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XUAN DUNG HO

Characterization of the genomic profile of osteosarcoma

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Department of Traumatology and Orthopaedics, Institute of Clinical Medicine, University of Tartu, Tartu, Estonia

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Supervisors: Professor Aare Märtson, MD, PhD
Department of Traumatology and Orthopaedics,
Institute of Clinical Medicine, University of Tartu, Tartu, Estonia

Associate Professor Katre Maasalu, MD, PhD
Department of Traumatology and Orthopaedics,
Institute of Clinical Medicine, University of Tartu, Tartu, Estonia

Professor Sulev Koks, MD, PhD
Department of pathophysiology, Institute of Biomedicine and Translational Medicine, Center of Excellence for Genomics and Translational Medicine; University of Tartu, Tartu, Estonia.
The Perron Institute for Neurological and Translational Science, Perth, WA, Australia.

Reviewers: Senior research fellow Neeme Tõnisson MD, PhD
Institute of Genomics, Estonian Genome Centre, University of Tartu, Tartu, Estonia

Associate professor Peeter Padrik MD, PhD
Department of Oncology, Institute of Clinical Medicine,
University of Tartu, Tartu, Estonia.

Opponent: Professor Thomas Rado, MD, PhD
Kadlec Clinic Hematology and Oncology, WA;
University of Washington, Seatle, WA, United States of America.

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following three original publications, which are referred to in the text by their Roman numerals (I-III):

I. Ho XD, Phung P, Q Le V, H Nguyen V, Reimann E, Prans E, Kõks G, Maasalu K, Le NT, H Trinh L, G Nguyen H, Märtson A, Kõks S. Whole transcriptome analysis identifies differentially regulated networks between osteosarcoma and normal bone samples. Experimental Biology and Medicine. 2017 Dec 19;242 (18):1802–11

II. S. Rubina Baglio, Tonny Lagerweij, Maria Pérez-Lanzón, Xuan Dung Ho, Nicolas Léveillé, Sonia A. Melo, Anne-Marie Cleton-Jansen, Ekaterina S. Jordanova, Laura Roncuzzi, Michelina Greco, Monique A.J. van Eijndhoven, Giulia Grisendi, Massimo Dominici, Roberta Bonafede, Sinead M.Lougheed, Tanja D. de Gruijl, Nicoletta Zini, Silvia Cervo, Agostino Steffan, Vincenzo Canzonieri, Aare Martson, Katre Maasalu, Sulev Koks, TomWurdinger, Nicola Baldini, and D. Michiel Pegtel. Blocking Tumor-Educated MSC Paracrine Activity Halts Osteosarcoma Progression. Clinical Cancer Research. 2017;23 (14):3721–34.

III. Ho XD, Nguyen HG, Trinh LH, Reimann E, Prans E, Kõks G, Maasalu K, Le VQ, Nguyen VH, Le NTN, Phung P, Märtson A, Lattekivi F, Kõks S. Analysis of the Expression of Repetitive DNA Elements in Osteosarcoma. Front Genet. 2017 Nov 30;8 (November):1–9.

Author's contributions:

Studies I and III: HO XD was involved in the design of the study, the collection of data and samples, sample analysis, and writing the papers.

Study II: HO XD was involved in the conception and design of the study, acquisition of data, analysis, and interpretation of data.

ABBREVIATIONS

AJCC	American Joint Committee on Cancer
ALP	Alkaline phosphatase
Bp	Base pair
BH	Benjamini-Hochberg
BS	Bloom Syndrome
cDNA	Complementary DNA
CSF1R	Colony-stimulating factor 1 receptor gene
CT	Centromeric
DBA	Diamond Blackfan anemia
DEGs	Differentially expressed genes
DLG2	Discs large MAGUK scaffold protein 2
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EUS- FNA	Endoscopic ultrasound-guided fine needle aspiration
FDR	False Discovery Rate
FFPE	Formalin-fixed and paraffin-embedded
FNA	Fine Needle Aspiration
GSEA	Gene set enrichment analysis
HCC	Hepatocellular carcinoma
HERVs	Human endogenous retroviruses
HSATII	Pericentromeric human satellite II
LDH	Lactate Dehydrogenase
LFS	Li- Fraumeni syndrome
LINEs	Long interspersed nuclear elements
LTR	Long terminal repeat
MAP	High dose methotrexate, anthracycline, and cisplatin
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
mTOR	The mammalian target of rapamycin
OS	Osteosarcoma
PCR	Polymerase Chain Reaction
PCT	Pericentric
PD- L1	Programmed death-ligand 1
PET	Positron Emission Tomography
PMI	Postmortem interval
RANK	Receptor Activator of Nuclear Factor κ B
RB	Hereditary Retinoblastoma
RB1	Retinoblastoma1
rdDNA	RNA-derived DNA
REs	Repetitive elements
RIN	RNA Integrity Number

RNA	Ribonucleic acid
RNA- ISH	RNA in situ hybridization
RT-PCR	Real-Time Polymerase Chain Reaction
RTS	Rothmund- Thomson syndrome
SINEs	Short interspersed nuclear elements
SNPs	Single nucleotide polymorphisms
TGF-β	Transforming growth factor-β
TKIs	Tyrosine kinase inhibitors
TE	Transposable elements
UV	Ultraviolet
VEGFRs	Vascular endothelial growth factor receptors
WS	Werner syndrome

1. INTRODUCTION

Osteosarcoma (OS), or osteogenic sarcoma, is a primary malignant bone tumor with a mesenchymal origin. It is characterized by the formation of immature bone or osteoid tissue by the tumor cells. In rare cases, OS can arise in soft tissue (Picci 2007).

OS is the most prevalent form of the primary malignant tumor to affect the skeletal system worldwide (Mirabello, Troisi, and Savage 2009a). OS also accounts for 20–40 % of all bone cancers (Whelan et al. 2012). The incidence rate of OS varies between different age groups. OS is known to occur mostly during adolescence. Its highest peaks of prevalence are with women between 10–14 years old and men between 15–19 years old. The second highest incidence peak is in adults older than 60 years old. Overall, OS incidence on average is about 3 cases per million per year (Whelan et al. 2012; Picci 2007; Mirabello, Troisi, and Savage 2009a; Mirabello, Troisi, and Savage 2009b). The incidence of OS is slightly higher in men than in women in most countries. The overall male-to-female ratio of OS varies by age from 1.01:1 in ages over 60 to 1.43: 1 in those in ages 0–24 (Mirabello, Troisi, and Savage 2009b). Clinically, pain and swelling were the most reported symptoms in patients with OS. General symptoms such as weight loss, fever, and anorexia are less common (Picci 2007). Over half of OS cases affect the long bones of the lower limb (56%) with the upper limbs (10%) and the pelvis (9%) being the next most affected sites (Whelan et al. 2012; Harrison et al. 2018).

The exact cause of OS is not yet determined, but some risk factors have been identified. Known risks include: rapid bone growth, radiation, and some OS susceptible genetic disorders such as Li-Fraumeni syndrome (LFS), hereditary retinoblastoma (RB), Werner syndrome (WS), Rothmund-Thomson syndrome (RTS) type 2, Bloom syndrome (BS), RAPADILINO syndrome, and Diamond Blackfan anemia (DBA) (Kansara et al. 2014; Ripperger et al. 2017). It is a highly metastasized cancer, as 80–90% of patients are believed to have micrometastasis at the time of diagnosis (Luetke et al. 2014). This can explain the improved outcome of OS with treatment after the introduction of chemotherapy in the late 1970s and early 1980s (J.Chou, S.Geller, and Gorlick 2008). Unfortunately, the prognosis of OS has remained poor and unchanged since the 1980s with the 5-year survival rate at 60–78% at the localized stage and only 20–30% at more advanced stages or the onset of metastatic disease (Durfee, Mohammed, and Luu 2016; Lisa Mirabello, Rebecca J. Troisi 2009; Kager et al. 2003; Picci 2007).

The number of studies on OS is rapidly growing. Increasing numbers of researchers are conducting research on and contributing to understanding molecular mechanism and discovering new therapeutic targets for this cancer, which is difficult due to the heterogeneity of the disease (Kansara et al. 2014). In 95% of OS tumors, the presence of mutations in the p53 pathway was noted. Trans-

locations and focal deletions were the most common forms of the TP53 mutations. RB1, DLG2, and ATRX gene mutations have also been identified (X. Chen et al. 2014; Kansara et al. 2014). Some microRNAs, such as with miR-206, miR-195, miR-340, miR-503, have also been found to be down-regulated as a result of OS and were associated with a poor prognosis of the disease. Other microRNAs may be upregulated, like miR-17 (Gao et al. 2014; Chong et al. 2014; Y.-P. Bao et al. 2013; Cai et al. 2015). Recent high-throughput technologies, implemented in OS studies, have identified the differential expression of genes in OS, but there are still many limitations and needed improvements with these studies in order to deliver a convincing conclusion. The limitations of those studies include small sample sizes, variations in control groups, and varied laboratory protocols and analysis techniques (Siddiqui et al. 2006; Z. Yang et al. 2014; Märtson et al. 2013). Heterogeneous findings from these studies raise questions as to how the design of these studies can be improved to yield more conclusive information.

Additionally, many in vitro and in vivo experiments assumed that TGF-β would promote cancer metastasis via its effects on the tumor microenvironment, enhanced invasive characteristics, and inhibiting the function of immune cells. These experiments have identified associations between TGF-β signaling and cancer progression which has motivated researchers to examine the complicated role TGF-β plays in tumorigenesis. Manipulation of these signals may offer a pathway for the interference of the metastatic mechanism that can be utilized for treatment of OS. TGF-β targeted therapies are being developed (Padua and Massagué 2009). However, the role of TGF-β is complicated and the results of anti-TGF-β therapies are diverse and challenging. Hence, more studies and research is needed in order to understand the function of TGF-β and perspective of TGF-β modulators in cancers, including OS.

In tumorigenesis, repetitive elements were found to correlate with some carcinomas, germinomas, and hematologic cancers. Some satellites such as ALR and or HSATII were suggested to be used as biomarkers for diagnosing some epithelial cancers but no similar study was done for OS (David Tsai Ting, Daniel A. Haber, Shyamala Maheswaran 2012). The investigation into the expression of repetitive elements in OS would broaden the knowledge of what repetitive elements can be seen in all types of cancer and it would suggest the potential role in the diagnosis of OS.

To develop more fundamental researches in OS, we aimed to set up a source of human OS samples to assist further studies on this disease and to provide an overview of some genetic changes in OS, which can suggest some biomarkers and lead to further targeted research in this field. We conducted the study focusing on gene expression of bone samples in OS where we compared paired samples of cancerous and normal bone tissue then we analyzed FFPE samples for changes in gene expression with chemotherapy. Our work was to describe the expression of TGF-β and its related genes in OS and to reveal the differential expression of repetitive elements in OS bone tissue.

2. LITERATURE OVERVIEW

2.1. The general aspect of osteosarcoma

2.1.1. Epidemiology

Among all types of cancers, osteosarcoma is rare, composing less than 1% of all cancers, but it is the most common primary bone cancer, especially among the young (Damron, Ward, and Stewart 2007; Mirabello, Troisi, and Savage 2009a; Whelan et al. 2012).

The age-standardized incidence rate of OS across 43 countries is relatively similar among men and women with rates ranging from 0.20 to 0.35/100,000 (Hung et al. 2014; Valery, Laversanne, and Bray 2015; Whelan et al. 2012). Though some studies have found OS to be more prevalent among men than women. For 2009, the overall world male to female ratio of OS was 1.43:1 for ages 1–24, 1.28:1 for ages 25–59, and 1.01:1 for ages 60 and up (Mirabello, Troisi, and Savage 2009b). The bimodal peak of OS incidence has been noted in most countries in both sexes, with the highest peak occurring around puberty and the second, lower peak, occurs among those 60 years and older (Savage and Mirabello 2011; Hung et al. 2014; Valery, Laversanne, and Bray 2015; Mirabello, Troisi, and Savage 2009a; Whelan et al. 2012).

2.1.2. Pathophysiology

OS can affect any bone, with the most common sites being at long bones of lower limb which accounts for 50–82% of cases (mostly distal femur and tibia) followed by the upper limb (mostly humerus at 10–14%) then pelvis (0–9%) (Mirabello, Troisi, and Savage 2009a; Whelan et al. 2012; Wiromrat et al. 2012; Sampo et al. 2011). The proportion of pelvic and axial skeleton primary tumors increases with age (5% at ages 10–19, 20% at ages 40–49; 28% at ages 70–79 (Whelan et al. 2012).

OS is divided into central or medullary and surface or peripheral OS. Medullary/central OS includes conventional or classic OS including osteoblastic (50%), chondroblastic (25%), and fibroblastic (25%); telangiectatic OS, well differentiated (low grade) OS and small-cell or round-cell OS. Peripheral OS originates on the bone surface, which contains parosteal (juxtacortical) OS (most common in this category); periosteal OS and high-grade surface OS (Fletcher and Unni 2002; Schajowicz, Sissons, and Sobin 1995). The most common subtype is conventional OS, which accounts for 75% to 85% of all OS cases (Marchiori 2014; Durfee, Mohammed, and Luu 2016; Picci 2007). Lungs are the most common metastatic sites of this potentially metastasized disease (Gorlick and Khanna 2010).

2.1.3. Etiology

The etiology of OS remains unclear. However, some risk factors have been identified. A high birth-weight and an above-average height are thought to increase the risk of developing OS (Mirabello et al. 2011). Paget disease has also been shown to increase the risk of secondary OS, especially in the elderly (Broadhead et al. 2011). Radiation has also been attributed to approximately 2% of OS cases. The increased incidence of radio-induced OS may be due to a better outcome with longer survival after primary radiotherapy (Picci 2007). Bone sarcoma obtained in rats and cell-free extracts from human OS suggests a possible viral origin of OS (Olson and Capen 1977; Picci 2007). Furthermore, several chemicals were associated with the development of OS, such as methylcholanthrene, beryllium oxide, chromium salts, zinc beryllium silicate, asbestos, and aniline dyes (Broadhead et al. 2011).

Several genetic syndromes and predispositions were also found to correlate with OS including Li-Fraumeni syndrome (LFS), Rothmund-Thomson syndrome (RTS) type 2, hereditary retinoblastoma (RB), Bloom syndrome (BS), Werner syndrome (WS), RAPADILINO syndrome, and Diamond Blackfan anemia (DBA), etc. (Calvert et al. 2012; Picci 2007; Lindsey, Markel, and Kleinerman 2017; Durfee, Mohammed, and Luu 2016).

2.1.4. Clinical presentation

Symptoms of OS are not specific and are easily neglected. They include pain at the affected site, pain at night, and a growing mass. If the pain gets worse with neither infection nor trauma, it is worrisome. A palpable mass, limited joint motion, increasing pain with movement or weight bearing, and a localized erythema warmth are all common clinical physical signs. Pathologic fracture appears in 5–10% of patients as the first sign of OS. General status such as malaise, fever, and weight loss are not early manifestation in children and they usually present only at an advanced stage (Lindsey, Markel, and Kleinerman 2017; Picci 2007; Durfee, Mohammed, and Luu 2016).

2.1.5. Workup

Initial investigation should be conducted with conventional radiographs with two planes. Radiographic images of OS are variable depending on subtype. Typical radiographic images of OS are often poorly marginated with the moth-eaten appearance of the bone with mixed amounts of cloudy, mineralized matrix and areas of bone resorption. A discontinuous periosteal reaction is commonly found with the presence of an associated soft tissue mass (Marchiori 2014; Lindsey, Markel, and Kleinerman 2017; Durfee, Mohammed, and Luu 2016; Clayer 2015). MRI is used to complement the plain radiograph in diagnosing OS in order to determine the extent of tumor invasion to the bone marrow and

the surrounding structures, the features of the soft tissue mass, and skip metastasis detection. For local staging, MRI is the most useful modality. Furthermore, MRI is used for evaluating the response of OS to neoadjuvant chemotherapy (Fox and Trotta 2013; Kubo et al. 2016; James S. Meyer, MD, * Helen R. Nadel, MD, Neyssa Marina, MD, Richard B.Womer and Kenneth L. B. Brown, MD, J.F. Eary, MD, Richard Gorlick, MD, Holcombe E. Grier, MD, R. Lor Randall, MD, FACS, Elizabeth R. Lawlor, MD, PhD, Stephen L. Lessnick, MD, PhD, Paula J. Schomberg, MD, and Mark D. Kailo 2008; Durfee, Mohammed, and Luu 2016). Computed tomography can be used to visualize calcification, periosteal bone formation, or cortical destruction. Computed tomography is more sensitive than a plain radiograph in detecting lung metastasis (Lindsey, Markel, and Kleinerman 2017; The ESMO/European Sarcoma Network Working Group 2014). Bone scans combined with computed tomography, or MRI, are used to evaluate the presence of bone metastasis (Lindsey, Markel, and Kleinerman 2017). Though, a PET scan is superior to computed tomography or a bone scan in detecting whole body metastasis, particularly soft tissues (lung, abdomen) (Durfee, Mohammed, and Luu 2016). Laboratory check-ups are not used for diagnosis, but the discovery of a high concentration of ALP and LDH predicts a poorer prognosis (Limmahakhun et al. 2011; Lindsey, Markel, and Kleinerman 2017; Marais et al. 2015; Durfee, Mohammed, and Luu 2016).

2.1.6. Diagnosis

The suspected bone tumor should be referred to an experienced center where standard biopsy and curative treatments can be performed. Core biopsy or open biopsy are recommended for diagnosing the disease. Core biopsy is increasingly used. An open biopsy will be prompted to confirm the diagnosis if core biopsy is non-contributive. Incisions are made longitudinally and never transversely; they should be matched with the operation field of definitive surgery (Lietman and Joyce 2010; James S. Meyer, MD, * Helen R. Nadel, MD, Neyssa Marina, MD, Richard B.Womer and Kenneth L. B. Brown, MD, J.F. Eary, MD, Richard Gorlick, MD, Holcombe E. Grier, MD, R. Lor Randall, MD, FACS, Elizabeth R. Lawlor, MD, PhD, Stephen L. Lessnick, MD, PhD, Paula J. Schomberg, MD, and Mark D. Kailo 2008; The ESMO/European Sarcoma Network Working Group 2014).

There are two systems for staging: Enneking and AJCC system (new version released for use as of 2018) (Amin, M.B., Edge, S., Greene, F., Byrd, D.R., Brookland, R.K., Washington, M.K., Gershenwald, J.E., Compton, C.C., Hess, K.R., Sullivan, D.C., Jessup, J.M., Brierley, J.D., Gaspar, L.E., Schilsky, R.L., Balch, C.M. and D.P., Asare, E.A., Madera, M., Gress, D.M., Meyer 2017; Durfee, Mohammed, and Luu 2016).

2.1.7. Treatment

The primary treatment approach for OS is multimodality management. In the 1970s, Jaffe announced the first success of methotrexate in treating the advanced disease, leading to the increasing use of chemotherapy for OS afterward (Durfee, Mohammed, and Luu 2016). In localized disease, multidrug perioperative treatment combined with curative operation remains as a standard treatment. Many regimens are permitted for the treatment of OS. MAP (high dose methotrexate, anthracycline, and cisplatin) is used as a referenced protocol. Curative surgery includes amputation and limb-sparing techniques. Amputated surgery was used widely in the past. Nowadays, limb-sparing surgery is indicated increasingly (Harrison et al. 2018; Durfee, Mohammed, and Luu 2016; Picci 2007; The ESMO/European Sarcoma Network Working Group 2014; Lindsey, Markel, and Kleinerman 2017).

The role of radiotherapy is minimal in OS. It can be indicated to treat the residual disease, in cases where limitedly resected surgery was performed due to the anatomical locations (Lindsey, Markel, and Kleinerman 2017; The ESMO/ European Sarcoma Network Working Group 2014). In metastatic disease, chemotherapy remains the principle modality. Isolated lung metastasis can be resected in adjunction with chemotherapy.

So far, targeted therapies such as mTOR inhibitors, RANK inhibitors, TKIs that inhibit VEGFRs, and immunotherapies (interferons, TGF-β modulators, anti-PD-L1 antibodies, etc) have been developed and trialed in different stages (Harrison et al. 2018; PosthumaDeBoer et al. 2011; Duval and Hamelin 2002; Kager, Tamamyan, and Bielack 2017; Heymann and Rédini 2013; Kansara et al. 2014). The results of these trials are promising but still, more testing needs to be conducted.

2.1.8. Prognosis

A marked improvement in survival rate was noted due to the introduction of chemotherapy during 70s-80s. The prior five-year survival rate of 20% increased up to 70% by 1980s, but little change has been observed since (Lisa Mirabello, Rebecca J. Troisi 2009; Whelan et al. 2012; Mirabello, Troisi, and Savage 2009a). The survival rates are higher in the following groups: females, youngest age group of 0–24, and those with a localized disease or extremity tumors (Lisa Mirabello, Rebecca J. Troisi 2009; Whelan et al. 2012).

2.2. RNA sequencing from bone originated samples

2.2.1. RNA isolation from fresh frozen bone

A biobank was developed to store the tissue samples to preserve their morphology, proteins, DNA, and RNA, which is easily degraded, for further use in other biologic studies. Even with freezing, RNA may degrade after 5 years.

Frozen tissue specimens can be preserved by keeping them in –80°C freezers or in liquid nitrogen (Riddick et al. 2003; Micke et al. 2006; Shabihkhani et al. 2014). RNA has been shown to remain stable by putting surgical specimens on ice (0°C) during transportation from the operation theater and stored in –80°C within 2–3 hours (Micke et al. 2006). DNA and Protein maintain for a longer time (Shabihkhani et al. 2014). RNA can be extracted successfully from frozen specimens with enough quality for further analysis through a series of protocols and kits available on the market (Linton et al. 2010). The quality and quantity of RNA extracted from freezing samples preserved by –80°C freezer or liquid nitrogen are equivalent (Auer et al. 2014). Isolating RNA from bone can be a challenge because bone is hard and rich in degradative enzymes and contains a low number of cells embedded in the highly mineralized tissue (Xin, Ling, and Nicola 2008; Carter et al. 2012; Ireland 2003). The present methods for isolating RNA from bone use several steps in which the frozen specimen is wrapped in foil, refrozen in liquid nitrogen, and then ground into powder by a hammer (Carter et al. 2012; Ireland 2003) or using a mortar and pestle with liquid nitrogen (Mantila Roosa, Liu, and Turner 2011; Xin, Ling, and Nicola 2008; Barbaric, Dalla-Pozza, and Byrne 2002). A new method of RNA isolation with a single step from the frozen bone specimen is relatively simple. It combines grinding the bone and the phenol-guanidinium based RNA isolation while maintaining nearly freezing temperatures. This method enhanced the yield of high-quality RNA by eight-fold, with RIN ranging from 6.7 to 9.2 (Carter et al. 2012).

2.2.2. RNA isolation from FFPE specimens

Formalin-fixed and paraffin-embedded (FFPE) tissue samples are very familiar to pathologists in histopathological analysis. They can be stored for a long period of time in the pathological archives. This kind of biospecimen is becoming a useful source for researchers using newly available techniques. In 1988, the first successful RNA isolation from 2.5-year-old FFPE samples was performed by Rupp and Locker, and since then many protocols have been introduced (Rupp and Locker 1988; Ribeiro-Silva, Zhang, and Jeffrey 2007).

The isolation of RNA from FFPE that had aged from several months to ten years or longer has been conducted successfully and with sufficient quality for gene analysis (Ribeiro-Silva, Zhang, and Jeffrey 2007; Penland et al. 2007; Hamatani et al. 2006). Many factors that affect the quality of RNA isolated from FFPE have been identified. They consist of prefixation conditions (cold ischemia time, decalcification), fixation (used buffer, duration of fixation, temperature, methods for speeding up the infiltration of formalin into the biospecimen), and processing and storage of FFPE samples (Bass et al. 2014; von Ahlfen et al. 2007). The limitations of gene expression analysis with FFPE samples vary at different steps: degradation of RNA can occur prior to formalin fixation; formalin fixation induces critical chemical modification of RNA; RNA

continues to fragment and degrade over time, even after dehydration and paraffin-embedding (Ribeiro-Silva, Zhang, and Jeffrey 2007). Because of this fragmentation and degradation over time, the RIN is too low with FFPE. Even with a low RIN, RNA can still be successfully analyzed using some molecular techniques. Using real-time RT-PCR reactions, researchers have managed to successfully amplify up to 80% of only-60-bp fragments (Ribeiro-Silva, Zhang, and Jeffrey 2007; Hamatani et al. 2006). Additionally, the quantity and quality seem to be independent in RNA extraction (Chung, Braunschweig, and Hewitt 2006). Although the quality of RNA isolated from FFPE older than ten years was shown to be similar to that of RNA isolated from recent samples aged only for several months, the quantity, consistency, and success rate of extractions was higher in the several-months-old group (Ribeiro-Silva, Zhang, and Jeffrey 2007).

Recently, advanced sequencing techniques make it possible for the RNA sequencing of low input RNA from FFPE specimens and even RNA with a RIN as low as 1.4 for gene expression analysis (Madabusi, Latham, and Andruss 2006). However, there are still many challenges associated with low RNA quality and recovery that need to be overcome (Stewart et al. 2017; Greytak et al. 2015; Hedegaard et al. 2014). Penland et al. showed that with FFPE, we can perform meaningful RNA expression analysis, but it should be noted that many samples are too degraded for analysis and there was a greater loss of information compared to frozen samples (Penland et al. 2007).

To improve the quality of RNA sequencing, some requirements should be met. PMI (postmortem interval) and cold ischemia time (the time between tissue removal from the body and fixation) should be kept as short as possible, ideally kept to less than four and twelve hours, respectively. The decalcification of specimens should be done with EDTA or by ultrasound if needed; time of fixation should be restricted between eight and forty-eight hours in a neutral buffered formalin at ambient temperature or 48°C. The tissue should not be thicker than 5 mm to speed up the inactivation of RNases, as other enzymes may affect the gene expression profile. It was recommended that the FFPE blocks should be examined within 1 year, and FFPE slices can only be kept at room temperature for a maximum of three months (Bass et al. 2014; von Ahlfen et al. 2007).

There are three principal steps for RNA extraction from FFPE samples: deparaffinization, which can be done by an organic compound such as xylene; elimination of cross-links between protein – RNA by proteinase K and liberating the RNA; recuperation of nucleic acids performed by phenol extraction or a column-based purification (Madabusi, Latham, and Andruss 2006). By modifying these three steps, several methods have been applied to the result of RNA purification (Okello et al. 2010; Ribeiro-Silva, Zhang, and Jeffrey 2007). Additionally, a fully automated RNA-purification method was applied to isolate RNA from FFPE samples and it showed the most reproducible method compared to semi-automated and manual methods in the gene expression analyses from FFPE aged between 3–20 years old (Bohmann et al. 2009).

2.2.3. RNA quality assessment

RNA is vulnerable to degradation because of the common presence of RNases in the environment. RNA quality has been shown to directly influence the distribution of gene expression levels (Imbeaud 2005). Thus, RNA handling must be done with care not only during isolation but also in choosing the method of quantification and subsequent analysis. To ensure the successful analysis of RNA, quantification of RNA should be done properly.

UV absorbance measurements with spectrophotometers, such as Nanodrop®, have been used to evaluate the purity of RNA for subsequent analysis. The ratio of absorbance at 260 and 280 nanometers is used to evaluate the purity of RNA. The ratio from 1.8–2.2 is normally judged as pure RNA (Doug Wieczorek 2012). Fluorescent dye-based quantification (using Qubit®, QuantiFluor™) is an alternative to the UV absorbance measurements to quantitate nucleic acids. It is more selective and accurate in quantitating nucleic acids than UV absorbance measurements (Thermo Fisher Scientific 2016; Doug Wieczorek 2012).

Agarose and Acrylamide Gel electrophoresis is also widely used in nucleic acids analysis. In which samples are loaded onto precast gels, and from there nucleic acid fragments move differently in speed according to its size through the gel matrix caused by the electrical current going through the gel. The visualization of separated fragments can then be done by fluorescent dye bound to the nucleic acid. In mammals, a 28S:18S rRNA ratio of 2:1 is commonly considered as good-quality RNA (Doug Wieczorek 2012).

In 1999, the Agilent 2100 bioanalyzer was introduced and has since been widely used in the evaluation of RNA quality. It is an automatically bioanalytical device which uses microfluidics technology to provide electrophoretic separations in an automated and reproducible manner (Mueller et al. 2000; Schroeder et al. 2006). Degradometer analysis and 'RNA Integrity Number' (RIN) algorithm analysis were developed for the analysis of RNA quality. They are both trustworthy and non-user-dependent methods for automatically assessing the degradation and integrity of RNA. The RIN system is slightly more informative (Imbeaud 2005). The latter software automatically generates a numerical value (RIN score) for each RNA sample based on its entire electrophoretic trace. The value ranges from 1 to 10, with 1 being totally degraded RNA and 10 being highly intact RNA (Mueller, Lightfoot, and Schroeder 2016; Schroeder et al. 2006; Imbeaud 2005; Doug Wieczorek 2012). It is free from instrument and concentration variability. Thus, RIN can be applied to make a comparison of samples among different laboratories (Schroeder et al. 2006). However, RIN values are not sensitive enough to judge the RNA quality of degraded FFPE specimens. They are also not a reliable predictive factor of successful library preparation (Doug Wieczorek 2012; Illumina 2016). We can also use DV200 metric to evaluate the FFPE RNA quality. The DV200 metric describes the percentage of RNA fragments that have more than 200 nucleotides, determined by a Fragment Analyzer or Bioanalyzer trace. It can adjust

precisely to determine the minimal RNA input needed for successful library preparation. RNA samples with DV200 < 30% are not recommended for experimentation (Illumina 2016). Real-Time Quantitative PCR and RT-PCR are increasingly used for nucleic acid quantification but are still quite expensive methods (Doug Wieczorek 2012).

2.2.4. RNA sequencing

The transcriptome represents a set of all RNA species in a tissue or a cell (Okazaki et al. 2002). This is a dynamic structure, where the total amount and types of transcripts change according to environmental factors and the current state of an organism (Wang, Gerstein, and Snyder 2009). The transcriptome sheds light on the functional mechanisms, development, and diseases of the genome (R. Chen et al. 2012). Transcriptome studies have gained more importance in the context of a cancer research. Cancer is connected to the unstable genome, thus investigation of gene expression and transcript structures reveal crucial information for oncology (Roychowdhury et al. 2011).

For transcriptome analysis, hybridization-based or sequencing-based approaches have been applied. However, one of the most powerful methods for transcriptome analysis is an RNA sequencing (RNA-Seq) approach. Based on the next generation sequencing (NGS) technology, RNA-seq is a rapid, accurate, and cost-effective approach, which allows not only the quantification and mapping of a transcriptome but also the determination of a gene's functional structure (Ruan et al. 2004; Costa et al. 2010; Wang, Gerstein, and Snyder 2009).

The workflow of an RNA-seq analysis is as follows:

Firstly, the complementary DNA (cDNA) library is created. The RNA, extracted from the tissue is fractionated according to RNA species. For this step, the hybridization with oligo(dT) or the selective degradation with nucleases may be used. Next, the cDNA library is prepared with a reverse transcription of target RNA species. The fragmentation of the library is done in order to reduce the length of analyzed sequences. Finally, adaptor ligation is conducted (Qian et al. 2014).

Sequencing of a cDNA library may be performed with different platforms: Illumina IG, Applied Biosystems SOLiD, and Roche 454 Life Science. SOLiD technology by Applied Biosystems uses sequencing by ligation technology. In the beginning, sequences are amplified with emulsion PCR. Primers are hybridized to template sequences. Probes are fluorescently labeled. For detection of fluorescence, a DNA ligase enzyme is used (Qian et al. 2014).

Data undergoes a bioinformatics analysis. For this purpose, a Bioconductor software (R-package) can be used. It can filter high-quality reads, perform mapping of a transcriptome via de novo assembly, or provide alignment to reference genome, if available. The edgeR, Deseq, and Deseq2 packages are among the most commonly used tools in Bioconductor for differential expression

analysis, which perform quantification of reads, disclosing the expression of the genes. Bioinformatic analysis can also supply information about single nucleotide polymorphisms (SNPs), fusion genes, and post-transcriptional gene regulation (Qian et al. 2014).

2.3. Differentially expressed genes in osteosarcoma

2.3.1. DEGs analysis

Recent advances in high-throughput sequencing technologies have assisted in conducting transcriptomic studies, especially for detecting the differential expression associated with specific conditions. Methods for differential gene expression analysis can be parametric or non-parametric. Many software tools have been developed for the analysis of gene expression, but still, there is no consensus in using them. An analysis of different tools such as baySeq, DESeq, DESeq2, EBSeq, edgeR, limma+voom, NOIseq, and SAMseq has shown the minimal influence of mapping tools on the final results (Z. H. Zhang et al. 2014; Oshlack, Robinson, and Young 2010; Costa-Silva, Domingues, and Lopes 2017).

2.3.2. DEGs in osteosarcoma

As high-throughput technologies continue to develop, the application of RNA sequencing has become more common in cancer research. This has led to promising results which contribute to a better understanding tumorigenesis and more biomarkers which can help guide diagnosing, prognostics, and treatment. This technique is increasingly used for studying OS, which is a very heterogeneous disease (Kansara et al. 2014). Results obtained from these studies were heterogeneous as well. In the transcriptome analysis of a single case of OS in an Estonian patient, 65 genes were found to be differentially expressed between tumor and normal bone in paired samples. 7 upregulated genes were found in normal tissue and 58 were upregulated in the cancer specimen (Märtson et al. 2013). In another study, the three differentially expressed genes GJA1, COL1A2, and COL5A2 were identified by Dajiang Wu et al. on the study of fourteen OS patients and six normal individuals. The study showed that COL1A2 and COL5A2 interact with several genes of the matrix metalloprotease (MMP) family, including MMP1, MMP2, MMP3, MMP14, TGF-β, and RUNX2 (D. Wu et al. 2014).

In an analysis of gene expression data from 8 published articles constituting 240 OS patients and 35 controls, Y Xiong et al. identified 979 DEGs in OS in comparison with normal tissues. Of the 979 DEGs, 472 were upregulated and 507 were downregulated DEGs. Ossification, bone development, and skeletal system developments were found to be significantly enriched annotations and may be involved in the progression of OS (Xiong et al. 2015). WWP1, EXT1,

LDHB, C8orf59, PLEKHA5, and CCT3 were highly upregulated while VWF was downregulated in OS compared to the control groups (Xiong et al. 2015).

In a study of 19 OS cell lines and four normal bone cell lines, 1170 DEGs were found with 530 upregulated genes and 640 downregulated genes. They suggested that RPL8, PLCγ1, PLCγ2, SYK, MAD2L1, AURKA, CDCA8, BUB1, and MELK may be correlated with OS (SUN, LI, and YAN 2015). A significantly higher number of differentially expressed genes was found between the 84 OS biopsies with MSCs and osteoblasts at 12,542 and 2,939 respectively (Kuijjer et al. 2012).

Differential gene expression was found to be associated with metastatic status and chemotherapy treatment. CXCR4 was found to be differentially expressed in metastasis and may serve as a prognostic factor in OS (Namløs et al. 2012; Salinas-Souza et al. 2013). From a cell lines study, AXL, TGFA, COLLA7A, and WNT5A were expressed more frequently in the three high-metastatic sublines than in the three low-metastatic (Nakano et al. 2003). Gene expression was also found to be differentially expressed after cytotoxic treatment. The myeloperoxidase gene, the thymine DNA glycosylase, and Hsp-60 were found significantly overexpressed after chemotherapy (Leonard et al. 2003).

High-throughput technologies are being used more frequently in OS studies. Diverse results have been obtained so far. This heterogeneity may come from the disease itself, as we mentioned in the beginning of this section, This may come from the variation of these studies in sample sizes, control groups, laboratory protocols, and analysis techniques (Z. Yang et al. 2014; Siddiqui et al. 2006). In order to validate these findings and to suggest biomarkers of OS, more studies need to be conducted.

2.4. Transforming growth factor-β (TGF-β)

2.4.1. TGF-β

The transforming growth factor-β (TGF-β) superfamily has more than 60 members identified in multicellular organisms at least 29 of which are found in humans. The TGF-β superfamily are polypeptides secreted to activate cellular responses during growth and differentiation (Feng and Derynck 2005). Three isoforms of TGF-β (TGF-β1-2-3) have been identified in human tissues; the major reservoir of these proteins is believed to be in the bone matrix (JENNINGS and MOHAN 1990). In bone, they are seen to be highly expressed by the epiphyseal growth plate, perichondrium, and the periosteum (M. Wu, Chen, and Li 2016; Lamora et al. 2016). The potency of TGF-β 3 is on average 3 to 10-fold more than TGF-β 1 or TGF-β 2 on a molar basis, which has been found in fetal rat bone and in rat OS cultures (Ten Dijke et al. 1990; CENTRELLA et al. 1994).

2.4.2. Role of TGF-β in tumorigenesis

The role of TGF-βs is complicated in oncogenesis. They are believed to regulate tumor initiation, progression, and metastatic development. The TGF-β paradox is such that TGF-βs act as both tumor suppressors and tumor promoters, depending on the cancer type and tumor development timing (Principe et al. 2014; Roberts and Wakefield 2003; Lamora et al. 2016). During the late stages of epithelial cancers, the TGF-β cascade promotes tumor progression principally by stimulating epithelial-to-mesenchymal transition, tumor invasion, metastatic dissemination, and/or the evasion of the immune system (Meulmeester and ten Dijke 2011; Katsuno, Lamouille, and Derynck 2013; Lamora et al. 2014).

2.4.3. TGF-β in osteosarcoma

In sarcoma, especially in OS, TGF-βs seem to have a pro-tumoral effect (Lamora et al. 2016). The concentration of TGF-βs has been shown at elevated levels in the sera of OS patients compared to the sera of healthy people (Lamora et al. 2014). Recently, TGF-β has been detected in association with extracellular vesicles, which are considered a mediator of cell-cell communication and EV associated TGF-β functions not as the same as soluble ones (Webber et al. 2010).

A variety of TGF-β inhibitors have been developed for testing. They modulate TGF-β production, TGF-β activation, and TGF-β signaling at different levels. These levels include the ligand level (antisense oligonucleotides such as Trabedersen, Belagenpumatucel-L), the ligand-receptor level (some monoclonal antibodies such as fresolimumab, IMC- TR1 [LY3022859]), and the intracellular level (TGF-β receptor kinase inhibitors such as Galunisertib). TGF-β inhibitor drugs are already being tested in both preclinical studies and in clinical trials (Neuzillet et al. 2015; Arjaans et al. 2012; Lamora et al. 2016). PET TGF-β has been studied with the aim to identify candidates for TGF-β inhibition treatment (Arjaans et al. 2012).

The role of TGF-β plays in cancer is not fully understood, particularly in OS, and needs further investigation. The perspective of TGF-β modulators in cancer treatment is a major source of motivation for researchers.

2.5. Repetitive DNA elements in cancers

2.5.1. Repetitive elements in general

Repetitive elements (REs), also known as repetitive DNA, are defined as sequences that occur multiple times in the genome. They differ in origin, arrangement, and size. They can be interspersed or next to each other to create tandem repeats. The size can vary from 1–2 bases to millions of bases (Padeken, Zeller, and Gasser 2015; Casa and Gabellini 2012). With advances in biotechnology,

people know more about REs. In 2001, the first sequencing of the human genome showed that REs accounted for at least 50% of the genome (Lander et al. 2001). But a dramatically higher proportional estimation of RE was discovered that constitutes two-thirds of REs in the human genome (Koning et al. 2011). REs were grouped into five classes: transposon-derived repeats, simple sequence repeats, segmental duplications, blocks of tandemly repeated sequences, and ribosomal gene clusters (Lander et al. 2001). They can also be classified into tandem repeats and transposable elements (Padeken, Zeller, and Gasser 2015). Tandem repeats include satellite DNA, minisatellite, and microsatellite (Padeken, Zeller, and Gasser 2015). While transposable elements consist of retrotransposon (class I) and DNA transposons (class II). Transposon-derived repeats accounts for about 45% of the genome. Retrotransposons are composed of long terminal repeat (LTR) and non-LTR retrotransposons. Non-LTR retrotransposons include long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs) (Lander et al. 2001; Rebollo, Romanish, and Mager 2012). Human endogenous retroviruses (HERVs) are the most important LTRs in human. HERVs are a family of viruses that integrated into the human genome and share common features with the present exogenous retroviruses (Nelson et al. 2003). They account for about 8% of the human genome (Cegolon et al. 2013; Lander et al. 2001). Structurally, HERVs are typically constituted of gag, pol and env regions sandwiched between the two long terminal repeats (Bannert and Kurth 2004; Mager and Stoye 2015; Nelson et al. 2003).

2.5.2. Repetitive elements in oncology

Recent research has identified additional roles of the repetitive elements. There have been particularly interesting findings of REs in many kinds of cancers and their role in tumorigenesis. REs have been shown to be associated with different kinds of epithelial, germinal, and hematologic cancers. Highly expressed CT (centromeric) and PCT (pericentric) sequences have been identified in cancerous tissues as compared to normal tissues in paired samples of the same patients of testicular, ovarian, liver, and lung cancers (Eymery et al. 2009).

A digital gene expression analysis of 15 cases showed the total level of all satellite transcripts was 21-times more highly expressed in pancreatic ductal carcinomas as compared to normal pancreas. The top differentially overexpressed satellite in malignant samples was the pericentromeric human satellite II (HSATII), while it was not detectable in normal pancreas and minimally expressed in other normal organs. An overexpression of HSATII was also found in other human malignant tumors such as lung, ovarian, kidney, and prostate cancers (Bersani et al. 2015; Ting et al. 2011). The level of GSATII, TAR1, and/or SST1 transcripts under the reference threshold indicates that a subject has a tumor (David Tsai Ting, Daniel A. Haber, Shyamala Maheswaran 2012; Bersani et al. 2015). Alpha human satellite DNA was 43 times higher in pancreatic cancer compared to normal pancreas (Ting et al. 2011).

Many retrotransposons have been found to be related to tumors. HERV-K HML-2 was thought to be associated with several cancers including melanoma (Schiavetti et al. 2002), leukemia, lymphoma (Contreras-galindo et al. 2008), breast tumors, (Feng Wang-Johanning et al. 2008; Pichon, Bonnaud, and Cleuziat 2006) testicular cancer (Pichon, Bonnaud, and Cleuziat 2006), and ovarian cancer (Feng Wang-Johanning et al. 2006). The HERV-E family correlated with prostate, kidney, ovarian, and uterine cancers (Gimenez et al. 2010; Feng Wang-Johanning et al. 2003). In colorectal cancer, HERV-H sequences were found to be overexpressed (Pérot et al. 2015).

An association between non-LTR retrotransposons and cancers was also discovered. LINE-1 was seen to be upregulated in pancreatic and prostate cancers (Criscione et al. 2014; Contreras-galindo et al. 2008). In colorectal cancer, De novo L1 insertions were noted (Solyom et al. 2012). This then suggests that L1-mediated retrotransposition is a potential source of mutations that can decrease the tumor suppression of somatic cells in hepatocellular carcinoma (Shukla et al. 2013). Differential expression of several SINE subfamilies was revealed in prostate cancer (Criscione et al. 2014).

A patent for the use of repetitive elements in epithelial carcinomas was approved by their associations. David Tsai et al. recommended to use several kinds of REs such as ALR, HSTAII as biomarkers in the detection, follow-up, and prognosis of several cancers (David Tsai Ting, Daniel A. Haber, Shyamala Maheswaran 2012). This encourages us to analyze the expression of REs in OS.

2.6. The general aspect of osteosarcoma management in Vietnam

2.6.1. Introduction of health care system in Vietnam

In Vietnam, the public healthcare system plays a key role in providing health services. The total number of health facilities (hospitals, healthcare center/unit) is about 13,508 including 182 private hospitals(Vietnam Ministry of Health 2015). However, the private system is increasingly competing with the governmental institutions. The public medical institutions in Vietnam are classified into four levels from 1–4, national level, provincial level, district level, and commune level (Sakano 2015; The National Assembly of Vietnam 2014). High-level hospitals are better equipped with modern machines necessary for OS management such as modern CTs, MRI, bone scan, PET scan, and accelerator. The typical cytotoxic drugs recommended for OS are now available in Vietnam.

The Vietnamese government is trying to increase the coverage of medical insurance for the whole population. The medical insurance cover rate was 76.5% in 2015 (Vietnam Ministry of Health 2015) and has risen to more than 86% by the end of 2017 (Anh 2017). The percentage of payment by medical insurance for medical care, including approved techniques and medications, ranges from 80–100% depending on the type of insurance. The prosthesis for

the limb-sparing surgery is not yet covered, which is too expensive for the Vietnamese (The National Assembly of Vietnam 2014). In general, the treatment of OS in Vietnam is covered by Vietnamese medical insurance except for prosthesis, which is used for the conservative surgery of OS.

2.6.2. Management of OS in Vietnam

There is no standard data set of OS available for the Vietnamese population. But, some local studies have been conducted at national level hospitals, which have generated some characteristics of OS in Vietnam. OS is primarily treated majorly in Ho Chi Minh City and Hanoi. A smaller number of patients have been treated at other national level hospitals in Hue, Can Tho, and Danang.

In Vietnam, OS is the most common type of bone cancer and accounts for more than 50% of malignant bone tumors (Le Chi Dung 2003; P. H. Nguyen, Le, and Phan 1998). In Vietnam, primary bone cancer affects 2.12 males and 0.92 females per 100 million people (Vo, Tran, and Doan 2000). It is more prevalent in men than women and mostly affects the people between the ages of 11 and 30 years old (89.2%) with a mean age of 18.3. The affected sites are commonly extremities, around 80%, in particular, the femur and tibia are the most common sites (Le Chi Dung 2003; P. H. Nguyen, Le, and Phan 1998; Q. D. Nguyen and Le 2002; Vo, Tran, and Doan 2000). The conventional subtype of OS is the most common. The signs and symptoms of OS are non-specific so patients and primary care doctors can easily misidentify the disease. Patients were diagnosed at a late stage in more than 90% of cases. The stage IIB of Enneking at the diagnosis was the most frequent at about 80% (Le Chi Dung 2003; P. H. Nguyen, Le, and Phan 1998; Nguyen, van-Thang 2005). Patients presented to the hospital for consultations due to pain, mass, and pathologic fractures. The most common reason to present to the hospital, in 81% of cases, was due to the appearance of mass (Q. D. Nguyen and Le 2002). The evolution and effectiveness of OS treatment in Vietnam are similar to the worldwide situation. The application of modern techniques in imaging diagnostics and anatomo-pathology with immunohistochemistry has improved the diagnosis quality. Before 2000, treatment of OS by only surgery accounted for 83.2% of cases. Chemotherapy was used in only 1.5% of cases. The five-year overall survival was 19.9%. In a 2000 study (Vo, Tran, and Doan 2000), Vo Tien Minh found that patients commonly underwent devastated surgery such as limb amputation or disarticulation. Conservative operation was only used in 12% of cases. The overall survival at two years and five years was 43.1% and 34.3% respectively (Vo, Tran, and Doan 2000). Another study examining 1996–2006 showed an increase in the use of chemotherapy with cisplatin and doxorubicin before and after the operation and also limb-sparing surgery was evaluated (Le et al. 2009). Wide tumorectomy with bone reconstruction performed by autografts, allografts, callosities, prosthesis, internal fixation, bone cement, or a combination of these techniques show promising result but still need to be im-

proved. Some unfavorable results including death, metastasis, recurrence, and infection, may be due to late-stage diagnosis and or a lack of equipment (Le et al. 2009).

Nowadays, the diagnosis of OS is usually confirmed by core biopsy or open biopsy. Chemotherapy combined with surgery remains the most common form of treatment in Vietnam. Multidrug chemotherapy regimens are used in Vietnam such as cisplatin and doxorubicin. High-dose methotrexate is not frequently used because of the lack of ability to measure its concentration in the serum. While limb-sparing surgery is increasingly used, amputation remains a popular treatment. Tumoral endoprosthesis replacement for the treatment of OS is limited.

2.7. Summary of the literature

OS is a rare disease that mainly affects young people. Treatment methods of the disease have remained unchanged for years and the prognosis of this highly metastatic cancer is still very poor. In recent years, there has been an increased demand for research examining the pathophysiology and mechanism of the disease in order to identify new biomarkers and develop targeted therapies for the disease. While there has been an increase in publications and studies looking at OS, there are still many unanswered questions that need to be clarified.

We would like to further contribute to the understanding of the disease by establishing an OS database, beginning with Vietnam and Estonia, that will form the basis for future molecular studies. We started the project by creating an overview description of gene expression changes in OS. We want to focus on TGF-β expression, which is believed to be involved in the metastatic process. Additionally, we aim to reveal the expression of repetitive DNA elements (REs) in OS, which have been suggested to be biomarkers for some epithelial cancers, as no observation of REs has been conducted for OS.

3. AIMS OF THE THESIS

We created a database of OS including biobank for genomics studies. The studies were conducted with the following aims:

1. To investigate and describe the differential expression of genes between malignant bone samples and normal adjacent bone samples and to reveal the changes in gene expression with chemotherapy.
2. To describe the TGF-β expression in OS and its related genes.
3. To describe the differential expression of repetitive DNA elements between malignant and normal bone samples.

4. METHODS AND MATERIALS

4.1. Study subjects

Table 1: General characteristics of involved osteosarcoma in the study

NO	Patient code	Type of sample	Age at diag-nosis	Gender	Site of tumor	Metastasis at diagnosis	Chemo-therapy	Study
1	OSVN001	fresh	16	female	femur	No	Yes	I, II, III
2	OSVN003	fresh	13	male	femur	No	Yes	I, II, III
3	OSVN004	fresh	16	female	femur	No	Yes	I, II, III
4	OSVN005	fresh	18	male	femur	No	Yes	I, II, III
5	OSVN006	fresh	18	male	femur	No	Yes	I, II, III
6	OSHN008	fresh	24	female	tibia	No	yes	I, II, III
7	OSVN008	fresh	52	male	femur	Yes	No	I, II, III
8	OSHN009	fresh	16	male	femur	No	Yes	I, II, III
9	OSHN010	fresh	20	female	femur	No	Yes	I, II, III
10	OSHN011	fresh	07	male	tibia	No	Yes	I, II, III
11	OSHN012	fresh	11	male	humerus	No	No	I, II, III
12	OSHN013	fresh	17	male	femur	No	No	I, II, III
13	OSHN014	fresh	16	female	tibia	No	Yes	I, II, III
14	OSVN015	fresh	15	male	tibia	No	Yes	I, II, III
15	OSHN015	fresh	8	female	tibia	No	Yes	I, II, III
16	OSHN016	fresh	20	male	femur	No	Yes	I, II, III
17	OSHN017	fresh	16	male	humerus	No	Yes	I, II, III
18	OSDN001	fresh	23	male	tibia	Yes	Yes	I, II, III
19	EE4878	FFPE	24	male	femur	NA	yes	I
20	EE6762	FFPE	51	male	tibia	NA	Yes	I
21	EE6065	FFPE	80	female	femur	NA	Yes	I
22	EE6311	FFPE	9	male	humerus	NA	No	I
23	EE1480	FFPE	18	female	tibia	NA	Yes	I
24	EE9244	FFPE	29	male	pelvis	NA	No	I
25	EE6921	FFPE	31	female	femur	NA	No	I
26	EE3447	FFPE	20	male	femur	NA	Yes	I
27	EE13536	FFPE	22	female	femur	NA	No	I
28	EE648	FFPE	32	male	femur	NA	No	I
29	EE8076	FFPE	19	male	femur	NA	No	I
30	VN26391	FFPE	52	male	femur	Yes	No	I
31	VN25065	FFPE	20	male	femur	No	No	I
32	VN23611	FFPE	23	male	fibula	No	No	I
33	VN21890	FFPE	15	male	femur	No	No	I

This study includes 18 bone paired samples collected from 18 Vietnamese patients with histologically confirmed OS underwent surgery (limb-sparing or amputation) and 15 FFPE samples. FFPE samples were collected from the biobanks of pathology departments of Tartu University hospital and Hue university hospital. These 15 patients were also histologically confirmed of the OS diagnosis. 11 FFPE samples were from Estonia and 4 were from Vietnam.

Of the 33 patients included in the study, ten (30%) were females and twenty-three (70%) were males. The age of patients ranged from 7 to 80 years with a mean age of 23.4. Basic characteristics of studied patients are described in table 1.

4.2. Collecting data and samples

4.2.1. Bone collection

Bone samples were collected during surgery, after the removal of bone tissue from the OS patients by surgeons. Two samples were collected from each patient, one at the affected site referred to as 'OS sample' and another normal piece (far from the tumor) referred to as 'control sample' or 'normal bone'. We used conical tubes to store the bone samples. They were coded and stored in a freezer at -80^{0}C till the time of experimentation. Bone samples were transported with dry ice.

4.2.2. Formalin-fixed paraffin-embedded (FFPE) tissue

FFPE blocks were retrieved from the biobanks of the pathology department of Hue University hospital in Vietnam and from Tartu University Hospital, Estonia. Ten-micrometer-thick slices were obtained from each FFPE for the experiment. These FFPE samples were used to analyze the gene expression changes with chemotherapy in the study I.

4.3. Samples analysis

4.3.1. RNA extraction from fresh frozen bone samples

Bone samples (40–50 mg) were ground with nitrogen by pestle and mortar into powder and pretreated with Trizol. The extraction of total RNA was performed using RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia CA, USA) following the manufacturer's instruction. Isolated RNA was dissolved in RNase-free water and kept in the freezer at –80^{o}C until sequencing. The RNA quality assessment was done by Agilent 2100 Bioanalyzer and the RNA 6000 Nano Kit (Agilent Technologies Inc., CA, USA).

4.3.2. RNA extraction from FFPE samples

For each FFPE sample, 6 ten-micrometer-thick slices were used. RNA was extracted with PureLink FFPE Total RNA Isolation Kit from Invitrogen (Invitrogen Corporation, Carlsbad, CA, USA) following the instructions.

4.3.3. RNA sequencing

50 nanograms of total RNA was used. First, it was amplified by using Ovation RNA-Seq System V2 (NuGen, Emeryville, CA, USA). The resulting cDNAs were then pooled in identical amounts and the pool was used to prepare the DNA fragment library with SOLiD System chemistry (Life Technologies Corp., Carlsbad, CA, USA). Sequencing was carried out using the SOLiD 5500W platform and DNA sequencing chemistry from Life Technologies Corp., Carlsbad, CA, USA. Using the Maxmapper algorithm implemented in the Lifescope software (Life Technologies, Ltd), raw reads were color-space mapped to the human genome hg19 reference. Mapping to multiple locations was allowed. The quality threshold was set to 10, increasing mapping confidence by more than 90. Readings with a score less than 10 were filtered out. The average mapping quality was 30. Analysis of the RNA content and gene-based annotation was done within the whole transcriptome workflow implemented in Lifescope.

4.4. Statistical analysis

Statistical analysis was performed using R program.

4.4.1. Deseq2 package and EdgeR package

For statistical analysis, DeSeq2 (for fresh samples) and edgeR (for FFPE samples and repetitive elements) packages for R were used (Love, Huber, and Anders 2014; Robinson, McCarthy, and Smyth 2010). DeSeq2 and edgeR are packages in R which are specially developed for RNA-seq or other count data. They allow for the testing of differential expression by using the negative binomial distribution and a shrinkage estimator to account for the distribution's variance (Anders and Huber 2010). The packages perform sample comparison and adjust the P-value to overcome multiple testing problems. Both packages use the Benjamini-Hochberg procedure, which controls for the false discovery rate (FDR) (Benjamini and Hochberg 1995).

4.4.2. Reactome analysis

We applied pathway enrichment analysis to show the functional relations among differentially expressed genes. We used the R/Bioconductor package *ReactomePA* for Reactome pathway analysis and visualization (Yu and He 2016). Enrichment analysis is a widely-used method to reveal the biological themes of differentially expressed genes. *ReactomePA* uses the hypergeometric model to assess whether the number of selected genes associated with the Reactome pathway is larger than expected. The p-values are calculated based on the hypergeometric model. After analysis, results were visualized using an enrichment map and category-gene-network tools (Fabregat et al. 2016; Yu and He 2016; Subramanian et al. 2005). This method was used to analyze the pathways enrichment of differentially expressed genes between the affected bone and normal bone and to detect the pathways related to genes expression changes with chemotherapy.

4.5. Ethical consideration

Our work was carried out in accordance with the Declaration of Helsinki. The protocols and informed consent forms used in this study were approved by the Ethics Review Committee on Biomedical Research at Hue University of Medicine and Pharmacy. The decision number 74/CN-BVYD was signed on 12/3/2014.

All the participants, or participant representatives, were given information about the disease; the aims of the study; their role as a participant in the study; how the samples would be collected, coded, transferred, stored, and analyzed; how the results will be used. Only patients with full acceptation were recruited. All the participants, or patient representatives, signed the informed consent. Bone samples and collected medical information were coded without carrying any personally identifying information. All fresh bone samples were labeled with an updated code that can be traced or linked back to subject only by the primary researcher.

FFPE samples from biobanks were anonymous and only disease information was available to the researchers.

5. RESULTS

5.1. Differentially expressed genes in osteosarcoma

5.1.1. Differential expression analysis between paired fresh tumor and normal samples

The pairwise analysis was applied to signals from 21,632 genes. The Benjamini-Hochberg (BH) adjustment implemented in R was used to eliminate false positives. We named these values the BH-adjusted p-values (FDR), which are shown in the column "padj" in tables 2 and 3. With an adjusted p-value below 0.05, we were able to identify 5,365 differentially expressed genes between the cancerous and normal bone, of which 3,399 genes were upregulated and 1,966 were downregulated. *BTNL9, MMP14, ABCA10, ACACB, COL11A1, and PKM2 (PKM)* were the most significant (the lowest FDR) differentially expressed genes.

5.1.2. The most downregulated and upregulated genes in OS

Based on the differences in fold change (log2fold) between tumor and control samples, we generated a list of genes of both the most upregulated and downregulated in tumor samples. It means that these genes got the lowest log2fold and largest log2fold respectively with a significant p-value. The most downregulated genes in OS were *BTNL9, DNASE1L3, CAMP, LEPR, MIR223, MS4A3, LTF, LCN2, MMP8, S100A12, S100A8, MPO, EPB42, HEMGN, AHSP, ABCA10, BPI, CEACAM6, and DEFA4* (Table 2).

Table 2. The most significantly downregulated genes in osteosarcoma

Symbol	Log2Fold Change	padj	Chr	Gene name
BTNL9	-1.54	1.20E-15	5	Butyrophilin-like 9
DNASE1L3	-1.45	2.90E-07	3	Deoxyribonuclease I-like 3
CAMP	-1.45	4.47E-06	3	Cathelicidin antimicrobial peptide
LEPR	-1.43	1.21E-09	1	Leptin receptor
MIR223	-1.43	6.84E-06	X	MicroRNA 223
MS4A3	-1.41	7.31E-06	11	Membrane-spanning 4-domains, subfamily A, member 3
LTF	-1.4	1.00E-05	3	Lactotransferrin
LCN2	-1.4	1.00E-05	9	Lipocalin 2
MMP8	-1.4	8.74E-06	11	Matrix metallopeptidase 8
S100A12	-1.39	1.17E-05	1	S100 calcium binding protein A12
S100A8	-1.39	1.17E-05	1	S100 calcium binding protein A8

Symbol	Log2Fold Change	padj	Chr	Gene name
MPO	-1.39	1.17E-05	17	Myeloperoxidase
EPB42	-1.39	8.52E-06	15	Erythrocyte membrane protein band 4.2
HEMGN	-1.39	1.03E-05	9	Hemogen
AHSP	-1.39	1.30E-05	16	Alpha hemoglobin stabilizing protein
ABCA10	-1.38	3.43E-11	17	ATP-binding cassette, sub-family A (ABC1), member 10
BPI	-1.38	1.16E-05	20	Bactericidal/permeability-increasing protein
CEACAM6	-1.38	5.56E-06	19	Carcinoembryonic antigen-related cell adhesion molecule 6
DEFA4	-1.37	1.63E-05	8	Defensin, alpha 4, corticostatin

The most upregulated genes in OS were *COL11A1, TGFBI, TREM2, COL2A1, COL10A1, HAPLN1, MMP14, PANX3, CTHRC1, STEAP1, COL3A1* (Table 3).

Table 3. The most significantly upregulated genes in OS

Symbol	Log2Fold Change	padj	Chr	Gene name
COL11A1	1.51	3.43E-11	1	Collagen, type XI, alpha 1
TGFBI	1.4	1.46E-08	5	Transforming growth factor, beta-induced, 68kDa
TREM2	1.39	7.91E-08	6	Triggering receptor expressed on myeloid cells 2
COL2A1	1.38	1.09E-05	12	Collagen, type II, alpha 1
COL10A1	1.35	5.05E-06	6	Collagen, type X, alpha 1
HAPLN1	1.26	1.60E-05	5	Hyaluronan and proteoglycan link protein 1
MMP14	1.24	2.86E-11	14	Matrix metallopeptidase 14 (membrane-inserted)
PANX3	1.22	8.83E-05	11	Pannexin 3
CTHRC1	1.21	3.90E-08	8	Collagen triple helix repeat containing 1
STEAP1	1.2	1.40E-07	7	Six-transmembrane epithelial antigen of the prostate 1
COL3A1	1.19	2.94E-07	2	Collagen, type III, alpha 1
CA12	1.18	5.99E-08	15	Carbonic anhydrase XII
GJB2	1.17	5.26E-05	13	Gap junction protein, beta 2, 26kDa
PLOD1	1.15	3.61E-10	1	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1
LEPRE1	1.15	2.78E-08	1	Prolyl 3-hydroxylase 1

Symbol	Log2Fold Change	padj	Chr	Gene name
PSAT1	1.13	2.78E-08	9	Phosphoserine aminotransferase 1
COL6A1	1.13	1.23E-06	21	Collagen, type VI, alpha 1
FGFBP2	1.12	0.0004	4	Fibroblast growth factor binding protein 2
CTSB	1.11	1.27E-07	8	Cathepsin B
UCHL1	1.1	7.90E-05	4	Ubiquitin carboxyl-terminal esterase L1

From the list of genes which got the lowest padj values, we chose 3 most upregulated (highest Log2FoldChange): COLL11A1, TGFBI, MMP14 and 3 most downregulated (lowest Log2FoldChange): BTNL9, LEPR and ABCA10 for illustration.

Visualization of the differential expression of these genes is demonstrated in figure 1 and figure 2, which plot the individual values of these significantly changed genes (including downregulated and upregulated ones).

Figure 1 illustrates three downregulated genes (*BTNL9, LEPR, ABCA10*) and the upregulated genes (*MMP14, COL11A1, TGFBI*) in OS specimens. Consistent patterns in the expression of above genes were evident.

Figure 2 shows the differential expression of BTNL9, LEPR, MMP14, and COL11A1 between cancerous and non-cancerous samples in pairs of each patient. Expression of each gene for every patient is illustrated by different colored lines. It indicates that the differential gene expression was consistent among different patients.

Table 4. The most significantly differentially expressed genes in OS

Symbol	Log2Fold Change	padj	Chr	Gene name
BTNL9	-1.54	1.20E-15	5	Butyrophilin-like 9
MMP14	1.24	2.86E-11	14	Matrix metallopeptidase 14 (membrane-inserted)
ACACB	-1.19	3.43E-11	12	Acetyl-CoA carboxylase beta
PKM2	1.02	3.43E-11	15	Pyruvate kinase, muscle
ABCA10	-1.38	3.43E-11	17	ATP-binding cassette, sub-family A (ABC1), member 10
COL11A1	1.51	3.43E-11	1	Collagen, type XI, alpha 1

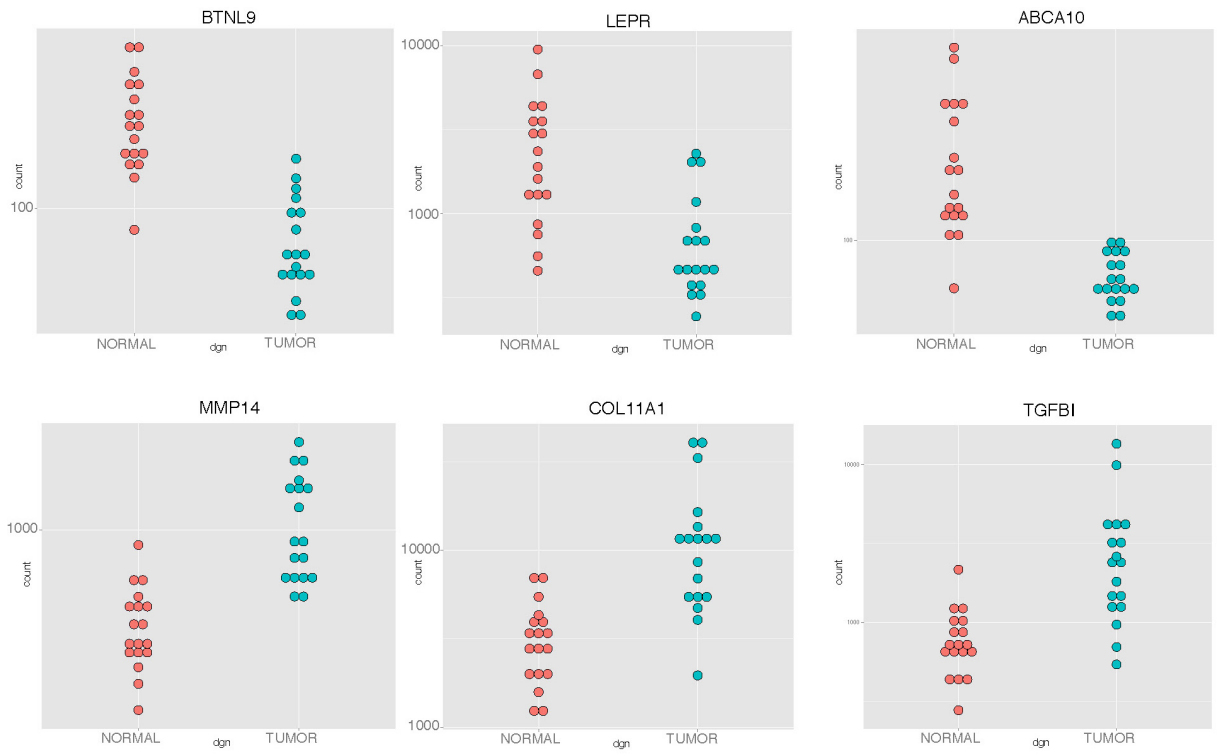

Figure 1: We illustrate 3 most upregulated genes (COLL11A1, TGFBI, and MMP14) and 3 most downregulated genes (BTNL9, LEPR, and ABCA10) from the top significant genes which have the lowest padj. The expression of these genes with the largest differences between cancerous and non-cancerous bone indicates consistency among different patients. *BTNL9, LEPR, ABCA10* were downregulated (log2foldchange<-1 with padj much less than 0.001) while *MMP14, COL11A1* and *TGF-βI* were upregulated in tumor samples (log2foldchange>1 with padj being much less than 0.001). The blue dots represent tumor samples and the red ones represent normal samples.

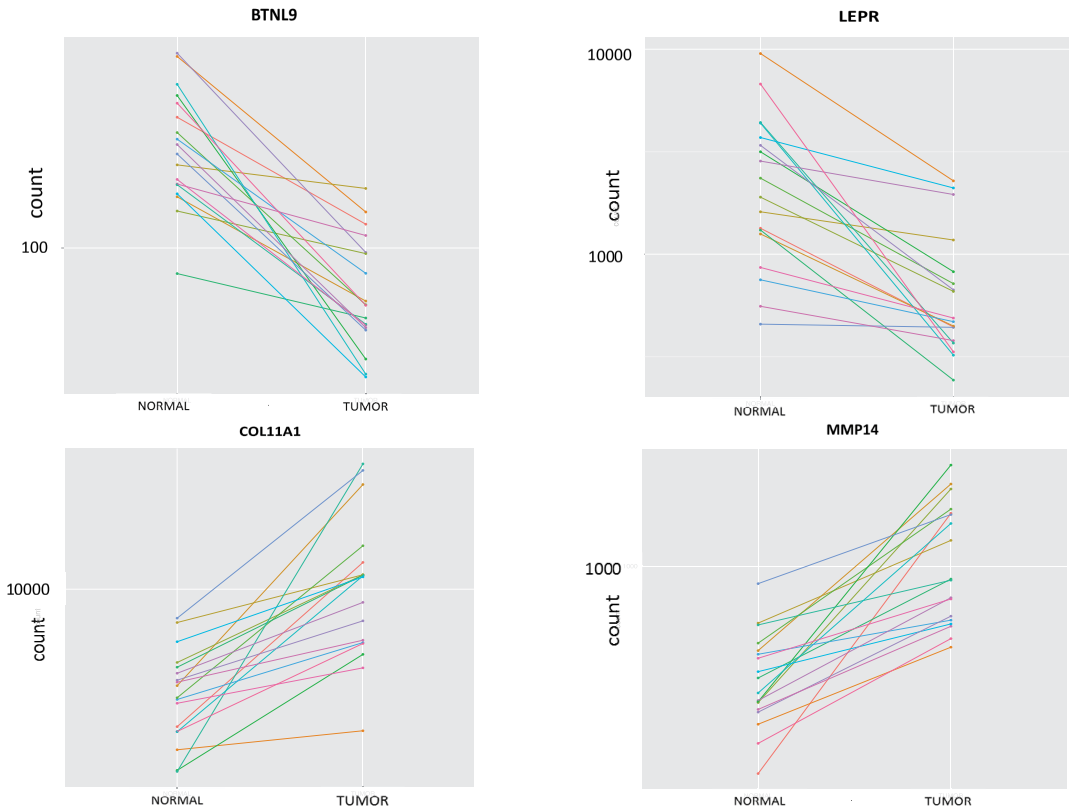

Figure 2. The expression of 4 genes was compared as pairwise between tumor and control samples. 18 lines demonstrate the 18 studied patients. The comparison indicates consistent changes between the tumor-normal paired data of the same person. Here we demonstrate the gene expression of BTNL9, LEPR, MMP14, and COLL11A1 where the expression is consistent through 18 patients.

5.1.3. Reactome analysis of fresh samples

By using the Reactome database (a manually curated resource describing chemical reactions, biological processes, and pathways), we aimed to describe the disease-specific transcriptome signature with a list of differentially expressed genes in paired-samples (Subramanian et al. 2005; Yu and He 2016; Fabregat et al. 2016). The analysis showed a significant enrichment of the pathways related to the collagen degradation, extracellular matrix organization, and erythrocyte activation. The detailed pathways are shown in table 5.

The eight most significantly activated pathways include: collagen degradation; erythrocytes take up carbon dioxide and release oxygen, erythrocytes take up oxygen and release carbon dioxide, O2/CO2 exchange in erythrocytes, degradation of the extracellular matrix and extracellular matrix organization. They are illustrated in the figure 3. They are all related to the degradation of the extracellular matrix or to hematopoiesis. Figure 4 shows genes from the pathways and their expression levels.

Table 5. The enrichment analysis of the gene expression profile in OS

ID	Description	Gene Ratio	BgRatio	p-value	p-adjust	q-value
1442490	Collagen degradation	11/89	62/6750	3.27E-10	1.89E-08	1.60E-08
1237044	Erythrocytes take up carbon dioxide and release oxygen	6/89	9/6750	3.61E-10	1.89E-08	1.60E-08
1247873	Erythrocytes take up oxygen and release carbon dioxide	6/89	9/6750	3.61E-10	1.89E-08	1.60E-08
1480926	02/C02 exchange in erythrocytes	6/89	9/6750	3.61E-10	1.89E-08	1.60E-08
1474228	Degradation of the extracellular matrix	13/89	109/6750	1.24E-09	5.22E-08	4.42E-08
1474244	Extracellular matrix organization	17/89	249/6750	1.77E-08	6.19E-07	5.24E-07
2168880	Scavenging of heme from plasma	5/89	12/6750	2.62E-07	7.86E-06	6.66E-06
2173782	Binding and Uptake of Ligands by Scavenger Receptors	7/89	40/6750	7.15E-07	1.88E-05	1.59E-05
1650814	Collagen biosynthesis and modifying enzymes	8/89	59/6750	8.51E-07	1.99E-05	1.68E-05
1474290	Collagen formation	8/89	70/6750	3.22E-06	6.76E-05	5.73E-05
2022090	Assembly of collagen fibrils and other multimeric structures	6/89	40/6750	1.19E-05	2.27E-04	1.92E-04

ID	Description	Gene Ratio	BgRatio	p-value	p-adjust	q-value
1592389	Activation of Matrix Metalloproteinases	5/89	27/6750	2.29E-05	4.00E-04	3.39E-04
3000178	ECM proteoglycans	7/89	67/6750	2.50E-05	4.04E-04	3.42E-04
109582	Hemostasis	17/89	450/6750	6.45E-05	9.67E-04	8.20E-04
983231	Factors involved in megakaryocyte development and platelet production	8/89	122/6750	1.88E-04	2.63E-03	2.23E-03
216083	Integrin cell surface interactions	6/89	83/6750	7.42E-04	9.74E-03	8.25E-03
375165	NCAM signaling for neurite out-growth	5/89	64/8750	1.47E-03	1.73E-02	1.46E-02
419037	NCAM1 interactions	4/89	38/6750	1.48E-03	1.73E-02	1.46E-02
5653656	Vesicle-mediated transport	8/89	184/6750	2.81E-03	2.99E-02	2.53E-02
5365859	RA biosynthesis pathway	3/89	22/6750	2.85E-03	2.99E-02	2.53E-02

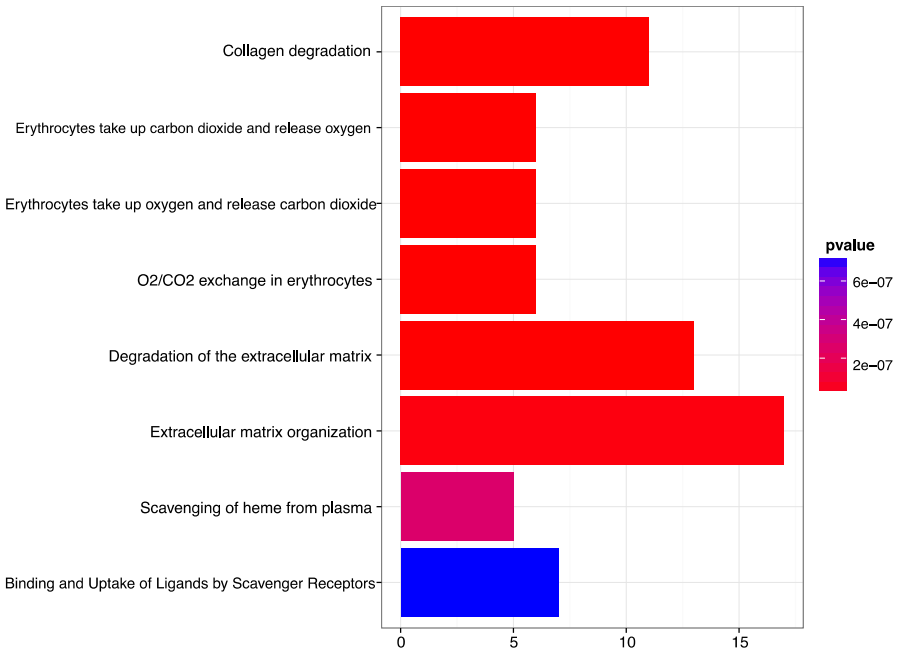

Figure 3. The most significantly activated pathways in OS.

The horizontal axis shows the number of genes found in every pathway while the vertical axis shows the different pathways. Their color depicts statistical significance on the activation where red is the most significant and blue is the least.

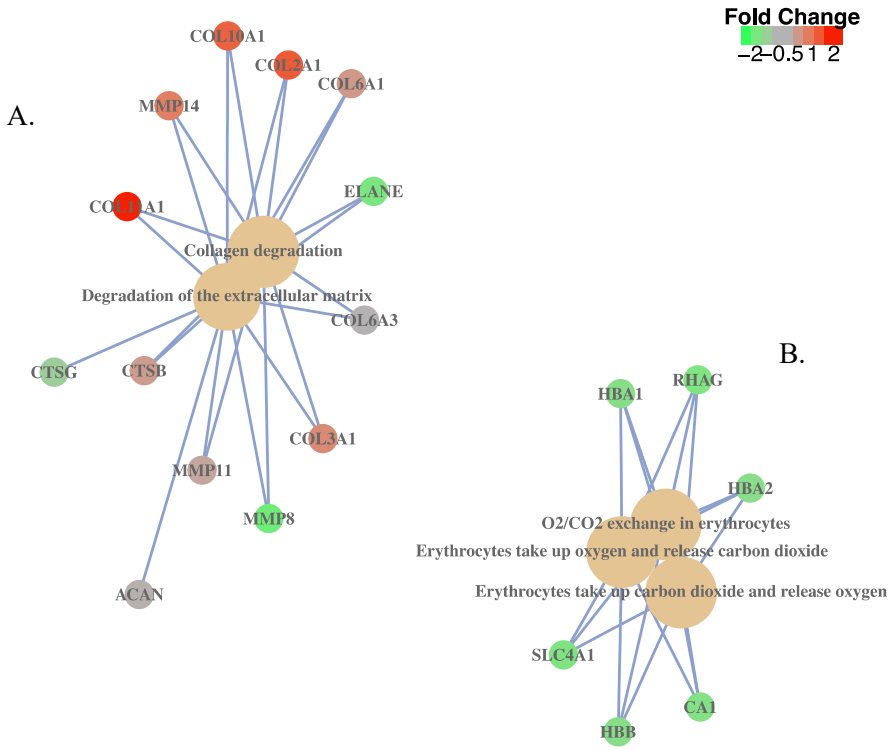

Figure 4. A map of the two most significantly altered genetic networks in OS. **A.** illustrates a group of genes interacting for collagen synthesis and degradation. They are composed of COL11A1, COL10A1, COL2A1, COL3A1, COL6A1, COL6A3, MMP8, MMP11, MMP14, CTSB, ACAN, CTSG, and ELANE. **B.** shows a hemato-poiesis interacting network that is composed of HBA1, HBA2, HBB, CA1, SLC4A1, and RHAG genes.

5.1.4. Gene expression changes induced by chemotherapy

To discover the transcriptional changes due to chemotherapy, we analyzed transcriptome from FFPE samples using edgeR package. We analyzed five pre-chemotherapy FFPE and ten post-chemotherapy specimens. Twenty-two genes were found to be expressed differentially with chemotherapy (padj <0.1). They all are upregulated genes with log2foldchanges of more than 1 (listed in table 6). Gene-set enrichment analysis of these 22 genes was done and significant pathways related to these genes were identified with padj <0.1. They include

collagen biosynthesis and modifying enzymes collagen formation; influenza viral RNA transcription and replication; eukaryotic translation elongation; integrin cell surface interactions; SRP- (the signal-recognition particle)-dependent cotranslational protein targeting to membrane; collagen degradation (table 7). Interestingly, most of the genes upregulated with chemotherapy were related to collagen formation and support osteogenesis.

Table 6. The most significantly upregulated genes in FFPE OS samples after chemotherapy

symbol	log2Fold Change	PValue	padj	genename
POSTN	3.16	2.59E-08	0.00	periostin. osteoblast specific factor
AMBN	4.07	1.80E-07	0.00	ameloblastin (enamel matrix protein)
SLC35F3	3.84	1.93E-07	0.00	solute carrier family 35. member F3
TAC3	3.9	1.71E-07	0.00	tachykinin 3
TYROBP	3.16	1.58E-07	0.00	TYRO protein tyrosine kinase binding protein
STX7	2.99	5.36E-07	0.00	syntaxin 7
HEATR1	2.12	5.18E-06	0.02	HEAT repeat containing 1
OGN	1.97	7.49E-06	0.02	osteoglycin
USP6	2.49	7.34E-06	0.02	ubiquitin specific peptidase 6
SPRR1B	2.53	9.96E-06	0.02	small proline-rich protein 1B
ASPN	1.94	1.54E-05	0.03	asporin
SULF1	1.07	1.52E-05	0.03	sulfatase 1
DNAJC14	2.11	2.24E-05	0.04	DnaJ (Hsp40) homolog. subfamily C. member 14
NOB1	2.25	2.75E-05	0.04	NIN1/RPN12 binding protein 1 homolog
FAP	2.21	3.48E-05	0.05	fibroblast activation protein. alpha
SP3	2.23	3.27E-05	0.05	Sp3 transcription factor
C8orf59	2.79	4.50E-05	0.05	chromosome 8 open reading frame 59
CCNB2	1.04	4.40E-05	0.05	cyclin B2
KRTDAP	3.24	4.54E-05	0.05	keratinocyte differentiation-associated protein
MRPS24	2.19	4.63E-05	0.05	mitochondrial ribosomal protein S24
C2orf89	2.34	6.99E-05	0.08	TraB domain containing 2A
RPS6	2.38	7.47E-05	0.08	ribosomal protein S6

Table 7. Results of the gene set enrichment analysis after chemotherapy

ID	Description	setSize	enrichment Score	p-adjust
1650814	Collagen biosynthesis and modifying enzymes	9	0.61	0.09
1474290	Collagen formation	10	0.54	0.09
168273	Influenza Viral RNA Transcription and Replication	22	0.52	0.09
156842	Eukaryotic Translation Elongation	21	0.56	0.09
216083	Integrin cell surface interactions	8	0.61	0.09
1799339	SRP-dependent cotranslational protein targeting to membrane	21	0.56	0.09
1442490	Collagen degradation	9	0.76	0.09

5.2. TGFB in osteosarcoma and its related genes' expression

From the data looking at the RNA-seq analysis of paired OS tissues and surrounding normal bone in the 18 OS patients, we focused on TGF-β expression and its related genes.

Surprisingly, TGF-β mRNA was not differentially expressed between normal and tumor samples (Figure 5), however, several TGF-β-induced genes were found upregulated (Figure 6 A and B; Figure 7 A và B). COL11A1 and TGF-βI were the top two upregulated genes among those genes (log2FC 1.51, P = 1.06 E-14 and log2 FC 1.40, P = 1.35E-11, respectively).

Gene set enrichment analysis (GSEA) of the most upregulated genes in OS samples with log2 FC > 1, P < 0.0001 revealed an overlap of 4 extracellular matrix genes that are induced early by TGF-β in fibroblasts (Verrecchia, Chu, and Mauviel 2001). They included three collagen genes known as definite TGF-β/SMAD3 targets (COL3A1, COL6A1, COL6A3) and MMP14 (Fig. 7B).

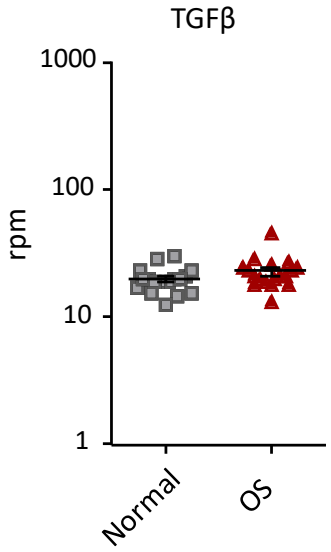

Figure 5. TGF-β expression among normal and osteosarcoma tumor bone samples TGF-β is not expressed differentially with significance between the tumoral and normal bone of the same patient.

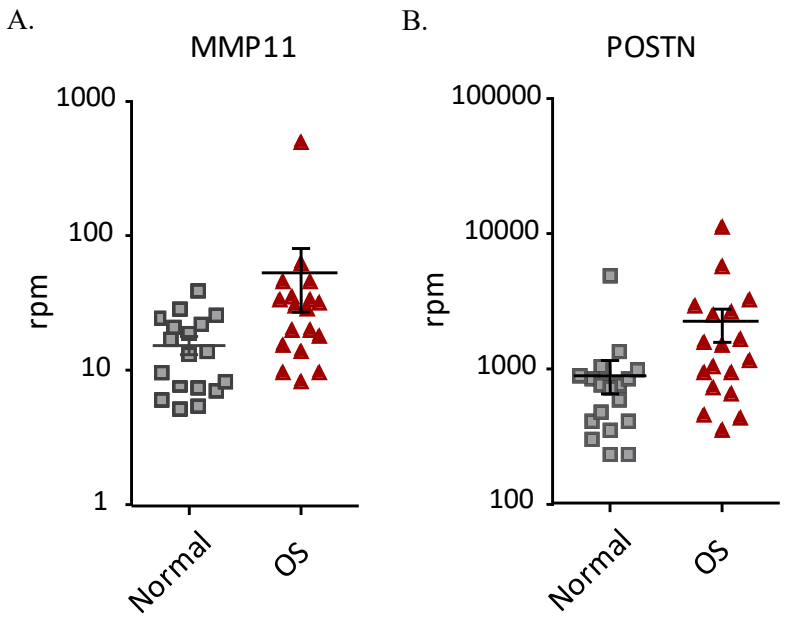

Figure 6. Differential expression of MMP11 and POSTN between normal bone and OS specimens.

MMP11 and POSTN were overexpressed in OS compared to normal bone.

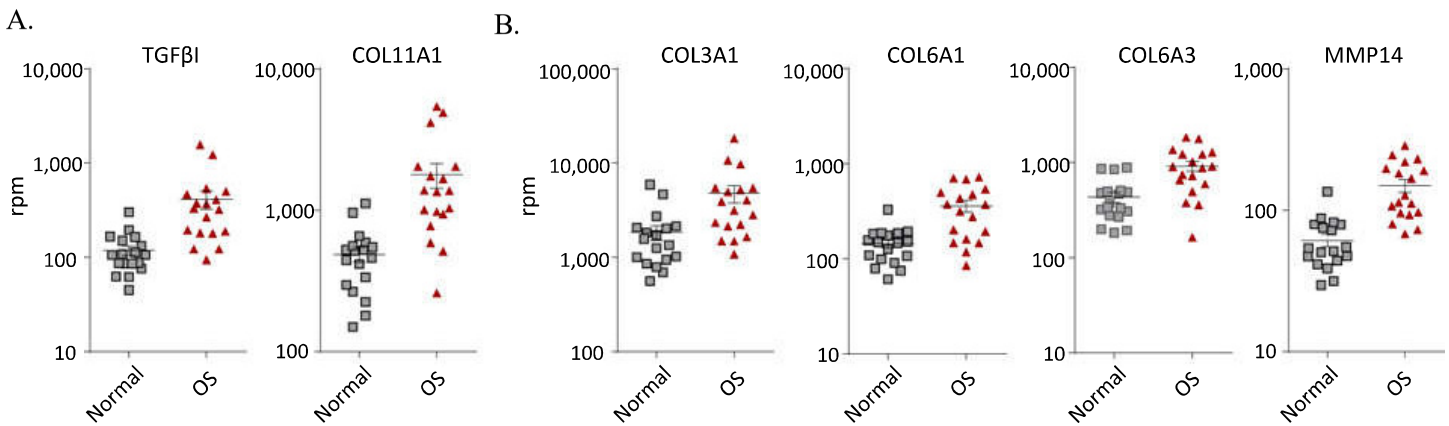

Figure 7. Differentially expressed TGF-β-induced genes in osteosarcoma frozen samples TGF-βI, COL11A1, COL3A1, COL6A1, COL6A3, and MMP14 were all upregulated in OS tissue compared to normal bone samples.

5.3. Repetitive DNA elements expressed differentially in osteosarcoma

We analyzed 1116 different repetitive elements from the Repbase. The analysis was done in R by EdgeR. We used the Benjamini-Hochberg (BH) adjustment to eliminate false positives. These BH-adjusted p-values (FDR) are shown in the column FDR. If we are to accept a fraction of false positives of five percent, then we can consider all REs with FDR less than 5%= 0.05 as significant ones. As a result of this analysis, eighty-two repetitive elements were found differentially expressed between the normal and cancer specimens, including 35 upregulated and 47 downregulated elements.

Ten elements with the lowest FDR, known as the most significant differential expression REs, are shown in table 8. Among those elements, THE1C-int, LTR5, MER57F, and MER87B had the highest significance. They are all HERVs which are a part of repetitive elements.

Table 8. The most significant repetitive elements which expressed differentially between affected bone versus normal bone in OS

Class	Family	Element	logFC	p-value	FDR
LTR	ERVL-MaLR	THE1C-int	0.612785	9.83E-10	1.07E-06
LTR	ERVK	LTR5	0.606272	4.58E-08	2.49E-05
LTR	ERV1	MER57F	0.943969	1.09E-07	2.98E-05
LTR	ERV1	MER87B	0.748537	1.01E-07	2.98E-05
RNA	RNA	7SK	0.9486	3.17E-07	6.91E-05
LTR	ERV1	MER34B-int	0.72914	4.78E-07	8.68E-05
LTR	Gypsy	MamGypLTR3	-0.41918	7.13E-07	0.000111
Satellite	centr	ALR_Alpha	1.291039	1.08E-06	0.000147
DNA	DNA	MER136	1.412429	1.27E-06	0.000154
Satellite	Satellite	HSATII	1.878344	3.66E-06	0.000366

The most upregulated elements that have the highest logFC (1.12–2.05) are listed in table 9. SAR, HSATII, _CATTC_n, MER136, ALR_Alpha, and _GAATG_n were the most strongly upregulated. The most downregulated elements were mostly the HERVs shown in Table 10.

Table 9. The most upregulated repetitive elements in OS

Class	Family	Element	logFC	p-value	FDR
Satellite	Satellite	SAR	2.052918	5.08E-05	0.003461
Satellite	Satellite	HSATII	1.878344	3.66E-06	0.000366
Satellite	Satellite	_CATTC_n	1.481369	3.7E-06	0.000366
DNA	DNA	MER136	1.412429	1.27E-06	0.000154
Satellite	centr	ALR_Alpha	1.291039	1.08E-06	0.000147
Satellite	Satellite	_GAATG_n	1.115843	0.000789	0.01829

Table 10. The most downregulated repetitive elements in OS

Class	Family	Element	logFC	p-value	FDR
DNA	TcMar	MamRep1161	-0.99091	6.38E-05	0.003862
tRNA	tRNA	tRNA-Ser-TCY	-0.94009	0.001438	0.026562
LTR	ERV1	HERV1_LTRe	-0.68333	0.000166	0.007247
LTR	ERVL-MaLR	MLT1E1-int	-0.64431	0.00306	0.043679
LTR	ERV1	HERV15-int	-0.61309	0.000353	0.010703
LTR	ERV1	MER51E	-0.5277	0.000964	0.02144
LTR	ERV1	MER83B	-0.43948	0.000292	0.010112
LTR	ERVL	LTR47B4	-0.43666	7.71E-05	0.0042
LTR	Gypsy	MamGypLTR3	-0.41918	7.13E-07	0.000111
LTR	ERV1	MER72B	-0.37858	0.000402	0.011198
LTR	Gypsy	LTR81	-0.35087	0.003015	0.043679
LTR	ERV1	LTR31	-0.34699	0.000763	0.01808

Figure 8 clearly illustrates the differential expression of repetitive elements in OS. The figure shows the most upregulated and the most downregulated ones with the range of logFC (A, B). We can see the difference in repetitive elements expression between OS and normal bone (C) from the PCA.

Figure 9 shows differential expression of repetitive elements in subgroups. The differential expression of REs was found particularly between the tumoral and control bone samples.

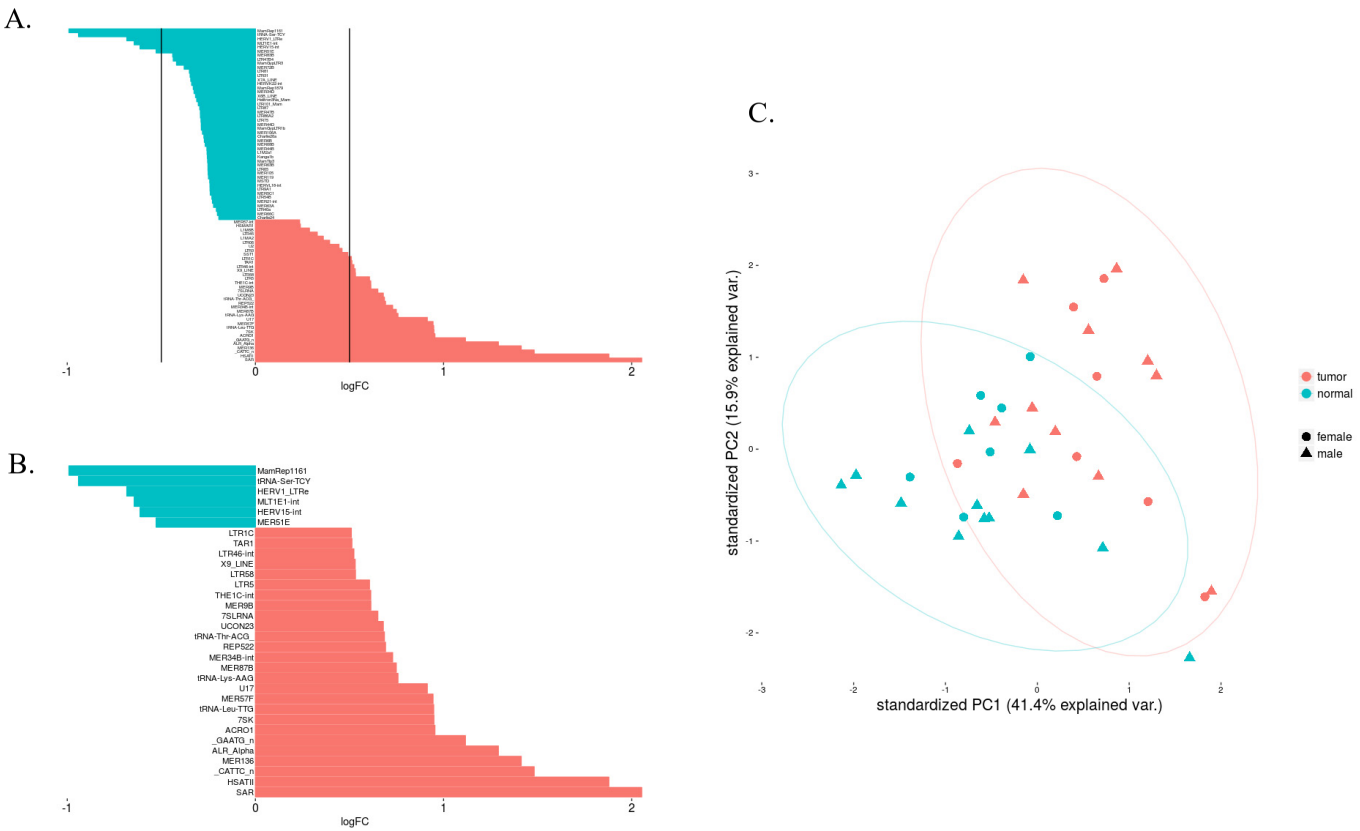

Figure 8. Differential expression of repetitive elements in OS
A. describes differentially expressed REs with significance, whereas **B.** illustrates only differentially expressed REs with |logFC| > 0.5. **C.** shows the difference between the expression of REs between cancerous vs non-cancerous samples.

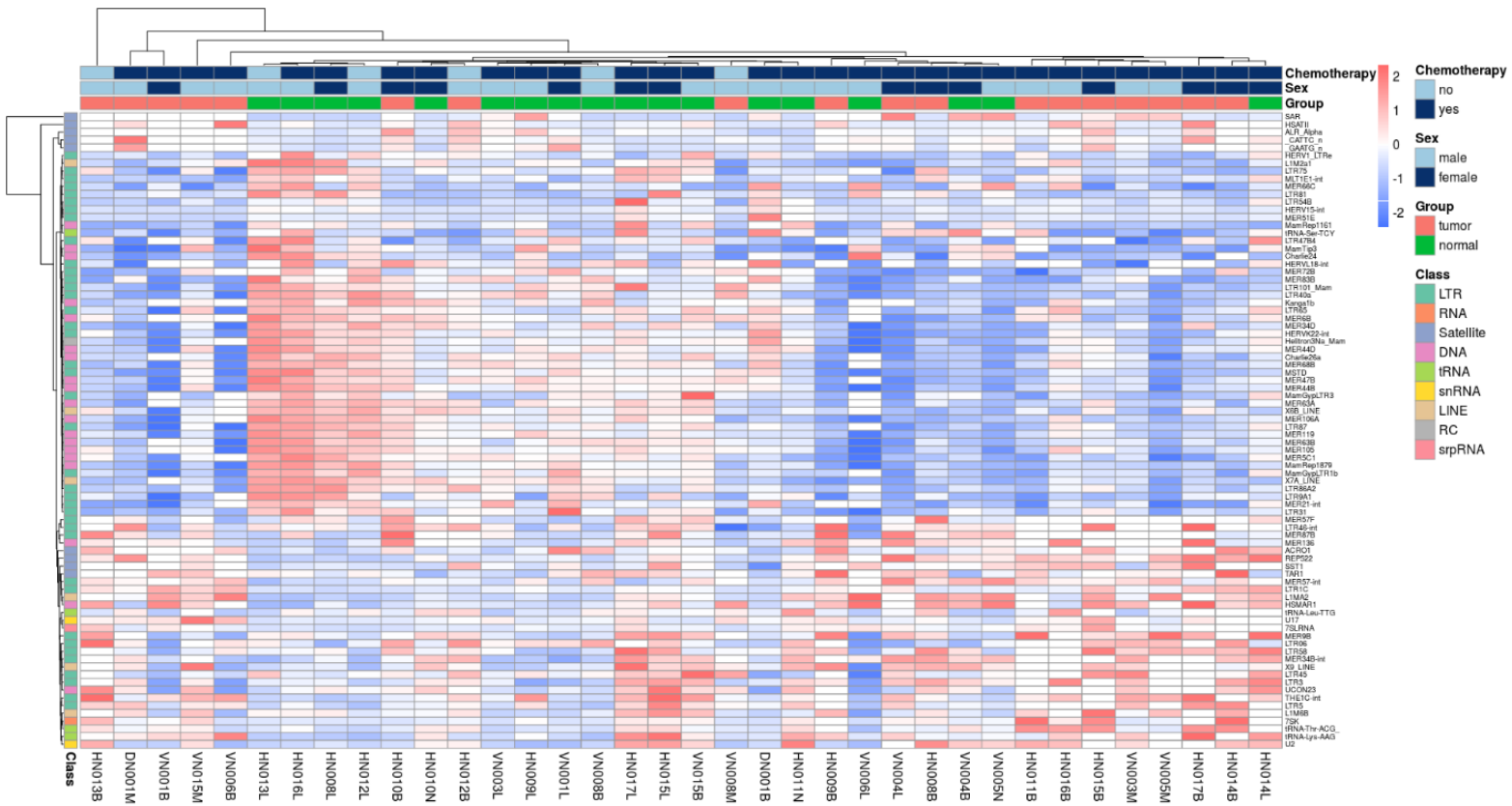

Figure 9. Heatmap of differentially expressed repetitive elements.

6. DISCUSSION

6.1. Genes expression differentially in osteosarcoma

The present study used RNAseq to analyze the differential gene expression between OS tissue and normal bone in 18 paired samples to overcome some known limitations of recent studies on DEGs in OS studies. We found 5,365 differentially expressed genes with an adjusted p-value below 0.05, of which 3,399 upregulated genes and 1,966 downregulated genes. A number of differentially expressed genes in OS varies among existing studies. Yang et al. found 979 differentially expressed genes in a meta-analysis of in vivo and in vitro studies (Z. Yang et al. 2014). A transcriptome analysis of a single case of OS in an Estonian patient identified only 65 differentially expressed genes (Märtson et al. 2013). A more abundant number of DEGs, about 1,608, was found in one GeneChip study of 10 OS samples and 2 normal tissue samples (Subramanian et al. 2005). Lu Sun et al. found 1170 DEGs, including 530 upregulated genes and 640 downregulated genes in a study that compared 19 OS cell lines and 4 normal bone cell lines (SUN, LI, and YAN 2015). Marieke L. Kuijjer et al. found 12,542 and 2,939 genes that were expressed differentially with significance when they compared the gene expression of 84 OS biopsies to 12 mesenchymal stem cells, and 3 osteoblasts respectively (Kuijjer et al. 2012). This was the highest number of DEGs found in an existing study. These variations are understandable due to the difference in study design, the sample size and control groups.

We analyzed paired tumor-normal samples with a relatively larger sample size as compared to studies of a similar design. Our study allowed the comparison of gene expression between the tumor and normal tissue from the same patient. This design substantially reduces biological variability and increases statistical power.

We found BTNL9, MMP14, ABCA10, ACACB, COL11A1, and PKM2 as the most significant differentially expressed gene. BTNL9 was not mentioned in previous OS studies. In our study, BTNL9 had the lowest padj value. Butyrophilin-like 9, also known as BTN3, BTN8, or VDLS1900, is located in 5q35.3. The function of BTNL9 has not yet been identified, but it is one of the 13 members of the human Butyrophilin family which plays a role in immune homeostasis (Arnett and Viney 2014).

MMP14 belongs to the MMP family, which is a hallmark of invasive cancers. In OS, MMP14 was revealed to interact with COL1A2 and COL5A2 (D. Wu et al. 2014). MMP14 was upregulated in breast cancer, mesothelioma, and lung cancer (Crispi et al. 2009; Stawowczyk et al. 2017; Têtu et al. 2006). It has also been associated with a worse prognosis (Têtu et al. 2006). Interestingly, inhibiting the MMP14 by targeting hemopexin, the domain of MMP14, showed promise as an approach in lung cancer treatment (Stawowczyk et al. 2017).

The ABCA10 is member 10 of the ATP binding cassette subfamily A and its location is on 17q24. It was downregulated in our study. ABCA10 was also found to be downregulated in colorectal and ovarian cancer (Hlavata et al. 2012; Elsnerova et al. 2016). The downregulation of ABCA10 found in OS fits with its expression in some others epithelial cancers mentioned above. It was noted that ABCA10 was correlated to the progression-free survival of ovarian cancer (Elsnerova et al. 2016). Further study should be done to confirm the role of ABCA10 as a prognostic marker in OS. Similarly, ACACB was downregulated and for the first time has been shown to be correlated to OS.

ACACB is acetyl-CoA carboxylase beta, which is also known as ACC2, and was located on chromosome 12. It may be involved in the regulation of fatty acid oxidation, rather than fatty acid biosynthesis (NCBI Resource Coordinators 2018). The role of lipid metabolism has been mentioned in cancer, in particular, involved Acetyl-CoA carboxylase (ACC 1 and 2) in some cancers (Luo et al. 2017). It was found that via the deactivation of ACC2 or ACACB, PHD3 loss in cancer enables a metabolic reliance on fatty acid oxidation (German et al. 2016).

The COL11A1 gene codes for the α1 chain of procollagen and mature collagen of type XI, which is an extracellular minor fibrillar collagen (Vázquez-Villa, García-Ocaña, Galván, García-Martínez, García-Pravia, Menéndez-Rodríguez, Rey, et al. 2015). COL11A1 was upregulated in this study. We have not found its correlation to OS in previous studies, but some family members were identified. COL1A2 and COL5A2 were identified to be upregulated in OS (D. Wu et al. 2014). Particularly, COL11A1/(pro)collagen 11A1 was significantly expressed by activated stromal cells of the desmoplastic reaction of human invasive carcinoma of the oral cavity, pharynx, head, neck, breast, lung, esophagus, stomach, pancreas, colon, and ovary (Vázquez-Villa, García-Ocaña, Galván, García-Martínez, García-Pravia, Menéndez-Rodríguez, González-del Rey, et al. 2015). The level of COL11A1/(pro)collagen 11A1 expression was correlated with the aggressiveness, progression, metastasis, and drug resistance of the epithelial cancers. It was suggested that COL11A1 regulates MMP3 and MMP9 via TGF-β and consequently, promotes cell proliferation, invasion, metastasis, and drug resistance. This pathway model should be further studied to develop targeted therapies for several cancers including OS (Vázquez-Villa, García-Ocaña, Galván, García-Martínez, García-Pravia, Menéndez-Rodríguez, González-del Rey, et al. 2015; Raglow and Thomas 2015).

PKM (pyruvate kinase muscle), also known as PKM2, was overexpressed in our study. This is similar to the previous findings in clear-cell renal carcinoma. In clear-cell renal carcinoma, PKM2 was upregulated and when it was suppressed in these cells the inhibition of the rapid proliferation, high glucose consumption, and high lactate production were induced (Huang et al. 2016). The role of PKM should be evaluated further in OS.

From the Reactome analysis, we found a significant enrichment of specific pathways in OS. The enrichment was focused on two main networks. The first group was the hemoglobin interacting network which was also discovered in a

recent OS study (Zhao et al. 2015). In our work, we identified the interacting network of hematopoiesis that is composed of HBA1, HBA2, HBB, CA1, and RHAG. The second network was collagen synthesis and degradation and its related genes were COL11A1, COL10A1, COL2A1, MMP14, COL3A1, COL6A1, MMP11, CTSB, ACAN, COL6A3, CTSG, MMP8, and ELANE. Several genes indicate the enrichment of the cancer progression and extracellular matrix remodeling. COL was thought to be possible featured genes of OS (Y. Zhang et al. 2015). A study on 6 cell lines samples (J. Yang and Wang 2016) discovered similar pathways that correspond with our discoveries such as the extracellular matrix organization and integrin cell surface interactions. Our findings on OS, which is a very highly metastatic disease, matched quite well with the present knowledge about the role of extracellular matrix remodeling in sarcoma progression and metastasis. Remodeling of ECM by collagen degradation and redeposition in interactions with matrix metalloproteinases (MMPs) promotes tumor infiltration, angiogenesis, invasion, and migration. Recent understanding of its mechanism has led to several clinical trials, especially for MMPs-inhibitor drugs, and an increasing interest in future treatment developments. Previous studies have suggested the further evaluation of MMPs serve as a potential marker of invasiveness and risk of metastasis (Jabłońska-Trypuć, Matejczyk, and Rosochacki 2016; Nerenberg, Salsas-Escat, and Stultz 2007; Lu et al. 2011).

We aimed to characterize the changes in gene expression with chemotherapy by analyzing the FFPE samples. We analyzed 5 samples with chemotherapy and 10 without chemotherapy. We made a comparison of gene expression with and without chemotherapy. Twenty-two genes were found to be upregulated significantly with chemotherapy (padj < 0.1 and log2foldchange >1. Collagen biosynthesis, modifying enzymes, collagen formation, and collagen degradation were among the most significant pathways. Apparently, these pathways lead to the degradation and remodeling of the extracellular matrix. This suggested that chemotherapy may induce the remodeling of ECM. P Leonard et al. also studied the differential expressed genes with chemotherapy and the myeloperoxidase gene, the thymine DNA glycosylase, and Hsp-60 were among the most highly expressed after chemotherapy (Leonard et al. 2003). We think that this discordance may come from the type of specimens used, as we used FFPE samples and they used biopsy samples. It may also come from the actual heterogeneity of OS, which needs more evaluation.

From our analysis of 18 paired patient samples, we found that the degradation and remodeling of ECM seem to be an important pathway in OS. The activation of collagen biosynthesis related pathways after cytotoxic treatment supports the fact that ECM degradation and remodeling may be an important mechanism of the disease.

6.2. TGF-β expression in osteosarcoma

The role of TGF-β in tumorigenesis is quite complicated, but it is believed that TGF-β plays an important role in progression and metastasis of cancer (Padua and Massagué 2009; Lamora et al. 2014; Katsuno, Lamouille, and Derynck 2013; Meulmeester and ten Dijke 2011) and has a critical role in the communication between cancer and stromal cells during disease progression (Calon et al. 2015).

In OS, TGF-β has been previously mentioned as an autocrine growth factor with a pro-tumoral effect (Lamora et al. 2016). Moreover, TGF-β mRNA expression in OS tissues has been linked to high-grade tumors (Franchi et al. 1998). The concentration of TGF-βs was higher in sera of OS patients than healthy control individuals (Webber et al. 2010). Surprisingly, our study did not show the differential expression of TGF-β at the mRNA level, but several TGF-β induced genes were highly upregulated in OS compared to normal bone control in paired samples. It raised the question whether TGF-β was expressed in another form. In fact, EV-associated TGF-β was detected and it functions not as the same as soluble TGF-β in several cancer cell lines (Webber et al. 2010). In OS, EVs carry elevated levels of TGF-β (S. Rubina Baglio, Tonny Lagerweij, Maria Perez-Lanzon, Xuan Dung Ho, Nicolas Leveille, Sonia A. Melo and Ekaterina S. Jordanova, Laura Roncuzzi, Michelina Greco, Monique A.J. van Eijndhoven1, Giulia Grisendi, Massimo Dominici, Roberta Bonafede, Sinead M. Lougheed, Tanja D. de Gruijl, Nicoletta Zini, Silvia Cervo, Agostino Steffa, Viincenzo Canzonieri, Aare M 2017). Another clue to explain the non-differential expression of TGF-β in OS when we used paired samples is that TGF-β is not from the tumor cells but from stromal cells (Cortini et al. 2016). This may explain the different level of TGF-β between the OS group and healthy group and why there was no difference of TGF-β expression between affected bone and normal bone in the same patient.

6.3. Expression of repetitive DNA elements in OS

Until our study, the expression of repetitive elements has not be analyzed in OS. In our work, 82 REs were found to be differentially expressed between OS and normal bone in paired samples with the significance of FDR <0.05. Of which, there were 35 upregulated and 47 downregulated elements. The list of the most significant REs that expressed differentially (with lowest FDR) consisted of THE1C-int, LTR5, MER57F, and MER87B. They are all LTRs or HERVs. THE1C-int is an ERV3, a retrovirus-like MaLR element. LTR5 is an ERV2, a clone of HERV-K18. MER57F and MER87B are ERV1 with 435 bp and 509 bp respectively (W. Bao, Kojima, and Kohany 2015; Jurka et al. 2005). When we reviewed the previous literature, LTR5 hypomethylation was shown to be involved in the systemic lupus erythematosus mechanism while THE1C-int, MER57F, and MER87B were not identified (Nakkuntod et al. 2013). With our

present findings, it is suggested that TE plays an important role in transcriptional control, genomic instability, chromosomal rearrangements, non-coding RNA regulation, and oncogenic activation (Anwar, Wulaningsih, and Lehmann 2017). HERVs presenting as a TE as shown to be associated with carcinogenesis in various cancers. Below, we have listed some interesting findings of HERVs expression in some cancers. These findings may support our results as we do not have similar studies to compare our results to.

In prostate cancer, some retrotransposons subfamilies were identified. HERV-E (and/or ERV3) env genes were expressed only in prostate cancer cells that were suggested as targets for immunotherapy (Feng Wang-Johanning et al. 2003). Additionally, HERV-K was also involved in prostate cancer. A study, utilizing a similar design as our present one, on 14 prostate cancer patients of different grades with paired samples found 475 retrotransposon subfamilies to be significantly differentially expressed in the affected tissue with FDR < 0.05. Among those, LTR was the most prevalent with endogenous retroviruses with ERV1 being the most represented (Ren et al. 2012; Criscione et al. 2014).

In ovarian cancer, HERV-K env was expressed only in cancer tissue at a high frequency of 90%. There was no expression found in normal and benign ovarian surface epithelial tissue. ERV3 and HERV-E were discovered to express simultaneously in the same ovarian cancer tissues and antibodies to HERVs were detected in the sera of ovarian carcinoma (Feng Wang-Johanning et al. 2006). This suggests the further evaluation of HERVs is necessary to confirm a new ovarian carcinoma screening tool and potentially serve as a new target for detection, diagnosing, and treatment. A similar study also found that HERV-K env expressed only in breast cancer tissues and cell lines but not in normal breast tissues. Therefore, it should be evaluated for use as a potential tumor marker (F Wang-Johanning et al. 2001). The expression of HERV-K Env proteins was shown in more than 85% of cases of breast cancer and induced both serologic and cell-mediated immune responses (Feng Wang-Johanning et al. 2008).

There has also been interesting findings showing an association between TEs and hepato-gastrointestinal cancers. About 30–50% of gastrointestinal cancers including gastric, pancreas, and colorectal cancers showed the overexpression of the HERV-H sequence (Wentzensen et al. 2007). Furthermore, the HERV-H gag gene locus on chromosome Xp22 was usually expressed in colon cancer samples (Alves et al. 2008). Among LTRs which were upregulated in HCC, LTR-007 was the most commonly expressed (Hashimoto et al. 2015).

In lymphoma, high levels of HERV-K were found in the plasma of patients and it decreased significantly with the successful treatment of lymphoma (Contreras-galindo et al. 2008). In Hodgkin's lymphoma, activation of endogenous LTR can be oncogenic; Hypomethylation of the THE1B LTR induced the activation of the CSF1R (colony stimulating factor 1 receptor gene) oncogene. CSF1R was highly expressed in these cancer cells. THE1B LTR, a MaLR family LTR retrotransposon, which is in the same subgroup with THE1C-int was also discussed in our results (Lamprecht et al. 2010). They also

detected HERV-K mRNA and proteins in melanoma tissues and cell lines (Büscher et al. 2005; Muster et al. 2003). HERVs have been studied in germinal cancers. HERV-K virus-like production was identified in teratocarcinoma cell lines (Boller et al. 1993; Löwer et al. 1993). Importantly, HERV-K Gag and Env proteins were upregulated in germinal cancers, and antibodies against these viral proteins were also detected (Sauter et al. 1996; Sauter et al. 1995). The involvement of HERs in many cancers supports our findings, which detected the hyperexpression of HERs found for the first time in OS. Further investigation should be conducted to reveal the role of TEs in OS.

We also generated a list of the most upregulated REs by ranking logFC (highest logFC) (table 9). SAR, HSATII and -CATTC-n were the top 3 most upregulated REs. SAR is a human satellite I DNA. Its 84 bp sequence is acagtatata atatatattt tgggtacttt gatattttat gtacagtata taatatatat tttgggtact ttgatatttt atgt (Jurka et al. 2005; W. Bao, Kojima, and Kohany 2015). To our knowledge, it has not been found in previous studies. This finding suggests to further investigate the role of SAR in the tumorigenesis of OS.

HSTAII is a pericentromeric human satellite II element. It was found overexpressed in some carcinomas including pancreatic adenocarcinoma, lung cancer, renal cancer, ovarian, prostate, and colon cancer (Ting et al. 2011; Bersani et al. 2015). In an analysis of 15 human pancreatic cancer samples, the total satellites transcripts were elevated 21-fold in cancerous samples compared to normal pancreas. Among the satellites, HSATII was highest differentially expressed with 131-fold, but it is undetectable in the normal pancreas or minimally expressed in other normal tissues (Ting et al. 2011). In a research study using RNA-ISH analysis of endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) of the pancreatic tumors, HSATII-positive cells were detected in 10 out of 10 cases of confirmed adenocarcinoma while only 2 cases were nondiagnostic with FNA (Ting et al. 2011). In colon cancer, the gain in HSATII copy was linked to a significant diminution of overall survival compared with no gain tumors (Bersani et al. 2015). An experiment was carried out by inhibiting HSATII rdDNA formation to treat colon cancer cell lines in three-dimensional conditions and mouse tumor xenografts which lead to a decrease in the size of tumors accompanying HSATII copy drop (Bersani et al. 2015). Promising findings of HSATII in several carcinomas support our results in the analysis of OS, suggesting the further study of HSATII in cancer.

(CATTC)n is a simple satellite repeat that was upregulated in human colon cancer simultaneously with HSATII and ALR/alpha. This finding completely matches with our results where we found similar simultaneous upregulation of these REs (Bersani et al. 2015).

Taken together with previous findings in epithelial, germinal, and hematologic cancers, our analysis of REs in OS shows that the expression of repetitive elements in OS may play a role in the pathophysiology of OS.

6.4. Limitations

The subjects of the study were mainly from Vietnam with samples used in 3 studies. 11 FFPE samples were retrieved from the pathology of the Tartu University hospital which served to analyze the expression changes of genes with chemotherapy in the study I of our work.

We had limitations to access all involved hospitals in Vietnam for a complete overview of OS in Vietnam. In Vietnam, the connection among OS-treatment hospitals has not been well established. Due to this lack of co-operation, it is difficult to get all OS patients for the study.

Moreover, as Vietnamese culture stresses the return of the body or any removed body parts to the family after operation, the bone collection needs to be quick and the size of the sample taken must be minimized. We then missed some cases in obtaining the fresh bone tissues.

7. CONCLUSION

1. In conclusion, 5,365 genes were discovered to be differentially expressed between the normal and OS tissues with an FDR adjusted p-value below 0.05. The most significantly differentially expressed genes were BTNL9, MMP14, ABCA10, ACACB, COL11A1, and PKM2. The degradation of collagen seems to be an important mechanism of OS and should be further studied to see if it serves as a biomarker of OS. The robust activation of several COL family genes supports their involvement in malignancy and their potential role in tumorigenesis. Twenty-two genes were found differentially overexpressed with chemotherapy. Collagen biosynthesis and modifying enzymes, collagen formation, as well as collagen degradation were among the most significant pathways. They were shown to induce the degradation and remodeling of the extracellular matrix. We can then assume that chemotherapy may induce the remodeling of ECM.
2. There was no difference in TGF-β expression between OS tissue and normal bone samples, but some TGF-β-induced genes such as COL11A1, TGF-βI, COL3A1, COL6A1, COL6A3, and MMP14 were upregulated.
3. Eighty-two repetitive elements were differentially expressed between the OS and normal control samples. Among these REs, thirty-five were upregulated and forty-seven were downregulated. The most upregulated REs in OS are SAR, HSATII, and simple repeat (CATTC)n. Our findings match what is presently known about the expression of HERVs and satellite DNA in epithelial, germinal, and hematologic cancers, which emphasizes a need for the further study of REs in OS.

8. MAIN PRACTICAL APPLICATIONS/FUTURE PERSPECTIVE

This is the first study on OS in Vietnam done in collaboration with different institutions. The study was conducted by sharing experiences and facilities among institutions. Especially, local staff in Hue was trained in building up and running a fundamental research project. This study provides the basis for further research in the detection of new markers, early diagnosis, and the discovery of new targets for more effective treatment options for this highly malignant disease. The preliminary results of this study have encouraged us to continue on this project in collaboration with the Hue University of Medicine and Pharmacy, Vietnam; University of Tartu, Estonia; The Cancer Center Amsterdam, VU University Medical Center, Amsterdam, the Netherlands in the near future.

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10. SUMMARY IN ESTONIAN

Osteosarkoomi geneetilise profiili iseloomustus

Sissejuhatus

Osteosarkoom (OS) on kõige enam levinud esmane luukoe kasvaja, moodustades ligikaudu 20–40% kõikidest pahaloomulistest luukasvajatest. OS-i avaldumus on ligikaudu 3 juhtu miljoni inimese kohta aastas. Enamikes riikides on haigestumus meeste seas mõnevõrra kõrgem võrreldes naistega. Sõltuvalt vanusest on keskmiselt meeste haigestumuse suhe naiste haigestumusse vahemikus 1.01 kuni 1.43. Põhiline haigestumise riskigrupp on puberteediealised noored. Keskmisest enam esineb OS-i ka eakamatel inimestel, kelle vanus ületab 60 eluaastat. OS-il puuduvad spetsiifilised sümptomid, mis raskendab kasvaja varajast diagnoosimist. Esimesteks põhilisteks sümptomiteks on tavaliselt valu ja paistetus kasvaja piirkonnas. Enamasti tekib kasvaja jäsemete pikkades luudes: 56% juhtumitest ala- ning 10% ülajäsemete luudes.

Käesolevaks hetkeks on teadmised OS-i tekkepõhjustest ja -mehhanismidest piiratud. On leitud, et haigestumise riski suurendavad väga kiire luukasv, kiiritus ning ka mõned geneetilised haigused nagu Li-Fraumeni südroom (LFS), pärilik retinoblastoom (RB), Werneri sündroom (WS), Rothmund-Thomsoni sündroomi (RTS) tüüp II, Bloomi sündroom (BS), RAPADILINO sündroom ja Diamond-Blackfani aneemia. OS-i puhul on metastaasid väga sagedased: eeldatavasti 80–90% patsientidest esinevad diagnoosimise hetkel mikrometastaasid. Seda toetab asjaolu, et enne keemiaravi kasutuselevõttu kirjeldati metastaase kopsudes ka pärast kasvaja kirurgilist eemaldamist ligikaudu 80% patsientidest. Keemiaravi kasutusele võtmine 1970. ja 1980. aastatel parandas oluliselt OS-i patsientide elulemust. Ühtlasi vähendas see oluliselt amputeerimiste osakaalu arenenud riikides. Küll aga on kaasaegse taristu puudumise tõttu amputeerimine peamine sekkumise vorm OS-i ravis ka tänapäeva Vietnamis. Olenemata kaasaegse meditsiini arengust, ei ole OS-i 5-aasta elulemus pärast 1980. aastaid oluliselt paranenud, olles 60–78% lokaliseerunud kasvajate puhul ning kõigest 20–30% kaugelearenenud ehk metastaseerunud staadiumis.

Biotehnoloogia, sh eriti oomika meetodite areng on toonud kaasa OS-i teemaliste publikatsioonide arvu kiire kasvu. Need uuringud viivad lähemale OS-i patofüsioloogia mõistmiseni ning uute ravimisihtmärkide leidmisele. Ühtlasi on OS-i uurimine viinud tõdemuseni, et tegemist on molekulaarsete mehhanismide tasemel vägagi heterogeense haigusega. Kõige sagedasemad on mutatsioonid p53 tuumor supressor valgu funktsioneerimisega seotud geenides, esinedes ligi 95% juhtudest. Neist kõige tüüpilisemad on *TP53* geeni translokatsioonid ja deletsioonid. Lisaks sellele on OS-i patogeneesiga seostatud mutatsioone leitud ka *RB1*, *ATRX* ja *DLG2* geenidest. OS-i kasvajakudedes on täheldatud ka mitmete mikroRNA-de vähenenenud ekspressiooni võrreldes terve koega, näiteks: miR-206, miR-195, miR-340 ja miR-503. Samas on tuvastatud mõnede mikroRNA-de suurenenud ekspressioon, sh miR-17. Hiljutiste ülegenoomsete

geenieksprессiooniuuringute raames on aga jõutud vastandlike tulemusteni. Selle põhjuseks võib olla nii haiguse heterogeensus kui ka uuringuplaanist tulenevad probleemid, sh liiga väiksed valimid, erinev kontrollgruppide komposit-sioon, laboratoorsete protokollide ning meetodite erinevused. Tegemist on prot-seduuriliste lahknevustega, mille ühtlustamine on vajalik enne kandvate järel-dusteni jõudmist.

Kuna OS-il on iseloomulik suure tõenäosusega metastaseeruda, on tegemist hea mudeliga metastaseerumise uurimiseks. OS uuringud on edendanud tead-misi *TGF-β* funktsioonidest ning toonud esile selle geeni osatähtsuse kasvajate arengus. *In vitro* ja *in vivo* katsete tulemusena on jõutud järeldusele, et TGF-β võib omada olulist rolli vähkkasvajate metastaseerumise protsessis reguleerides kasvajate invasiivseid omadusi, immuunrakkude inhibitsiooni ning kasvajatele soodsa mikrokeskkonna väljakujunemist. Seetõttu on tegemist signaalirajaga, mille manipuleerimine võimaldaks oluliselt inhibeerida metastaseerumise meh-hanisme. TGF-β on atraktiivseks sihtmärgiks ravimiarenduses, kuid tulenevalt antud valgu mitmekesistest funktsioonidest, on ka anti-TGF-β teraapia tulemu-sed olnud seni varieeruva edukusega. Sellegipoolest on TGF-β modulaatorite näol tegemist paljulubavate ravimikandidaatidega nii OS kui ka teiste vähk-kasvajate puhul.

On leitud, et lisaks traditsioonilistele geneetilistele teguritele, võivad mitme-te vähkkasvajate puhul patogeenset efekti omada ka genoomsed korduselemen-did. Korrelatsioone korduselementide ekspressiooni ja vähkkasvajate patoge-neesi vahel on leitud näiteks pahaloomuliste epiteelkudede, idurakkude ja hema-toloogiliste kasvajate puhul. Nende vähkkasvajate korral on satelliitelementide ekspressiooni tõstetud esile kui võimalikku biomarkerit, kuid seni ei ole OS-i puhul sarnaseid uuringuid läbi viidud. Seni kirjeldatud korrelatsioonid lubavad oletada, et genoomsed korduselemendid, sh endogeensed retroviirused, võivad mõjutada vähkkasvajate teket ja arengut. Korduselementide ekspressiooni kir-jeldamine OS-i kontekstis avardaks teadmisi nende võimalikust rollist ka teiste vähkkasvajate puhul.

Käesoleva doktoritöö eesmärk oli uurida OS-i genoomi ja transkriptoomi, et tuvastada biomarkerite kandidaate ja uusi võimalikke haigusmehhanisme. Kes-kenduti ülegenoomsele geenide ekspressiooni võrdlemisele kasvajakoe ja sama-de indiviidide terve koe vahel, võttes arvesse ka võimaliku keemiaravi mõju. Põhilises fookuses oli TGF-β ning antud signaalirajaga seotud geenid. Ühtlasi kirjeldati seni vähe uuritud genoomsete korduselementide ekspressiooni.

Eesmärgid:

1. Tuvastada osteosarkoomi koes erinevalt ekspresseerunud geenid võrreldes samade indiviidide terve luukoega, võttes ühtlasi arvesse, kas patsiendid on saanud keemiaravi või mitte.
2. Kirjeldada TGF-β ja antud signaalirajaga seotud geenide ekspressiooni.
3. Tuvastada osteosarkoomi koes muutunud ekspressiooniga korduselemendid, sh endogeensed retroviirused.

Materjalid ja meetodid:

Proovid koguti 33 patsiendilt, kelle puhul oli OS histoloogiliselt kindlaks tehtud. Neist kümme (30%) olid naised ning 23 (70%) mehed. Patsientide vanus jäi vahemikku 7 kuni 80 eluaastat ning keskmine vanus oli 23.4 aastat. 18 patsiendi puhul oli tegemist hiljuti kogutud külmutatud proovidega ning 15 patsiendi korral olid tegemist formaliinis fikseeritud parafiini sisestatud koeproovidega.

Luukoe proovid koguti kasvajate kirurgilise eemaldamise käigus. Igalt patsiendilt koguti kaks proovi: OS proov kasvajakoest ning võrdluseks terve luukoe proov kasvajast võimalikult kaugelt. Proovid märgistati ning hoiustati -80 ^{o}C juures kuni katsete teostamiseni. Külmutatud proovide transport toimus kuival jääl. Formaliinis fikseeritud parafiini sisestatud koeproovid saadi patoloogia osakondade arhiividest nii Vietnamis kui ka Tartu Ülikooli Kliinikumist. RNA eraldamiseks lõigati parafiinblokkidest 10µm paksused lõigud.

Külmutatud koeproovid töödeldi pulbriks kasutades uhmrit, nuia ja vedelat lämmastikku. Saadud materjal töödeldi Trizol reagendiga ning kogu RNA eraldamiseks kasutati RNeasy Fibrous Tissue Mini Kit'i vastavalt tootja juhistele. Parafiini sisestatud proovide puhul eraldati RNA iga proovi kohta kuuest 10µm paksusest lõigust kasutades selleks PureLink FFPE Total RNA Isolation Kit'i vastavalt tootja juhistele. Eraldatud RNA lahustati RNaasi vabas vees ning hoiustati -80 ^{o}C juures. RNA kvaliteedi hindamiseks kasutati RNA 6000 Nano Kit'i ja Agilent 2100 Bioanalyzer instrumenti. RNA proovide sekveneerimine toimus SOLiD^{TM} 5500W sekvenaatoriga.

Andmete statistiliseks analüüsiks kasutati R'i keskkonda. Ekspressiooni andmeid analüüsiti kasutades DESeq2 ja edgeR pakette. Tulemuste statistilist olulisust hinnati FDR (ingl k *False Discovery Rate*) põhjal. Erinevalt ekspresseerunud geenide klasterdumist signaali- ja metabolismiradade lõikes analüüsiti kasutades ReactomePA paketti.

Tulemused

Võrreldes OS-i kasvajakoe ja terve luukoe ekspressiooni andmeid, leiti 5 365 erinevalt ekspresseerunud geeni, mis olid statistiliselt olulised (FDR $\leq$ 0.05). Neist 3 339 (63%) ekspressiooni olid kasvajakoes kõrgemad ning 1 966 (37%) geeni ekspressioonid madalamad. Kõige väiksema FDR väärtusega ehk statistiliselt kõige olulisemad olid *BTNL9*, *MMP14*, *ABCA10*, *ACACB*, *COL11A1* ning *PKM2 (PKM)* geenid. Kõige enam oli ekspressioon kasvajakoes suurenenud *BTNL9, DNASE1L3, CAMP, LEPR, MIR223, MS4A3, LTF, LCN2, MMP8, S100A12, S100A8, MPO, EPB42, HEMGN, AHSP, ABCA10, BPI, CEACAM6* ja *DEFA* geenide puhul. Kõige enam oli OS-i koes vähenenud *COL11A1, TGFBI, TREM2, COL2A1, COL10A1, HAPLN1, MMP14, PANX3, CTHRC1, STEAP1* ja *COL3A1* geenide ekspressioon. Signaali- ja metabolismiradadesse klasterdumise analüüsi tulemusena selgus, et kõige enam mõjutasid geenide ekspressiooni muutused kollageeni lagundamise, ekstratsellulaarse maatriksi

kujundamise ja erütrotsüütide aktivatsiooniga seotud signaali- ja metabolismiradasid.

Tuvastamaks muutusi geenide ekspressioonis pärast keemiaravi, analüüsiti viite keemiaravi eelselt ning kümmet keemiaravi järgset parafiini sisestatud koeproovi. Statistilise olulisuse kriteeriumi FDR ≤ 0.1 korral leiti 22 erinevalt ekspresseerunud geeni: *POSTN, AMBN, SLC35F3, TAC3, TYROBP, STX7, HEATR1, OGN, USP6, SPRR1B, ASPN, SULF1, DNAJC14, NOB1, FAP, SP3, C8orf59, CCNB2, KRTDAP, MRPS24, C2orf89* ja *RPS6. Kõikide* loetletud geenide ekspressioon oli keemiaravi järgselt kasvajakoes suurenenud. Signaali- ja metabolismiradadesse klasterdumise analüüsi tulemuseks oli vaid üks statistiliselt oluline (korrigeeritud p-väärtus ≤ 0.1) metabolismirada, milleks oli kollageeni moodustamine.

18 patsiendilt võetud kasvajakoe ja terve luukoe proovide puhul keskenduti *TGF-β* ja sellega seotud geenide ekspressiooni uurimisele. Vastupidiselt eeldustele ei leitud, et *TGF-β* geeni ekspressioon erineks statistiliselt olulisel määral kasvajakoe ja terve luukoe võrdluses. Sellegipoolest tuvastati mitme *TGF-β* poolt indutseeritud geenide suurenenud ekspressiooni. Lähtudes statistilisest olulisusest ja ekspressiooni muutumise ulatusest, olid neist kõige olulisemad *COL11A1* (ekspressioon suurenenud 2.8 korda; $p = 1.06 \times 10^{-14}$) ja *TGF-βI* (ekspressioon suurenenud 2.6 korda; $p = 1.35 \times 10^{-11}$).

Korduselementide ja endogeensete retroviiruste puhul tuvastati statistiliselt oluline ekspressiooni erinevus kasvajakoe ja terve luukoe vahel 82 elemendil 1116-st. Nendest 35 (43%) korral oli ekspressioon kasvajakoes kõrgem kui terves luukoes ning 47 (57%) elemendi puhul oli ekspressioon kasvajakoes vähenenud võrreldes terve luukoega. Statistiliselt kõige olulisemad elemendid olid inimese endogeensed retroviirused (HERV): THE1C-int, LTR5, MER57F ja MER87B. HERV-ide ekspressioon oli valdavalt kasvajakoes võrreldes terve luukoega vähenenud. Leitud transponeeruvate elementide ja satelliit-järjestuste ekspressioon oli aga kasvajakoes suurenenud võrreldes terve luukoega. Nendest kõige olulisemad olid SAR, HSATII, (CATTC)n, MER136, ALR_Alpha ja (GAATG)n.

Kokkuvõte

– Leiti 5 365 geeni, mille ekspressiooni erinevused OS-i kasvajakoe ja terve luukoe vahel olid statistiliselt olulised (FDR ≤ 0.05). Nende seast kõige väiksema FDR väärtusega ehk statistiliselt õige olulisemad tulemused olid muutused *BTNL9, MMP14, ABCA10, ACACB, COL11A1* ja *PKM2* geenide ekspressioonis. Erinevalt ekspresseerunud geenide signaali- ja metabolismiradade analüüsi tulemusena leiti, et kollageeni biosünteesi ja lagundamise seotud geenid olid statistiliselt olulisel määral üleesindatud. Käesolevate tulemuste põhjal võib järeldada, et kollageeni lagundamise protsess võib omada olulist rolli osteosarkoomi patogeneesis ning väärib edasist uurimist nii potentsiaalse biomarkerite allikana kui ka funktsionaalsel tasemel. Lisaks leiti keemiaravist tingitud ekspressiooni erinevused 22 geeni puhul, mille

tulemusena kerkis samuti esile kollageeni biosünteesi protsess. Sellest johtub, et keemiaravi tulemusena võib toimuda ekstratsellulaarse maatriksi remodelleerimine.

– Käesoleva töö tulemusena ei leitud, et *TGF-β* geeni ekspressioon erineks statistiliselt olulisel määral kasvajakoe ja terve luukoe võrdluses. Kasvajakoes olulised suurenenud ekspressioon tuvastati aga mitme *TGF-β* poolt indutseeritud geeni puhul, näiteks: *COL11A1, TGFβI, COL3A1, COL6A1, COL6A3* ja *MMP14*.
– Antud doktoritöö tulemusena kirjeldati esmakordselt erinevusi korduselementide ekspressioonis OS-i kasvajakoe ja terve luukoe võrdluses. Leiti 35 korduselementi, mille ekspressioon oli kasvajakoes suurenenud ning 47 elementi, mille ekspressioon oli kasvajakoes vähenenud. Kõige enam oli ekspressioon OS-i proovides suurenenud satelliitjärjestuse SAR ja HSATII ning lihtsa kordusjärjestuse (CATTC)n puhul. Leitud tulemused täiendavad eelnevaid teadmisi korduselementide ja inimese endogeensete retroviiruste ekspressiooni muutustest pahaloomulistes epiteelkudede, idurakkude ja hematoloogiliste kasvajates ning avavad uusi potentsiaalseid uurimise suundasid nii OS-i kui ka teiste kasvajate kontekstis.

11. SUMMARY IN VIETNAMESE

Tổng quan ung thư tạo xương

Ung thư tạo xương là bệnh hay gặp nhất trong số các ung thư xương nguyên phát, chiếm khoảng 20-40% tất cả u xương ác tính. Tỉ lệ mới mắc của ung thư tạo xương cao hơn ở nam so với ở nữ giới tại hầu hết các quốc gia và ở mức trung bình là 3 trường hợp trên 1 triệu người hàng năm. Bệnh xảy ra chủ yếu ở người trẻ quanh độ tuổi dậy thì và tạo nên đỉnh cao nhất về tỉ lệ mới mắc, đỉnh cao thứ 2 thấp hơn về tỉ lệ mới mắc bệnh này ở tuổi già sau 60 tuổi. Tỉ số nam-nữ mắc ung thư tạo xương biến thiên từ 1.01 đến 1.43:1.

Triệu chứng của ung thư tạo xương rất không đặc hiệu do đó bệnh dễ bị bỏ sót bởi bác sỹ chăm sóc ban đầu. Đau và sưng là triệu chứng hay gặp nhất cần được thăm khám và xét nghiệm chuyên sâu. Bệnh gặp phần lớn ở xương dài chi dưới (56%), chi trên (10%). Nguyên nhân và cơ chế bệnh sinh của ung thư này vẫn còn chưa được biết nhiều.

Một số yếu tố nguy cơ của bệnh như tăng trưởng nhanh, xạ trị và một số bệnh di truyền mắc phải như hội chứng LI-Fraumeni, bệnh u nguyên bào võng mạc di truyền, hội chúng Werner, hội chứng Rothmund-Thomson típ 2, hội chứng Bloom, hội chứng RAPADILINO và thiếu máu Diamond Blackfan. Ung thư tạo xương là bệnh có tính di căn cao. Có khoảng 80-90% bệnh nhân có vi di căn ở thời điểm chẩn đoán. Điều này giải thích được khoảng 80% bệnh nhân tiến triển di căn phổi sau phẫu thuật đơn thuần trước thời kỳ điều trị hóa chất.

Tỉ lệ sống thêm của bệnh nhân ung thư tạo xương được cải thiện rõ sau khi ứng dụng điều trị hóa chất vào cuối thập niên 1970 và đầu thập niên 1980. Nhờ hiệu quả của hóa trị, phẫu thuật bảo tồn được thực hiện tốt và rộng rãi, giảm đáng kể phẫu thuật cắt cụt. Tuy nhiên, tiên lượng của ung thư tạo xương vẫn còn xấu và không thay đổi từ sau những năm 80 với tỉ lệ sống thêm 5 năm ở mức 60-78% cho giai đoạn khu trú và chỉ 20-30% cho bệnh giai đoạn tiến xa hay di căn.

Với các tiến bộ gần đây trong công nghệ sinh học, đặc biệt với ứng dụng nhiều kỹ thuật giải trình tự thông lượng cao, số lượng các nghiên cứu ung thư tạo xương gia tăng đáng kể nhằm hiểu rõ hơn về cơ chế bệnh sinh và tìm đích phân tử cho điều trị mới. Kiến thức về cơ chế bệnh ung thư tạo xương đã được mở rộng và đặc tính không ổn định về mức phân tử được ghi nhận ở ung thư tạo xương. Đột biến con đường p53 ghi nhận trong 95% u, mà phần lớn đột biến liên quan TP53 là chuyển đoạn hay mất đoạn. Một số đột biến gene RB1, ATRX và DLG2 cũng được ghi nhận. MicroRNA giảm biểu hiện được cho có liên quan đến tiên lượng bệnh xấu như miR-206, miR-195, miR-340, miR-503, trong khi một số tăng biểu hiện. Gần đây, sự biến đổi về gen trong ung thư tạo xương đã được nghiên cứu với các kỹ thuật mới hiện đại. Biểu hiện khác biệt của gen trong ung thư tạo xương được tìm thấy khác nhau giữa các nghiên cứu có thể do tính không đồng nhất của bệnh hay do hạn chế về thiết kế như mẫu nhỏ, khác nhau nhóm chứng, khác kỹ thuật làm thí nghiệm và kỹ thuật phân

tích. Câu hỏi được đặt ra là làm sao để cải thiện được thiết kế nghiên cứu nhằm mang lại kết quả đáng tin cậy.

Được biết như là một bệnh có khả năng di căn cao, cơ chế di căn trong ung thư tạo xương là hình mẫu tốt để nghiên cứu. Nhiều thí nghiệm in vitro và in vivo cho rằng TGF-β có thể thúc đẩy ung thư di căn thông qua hiệu ứng trên vi môi trường u, tăng cường đặc tính xâm lấn và ức chế chức năng tế bào miễn dịch. Người ta tìm thấy nhiều mối liên quan giữa TGF-β và sự tiến triển của bệnh ung thư và nó thúc đẩy nhiều nghiên cứu nhằm hiểu về cơ chế phức tạp của TGF-β trong sinh ung. Nó gợi ý có con đường ảnh hưởng đến cơ chế di căn. Liệu pháp điều trị nhắm TGF-β đã được phát triển. Tuy nhiên, vai trò của TGF-β rất phức tạp và kết quả của thuốc kháng TGF-β rất khác nhau và đầy thách thức. Do đó, chức năng của TGF-β cũng như các thuốc điều hòa TGF-β đầy hứa hẹn thu hút nhiều nhà nghiên cứu thực hiện trong ung thư nói chung bao gồm cả ung thư tạo xương. Trong cơ chế sinh ung, các yếu tố lặp (repetitive elements) được phát hiện có mối liên quan đến các ung thư biểu mô, u mầm, ung thư máu. Một số vệ tinh (satellites) được đề xuất sử dụng như là chất chỉ điểm sinh học nhưng không có nghiên cứu tương tự được thực hiện với ung thư tạo xương. Nghiên cứu về sự biểu hiện của các yếu tố lặp trong ung thư tạo xương trở nên thú vị để mở rộng thêm kiến thức về vài trò của các yếu tố lặp trong tất cả các phân loại ung thư khác nhau.

Với mong muốn tạo lập một nguồn mẫu bệnh phẩm ung thư tạo xương ở người cho nhiều nghiên cứu hơn trong ung thư tạo xương và nhằm mô tả tổng quan một số biến đổi về gen trong ung thư tạo xương, tìm các chất chỉ điểm sinh học có thể có và để định hướng cho các nghiên cứu chuyên biệt hơn cùng chủ đề này. Chúng tôi thực hiện nghiên cứu này sử dụng mẫu bệnh phẩm xương tươi và mẫu khối nến để tìm hiểu sự biểu hiện gen và các yếu tố lặp ở ung thư tạo xương với các mục tiêu cụ thể như sau:

Mục tiêu:

1. Tìm hiểu và mô tả sự biểu hiện khác biệt của gen ở mô xương bị ung thư so với mô xương lành của cùng bệnh nhân và sự thay đổi biểu hiện gen sau hóa trị.
2. Mô tả sự biểu hiện của TGF-β trong ung thư tạo xương và các gen liên quan
3. Mô tả sự biểu hiện khác biệt của các yếu tố lặp trong ung thư xương so sánh mô xương bệnh và mô xương lành của cùng bệnh nhân.

Đối tượng và phương pháp nghiên cứu

Chúng tôi nghiên cứu 33 bệnh nhân ung thư tạo xương đã được xác minh về mặt mô học. Trong đó, có 10 (30%) bệnh nhân nữ và 23 (70%) bệnh nhân nam. Tuổi của bệnh nhân từ 7 đến 80 tuổi với độ tuổi trung bình là 23.4. Mười tám bệnh nhân có mẫu xương tươi đông lạnh và 15 bệnh nhân với mẫu khối nến.

Mẫu xương tươi được lấy từ mẫu bệnh phẩm sau mổ, được lấy ngay sau khi phẫu thuật viên cắt bỏ được phần bị bệnh. Với mỗi bệnh nhân, hai mẫu xương được thu thập, một mẫu tại vị trí u và một mẫu mô xương lành xa u làm đối chứng. Chúng tôi dùng ống hình phễu để giữ mẫu xương. Các ống đựng mẫu được đánh mã số và được giữ ở tủ đông nhiệt độ âm 80^{o}C cho đến lúc phân tích. Vận chuyển mẫu xương bằng đá khô. Các khối nến được lấy từ khu vực lưu trữ mẫu của các khoa giải phẫu bệnh ở bệnh viện Đại học Y Dược Huế và tại bệnh viện Đại học Tartu, Estonia. Các lát cắt dày 10 micromet từ khối nến được thu thập để tách RNA.

Mẫu xương được nghiền thành bột nhờ nitrogen sử dụng cối và chày và xử trí bằng Trizol. Tách chiết RNA toàn bộ bằng RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia CA, USA) theo hướng dẫn của nhà sản xuất. Với mẫu nến, sử dụng 6 lát cắt có độ dày 10 micromet để tách RNA dùng PureLink FFPE Total RNA Isolation Kit from Invitrogen (Invitrogen Corporation, Carlsbad, CA, USA) theo hướng dẫn. RNA chiết tách được hòa loãng trong nước không có RNase và giữ ở -80^{o}C. Đánh giá chất lượng RNA bằng máy Agilent 2100 Bioanalyzer và máy RNA 6000 Nano Kit (Agilent Technologies Inc., CA, USA). Giải trình tự bằng SOLiD 5500W platform và hóa chất giải trình tự DNA của Life Technologies Corp., Carlsbad, CA, USA.

Phân thích thống kê sử dụng phần mềm R. Gói Deseq2 và edgeR trong R được dùng để phân tích sự biểu hiện khác biệt của gen. Cả hai gói này đều dùng Benjamini-Hochberg để kiểm soát tỉ lệ phát hiện nhầm (FDR). Chúng tôi sử dụng gói R/Bioconductor ReactomePA để phân tích và biểu diễn con đường phản ứng protein (Reactome pathway analysis and visualization). Phương pháp này được dùng để phân tích các con đường sinh học liên quan đến nhóm các gen biểu hiện khác biệt giữa mô xương bệnh và mô xương lành và phát hiện được các con đường sinh học liên quan đến biểu hiện gen biến đổi theo hóa trị.

Kết quả

Với padj <0.05, chúng tôi phát hiện ra 5.365 gen biểu hiện khác nhau giữa mô xương ác tính và mô xương lành. Trong đó có 3.399 và 1.966 gen tăng biểu hiện và giảm biểu hiện theo thứ tự. BTNL9, MMP14, ABCA10, ACACB, COL11A1, and PKM2 (PKM) là các gen có biểu hiện khác biệt có ý nghĩa nhất (FDR thấp nhất).

Các gen giảm biểu hiện nhất (log2fold thấp nhất) trong ung thư tạo xương là BTNL9, DNASE1L3, CAMP, LEPR, MIR223, MS4A3, LTF, LCN2, MMP8, S100A12, S100A8, MPO, EPB42, HEMGN, AHSP, ABCA10, BPI, CEACAM6, DEFA. Các gen tăng biểu hiện nhất (log2fold cao nhất) trong ung thư tạo xương là COL11A1, TGFBI, TREM2, COL2A1, COL10A1, HAPLN1, MMP14, PANX3, CTHRC1, STEAP1, COL3A1.

Phân tích con đường sinh tổng hợp ghi nhận các con đường ý nghĩa nhất liên quan đến thoái hóa collagen, tổ chức chất ngoại bào và hoạt hóa hồng cầu. Tám con đường sinh tổng hợp đáng chú ý nhất gồm thoái biến collagen, hồng cầu

tiếp nhận CO_2 và nhả oxy, hồng cầu nhận oxy và nhả CO_2, trao đổi Oxy/ CO_2 trong hồng cầu, thoái hóa chất nền ngoại bào và tổ chức chất nền ngoại bào. Tất cả chúng đều liên quan đến sự thoái hóa chất nền ngoại bào hay sự tạo máu.

Năm mẫu nến trước hóa trị và mười mẫu nến sau hóa trị đã được phân tích để thấy sự thay đổi biểu hiện gen liên quan đến hóa trị. Hai mươi hai gen được tìm thấy có biểu hiện khác biệt với hóa trị (padj<0.1). Chúng bao gồm POSTN, AMBN, SLC35F3, TAC3, TYROBP, STX7, HEATR1, OGN, USP6, SPRR1B, ASPN, SULF1, DNAJC14, NOB1, FAP, SP3, C8orf59, CCNB2, KRTDAP, MRPS24, C2orf89, RPS6. Các gen này đều tăng biểu hiện với hóa trị. Thực hiện phân tích tăng cường tổ hợp gen (Gene-set enrichment analysis) của 22 gen này cho thấy các con đường sinh tổng hợp liên quan đến các gen này với padj<0,1. Enzyme sinh tổng hợp và biến đổi collagen, sự hình thành collagen, thoái hóa collagen là các con đường sinh tổng hợp liên quan hóa trị có ý nghĩa nhất. Điều thú vị là hầu hết các gen tăng biểu hiện với hóa trị có liên quan đến tổng hợp collagen và hỗ trợ quá trình tạo xương.

Từ dữ liệu giải trình tự RNA của các cặp mẫu bệnh phẩm ung thư xương và mô xương lành của 18 bệnh nhân ung thư tạo xương, chúng tôi tập trung phân tích sự biểu hiện của TGF-β và các gen liên quan. Rất ngạc nhiên là TGF-β không biểu hiện khác biệt giữa u và xương lành. Tuy nhiên một số gen liên quan TGF-β tăng biểu hiện. Trong đó, COLL11A1 và TGF-βI tăng biểu hiện nhất (log2FC 1.51, p= 1.06 E-14 và log2 FC 1.40, p = 1.35E-11, theo thứ tự).

Chúng tôi cũng phân tích 1116 yếu tố lặp khác nhau từ Repbase. Chúng tôi phát hiện 82 yếu tố lặp biểu hiện khác biệt giữa mô lành và mô ung thư tạo xương. Trong số đó, có 35 yếu tố lặp tăng biểu hiện và 47 yếu tố giảm biểu hiện. THE1C-int, LTR5, MER57F, MER87B là các yếu tố lặp có biểu hiện khác biệt ý nghĩa nhất. Chúng đều là HERV, một phân nhóm của yếu tố lặp. Các yếu tố tăng biểu hiện nhất gồm có SAR, HSATII, _CATTC_n, MER136, ALR_Alpha, _GAATG_n. Các yếu tố lặp giảm biểu hiện hầu hết là HERV.

Kết luận

- Chúng tôi phát hiện 5.365 gen biểu hiện khác biệt giữa mô xương lành và mô xương bệnh với FDR hiệu chỉnh p<0.05. Trong số đó, BTNL9, MMP14, ABCA10, ACACB, COL11A1 và PKM2 là các gen biểu hiện khác biệt có ý nghĩa nhất. Sự thoái biến collagen dường như là cơ chế quan trọng của ung thư tạo xương và cần được nghiên cứu thêm để sử dụng như là chất chỉ điểm của ung thư tạo xương. Hai mươi hai gen biểu hiện khác biệt liên quan đến hóa trị. Enzyme sinh tổng hợp và biến đổi collagen, sự hình thành collagen, thoái hóa collagen là các con đường sinh tổng hợp liên quan hóa trị có ý nghĩa nhất. Chúng dẫn đến sự thoái biến và sự tái tạo chất nền ngoại bào. Chúng ta có thể cho rằng hóa trị có thể gây ra sự tái tạo chất nền ngoại bào.
- Không có sự khác biệt về biểu hiện của TGF-β giữa mô ung thư xương và mô thường nhưng một số gen liên quan TGF-β như COL11A1, TGF-βI, COL3A1, COL6A1, COL6A3, and MMP14 tăng biểu hiện.

– Tám mươi hai yếu tố lặp có hiểu hiện khác biệt giữa mô thường và mô ung thư. Trong đó, có 35 yếu tố tăng biểu hiện và 47 yếu tố giảm biểu hiện. SAR, HSATII và đoạn lặp đơn giản (CATTC)n tăng biểu hiện trong ung thư tạo xương. Kết quả của nghiên cứu này bổ sung thêm những phát hiện về biểu hiện của HERV và vệ tinh DNA trong ung thư biểu mô, mầm và ung thư máu và nó là vấn đề thú vị để nghiên cứu thêm trong ung thư tạo xương.

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PUBLICATIONS

CURRICULUM VITAE

Name: Xuan Dung HO
Date of birth: September 5, 1983
Place of birth: Thua Thien Hue, Vietnam
Citizenship: Vietnamese
Telephone: +84 982558945
E-mail: hxdung@huemed-univ.edu.vn

Education:

2013 till now	Ph.D. student at the University of Tartu, Estonia.
2015–2016	Interuniversity diploma (DIU- diplome interuniversitaire) in onco-gynecology, Limoges University, France.
10/2015	Certificate of specialization in family medicine, Ho Chi Minh University of medicine and pharmacy, Ho Chi Minh City, Vietnam.
2010–2012	Diploma of medical specialization formation (DFMS-diplôme de formation médicale spécialisée) in medical oncology at the faculty of medecine of Paris Descartes (Paris 5), Paris, France.
2001–2007	MD, general practitioner, Hue college of medicine and pharmacy, Hue University, Hue city, Vietnam.
2001	Bachelor's degree at Quoc Hoc high school, Hue city, Vietnam.

Languages

Vietnamese	Native
English	Very good
French	Very good

Professional employment:

At present	Medical oncologist, Hue university hospital, Hue city, Vietnam. Lecturer of oncology department, Hue University of Medicine and Pharmacy, Hue city, Vietnam.
11/2011–10/2012	Resident at the department of medical oncology, Tenon Hospital, Paris, France.
11/2010–10/2011	Resident at the department of medical oncology, Avicenne Hospital, Bobigny, France.
8/2007–10/2010	Physician at the department of oncology, Hue university hospital, Hue city, Vietnam. Lecturer of oncology department, Hue University of Medicine and Pharmacy, Hue city, Vietnam.

Membership in the scientific organization

Member of the American society of clinical oncology
Member of Vietnam society of oncology
Member of Thua Thien Hue province of oncology

International publications

1. Reimann E, Kõks S, Xuan Dung Ho, Maasalu K, Märtson A. Whole exome sequencing of single osteosarcoma case – interactive analysis with WT RNA-seq data. Hum Genomics. 2014 Dec 11;8:20
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ELULOOKIRJELDUS

Nimi: Xuan Dung HO
Sünniaeg: 5. september 1983
Sünnikoht: Thua Thien Hue provints, Vietnam
Kodakondsus: Vietnami Sotsialistlik Vabariik
Telefon: +84 982558945
E-mail: hxdung@huemed-univ.edu.vn

Haridus

alates 2013	Tartu Ülikool, doktorantuur, arstiteadus
2015–2016	Limoges'i Ülikool, Limoges, Prantsusmaa, onkogünekoloogia erialaõpe (DIU-diplôme interuniversitaire)
2015	Meditsiini ja farmaatsia Ülikool, Ho Chi Minh, Vietnam, spetsialiseerumine peremeditsiini alal
2010–2012	Descartes'i ülikooli (Paris 5) Pariis, Prantsusmaa, meditsiiniteaduskond, onkoloogiaalane spetsialiseerumine (DFMS-diplôme de formation médicale spécialisée)
2001–2007	Hue meditsiini ja farmaatsia ülikool, Hue, Vietnam, arstiõpe (üldarst)
2001	Quoc Hoc keskkool, Hue, Vietnam, keskharidus

Keelteoskus

Vietnami keel	emakeel
Inglise keel	väga hea
Prantsuse keel	väga hea

Töökogemus

alates 2012	Hue ülikooli haigla, Hue, Vietnam, onkoloog
alates 2007	Hue meditsiini ja farmaatsia ülikool, Hue, Vietnam, onkoloogia osakond, lektor
2010–2011	Tenon'i haigla, Pariis, Prantsusmaa, onkoloogia eriala resident
2010–2011	Avicenne'i haigla, onkoloogia osakond, Bobigny, Prantsusmaa, onkoloogia eriala resident
2007–2012	Hue ülikooli haigla, onkoloogia osakond, Hue, Vietnam, raviarst

Organisatsiooniline ja erialane tegevus

Ameerika kliiniliste onkoloogide seltsi liige
Vietnami onkoloogia seltsi liige
Thua Thien Hue provintsi onkoloogia ühingu liige

Rahvusvahelised publikatsioonid

1. Reimann E, Kõks S, Xuan Dung Ho, Maasalu K, Märtson A. Whole exome sequencing of single osteosarcoma case – interactive analysis with WT RNA-seq data. Human Genomics. 2014 Dec 11;8:20
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