catenin, the staining is mainly cytoplasmic in OS tissue. Colon cancer tissue served as positive tissue control and Spleen tissue was used as negative tissue control. Images are representative data of four (4) optimization experiments.

3.14. IHC staining of ABC in OS Tumor Tissue Array (TMA)

To further explore the correlation of nuclear ABC staining with OS progression, we used a commercially available OS tumor tissue array (TMA) which was comprised of 40 embedded histologically confirmed primary OS samples (adults and children) each in duplicate (Folio BioSci). The patient information provided by Folio BioScience included information about tumor stage, tumor pathology, age and sex of patients. Figure 25. shows representative OS cores with ABC nuclear staining. ABC nuclear staining was detected in 34 (85%) out of 40 tumor cores (Table 5). 6 (15%) out of 40 tumor cores were negative for nuclear ABC staining. Among the positive tumors, 29 (85%) showed 1+ (<= 30% positive nuclear staining cells), 3 tumors (10.30%) showed 2+ (31% -60% positive nuclear staining cells) and 2 (7%) had 3+ (61%-100% positive nuclear staining) staining. The representative cores were from tumors of stages IA, IB, IIA and IIB. There were no cores from stage III tumors in this array. Also, all patients from children and AYA group (n=15) stained positive for nuclear ABC staining whereas all cores which stained negative for nuclear ABC belonged to the adult group.

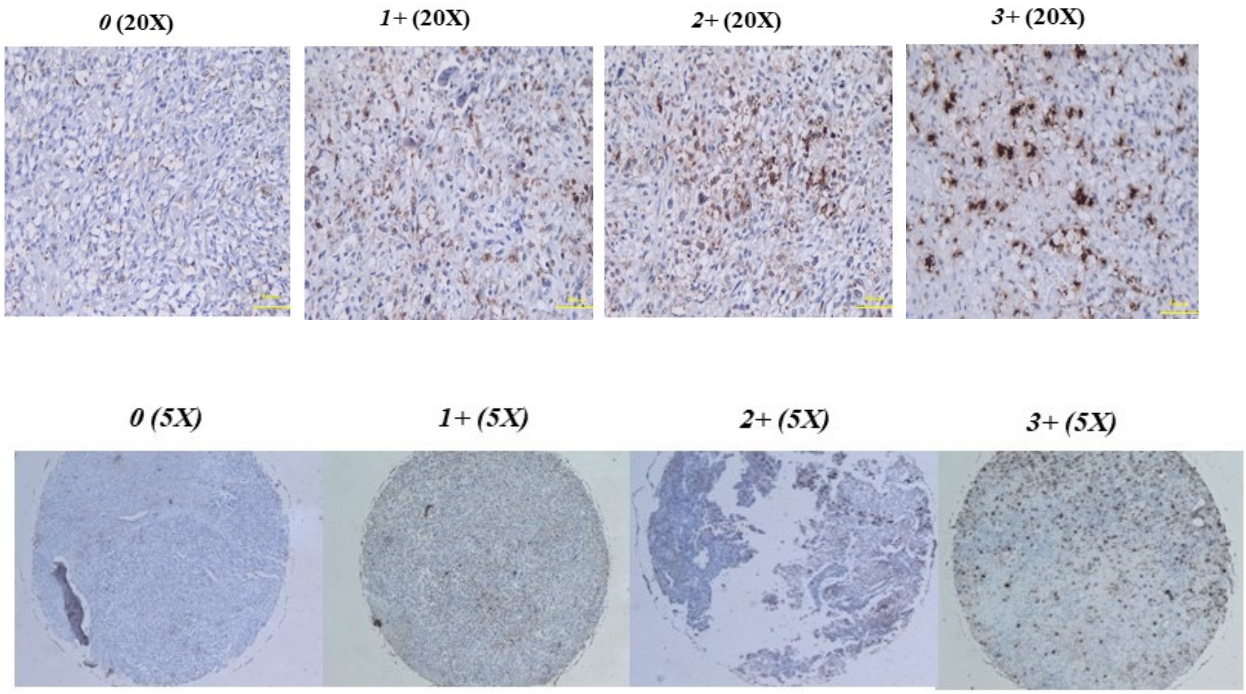


Figure 25. Representative images of ABC staining in OS TMA.

Representative expression of nuclear ABC staining in OS TMA: 0 (no nuclear staining); 1+ (≤30%); 2+ (31-60%); 3+ (>60%). These images were taken at x20 and x5 by Dr. Consolato Sergi.

Table 6. Summary of clinicopathological data and statistical analysis for ABC immunostaining results on OS TMA.

	Total	ABC positive nuclear staining			ABC negative nuclear staining	P value
Total	**40**	**34 (85%)**			**6 (15%)**	
Gender						
Male	**27**	**22 (81.5%)**			**5 (18.5%)**	
Female	**13**	**12 (92.3%)**			**1 (7.7%)**	**0.64[a]**
Age groups						
Children and AYA	**15**	**15 (100%)**			**0 (0%)**	
Adults	**25**	**19 (76%)**			**6 (24%)**	
Mean Age (years)		**28.91**			**44.67**	**0.01[b]**
Scores / Tumor Stage		**1+**	**2+**	**3+**	**0**	
IA	**2**	**1**	**0**	**0**	**1**	**0.04[c]**
IB	**3**	**1**	**1**	**0**	**1**	
IIA	**9**	**6**	**0**	**1**	**2**	
IIB	**26**	**21**	**2**	**1**	**2**	

[a] Indicates the P value for nuclear ABC staining in males vs females

[b] Indicates the P value for difference between mean ages of patients in positive and negative ABC staining groups.

[c] Indicates the P value for nuclear ABC staining across different stages of OS tumor (IA, IB, IIA & IIB).

3.15. Statistical Analysis of positive nuclear ABC staining in OS TMA

As summarized in Table 5 above, the OS tumor cores comprised of samples from 27 males and 13 females and 22 of the 27 males and 12 of the 13 females stained positive for nuclear ABC. Association between sex and nuclear ABC staining was tested via Fisher's exact test and the results showed no significant association between sex of patients and their nuclear ABC staining ($p=0.64$). We also found that patients with positive nuclear ABC staining were, on average, younger (mean age = 28.91 years) than patients with negative nuclear ABC staining (mean age = 44.67 years). Statistical analysis (non-parametric Mann-Whitney test) showed that this correlation between average age and ABC positive nuclear staining was significant ($p=0.01$). We also looked at the relationship between positive ABC nuclear staining and OS tumor stage. Positive ABC nuclear staining was present in 24 of 26 (92%) stage IIB OS tumors, 7 of 9 (77%) of the stage IIA tumors, 2 of 3 (66%) of the stage IB tumors and 1 of 2 (50%) of the stage IA tumors. Association between ABC nuclear staining and tumor stage was statistically tested using Kruskal Wallis Trend test and the results showed that positive nuclear ABC staining was significantly ($p=0.04$) correlated with stage of tumor.

Chapter 4

Discussion

4.0. Discussion

OS is a rare but aggressive primary malignancy of the bone which affects children and young adults <25 years of age. Several genetic and environmental factors are known to predispose individuals to OS development, but no single underlying cause has been identified for this malignancy. Many studies have implicated Wnt/β-catenin to be dysregulated in OS, but the role of its effector molecule β-catenin in OS progression has remained inconclusive (Guo, Rubin, Xie, Zi, & Hoang, 2008; Guo *et al*., 2007; B. H. Hoang, Kubo, Healey, Sowers, *et al*., 2004; B. H. Hoang, Kubo, Healey, Yang, *et al*., 2004; Kansara *et al*., 2009; C. H. Lin *et al*., 2013; Rubin *et al*., 2010). Thus, in an attempt to better understand OS development, our study evaluates the role of ABC and β-catenin in OS progression and investigates the use of ABC as a potential marker for predicting OS invasion and metastasis. We used a model of two paired OS cell lines and showed that ABC had significantly higher protein levels and increased transcriptional activity in more metastatic OS cell lines compared to parental ones, with no difference in β-catenin levels. These results were supported by an increased localization of ABC in the nucleus, where it possibly acts as a transactivation factor, in more metastatic SaOS2-LM7/HOS-143B compared to parental SaOS2/HOS cells, respectively. With these findings of upregulated nuclear ABC in more metastatic OS cell lines, we proceeded to evaluate immunohistochemical staining of ABC in an OS TMA. Our results showed positive nuclear ABC staining in 85% of OS samples. Moreover, using our TMA results, we performed statistical analysis to understand how ABC levels can be correlated with different clinical and patient variables. Hence, to the best of our knowledge, this is the first study which investigates role of ABC in OS progression and proposes its potential use as a biomarker.

For our study, an *in vitro* model comprising of two pairs of OS cell lines was used. Each pair consisted of one parental or less invasive/metastatic cell line and a second daughter or more invasive-metastatic cell line. These paired cell lines are representative of OS progression, allowing us to investigate the changes that occur as the malignancy progresses. The cell lines included – *Pair 1* (i) SaOS2 and its metastatic counterpart (ii) SaOS2-LM7 and *Pair 2* (i) HOS and its metastatic counterpart (ii) HOS-143B. It is Important to note that in our model each pair consists of a parental line and a metastatic line of same lineage and this eliminates biases in differences due

to cell type as well as any patient-specific differences. However, our model of paired cell lines has the limitation of being *in vitro* and might not mimic the exact human condition and the microenvironment which is essential to tumor growth and development. In addition to this, our study involves the use of a transformed cell line (HOS-143B) and such oncogenic transformation of tumor cells can interfere with the molecular signaling pathways that are contributing to tumor development. Hence, this interference could be confounding to the accurate study of potentially mutated genes in OS (Ek *et al*., 2006; McGary *et al*., 2003). We used gelatin zymography to confirm invasive/metastatic potential of the OS cell lines by checking for their MMP2 and MMP9 activity. MMPs are gelatinases which when activated, digest components of the extracellular matrix, allowing cells to invade through the matrix and metastasize to other parts of the body. In fact, studies have shown that high MMP2 and MMP9 expression are correlated with poor OS prognosis (J. Wang *et al*., 2014; Wen, Liu, Yu, & Liu, 2014). Our results show a significantly higher MMP2 (active form) activity for SaOS2-LM7 compared to SaOS2 (Figure 8A). For the other pair, MMP9 had an increased activity in HOS-143B compared to HOS (Figure 8B). This increased activity of MMPs in invasive-metastatic SaOS2-LM7 and HOS-143B also explains the increased invasiveness in these cell lines compared to SaOS2 and HOS, respectively (Figures 8A & 8B). These results substantiated our use of paired OS cell lines as a model of OS progression.

To determine how β-catenin and ABC levels change as OS progresses, we performed Western blot analysis of total cellular β-catenin and ABC levels in all four OS cell lines. Our results showed significantly higher levels of ABC in invasive-metastatic SaOS2-LM7 and HOS-143B compared to parental SaOS2 and HOS cell lines respectively. However, no difference could be seen in β-catenin levels within each pair of OS cell lines (Figures 3A & 3B). Since the transcriptional activity of β-catenin and ABC is executed within the nucleus, we further investigated levels of β-catenin and ABC in cytoplasmic and nuclear extracts of OS cell lines. Interestingly, our results showed significantly higher nuclear levels of ABC in invasive-metastatic OS cell lines compared to their parental counterparts respectively. Cytoplasmic ABC and cytoplasmic and nuclear β-catenin levels displayed no significant difference between SaOS2-LM7/HOS-143B and SaOS2/HOS cells, respectively (Figures 4A & 4B). Our findings are corroborated by Staal *et al*.'s study which demonstrated that accumulation of β-catenin is insufficient for transducing Wnt/β-catenin signals.

The study showed that it is the increase in the N-terminally dephosphorylated form of β-catenin, ABC, that transmits signals of the Wnt/β-catenin pathway (Staal *et al*., 2002). Thus, increase in nuclear ABC possibly constitutes a key oncogenic step in malignancies where the Wnt/β-catenin pathway is dysregulated. Overall, specific increase in nuclear levels of ABC in invasive-metastatic OS cell lines support our hypothesis of potential oncogenic role of ABC in OS progression *in vitro*, via its transcriptional activity, with no change in β-catenin levels.

In addition to investigating β-catenin and ABC levels in OS cell lines, we also observed localization pattern and intensity of these proteins via immunofluorescence (IF). Our results showed distinct localization of ABC in nuclei of OS cells, with increased nuclear presence of ABC in SaOS2-LM7 and HOS-143B compared to SaOS2 and HOS, respectively (Figures 5A & 5B). Moreover, β-catenin staining could be seen throughout the cytoplasm, without distinction in its localization and fluorescence intensity between SaOS2-LM7/HOS-143B and SaOS2 and HOS cell lines. These results corroborate our western blot results of observing significantly higher nuclear ABC in invasive-metastatic OS cell lines than parental ones, with no difference in β-catenin levels. Since IF imaging visualized a limited number of cells at higher magnification, we performed high content analysis which measures fluorescence intensity for a large number of cells at 10x magnification. Similar to IF results, we saw increased fluorescence intensity in high content analysis for ABC in total cellular and nuclear compartments of SaOS2-LM7 and HOS-143B in comparison to SaOS2 and HOS, respectively (Figures 6A & 6B). However, it is important to note that high content alone would not be sufficient to show significant differences in levels of ABC between parental and metastatic OS cell lines. This is because in this technique, it is difficult to distinguish between background fluorescence staining and target fluorescence signal, resulting in possible false positives and overall quantification errors. Therefore, we used high content analysis to supplement our Western blot and IF results which show increased cellular and nuclear ABC in metastatic OS cell lines compared to parental cells. Also, we did not observe any significant changes in ABC protein levels between cytoplasmic extracts of SaOS2-LM7/HOS-143B and SaOS2/HOS cell lines in our western blot results (Figures 4A & 4B). This potentially means that the increase seen in cellular ABC levels in SaOS2-LM7 and HOS-143B compared to SaOS2 and

HOS cell lines (western blot, IF and high content results) can be attributed to higher ABC levels in the nuclei of the former two cell lines compared to the latter ones.

While we observed upregulated ABC levels in invasive-metastatic OS cell lines compared to their parental counterparts, it was also important to measure how ABC/β-catenin-TCF mediated transcriptional activity changes with OS progression. As can be seen from the TOPFlash reporter assay results, the activity was significantly higher in HOS-143B cells compared to parental HOS cells *in vitro* (Figure 15). Since no difference in total β-catenin levels was observed between these cells in western blot results, the higher TCF transcriptional activity in HOS-143B cells can be likely explained by higher nuclear ABC levels in these cells compared to parental HOS cells. In addition, we also quantified mRNA expression of downstream target genes of ABC/β-catenin including MMP2, MMP9, cyclin D1 and VEGFA. Our qRT-PCR results demonstrated significantly higher activity of all four target genes in SaOS2-LM7 compared to SaOS2 (Figure 14A). For the other pair, except MMP9, there was increased mRNA expression of MMP2, cyclin D1 and VEGFA in HOS-143B compared to HOS (Figure 14B). As discussed before, since no change in cellular β-catenin levels was observed between SaOS2-LM7/HOS-143B and SaOS2 and HOS cell lines, the significant increase in mRNA expression of target genes in SaOS2-LM7 and HOS-143B can be attributed to increased nuclear ABC levels in these cell lines compared to the parental ones. It should also be pointed out that the gene expression changes of MMP2 and MMP9 shown in Figures 13A and 13B do not correlate stringently with the MMP enzyme activities shown in Figure 8A and 8B. This can be explained by the fact that mRNA expressions are not always directly proportional to protein levels or activity. Hence, an increased/decreased mRNA expression may not be represented by an equivalent/parallel change in protein levels or activity.

It was also observed that the increase in TCF-mediated activity as measured by TopFlash reporter assay and in mRNA expression of target genes is significantly more robust (4-15 fold) in comparison to the fold change observed in cellular/nuclear ABC levels. This phenomenon, where modest changes in ABC levels result in a significant transcriptional efficacy has been reported previously as well (Staal *et al*., 2002). The same study also suggested that the phosphorylation status of ABC plays a crucial role in the efficiency with which ABC is transported to the nucleus,

where it carries out its transcriptional activities (Staal *et al*., 2002). It is also possible that this partially phosphorylated state of ABC affects the efficacy with which it binds to TCF and other transcription factors, but more research needs to be done to further clarify these mechanisms. Overall, our current findings support the notion that nuclear ABC, and not β-catenin, play a role in OS progression.

The initiation and development of a tumor involves deregulation of a multitude of signaling pathways, overexpression of oncogenic proteins and/or inhibition of tumor suppressors. This means that in most cases, the development of a tumor cannot be caused by deregulation of just one protein or even a singular pathway. Since our results show a significant increase in nuclear ABC levels and its activity in invasive-metastatic OS cell lines compared to the parental ones, we found it essential to investigate if overexpression of ABC in parental OS cell lines causes the phenotype of these cells to become more invasive. Therefore, an ABC overexpression construct was designed and transfected in parental SaOS2 cell line. Along with this, SaOS2 cells were also transfected with β-catenin and empty-vector GFP constructs. Our results show successful transfection of all three plasmids in SaOS2 cell line (Figure 17A). In fact, our co-localization results confirmed that the transfected ABC-GFP localized mainly to the nuclear and peri-nuclear regions whereas the β-catenin-GFP was present in the cytoplasmic as well as nuclear regions of the cell (Figure 18). The overexpressed proteins appear to co-localize well with their respective endogenous proteins. However, the co-localization experiments need to be repeated to confirm the co-localization of the proteins.

Even though studies have established the dysregulation of Wnt/β-catenin signaling in OS, research on the role of β-catenin in OS has been limited and controversial. Most of these studies have investigated the role of β-catenin, and not ABC, in OS. These studies have investigated cellular localization of β-catenin *in vitro*, *in vivo* and in OS patient samples. Investigation of β-catenin in murine models of OS have shown contradictory findings of its association with OS progression. As discussed earlier, a study by Iwaya *et al*., proposed that increased nuclear β-catenin is associated with OS progression in a murine OS model (Iwaya *et al*., 2003). In contrast, another study using an OS murine model demonstrated that decreased metastatic potential was correlated

with increased cytoplasmic β-catenin (Kidani *et al*., 2014). Even though both studies report contradictory findings, it is important to note that while Kidani *et al*.'s study was about cytoplasmic β-catenin, Iwaya *et al*. focused on nuclear β-catenin, and it is in the nucleus where β-catenin acts as a transcriptional regulator and thus can execute its potential oncogenic activities. Hence, localization of β-catenin can play a crucial role in determining its function and eventually its potential role in cancer progression as well.

Similar to the studies done with the murine models, studies investigating β-catenin in human OS cell lines and OS clinical samples have also been inconclusive about their findings. As discussed earlier, Cai *et al*. concluded no positive association between nuclear β-catenin accumulation and OS progression in a panel of OS cell lines (Yongping Cai *et al*., 2010). For OS clinical samples, studies by Du *et al*., and Lu *et al*., have shown positive cytoplasmic and/or membranous staining of β-catenin in 32/46 and 66/96 samples tested, respectively (G. Bacci *et al*., 2001; Du *et al*., 2014; Lu *et al*., 2015). These findings show accumulation of β-catenin as a common event in OS, but no significant correlation appears to exist between β-catenin levels and OS progression. Also, important to note is that some studies attempt to correlate cytoplasmic β-catenin levels with OS progression. An example would be Lu *et al*.'s study in which cytoplasmic β-catenin was found to be associated with metastasis and decreased patient survival (Lu *et al*., 2015). However, as discussed previously, the presence and association of nuclear β-catenin would possibly be more relevant in correlating its oncogenic activities with OS progression.

Cumulatively, these studies focus on correlation between β-catenin and OS progression, and not on ABC, which is the focus of our study. ABC, which is suggested to be transcriptionally more potent than β-catenin, can also potentially contribute to oncogenic activity of Wnt signaling, resulting in OS progression (Staal *et al*., 2002).

We also investigated the localization pattern and staining intensity for ABC and β-catenin in OS patient samples. Our results show a distinct nuclear localization of ABC in OS tissue sample and a more cytoplasmic and membranous staining for β-catenin (Figure 23). These results are

consistent with studies by Du *et al*. and Lu *et al*., both of which show cytoplasmic β-catenin staining in OS samples. Since we observed increased nuclear ABC levels in invasive-metastatic OS cell lines compared to parental ones, we further went on to assess ABC staining in an OS tissue microarray (TMA). For this purpose, we used a commercially available TMA which comprised of 40 embedded histologically confirmed primary OS samples in duplicate and included information on age, sex and stage of the tumors. The cores belonged to stages IA, IB, IIA and IIB of OS. Nuclear ABC staining was detected in 34 (85%) out of 40 tumor cores (Table 5). Statistical analysis showed that there was no positive correlation between the sex of patients and nuclear ABC staining ($p=0.64$). However, statistical analysis showed that, on average, positive nuclear ABC staining was seen mainly in tumors from younger patients ($p=0.01$). Further, there was significant association between stage of tumor and positive nuclear ABC staining ($p=0.04$). Our results underscore that there are significant correlations between positive nuclear ABC staining and tumor stage and age of patients. However, our findings are limited due to a small patient cohort size of our TMA, access to only limited patient information which does not include important information such as presence of metastasis, necrosis, etc. and an unequal distribution of number of patients in each stage of tumor. Moreover, the TMA did not consist of any OS tumors from stage III, which is also a limitation for our results. It is also of importance to note that all ABC negative tumors in our cohort were from adult patients and all tumors obtained from the children and adolescent and young adult (AYA) groups stained positively for nuclear ABC. Thus, these findings support prospective studies asking the question "can positive nuclear ABC staining serve as a prognostic marker in children and AYA with OS?"

In conclusion, our findings highlight that ABC is associated with OS progression *in vitro* and propose its potential use as a prognostic marker for OS. However, more studies consisting of a larger patient cohort size representative of all stages of disease and available clinicopathologic information such as metastasis, relapse, etc. are needed to establish a stronger basis for use of ABC as a marker for OS progression.

Chapter 5

Future Directions

5.0. Future Directions

5.1. Improvements for current study

Our current findings provide evidence for involvement of ABC in OS and show preliminary data for use of ABC as a potential marker for predicting OS progression. However, our results can be further strengthened by making some improvements to the existing data.

(i) *Co-localization of endogenous and transiently transfected ABC and β-catenin in SaOS2 cells*

Our co-localization results show that the ABC-GFP an d βcat-GFP transfected proteins have similar cellular localization patterns as seen for endogenous ABC and β-catenin respectively. However, our current confocal microscopy images can be further improved by capturing areas with a greater number of cells that show fluorescence intensities for both, GFP (for cells that are transfected with ABC-pEGFP-C2/βcat-pEGFP-C2) and Alexa-555 (for endogenous ABC/β-catenin tagged with anti-Alexa-555 secondary antibody). Seeing a similar co-localization pattern in an increased number of cells will strengthen our current findings for this experiment. Moreover, this experiment has been performed twice, and needs more repeats (at least n=3) to consolidate our current results. Thus, our current findings of similar localization pattern for transfected and endogenous ABC and β-catenin proteins can be made more sound by improving the images and by adding more repeats to this experiment.

(ii) *Western blot analysis of ABC-GFP, βcat-GFP and GFP proteins in SaOS2*

Our Western blot results for anti-GFP antibody show bands at ~119 kDa (ABC-GFP and βcat-GFP proteins) and 27 kDa (GFP protein) respectively. However, there is also presence of non-specific bands that can be seen in our results. Hence, the Western blot protocol should be modified to optimize primary and secondary antibody dilution and the number of washes performed to reduce the presence of non-specific background bands. Also, this experiment needs to be repeated twice and more repeats (at least n=3) need to be performed to consolidate our results.

5.2. Prospective studies

(i) *Validation of role of ABC in promoting the invasive/metastatic phenotype in OS*

Our study aims to evaluate ABC's role in promoting OS progression by overexpressing ABC and observing consequent changes in OS-associated cell properties and in transcriptional activity of ABC. To investigate this, we created an ABC over-expression construct (ABC-pEGFP-C2) which has successfully been transfected into SaOS2 cells (which express less endogenous ABC). β-catenin-pEGFP-C2 and pEGFP-C2 constructs were also transfected in SaOS2 cells in parallel. Protein levels of these constructs have been observed by (i) fluorescence microscopy due to GFP fluorescence tag on over-expressed proteins and (ii) Western blot analysis by blotting against anti-GFP and anti-β-catenin antibodies. Following this, ABC-pEGFP-C2 transfected SaOS2 cells will be compared with β-catenin-pEGFP-C2 and empty vector pEGFP-C2 transfected SaOS2 cells for gene/protein expression changes, transcriptional activity and properties associated with OS progression. qRT-PCR and Western blot analysis will be carried out to study changes in gene expression/protein levels of OS-specific targets of ABC including RANKL, OPG, VEGFA, cyclin D1, MMP2 and MMP9. Also, changes in ABC transcriptional activity, following transfection of constructs, will be studied via TopFlash assay. The OS associated properties will also be investigated including colony formation, migration, invasion, survival and proliferation of cells. These properties will be observed through functional assays including (i) colony formation assay, (ii) scratch assay for migration, (iii) Transwell® Invasion assay for invasion, (iv) MTT assay for cell survival and (v) ki67 vs Caspase-3 index for cell proliferation.

In addition to this, an important future step will be to include *in vivo* studies for investigating ABC's role in OS development and metastasis. For this, SaOS2 cells infected with lentiviral vectors which stably express the firefly luciferase gene will be established. Our model will consist of three groups of immunocompromised mice including (i) luciferase-expressing SaOS2 cells (SaOS2-luc), (ii) SaOS2 transduced with both luciferase and ABC-eGFP-C2 (SaOS2-ABC-luc) and (iii) SaOS2-LM7 cells expressing luciferase (SaOS2-LM7-luc). Growth of OS and subsequent metastasis will be monitored *in vivo* by bioluminescence produced by luciferase's reaction with its substrate luciferin. Based on our current findings, we expect our SaOS2-ABC-luc model to show more rapid tumor growth and increased metastasis when compared to SaOS2-luc tumors. Since

SaOS2-LM7 cells are known to be more metastatic than SaOS2, we expect to see similar metastatic potential for SaOS2-ABC-luc and SaOS2-LM7-luc tumors. Hence, if expected results are observed, they will support our current findings of upregulated ABC levels and enhanced ABC activity in invasive-metastatic OS cell lines compared to parental ones.

(ii) Validation of ABC as a marker of invasive/metastatic behaviour in OS

At present, we evaluated positive nuclear ABC staining across 40 unique OS patient samples present on a TMA. Our results showed that 85% (34 out of 40) of the samples stained positive for nuclear ABC with 15% (6 out of 40) showing no nuclear ABC staining. We also performed correlational analysis and saw an association for nuclear ABC staining with patients' age and stage of tumor. However, our current findings are limited due to a small sample size (n=40) of OS patients and availability of limited patient information including sex, age, pathology diagnosis and stage of OS tumor. Therefore, we aim to expand our current study by increasing our sample size. Power calculations show that one-way ANOVA with 84 patients will achieve 95% power to detect a difference in ABC levels in OS tumor tissues. In addition to this, we will have access to important clinicopathologic information regarding patients for both, at time of diagnosis and at follow-ups. These variables will include age at diagnosis/resection/post-resection interventions, sex, presenting signs and symptoms, tumor location and metastatic sites at diagnosis, tumor size, qualitative radiologic characteristics, soft tissue extension, therapy type and duration, local and/or metastatic disease progression at follow up clinic visits, tumor or treatment complications, and survival. Paraffin embedded OS tumor samples will be used to generate TMAs. The TMA will be stained for ABC and scored independently by two pathologists who are blinded to clinical information/outcomes pertaining to the patients. After the scoring is complete, ABC levels will be correlated to the clinico-pathologic features of the tumors (grade, histologic type) (unpaired Student's *t* test) and to selected clinical and demographic variables. Important to note is that β-catenin levels will be evaluated in parallel. Thus, with these prospective findings, we will be more informed about potential use of ABC as a biomarker for OS metastasis, chemotherapy sensitivity and survival outcome for patients.

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