ROLE OF RUNT-RELATED TRANSCRIPTION FACTOR 2 (RUNX2) AND WW DOMAIN CONTAINING OXIDOREDUCTASE (WWOX) GENES IN OSTEOSARCOMA: A MOLECULAR STUDY

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Protocol

Arabic summary

ABC	Avidin-Biotin-Peroxidase Complex
AJCC	American Joint Committee on Cancer
AML	Bcute myeloid leukemia
BMP/TGFb	Bone morphogenetic protein/ transforming growth factor b
BSP	Bone sialoprotein
CBF α	Core-binding factor-α
CCD	Cleidocranial dysplasia
CT	Cycle threshold
d NTP	Nucleoside triphosphates containing deoxyribose
DAB	3′3-diaminobenzidine HCl chromogen
DALY	Disability adjusted life year
DEPC	Diethylpyrocarbonate
DFS	Disease free survival
DTT	Dithiothreitol
EGFR	Epidermal growth factor receptors
EURAMOS	E**ur**opean and **Am**erican **O**steosarcoma **S**tudy Group
F	Female
FFPE	Formalin fixed paraffin embedded blocks
FGF	Fibroblast growth factor
FNCLCC	French Federation of Cancer Centers
FRET	Fluorescence resonance energy transfer
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
H	Kruskal-Wallis
H& E	Haematoxylin & Eosin
H2O2	Hydrogen peroxide
HD	Homozygous deletion

INTRODUCTION

Osteosarcoma (OS) is one of the significant causes of morbidity and mortality, especially among young people. [1] The World Health Organization (WHO) measured the disability adjusted life year (DALY) to assess the overall burden of the disease. An average of 17 years of disability per patient caused by sarcomas was found compared to 6.5 years for bowel, breast and lung cancers making sarcomas a major public health problem. [2]

In spite of the recent improvements in the treatment modalities including neoadjuvant or adjuvant chemotherapy and radiotherapy, the 5 year-survival of OS patients is still low (25-30%), especially for patients with metastasis. [3, 4]

Innovative treatment modalities are needed for further improvement of the survival of OS patients. Understanding the genetic and molecular alterations of OS is essential to generate new therapies. Novel treatments target the signal transduction pathways included in OS development and progression.[3]

Runt related proteins 2 (***RUNX2***) and WW domain containing oxidoreductase (***WWOX***) genes are two important genes implicated in both bone development as well as OS oncogenesis. [5]

RUNX2 is an important transcription regulator. It is a critical regulator of osteogenesis and cell differentiation. ***RUNX2*** genetic aberrations were detected in OS and many other cancers.[6]

WWOX is a tumor suppressor gene, it is found in all eukaryotes and play an important role in the regulation of a wide variety of cellular functions such as protein degradation, transcription, and RNA splicing.[7]

WWOX has also a central role in osteoblast differentiation. Its complex functionality in bone homeostasis suggests that its deregulation could contribute to the pathogenesis of OS. ***WWOX*** has been shown to partner with several proteins implicated in the pathogenesis of OS including ***RUNX2***.[8]

We attempted to study both gene expression abnormalities in a cohort of Egyptian patients suffering from OS.

REVIEW OF LITERATURE

OS is the most common primary malignant, non hematopoietic, bone tumor worldwide. [1] In the United States, it represents 55% of malignant bone tumors. [9] In Egypt, according to the National Cancer Institute (NCI), OS is the most common primary malignant bone tumor, constituting (47.75%), followed by Ewing's sarcoma (17.57%), chondrosarcoma (14.86%) and Non-Hodgkin lymphoma (9.01%). [10]

OS shows bimodal age distribution worldwide, it is rarely diagnosed before the age of five, but the incidence increases with age until around puberty during the period of active bone growth. [11] This primary peak is followed by a decrease and plateau in incidence in individuals between 25 and 60 years of age. A second peak is observed during the seventh and eighth decades of life. Males' incidence is higher than females. [1]

OS occurs in the long bones of the appendicular skeleton, (42%) occurs in the femur, (75%) of which occur in the distal femur, (19%) in the tibia, of which (80%) occur in the proximal tibia, (10%) occurs in the humerus, (90%) of which in the proximal part. Other locations of note are the jaw (8%) and the pelvis (8%). OS occurs mainly in the metaphysis and rarely in the diaphysis and the epiphysis. [12]

The most recent WHO classification of bone tumors (2013) classifies OS into: conventional (which is further classified as osteoblastic (50%), chondroblastic (25%) and fibroblastic (25%) variants), low grade central, telangiectatic, small cell, paraosteal, periosteal and high grade surface OS. Other variants includes: giant cell rich, osteoblastic OS sclerosing type, OS resembling osteoblastoma, chondromyxoid fibroma like OS, chondroblastoma like OS, clear cell OS, epithelioid OS and secondary OS. [13]

According to WHO and College of American Pathologists, the French Federation of Cancer Centers (FNCLCC system) 3-tiered, and Broder's 4 tiered grading systems offer the best combination of ease of use, interobserver agreement and predictive power, to be used for grading bone and soft tissue sarcomas. [13] Staging is the most important prognostic factor. American Joint Committee on Cancer (AJCC), TNM (Tumor size, lymph node and metastasis) is described in table 1. [14]

Table 1: the seventh TNM classification of malignant bone tumors (2010) [15]:

Primary tumor (T)	TX: primary tumor cannot be assessed
	T0: no evidence of primary tumor
	T1: tumor ≤8 cm in greatest dimension
	T2: tumor > 8 cm in greatest dimension
	T3: discontinuous tumors in the primary bone site
Regional lymph nodes (N)	NX: regional lymph nodes cannot be assessed
	N0: no regional lymph node metastasis
	N1: regional lymph node metastasis
Distant metastasis (M)	MX: distant metastasis cannot be assessed
	M0: no distant metastasis
	M1: distant metastasis

	M1a: lung, M1b: other distant sites	
Stage IA	T1 N0M0	Grade 1-2/4, Grade 1/3
Stage IB	T2, T3 N0M0	Grade 1-2/4, Grade 1/3
Stage IIA	T1N0M0	Grade 3-4/4, Grade 2-3/3
Stage IIB	T2N0M0	Grade 3-4/4, Grade 2-3/3
Stage III	T3N0M0	Grade 3-4/4, Grade 2-3/3
Stage IVA	Any T N0,NX M1a	Any grade
Stage IVB	Any T N1 Any M Any T Any N M1b	Any grade Any grade

The etiology of OS is not well understood. Sporadic cases occur due to the inactivation of the tumor suppressor gene as ***P53*** and overexpression of oncogenes. OS exhibits both numerical and structural chromosomal abnormalities. Numerical chromosomal abnormalities associated with OS include loss of chromosomes 9, 10, 13, and 17 as well as gain of chromosome 1. OS is also associated with certain genetic predisposition syndromes with different mutations such as: Li-Fraumeni Syndrome, Familial retinoblastoma syndrome, Rothmund Thomson Syndrome, Werner Syndrome, Bloom Syndrome and Diamond Blackfan Anemia. [2, 16, 17]

Rare causes of secondary OS include individuals with Paget's disease of bone and following therapeutic radiation, previous bone infarction, chronic osteomyelitis, preexisting primary benign lesion as osteochondroma, enchondroma, fibrous dysplasia, giant cell tumor, osteoblastoma, aneurysmal bone cyst and unicameral bone cyst. [13]

The most common clinical presentation of OS is pain which is deep and progressive, compromising the range of motion and enlarging palpable mass. In 5% to 10% of cases, the presenting feature can be a pathological fracture through the destructive mass. [13]

Historically, amputation was considered as the sole accepted treatment with poor survival rate of 10%. Later in 1931, many other treatment modalities were introduced, in view of the poor prognosis of the surgical management, as radiotherapy of primary tumor with elective surgery in the absence of metastasis. [18] Many chemotherapeutic agents were used since 1960 in the treatment of OS with different combinations. Treatment of the primary tumor, preoperative neo-adjuvant therapy was introduced by European and American Osteosarcoma **S**tudy Group. (EURAMOS) with detection of necrosis as an indicator for the response to therapy [19] followed by surgery with optimal safety margins and post operative chemotherapy. New techniques as proton or carbon ion radiotherapy had acceptable local treatment in special cases with ongoing researches on the immunotherapy.[19]

The multi-agent chemotherapy together with gradually-improved surgical techniques in the 1970s had evolved the treatment of OS with five times increase in the survival rate reaching 70% in non metastatic cases and 30% for cases with pulmonary metastasis.[3]

Personalized medical care and the development of biologically tailored therapy based on molecular diagnosis, within a multidisciplinary approach remains the hope for further advancement in the treatment of OS with better survival. [3, 19].

Bone is a dynamic organ that undergoes significant turnover as compared to other organs in the body with complex intercellular signaling. [20] Molecular understanding of the disease will help in the development of targeted therapy. Ongoing studies continue to detect genes whose protein products may play a role in OS oncogenesis and may have potential role as therapeutic targets. OS harbors many genetic abnormalities. Recently, two important genetic aberrations in OS were discovered ***RUNX2*** and ***WWOX*** genes.[5]

Mammalian Runt related proteins (***RUNX***) family includes three members which are tissue-specific transcription factor genes. They are named by the family names core-binding factor-α (CBFA, sequence-specific DNA-binding proteins), acute myeloid leukemia (AML, based on genetic studies of leukemia-related chromosomal translocations), and mouse polyoma enhancer binding protein 2α (PEBP2α, murine cDNAs isolated for polyoma enhancer-binding proteins) according to their types.[6] ***RUNX1/AML1/CBFA2/PEBP2αB*** (***RUNX1***) which has a role in the hematopoiesis was discovered as a chromosomal translocation target in chronic myelogenous and acute myeloid leukemias, ***RUNX2/AML3/CBFA1/PEBP2Aa*** (***RUNX2***) is essential for the osteoblast differentiation and regulates the expression of many genes during bone development,[21] while ***RUNX3/AML2/CBFA3/PEBP2αC*** (***RUNX3***) is essential for development of neuronal networks and the gastrointestinal tract, its inactivation was found to be associated with gastric cancer. [6] The Nomenclature Committee of the Human Genome Organization (HUGO) gave the term '***RUNX'*** to refer to genes encoding the runt-related proteins. [22]

The Runt domain of Runx proteins confers the ability for binding to DNA and for heterodimerisation with Core Binding Factor to form the CBF complex which increases the DNA-binding capacity of the ***RUNX*** partner. [6, 23]

RUNX2 gene occupies the locus 6p12-p21, which is about 220 kbp. There are two promoters for ***RUNX2*** gene P1 and P2 which give rise to two transcripts. Type I isoform MRIPV /p56 of ***RUNX2*** is expressed from P2 promoter and is required for the chondrocyte hypertrophy and maturation. Type II isoform MASNS/ p57 is expressed from P1 promoter, it is the osteoblast specific form of the protein (Figure 1).[6]

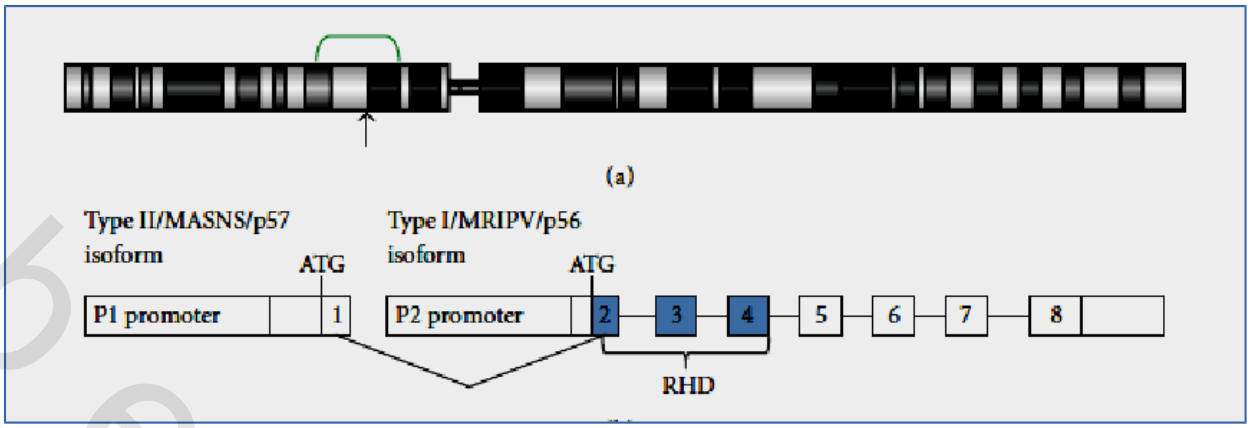


Figure 1: The promoters and transcripts of RUNX2 gene.[6].

RUNX2 is expressed in osseous and non-osseous tissues including endothelial cells and many epithelial cells as breast, ovary and prostate. [24]

RUNX2 is an important transcription factor and regulatory gene through multiple interactions.[25] It recruits chromatin remodeling proteins and assembles complexes at ***RUNX2*** regulatory elements in genes to either activate or repress gene transcription. [26]

RUNX2 is a master regulator of osteogenic differentiation and bone formation. ***RUNX2*** transcriptional activity is necessary for all stages of osteogenesis. It plays an essential role in the commitment of pluripotent mesenchymal stem cells (MSCs) to the osteoblastic lineage. ***RUNX2*** regulates the osteoblast lineage maturation and differentiation. It interacts with many other coactivators and corepressors and inhibits adipogenic or chondrogenic differentiation of MSCs to promote the preosteoblastic pathway and osteoblat lineage determination.[27] ***RUNX2*** can change the phenotype of a cell, e.g., from a non-osseous adipocyte to an osteoblast. It controls the recruitment of stem cells into the chondrogenic and osteogenic phenotype. [25]

RUNX2 controls bone matrix deposition, including osteopontin, bone sialoprotein (BSP), osteocalcin (OC), fibronectin and collagen I.[21, 28] Runx2-pRb (retinoblastoma) complex coactivates the transcription of OC. [6]

Mice heterozygous for mutant ***RUNX2*** develop the autosomal dominant disease cleidocranial dysplasia (CCD) like phenotype. Human CCD is characterized by defects in cranial development, small or missing collar bones, dental abnormalities and a shorter stature. [29] Homozygous mutation is deficient in osteoblasts and neovascularisation due to lack of differentiation of the MSCs. [6]

RUNX2 expression is regulated by many factors. In stem cells, it is regulated by Indian hedgehog (***IHH***) gene, bone morphogenetic protein/ transforming growth factor b (BMP/TGFb), lymphoid enhancer binding factor 1 (LEF1) and Wnt proteins β-catenin. The latter activates fibroblast growth factor 18 (***FGF18***) genes which inhibits chondrogenesis and stimulates osteogenesis (Figure 2).[30]

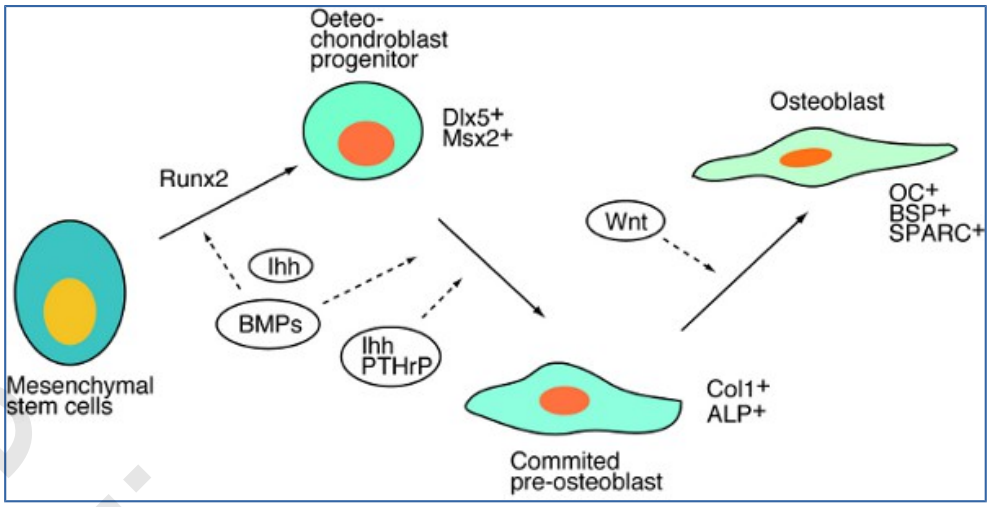


Figure 2: Regulation of RUNX2 and its interaction with other factors during osteogenesis.[6]

RUNX2 has a role in the terminal maturation of preosteoblast and their differentiation from chondrocytes as a prerequisite for endochondral ossification (Figure 3).[31] During endochondral ossification, ***RUNX2*** transcriptional activation is regulated by ***IHH*** and insulin growth factors (***IGFs***).[32] During osteogenesis, ***RUNX2*** expression is regulated by different transcription factors in each stage of differentiation.[33]

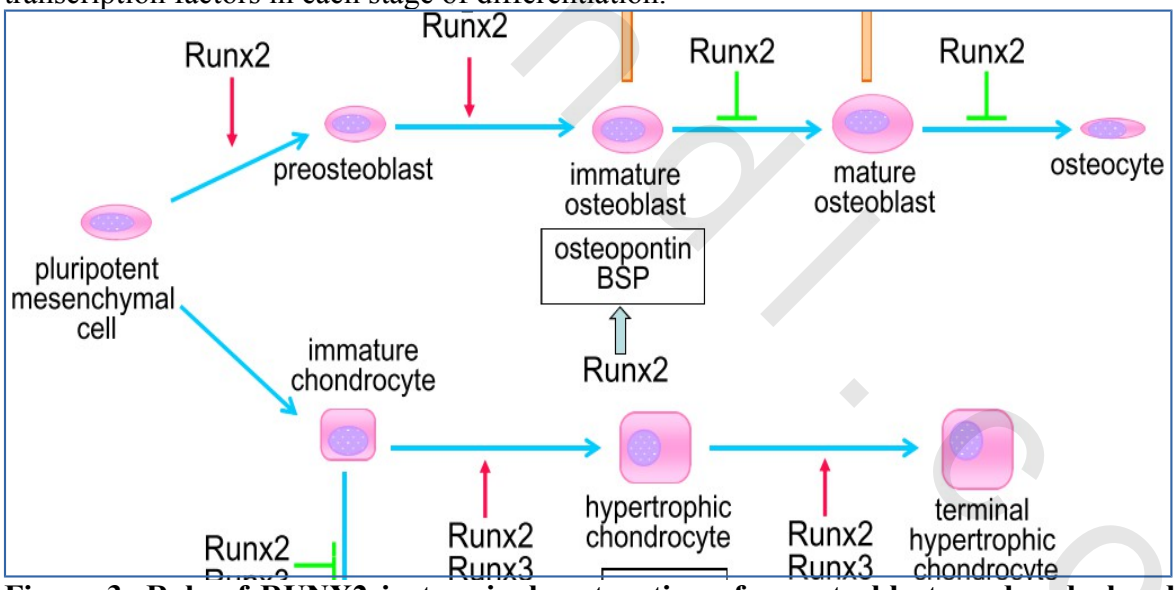


Figure 3: Role of RUNX2 in terminal maturation of preosteoblasts and endochondral ossification. [21]

RUNX2 activity differs according to the phase of the cell cycle. It affects the cell cycle progression in G1 phase suppressing the preosteoblast proliferation. ***RUNX2*** activity is maintained at high levels into the G0 phase if quiescence is induced, then inactivated in G1/S phase transition and regains its activity back after the mitotic phase. ***RUNX2*** regulates epigenetic protein-encoding genes during mitosis.[21] ***RUNX2*** regulation of cell cycle is tissue specific.[34]

Retinoblastoma ***(RB)*** gene is important for the control of the cell-cycle. Hpophosphorylated Rb protein binds and inactivates the transcription factor E2F until the CDK4/cyclin D complex phosphorrylates Rb.[2] ***RUNX2*** mediates cell-cycle exit for terminal osteoblastic differentiation and expression of complete phenotypic markers. It mediates the hypophosphorylated form of Rb necessary for cell-cycle exit by binding to it forming Runx2-pRb complex. ***RUNX2*** also induces high levels of p27 which causes dephosphorylation of Rb as well as inhibiting S-phase cyclin-dependent kinases to promote cell-cycle exit.[6]

RUNX2 has tumor like suppressor behavior due to its regulatory effect on cell cycle, its binding to ***PRB*** and ***P53***.[6] ***RUNX2*** also plays a role in apoptosis through the activation of the proapoptotic gene ***BAX***. [35] This role of ***RUNX2*** in programmed cell death is absent in normal osteoblasts.[35]

The suppressive function of ***RUNX2*** makes it inactivated in certain cancer types, meanwhile, it contributes to the tumorigenic and metastatic potential of other cancers.[36]

Depending on cellular context, ***RUNX2*** could be considered as tumor suppressor gene or oncogene.[36] ***RUNX2*** plays an important role in the growth and metastasis of many cancers. It is overexpressed in many cancers as prostate, breast, colon, epithelial ovarian malignant tumors, pancreatic and thyroid cancers.[28]

RUNX2 promotes cancer cell survival and growth by the activation of the expression of ***IHH*** and the interaction with the ***TGFβ/BMP*** a finding evident in metastatic breast cancer. Overexpression of ***RUNX2*** in malignant tumors leads to the activation of parathormone related protein (PTHrP) and interleukin 8 (IL-8) which stimulates bone osteolysis by receptor activator of nuclear factor κβ (RANKL).[37]

OS is linked to loss of osteoblastic growth and differentiation [38]. The function of ***RUNX2*** in OS has not yet been identified, but due to the complex functionality of ***RUNX2*** in developing osteoblasts, it could be deregulated in OS (Figure 4).[6]

In developing osteoblasts, expression of ***RUNX2*** normally decreases during maturation, and overexpression of the gene leads to a higher rate of bone turnover (Figure 4). [39] While elevated expression of ***RUNX2*** in OS with an unfavorable chemotherapy response is consistent with its oncogenic potential ***RUNX2*** expression level was different in the different OS cell lines and this causes difference in their growth rates.[40]

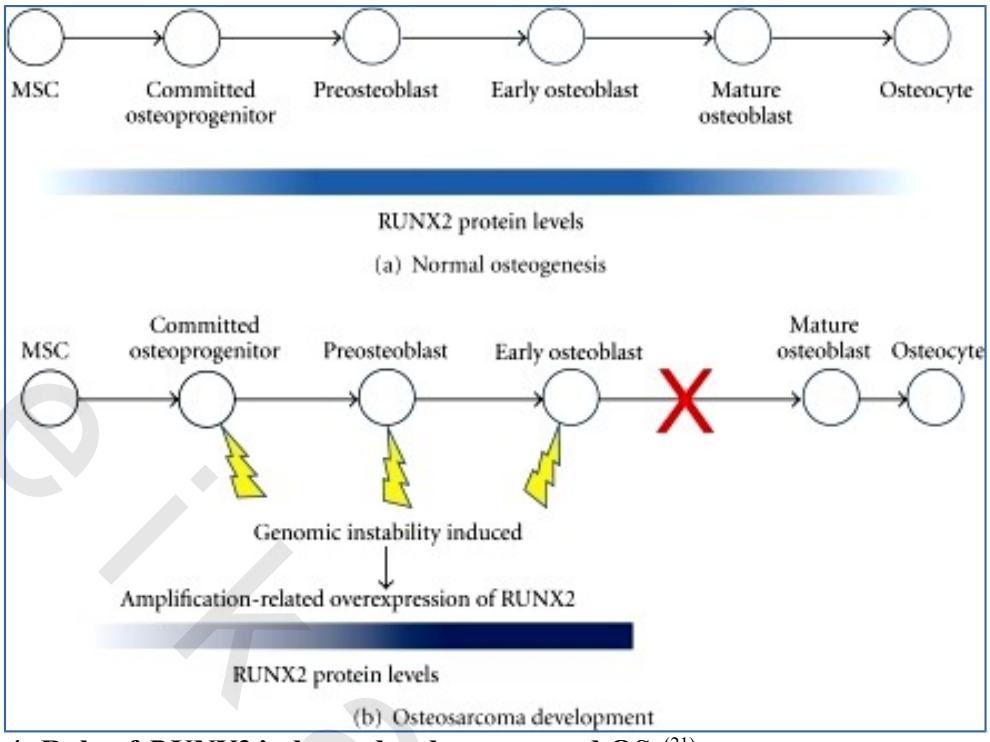


Figure 4: Role of *RUNX2* in bone development and OS. [21]

Many proteins interact with ***RUNX2*** and modulate its activity, one of these factors is ***WWOX*** gene [20].

The WW domain family is classified into four groups. WW domain containing oxidoreductase (***WWOX***) also known as **FOR** or ***WOX1*** belongs to the largest group.[21]

WWOX gene encodes a 46 KDa tumor suppressor gene.[24] It is present in 16q23.3–24.1 region, spans a genomic locus of >1 megabase that encompasses nine exons and encodes an open reading frame of 1245 bases.[17] ***WWOX*** spans a chromosomal area characterized by a very high incidence of allelic loss and chromosomal rearrangements as it occupies the second most active fragile site FRA16D. Its presence in this fragile site causes its frequent inactivation early in the neoplastic progression.[24, 41] ***WWOX*** loss of heterozygosity (LOH), homozygous deletion (HD), chromosomal translocation and hypermethylation are detected in different malignancies.[42] Being in a fragile site, environmental factors contribute to its genetic alterations as Aflatoxin, UV-light exposure and tobacco smoking.[43]

The highest ***WWOX*** gene expression is in the hormonally active tissues as the testis, the ovary and the prostate, indicating its role in steroid metabolism. It is also expressed in chondrocytes and osteoblasts of limbs, calvarium and vertebral bone.[17] WW domain is present in all eukaryotes and plays many cellular roles in RNA splicing and transcription, protein degradation, receptor signaling, and control of the cytoskeleton. [7]

WWOX protein sequence contains two WW domains (WW1 and WW2) and a short chain dehydrogenase/reductase domain (SDR) central domain (Figure 5). WW2 and SDR play a role in the stability and/or half-life related behavior. [7, 44]

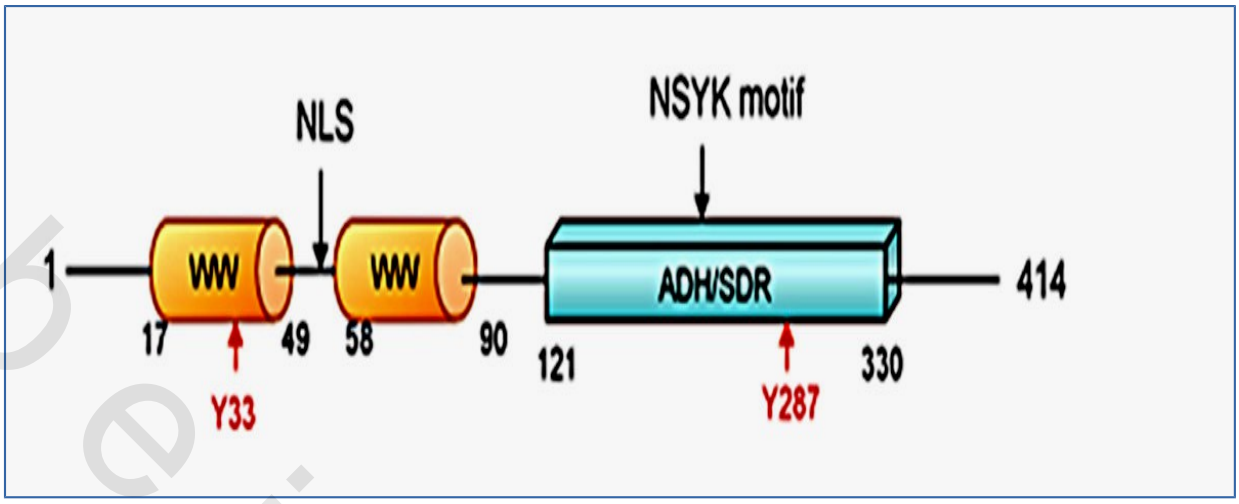


Figure 5: Structure of Wwox protein. [44]

Many proteins have been demonstrated to interact with its first WW domain; among these ligand containing proteins are p73, Ap2α, Ap2γ, ErbB4, Jun and Runx2 (Figure 6).[42] This makes Wwox protein a part of a protein signaling network which suppresses the oncoproteins implicated in the pathogenesis of cancer.[45]

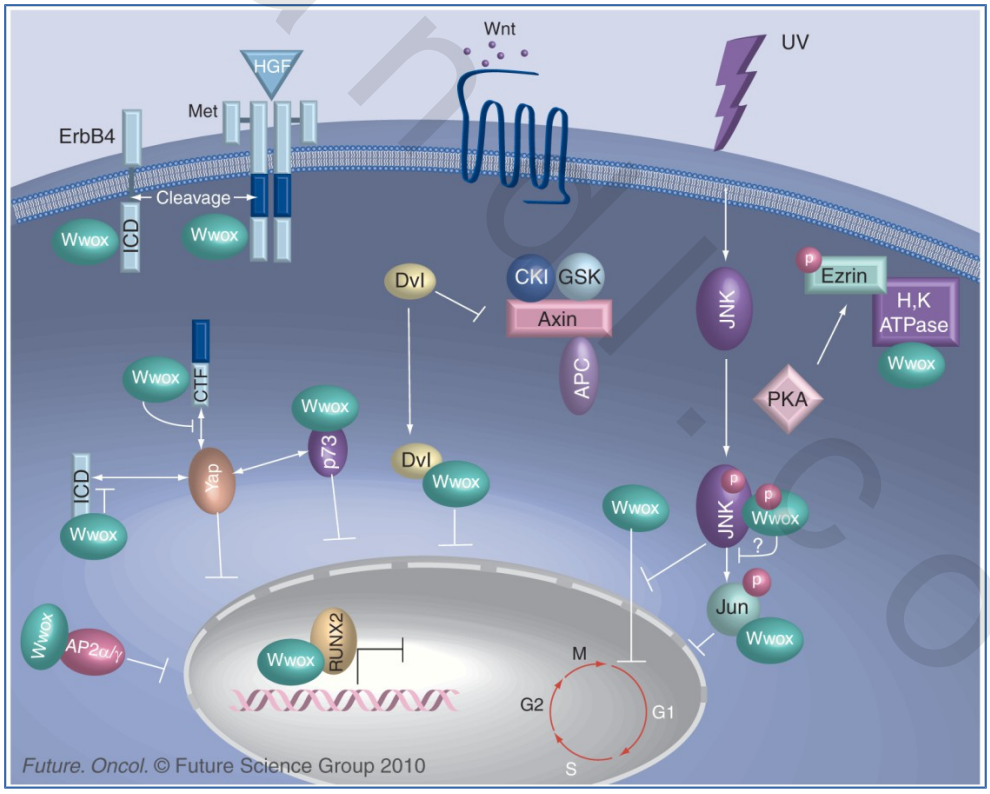


Figure 6: Participation of Wwox in many signal pathways through protein-protein interactions. [42]

WWOX is a tumor suppressor gene; its expression is lost in many human cancers as non small cell lung cancer, breast, bladder and gastric cancers. (Figure 7)[5] ***WWOX*** tumor suppressor function is mediated through its ability to interact, through its WW1 domain, with distinct proteins in multiple cellular pathways.[44]

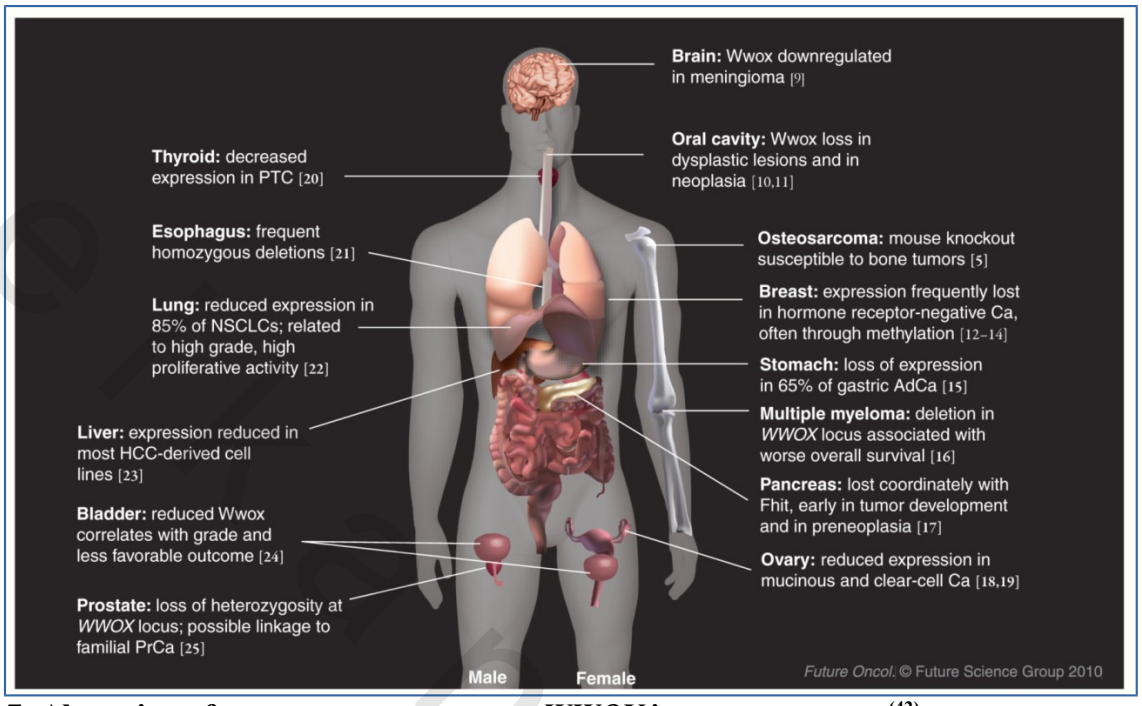


Figure 7: Alteration of tumor suppressor gene *WWOX* in many cancers. [42]

Recent studies demonstrated the role ***WWOX*** plays in the inhibition of Wnt-catenin signaling pathway, which is important in the development and progression of many cancer types, through the physical binding of SDR domain of ***WWOX*** to the proteins of β-catenin sequestering them in the cytoplasm and inhibiting their transcriptional activity.[46]

Wwox binds to ErbB4 tyrosine receptor kinase which plays an important role in cell differentiation and proliferation. After binding, ErbB4 is sequestered in the cytoplasm with inhibition of further translocation in the nucleus. This sequestration in the cytoplasm was proved to be important effector of Tamoxifen induced apoptosis in breast cancer.[47]

Upon exposure to UV radiation, ***WWOX*** expression is reduced due to its presence in the chromosome fragile site while ***JUN*** is activated which could be considered as a mechanism in skin carcinogenesis. ***WWOX*** binding to ***JUN*** regulates the transactivation of the latter inhibiting its carcinogenic effect.[48]

WWOX expression can inhibit the dissemination of ovarian cancer by the reduction of membrane associated integrin-α 3 which mediates the adhesion of ovarian malignant cells.[49] ***WWOX*** interacts with ***EZRIN***, a linker between actin cytoskeleton and the plasma cell membrane. This prevents the generation of the propulsive force of the cell and the mediation of malignant cells migration and metastasis.[50]

WWOX-P73 interaction increases the apoptotic activity mediated by ***P73*** through many pathways. ***WWOX*** competes with other WW containing proteins which bind and degrade ***P73*** as itchy E3 ubiquitin protein ligase (ITCH). [51] ***WWOX*** leads to the sequestration of ***P73*** in the cytoplasm and enhances its cytoplasmic apoptotic activity by the enhancement of the Tumor Necrosis Factor-Alpha-Related Apoptosis-Inducing Ligand (TRAIL).[51, 52] ***WWOX*** mediates the stability of ***P53***, in response to stress or apoptotic stimuli, Wwox becomes phosphorylated which allows complex formation with activated p53. The p53-Wwox complex translocates to the mitochondria and nuclei to mediate apoptosis.[53]

WWOX also mediates apoptosis in cancers by inhibition of Bcl2 and promotion of the expression of caspase mediated apoptotic activity.[54]

WWOX is essential for normal bone development. ***WWOX*** is expressed in osteoblasts, osteoclasts, osteochondroprogenitor cells and mature chondrocytes. ***WWOX*** gene is essential in regulating the proliferation, maturation of osteoprogenitor cells and mineralization during bone formation. ***WWOX*** role in osteoclast is minimal.[17] In ***WWOX*** homologous deficient mice, gonadal atrophy and bone growth retardation occurs with decrease bone formation and increased bone resorption manifested as osteopenia and osteoporosis.[55]

WWOX inactivation is important to develop OS. 30% of ***WWOX*** deficient mice develop spontaneous tumors.[17] Deletion of ***WWOX***, as a tumor suppressor gene, is one of the main mechanisms involved in ***WWOX*** inactivation in OS.[5] Other genetic anomalies of ***WWOX*** gene in cancers include epigenetic modification of the ***WWOX*** promoter by methylation.[45]

WWOX role in normal osteogenesis and OS development is mediated, mainly, through its interaction with ***RUNX2***, the master factor of osteogenesis.[55]

WWOX binds physically to ***RUNX2*** and suppresses its transcription activity.[55] Absence of ***WWOX*** contributes to increased ***RUNX2*** expression which affects bone growth, metabolism and initiates OS tumorigenesis.[24]

Since the development of targeted therapy, many genes were considered as targets for therapy in different malignancies with good response to therapy. As examples of these targeted therapy Her2/neu in breast cancer and epidermal growth factor receptors (EGFR) in non small cell lung cancer.

WWOX and ***RUNX2*** can be considered as new targets for gene therapy development. Ectopic ***WWOX*** expression promotes the caspase mediated apoptotic activity in ***WWOX*** knockout mice, diminishes the tumor progression and its metastasis in OS cell lines.[5, 24, 54] Also, ectopic expression of ***WWOX*** in breast cancer cell lines reduced the expression of ***RUNX2*** and its target genes, including ***VEGF*** and ***OC***[55]

In 2009, ***RUNX2*** expression in malignant tumors was modulated and its anticancer effect was decreased by declining its transcriptional activity in prostatic carcinoma cell lines by the drug FTY720, demonstrating its importance in drug targeted therapy.[56]

Molecular studies of OS are important for tissue engineering and gene therapy. Molecular techniques are important to investigate the role of different genes in tumorigenesis,

tumor growth and metastasis. They are also important in the measurement of the prognostic and predictive values of each gene. One of these molecular techniques is the quantitative real time polymerase chain reaction (qRT-PCR). It proved to be a biological tool which can be used as a routine instrument to gain knowledge about the biology and behavior of the tumors. qRT-PCR is a quick, quantitative, sensitive method to measure a target nucleic acid.[57]

qRT-PCR combines amplification and detection of the amplicon. Many detection systems are used in qRT-PCR: the intercalating dyes as SYBR green, the hybridization system in the form of degradable probe as TaqMan or displaced probes as molecular Beacons. Other types include fluorescence resonance energy transfer (FRET) hybridization probe, scorpions, sunrise primers and light upon extension (LUX)**.** The use of fluorescent dye in the form of the intercalating dye as SYBR green or fluorogenic dye labeling the primer or probe allows the generation of easily detectable fluorescent signals [57]. SYBR green and TaqMan are the two most frequently used methods.

SYBR green is a dye which binds to double stranded DNA and fluoresces to monitor the synthesis of DNA during qRT-PCR reactions. SYBR green use two PCR primers: one to amplify a specific region in the target DNA and the other one to detect the amplified product. The importance of the primer and amplicon designs are important to avoid the affection of the efficiency of the experiment. [58]

TaqMan uses the principle of fluorescence resonance energy transfer (FRET). TaqMan probes are short probes labeled with a reporter donor (reporter) dye at the 5' end and an acceptor (quencher) dye at the 3' end. During PCR's extension phase, the 5' endonuclease activity of Taq polymerase, releases the reporter dye from the quencher dye and detects the fluorescence released which is proportionate to the number of amplicons generated during the reaction TaqMan is more specific than SYBR green (Figure 8).[58]

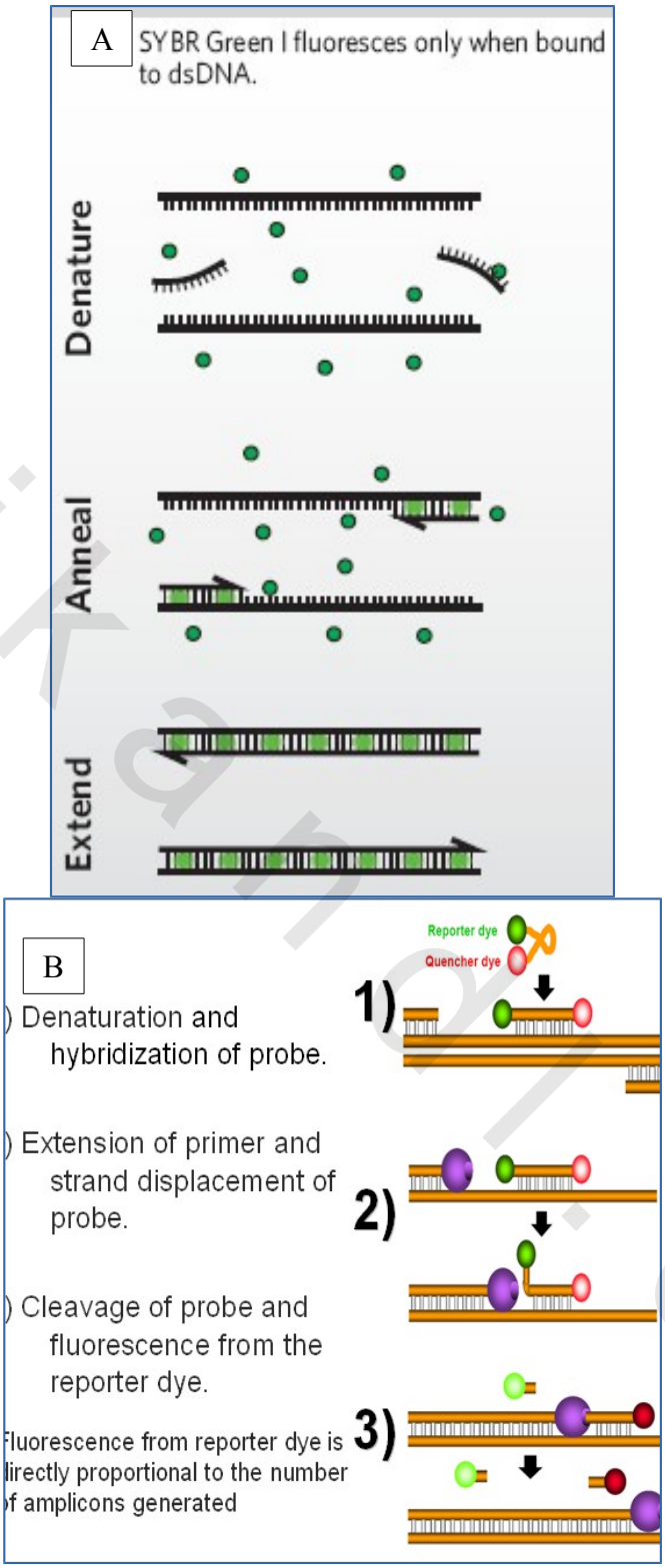


Figure 8: SYBR green (A) and TaqMan (B) techniques of qRT-PCR. [58]

qRT-PCR is useful in many aspects in cancer researches including the gene expression, mutation, amplification and loss. Identification of BRCA1/BRCA2 mutations for breast cancer susceptibility. Measurement of the viral load, to monitor oncogenic viruses.[59] Detection of the minimal residual disease after the treatment. Detection of micrometastasis in sentinel lymph node.[60] From the previous and ongoing clinical applications of qRT-PCR, it was proved to be a biological tool which can be used as a routine instrument to gain knowledge about the biology and behavior of tumors.[61]

The main drawbacks and limitations of the technique are the variability of the results due to the multiple steps. This do not underestimate the role of qRT-PCR as a valuable technique for the detection and quantitation of the differences in RNA expression, in case of small amount of starting RNA material as those obtained from the microdissection.[57]

Immunohistochemistry (IHC) is a complementary diagnostic method with variable useful applications including the diagnosis of non differentiated malignancies, the subtyping of tumors as in lymphomas, identification of the primary tumor in metastatic cases and research of predictive factors and prognostic markers. The drawbacks of IHC are the technical and interpretative bias. IHC is a simple, relatively low cost and highly effective tool.[62]

The study of ***RUNX2*** and ***WWOX*** molecular and IHC basis is important due to many reasons: the paradoxical role of ***RUNX2*** as tumor suppressor gene and oncogene in different tissue types, the relation between ***RUNX2*** and ***WWOX***, their importance in both the osteogenesis as well as the pathogenesis of many cancers including OS. The study of these genes will provide an insight on the pathogenesis of OS and the role these genes could play as candidate genes in targeted therapy.[6]

AIM OF THE WORK

The aim of the present study is to detect Runt related transcription factor 2 (***RUNX2***) and WW domain containing oxireductase (***WWOX***) gene mutations genetic abnormalities in OS and their correlation with clinicopathological parameters.

MATERIALS

Patients and tissue specimens:

Formalin-fixed, paraffin-embedded (FFPE) blocks from 30 patients who were diagnosed with primary osteoblastic OS, by incisional biopsies, during the period from 2008 to 2013 were retrospectively retrieved from the Surgical Pathology Laboratories of Alexandria Faculty of Medicine and National Cancer Institute (NCI), Cairo.

Inclusion criteria were:

1) Adequacy of tissue in the FFPE tumor blocks
2) Absence of previous neoadjuvant chemotherapy (all patients were chemotherapeutic-naïve).
3) Osteoblastic OS by histopathology.

The 30 FFPE blocks were included in the IHC study, while only 20 cases of which were included in the molecular study. In addition, 10 FFPE blocks of normal bone biopsies obtained from viable parts of non-pathologically fractured bones, from other patients, served as a control group for both IHC and molecular studies.

The research was approved by the ethical committees at the Faculty of Medicine, University of Alexandria and the NCI, Cairo.

METHODS

1- Clinicopathological data:

Clinicopathological and radiological data of the patients including age, sex, site, histopathological type and tumor grade were collected from the pathological files at the Surgical Pathology Laboratories of Alexandria Faculty of Medicine and NCI, Cairo.

Tumor stage was assessed according to AJCC, 7th TNM staging system for malignant bone tumors. (15)

2- Follow up of the patients:

Follow up information including recurrence; metastasis and survival of the patients were collected from the Clinical Oncology and Nuclear Medicine Department, Main University Hospital, Alexandria Faculty of Medicine, and NCI, Cairo.

Patients were followed up according to the National Comprehensive Cancer Network (NCCN) guidelines. Surveillance intervals of the patients were every 3 months in years 1 and 2 after diagnosis, every 6 months thereafter. (63)

In the current study, follow up of the disease progression of the patients ranged from18 to72 months (*M*= 45 months, *SD*= 38.18).

3- Histopathological examination:

Five microns (μ) thick sections, were cut from each FFPE block, stained by conventional hematoxylin and eosin (H&E) stain and assessed for the following:

a) Confirmation of the diagnosis and subtype.
b) Confirmation of the tumor grade (13)
   The French Federation of Cancer Centers (FNCLCC system) 3 tiered grading system was used depending on the following:
   i- Tumor differentiation
   ii- Mitosis:
      Score 1: 0 to 9 mitoses per 10 High Power Fields (HPFs)
      Score 2: 10 to19 mitoses per 10 HPFs
      Score 3: 20 or more mitoses per 10 HPFs.
   iii- Tumor necrosis:
      0: No tumor necrosis
      1: Less than or equal to 50% tumor necrosis
      2: More than 50% tumor necrosis.

The summation of the different scores produced the three tiered grading system:
Grade 1: 2-3
Grade 2: 4-5
Grade 3: 6-8

4- IHC procedure:

IHC was done using an Avidin-Biotin-Peroxidase Complex (ABC) methodology [64]. The most representative paraffin blocks were identified by examination of H&E stained sections. From each FFPE blocks of normal bone and tumor, 3 µ thick sections were cut onto positive charged glass slides for IHC.

A- Steps of IHC were done as the following:

1- Deparaffinization and rehydration :

The tissue sections were deparaffinized in xylene for 10 minutes followed by immersion in descending grades of alcohol (100%, 95% and 75%).

2- Antigen retrieval:

Antigen retrieval was done by citrate buffer at pH6.0 in a microwave within steamer for 20 minutes at 700 watts.

3- Endogenous peroxidase blocking:

Blocking was done by 3% hydrogen peroxide (H2O2) for 10 minutes.

4- Incubation with primary antibody:

In a humid chamber, the tissue sections were incubated overnight at 4ºC with the primary antibodies. ***RUNX2*** mouse monoclonal primary antibody (Santa Cruz Biotechnology, Inc, Texas, USA) at a concentration of 1:50 and ***WWOX*** polyclonal primary antibody (Abcam Inc., USA) at a concentration of 1:100.

5- Incubation with the secondary antibody:

At RT, in humid chamber, the tissue sections were covered by UltraVision biotinylated goat anti-polyvalent secondary antibody for 15 minutes and by streptavidin peroxidase for half an hour.

Wash by Phosphate Buffer Saline (PBS) buffer was performed for 3 times, 5 minutes each following each of the previous steps.

6- Visualization**:**

Tissue sections were incubated with 3′3-diaminobenzidine HCl chromogen (DAB) and substrate at RT for 10 minutes, and then washed by tap water.

7- Counterstaining was done by Harris Hematoxylin and cover slipping followed. [64]

In each run, positive control (skin for Wwox and placenta for Runx2) as well as negative control (by omitting the primary antibody) were included.

B- IHC scoring:

Using the light microscope (Leica), IHC was scored for ***WWOX*** (cytoplasmic) and ***RUNX2*** (nuclear) staining using total scoring system, which was based on the intensity multiplied by the percentage of staining in tumor cells.

The percentage of staining was scored as: 0% (score 0), 1-10% (score 1), >10–25% (score 2), >25–50% (score 3), >50–75% (score 4) and >75% (score 5).

Staining intensity was scored as follows: no staining (score 0), mild (score 1), moderate (score 2) and intense (score 3). In addition to the total score, for statistical purposes, scores were also categorized into two groups: negative (0-1) and positive (2-15). [65]

Positive staining of other tissues detected in the sections as skeletal muscle was considered as positive internal control for both antibodies. [5, 29]

5- Quantitative Real time- PCR (qRT-PCR):

i- RNA Extraction from FFPE blocks:

From tumor and control FFPE blocks, at least 3 sections were cut, 10 µ thick each, and placed in an autoclaved plastic microtube (1.5 ml). Total RNA was extracted using the Invisorb Kit ® InviTrap Spin Tissue RNA Mini Kit (STRATEC Molecular GmbH, D-13125 Berlin)[66] according to the manufacturer's protocol as follows:

1- Deparaffinization, disruption and homogenization of the starting material:
-Microtubes were washed in xylene for 3 times and centrifuged at 13,000 rpm for 3 minutes. The wash was associated with mechanical cutting and grinding.
-Samples were dried from xylene and absolute alcohol was added.
-Drying from alcohol followed.

2 - Lysis:
-Lysis was performed by the addition of proteinase K (20µl) with *dithiothreitol* (DTT) (20 mMol in 160 µl RNase free Tris (TE) Buffer) to the samples in the microtubes.
-The microtubes were put in water bath for 10 minutes at 48ºC and kept for other 10 minutes at 80ºC.

3 - Selective binding of the genomic DNA to a specific carrier:
-The tubes were removed from the water bath.
-600µl of DTT containing lysis solution were added in the tubes (vigorously mixed before addition).
-The samples were transferred into 2 ml receiver tube.
-The 2 ml receiver tubes containing the lysate were centrifuged for 2 minutes at maximum speed.
-500 µl of the supernatant were transferred in a new 2 ml receiver tube and 330 µl 96-100% ethanol was added and pipetted thoroughly to mix the lysate completely with the ethanol.

4 - Transfer of the supernatant:
-The lysate was transferred into the RNA binding RTA Spin filter.
5 - Binding of the total RNA to the membrane:

- The lysate was incubated for 1 minute and centrifuged for 1 minute at 10.500 rpm.
- The flow was discarded and the RTA spin filter was placed back into RTA receiver tube.

6 - Washing of the membrane and elimination of contaminants and ethanol:
-First wash of the RTA spin filter was done by adding 500 µl wash buffer R1 onto RTA spin followed by centrifugation for 30 seconds at 10.500 rpm.
-The flow and the RTA receiver tube were discarded.
-The RTA spin filter was transferred in a new RTA receiver tube.
-700µl of second wash buffer R2 was added onto RTA spin filter, pipetted and centrifuged for 30 seconds at 10.500 rpm.
-The flow was discarded and the RTA receiver tube was reused.
-The second wash was repeated once.
-The flow was discarded and the RTA receiver tube was reused.
-For the elimination of any remnants of ethanol or contaminants.
-The RTA spin was centrifuged for 5 minutes at maximum speed for 4 minutes.
-RTA receiver tube was discarded.
7 - Elution of pure total RNA:
-The RTA spin filter was transferred into RNase free elution tube.
-40 µl of elution buffer R was added directly onto the membrane of the RTA spin filter.
-Incubation for 2 minutes and centrifugation for 1 minute at 10.500 rpm was done.
-The RTA spin filter was discarded and the eluted total RNA was incubated at -80ºC.

Precautions for proper handling of RNA in molecular techniques:

- Sterile gloves and equipments in all steps are used and skin contact with any of them should be avoided.
-Time needed for the preparation is decreased.
- RNase-free disposable plastic ware and RNase free distilled water in the preparation of the buffers are used.
-Glassware that comes in contact with RNA is treated by 0.1% Diethylpyrocarbonate (DEPC) solution and heated for 4 hours at 200°C.
-Contamination with any RNase biological sources or other plastic ware is avoided.

ii- Assessment of the quantity and the quality of RNA:

Using 0.5- 2 µL of the RNA sample, the concentration and the quality of the RNA samples were assessed by the NanoDrop 2000 Spectrophotometer (Thermo scientific, USA). Sample purity ratios (260/280 nm) were measured by the software and A260: A280 ratio greater than 2 indicated pure RNA.

iii- Reverse transcription of RNA to cDNA.

First strand cDNA synthesis was done using SuperScript™III First-Strand Synthesis SuperMix for qRT-PCR (Bio-Rad, Milano, Italy)[67] from the previously extracted RNA following the manufacturer's protocol:

1- In a nuclease free microcentrifuge tube, a mixture of 10pg to 500 ng RNA, 1 µl of oligo (dT), 1 µl 10 mM nucleoside triphosphates containing deoxyribose (dNTP) Mix and sterile distilled water was added.

2- The mixture was heated to 65 ºC for 5 minutes and incubated on ice for at least 1 minute. Then, contents of the tube were collected by brief centrifugation.
3- 4 µl 5x first strand buffer, 1 µl 0.1 M DTT, 1 µl RNase out, recombinant RNase inhibitor and 1 µL of superscript III RT (200 units/µl) were added to the mixture followed by gentle pipetting for mixing.
4- Incubation of the mixture at 50ºC for 30 minutes was followed.
5- Inactivation of the reaction by heating at 70ºC for 15 minutes was done.
6- cDNA was then stored at -20 ºC.

iv- SYBR green:

RUNX2 RNA expression was detected by SYBR green using predesigned RUNX2 primer (Hs_RUNX2_1_SG QuantiTect primer assay (249900), Qiagen, Hilden, Germany). The apparatus used was stratagene MAX3000P (Applied Biosystems, Inc., Foster City, CA, USA). According to the manufacturer's protocol[68]; master mix was prepared. It included 12.5 µl 2x QuantiTect SYBR Green PCR Master Mix, 2.5 µl 10x QuantiTect Primer Assay, variable amount of cDNA in a final concentration of ≤100 ng/reaction and variable amount of RNAse free water. Glyceraldehyde 3-phosphate dehydrogenase (***GADPH***) was used as as an internal control, reference gene. The mix was mixed thoroughly and divided in tubes. The real time cycler was programmed for 15 minutes at 95°C for PCR initial activation step (hot start) followed by 3 step cycling of denaturation for 15 seconds at 94°C, annealing for 30 seconds at 55°C and extension for 30 seconds at 72°C. Number of cycles was 40-45.

In each run, no template control (NTC), a tube which included all the components of the reaction except for the template to detect the contamination was added. Normal bone was added in each run as a calibrator.

v- TaqMan

WWOX RNA expression was detected by TaqMan using predesigned ***WWOX*** probe Hs_WWOX_QF_1 QuantiFast probe assay (Qiagen Hilden, Germany) (243132). The apparatus used was Rotor- gene Q (Qiagen, Hilden, Germany). According to the manufacturer's protocol[69]; master mix was prepared. It included 25 µl 2x QuantiFastMultiplex PCR Master Mix (w/o ROX), 1.25 µl 20xQuantiFastProbe Assay (FAM), 1.25 µl 20xQuantiFastProbe Assay (MAX), 0.5 µl Rox dye solution, ≤100 ng/ reaction cDNA and variable amount of RNAse free water. Glyceraldehyde 3-phosphate dehydrogenase (***GADPH***) was used as an internal control, reference gene.

The mix was mixed thoroughly and divided in tubes. The real time cycler was programmed for 5 minutes at 95°C for PCR initial activation (hot start) step followed by 2 step cycling of denaturation for 30 seconds at 95°C, annealing and extension for 30 seconds at 60°C. Number of cycles was 40-45.

In each run, no template control (NTC), a tube which included all the components of the reaction except for the template to detect the contamination was added. Normal bone was added in each run, as a calibrator.

vi- qRT-PCR interpretation and analysis:

qRT-PCR assays were carried out in triplicate: "Delta Delta CT" (ΔΔCT) was used to assess the fold change in gene expression normalized to an endogenous reference gene (GADPH) and relative to the normal bone $2^{-\Delta\Delta CT}$. Mean cycle threshold (CT value) was calculated and used to determine the delta CT (ΔCT) for each sample as follows:

ΔCT= CT for the gene of interest - CT of the internal control gene (GADPH).

Then the delta delta CT (ΔΔCT) was calculated as follows:

ΔΔCT = (ΔCT for sample A - ΔCT for sample B), where sample A is the tumor and sample B is the calibrator (normal bone). For statistical analysis, ΔΔCT and not the raw CT data were used and then the data were expressed as relative expression units. [70]

Statistical analysis:

Data were analyzed using the SPSS® Statistics 20.Qualitative data were described using number and percent. Association between them was tested using Chi-square test; if more than 20% of the cells have expected count less than 5; correction was conducted using either *Firsher's* Exact test or *Monte Carlo* correction. The distributions of quantitative variables were tested for normality using *Kolmogorov-Smirnov* test which revealed significant deviation from normality. They were described using mean (*M*) and standard deviation (*SD*), median (*Mdn*), minimum (*min*.) and maximum (*max*.). *Mann-Whitney* (*U*) and *Kruskal-Wallis* (*H*) tests were used to compare them between two groups and more than two groups respectively. *Spearman's rho* (*ρ*) correlation was used to test the relation between quantitative or qualitative ordinal variables. Significance test results was quoted as two-tailed probabilities, and judged at the 5% level**.**

RESULTS

Clinicopathological data of the patients:

The current study included 30 cases of primary osteoblastic OS obtained from chemotherapeutic-naïve Egyptian patients (the age of the cases was bimodal and ranged between 15 and 57 years, median age=19 years, M: F ratio= 2:1). All relevant clinicopathological data of the studied cases were illustrated in table 2.

In addition, 10 normal bone biopsies (age ranged between 18 and 50 years, median age= 20 years, M: F ratio = 2:1), obtained from viable non-pathologically fractured bones served as a control group.

Follow up of the patients:

During the follow up period (range=18-72 months, *M*= 45 months, *SD*= 38.18), 6 (20%) patients had local recurrence, 11 (37%) patients had pulmonary metastasis, and 8 (27%) patients succumbed to the disease. The disease free survival (DFS) time ranged from 6 to 72 months with an estimated mean survival time of 56 months (95%CI=46.4, 65.4).

Histopathological examination:

Osteoblastic OS was formed of neoplastic cells and variable amount of osteoid. The malignant cells showed, according to their degree of differentiation, different degrees of pleomorphism, hyperchromatism, prominent nucleoli and abnormal mitotic figures with variable amount of eosinophilic cytoplasm.

OS grade 2 was diagnosed in 7/30 cases (23%), these cases showed mild to moderate degree of differentiation, absent or <50% necrotic areas and absent or <10 mitotic figures/10HPF (Figure 9)

OS grade 3 was diagnosed in 23/30 cases (77%), they showed high degree of cellular pleomorphism, bizarre tumor giant cells, prominent nucleoli, brisk mitotic figures including abnormal forms and wide areas of necrosis. Variable amount of osteoid were present between the malignant cells. (Figures 10 and 11).

The osteoid was deposited as primitive, disorganized trabeculae producing lace-like pattern in 8/30 (27%) of the cases (Figures 12 and 13) or broad, large sheets of coalesced trabeculae in 5/30 (17%) of the cases (Figure 14). 17/30 cases (56%) showed mixed patterns of lace like and coarse osteoid deposition. Variable amount of hemorrhage and necrosis were present in all cases (100%).

Malignant cells were seen infiltrating the surrounding soft tissues including fat and skeletal muscles in 10/ 30 cases (33.3%) (Figure 15).

Table 2: Clinicopathological features of 30 and 20 human OS patients:

	30 cases included in IHC		20 cases included in qRT-PCR	
	No.	**(%)**	**No.**	**(%)**
Gender				
Male	20	(67)	13	(65)
Female	10	(33)	7	(35)
Site				
Femur	20	(67)	12	(60)
Tibia	6	(20)	5	(25)
Humerus	4	(13)	3	(15)
Histopathological grade				
2	7	(23)	4	(20)
3	23	(77)	16	(80)
TNM Stage				
II	10	(33)	10	(50)
III	20	(67)	10	(50)
Recurrence*				
Absent	24	(80)	18	(90)
Present	6	(20)	2	(10)
Metastasis				
Absent	19	(63)	15	(75)
Present	11	(37)	5	(25)
Survival				
Alive	22	(73)	16	(80)
Dead	8	(27)	4	(20)

**Recurrence was not included in statistics of qRT-PCR due to limited number of positive cases.*

**For statistical purposes, overall survival and not (DFS) was used in the statistics due to the small number of events of the latter.*

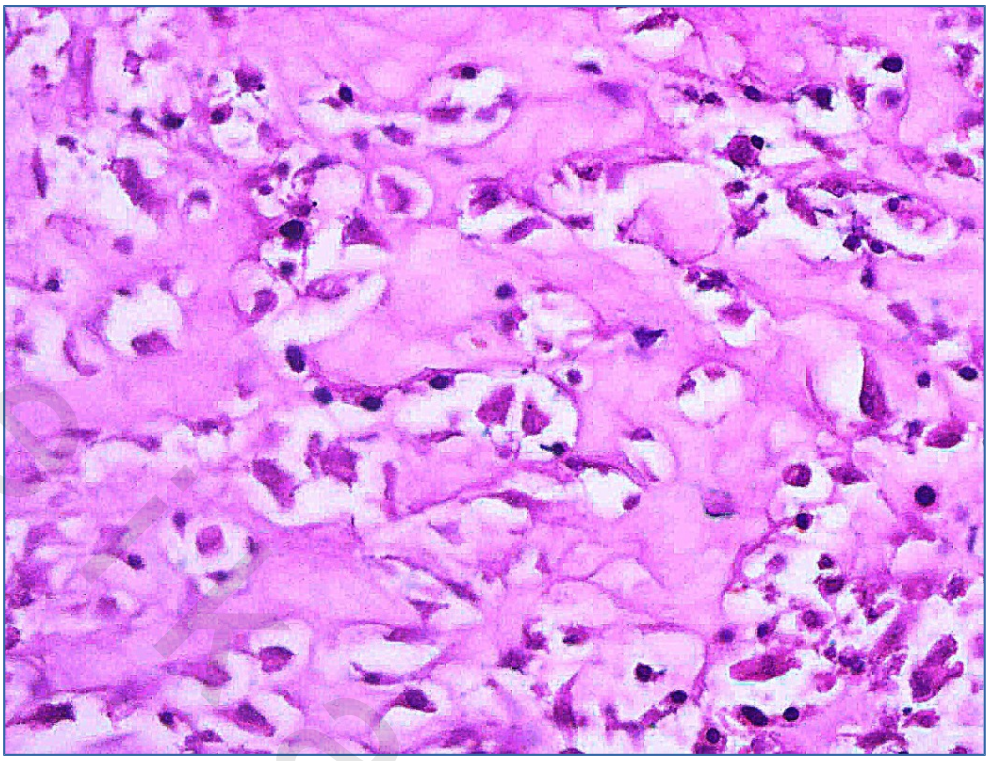


Figure 9: A case of osteoblastic OS, grade 2 showing malignant osteoblasts with intervening osteoid (H& E X 400).

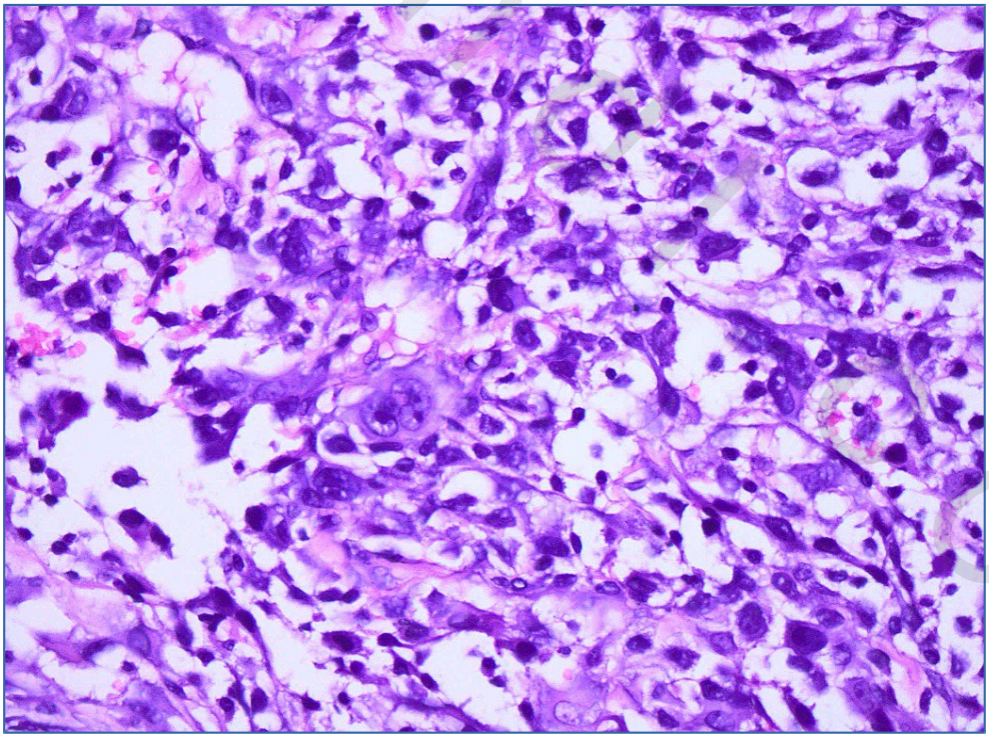


Figure 10: A case of osteoblastic OS, grade 3 showing pleomorphic malignant osteoblasts with occasional prominent nucleoli (H&E X 400).

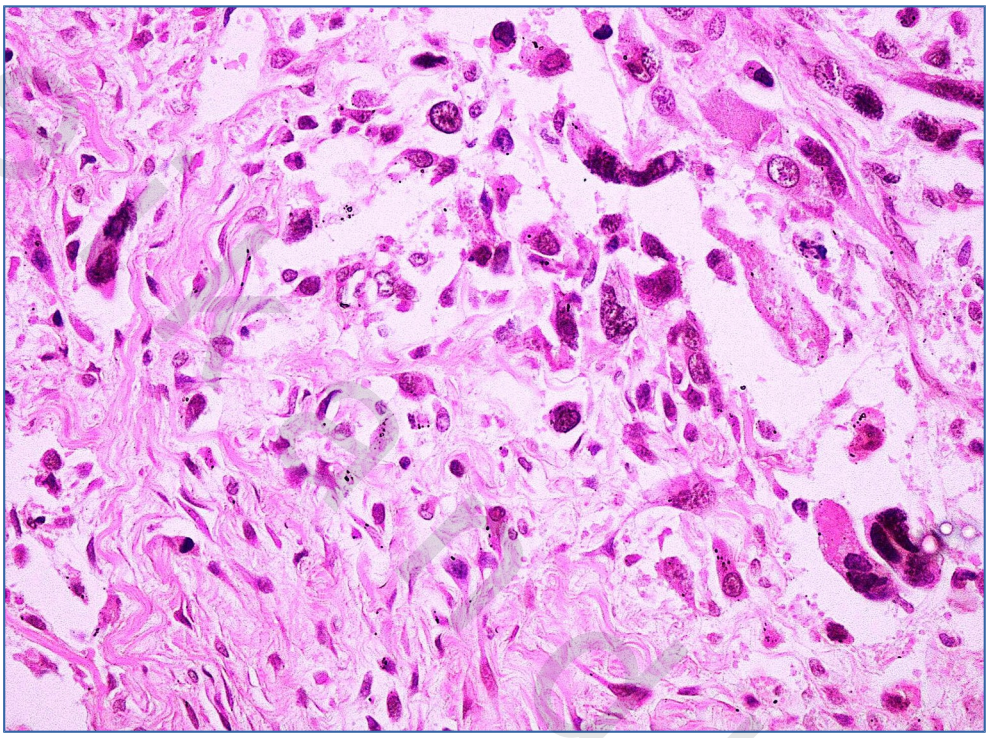


Figure 11: A case of osteoblastic OS, grade 3, showing malignant bizarre giant tumor cells and abnormal mitotic figures (H& E x 400).

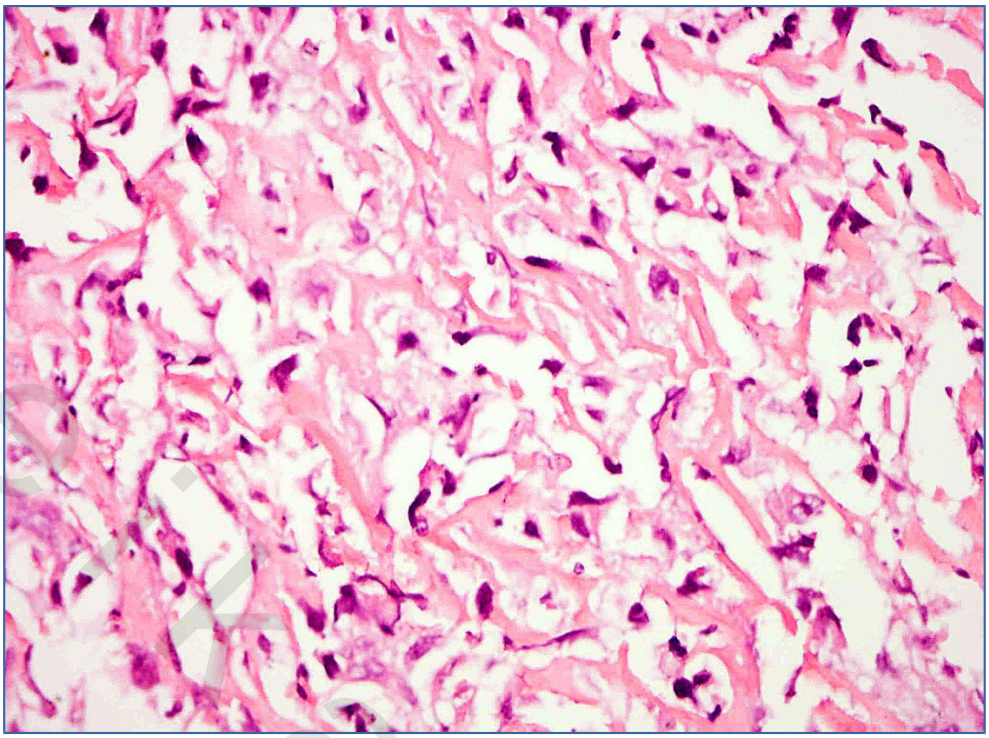


Figure 12: A case of osteoblastic OS showing lace like appearance of osteoid (H& E x 400).

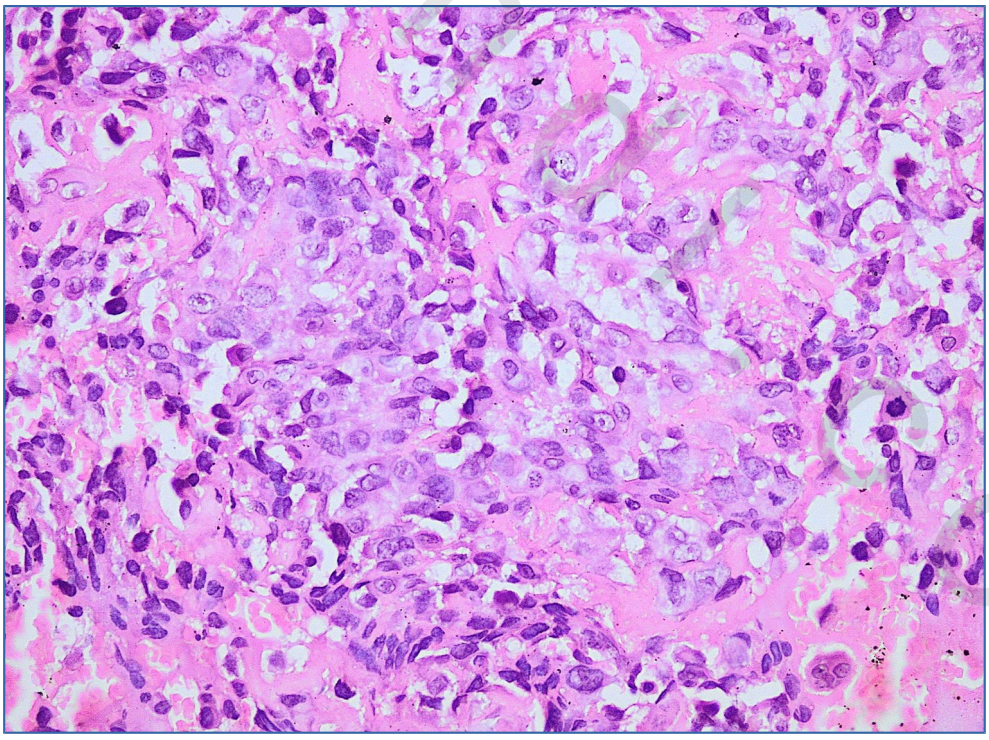


Figure 13: A case of osteoblastic OS showing lace like appearance of osteoid (H& E x 400).

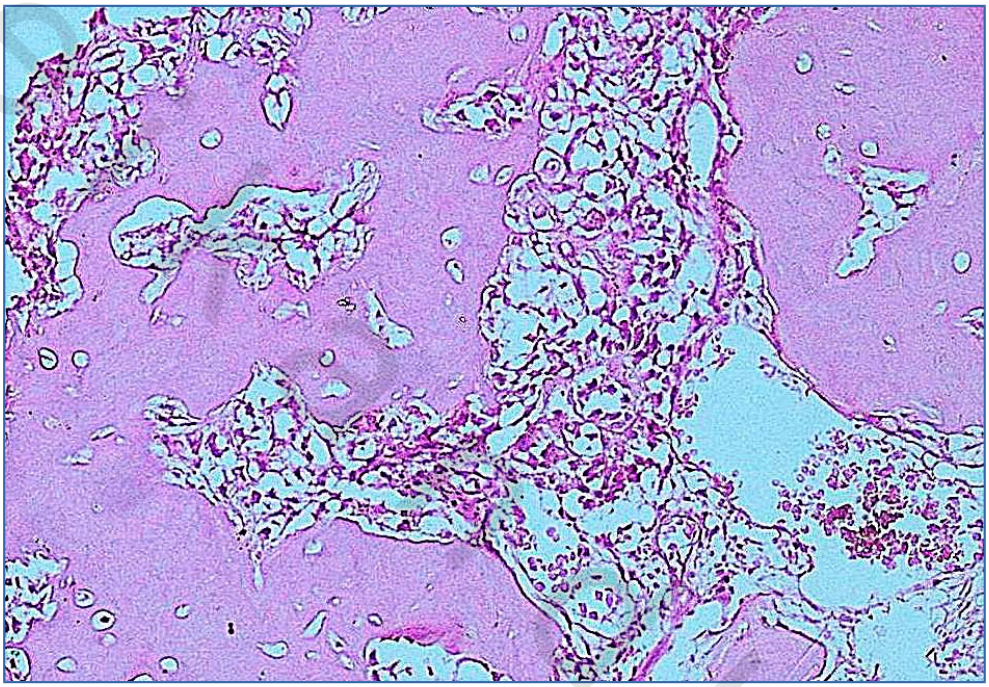


Figure 14: A case of osteoblastic OS showing malignant osteoblasts arranged on the surface of coarse osteoid (H& E x 200).

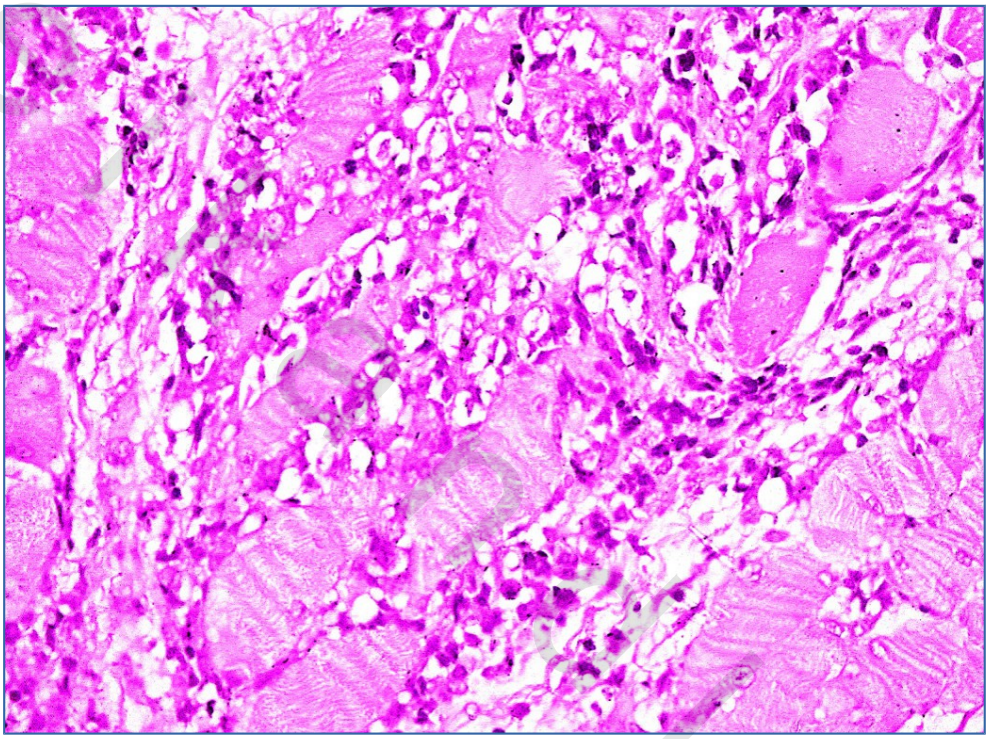


Figure 15: A case of osteoblastic OS, showing malignant osteoblasts infiltrating the adjacent skeletal muscles (H& E X 400).

Wwox and Runx2 immunohistochemical protein expression:

IHC was assessed in the 10 control cases and the 30 osteoblastic OS cases.

In OS cases, Runx2 protein expression was detected in 27/30 (90%) and was negative in 3/30 (10%) cases. Regarding Runx2 positive OS cases, 21 (78%) showed strong nuclear staining intensity in > 25% to >75% of tumor cells, 5 (17%) cases showed moderate intensity in >50% to 75% of tumor cells and a single case (5%) showed weak intensity in >75% of tumor cells. (Figures 16-24)

According to the total staining score, median expression of Runx2 in normal bone was = 1 (*Min.-Max.* = 0-8) which was significantly lower than median expression of Runx2 in OS cases=13 (*Min.-Max.* = 0-15) *(U*=3.5, p <.001). According to IHC groups, in normal bone, Runx2 protein expression was negative in 8/10 (80%) and was expressed in 2/10 (20%). The positive cases showed weak to moderate nuclear staining intensity in >25% to 75% of osteoblasts.

In OS cases, Wwox protein expression was negative in 22 cases (73%) and was detected in the remaining 8 cases (27%). Single positive Wwox OS case (12%) showed moderate cytoplasmic staining intensity and 7 cases (88%) showed weak staining intensity. 6 of the Wwox positive cases (75%) showed both cytoplasmic and nuclear staining. (Figures 25-28).

According to the total staining score, median expression of Wwox in normal bone was =7 (*Min.-Max.* = 4-10) which was significantly higher than median expression of Wwox in OS cases= 0 (*Min.-Max.* = 0- 6) *(U*=3.5, p <.001). According to IHC groups, in normal bone, Wwox protein expression was detected in 10/10 control cases (100%) with moderate to strong cytoplasmic staining intensity in all cases (100%).

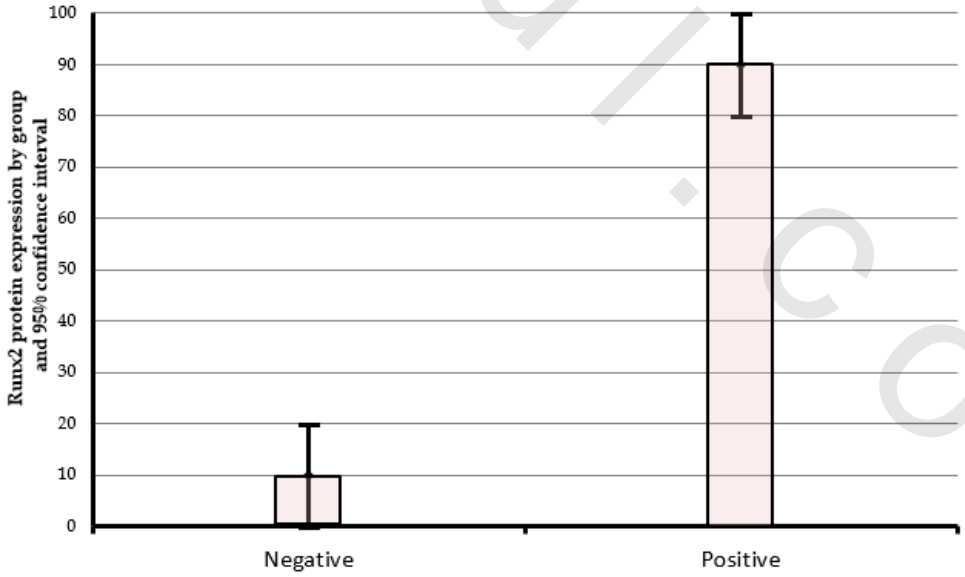


Figure 16: Bar chart showing the frequency of Runx2 protein expression in OS cases according to groups.

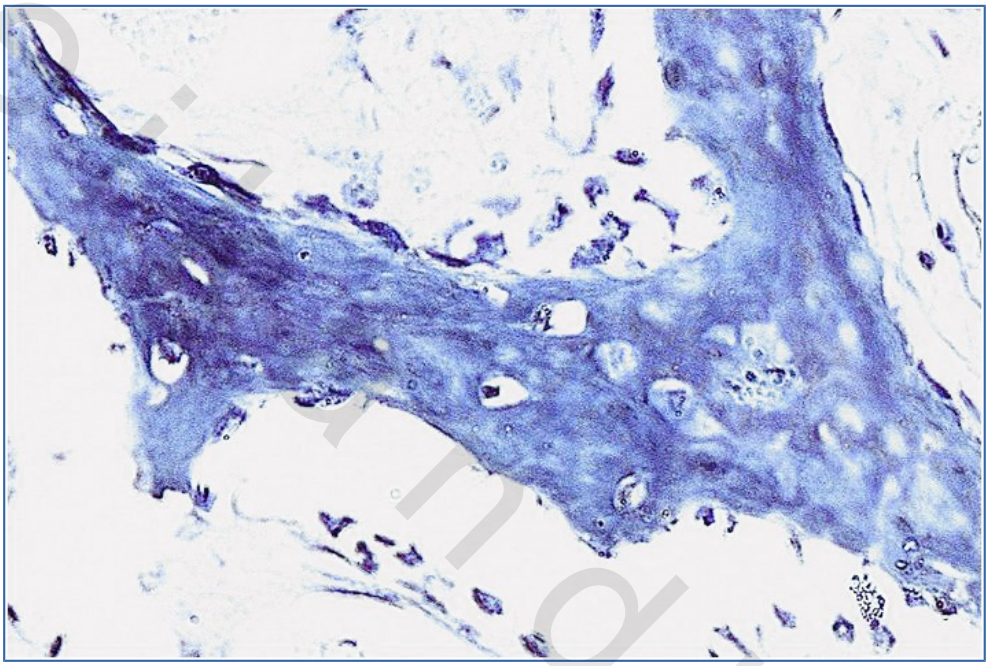


Figure 17: Negative Runx2 expression in normal bone (x 400).

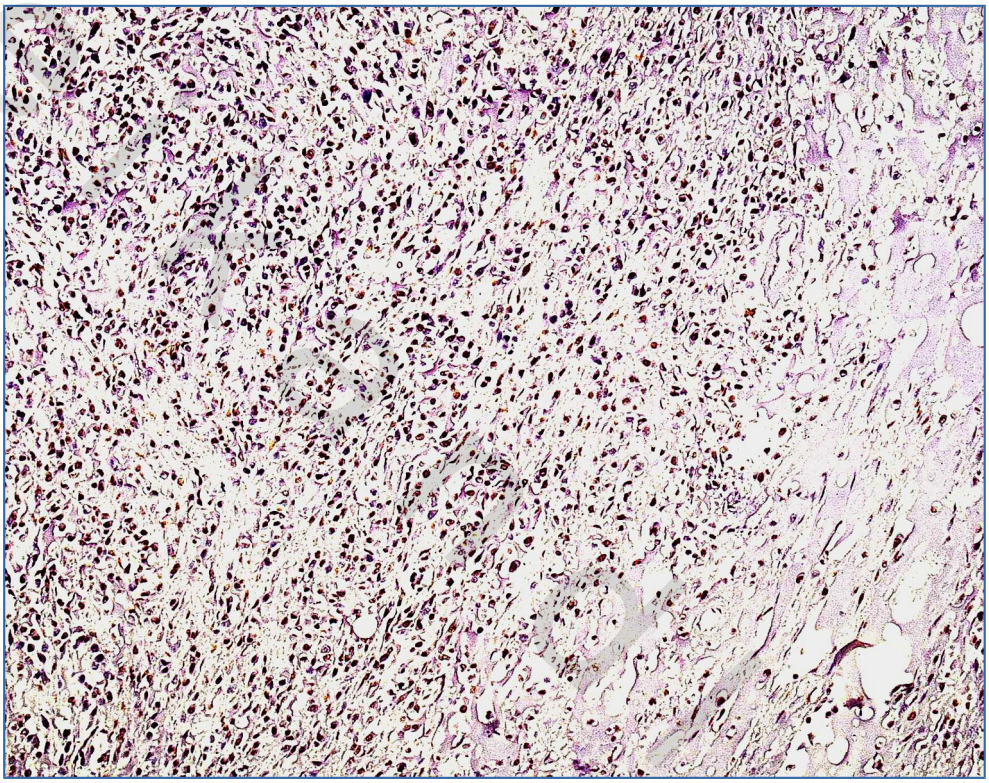


Figure 18: A case of osteoblastic OS, grade 2 showing strong nuclear staining for Runx2 (x 100).

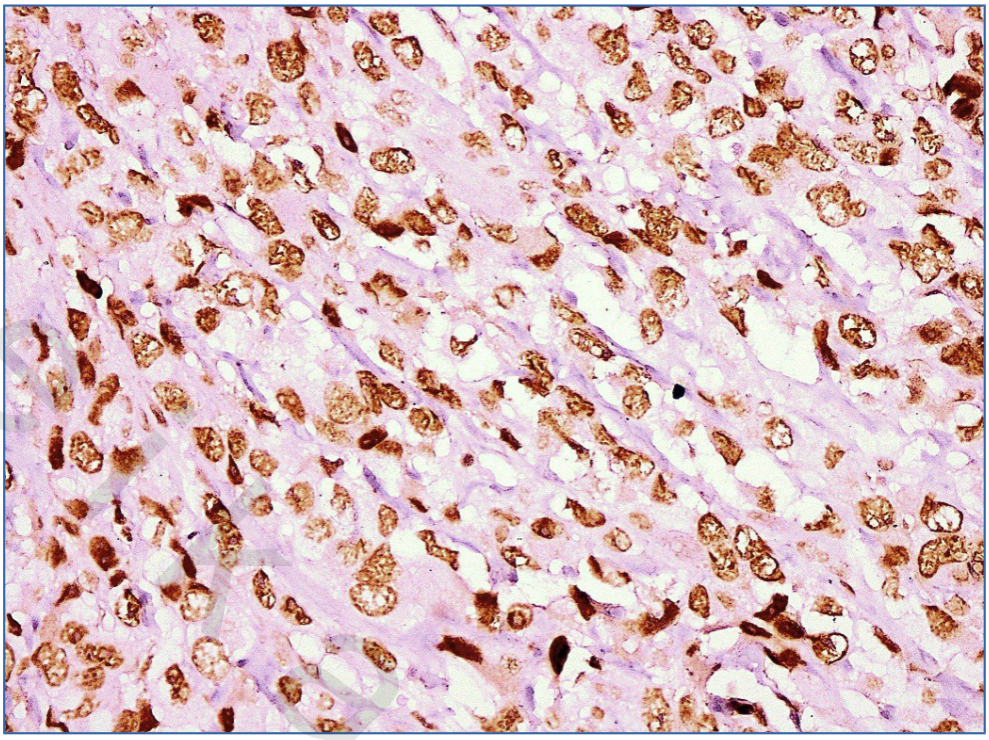


Figure 19: A case of osteoblastic OS, grade 2 showing strong nuclear staining for Runx2 (x 400).

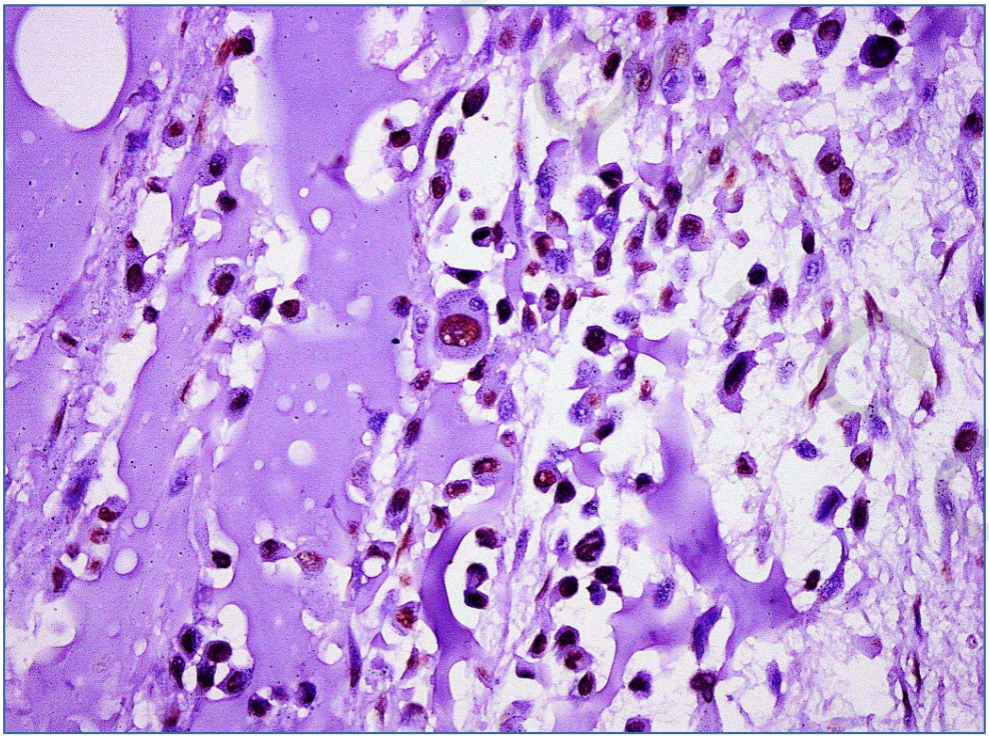


Figure 20: A case of osteoblastic OS, grade 3 showing strong nuclear staining for Runx2 (x 400).

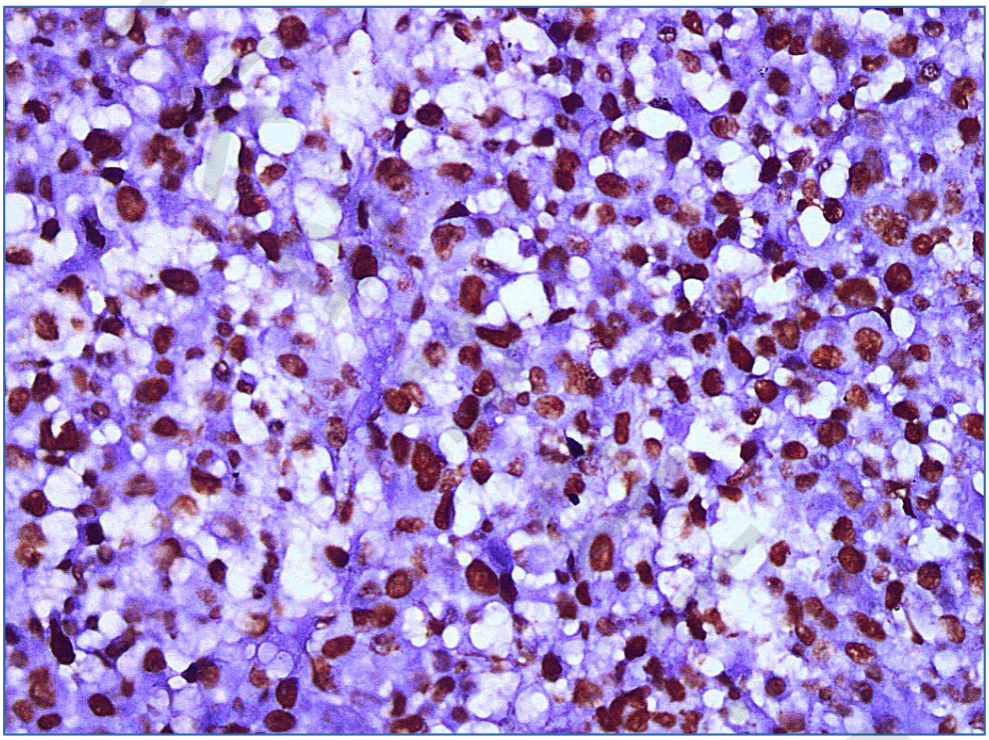


Figure 21: A case of osteoblastic OS, grade 3 showing Runx2 strong nuclear staining (x 400).

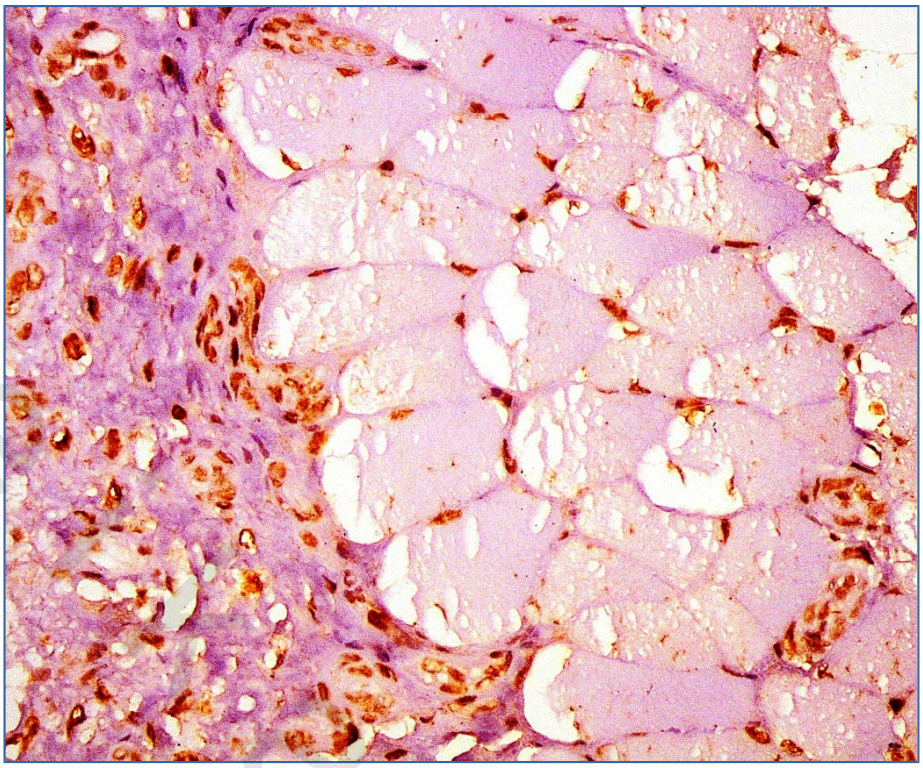


Figure 22: A case of osteoblastic OS, grade 2 showing Runx2 strong nuclear staining. Note the infiltration of the adjacent skeletal muscles which show positive nuclear staining for Runx2 (internal control)(x 400)

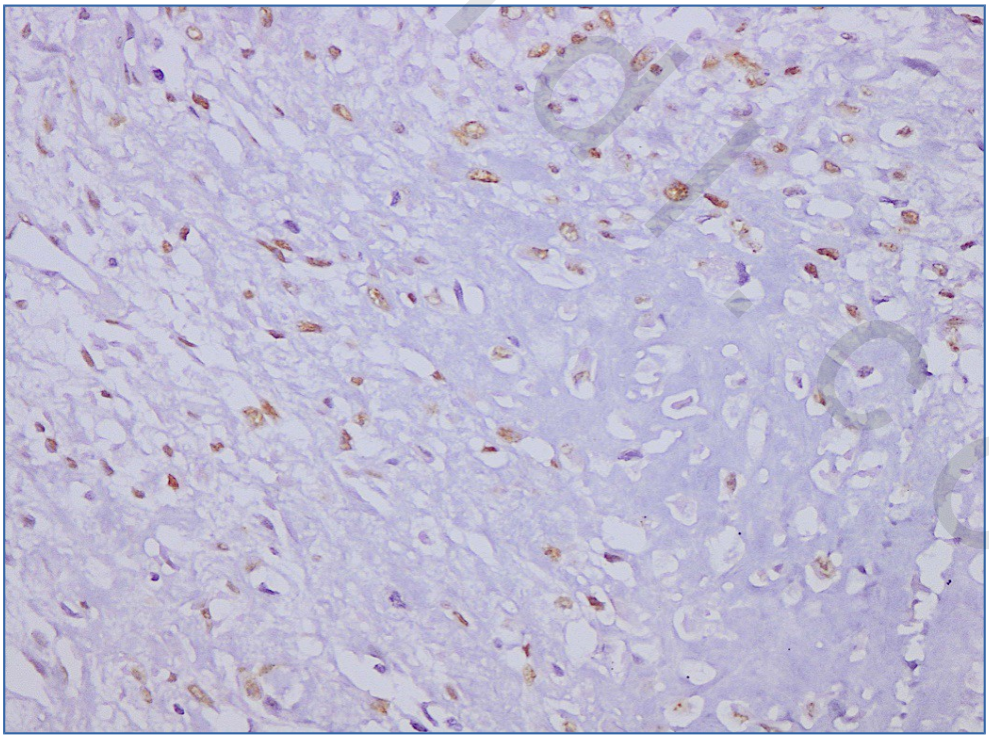


Figure 23: A case of osteoblastic OS, grade 2 showing moderate Runx2 nuclear staining (x 400).

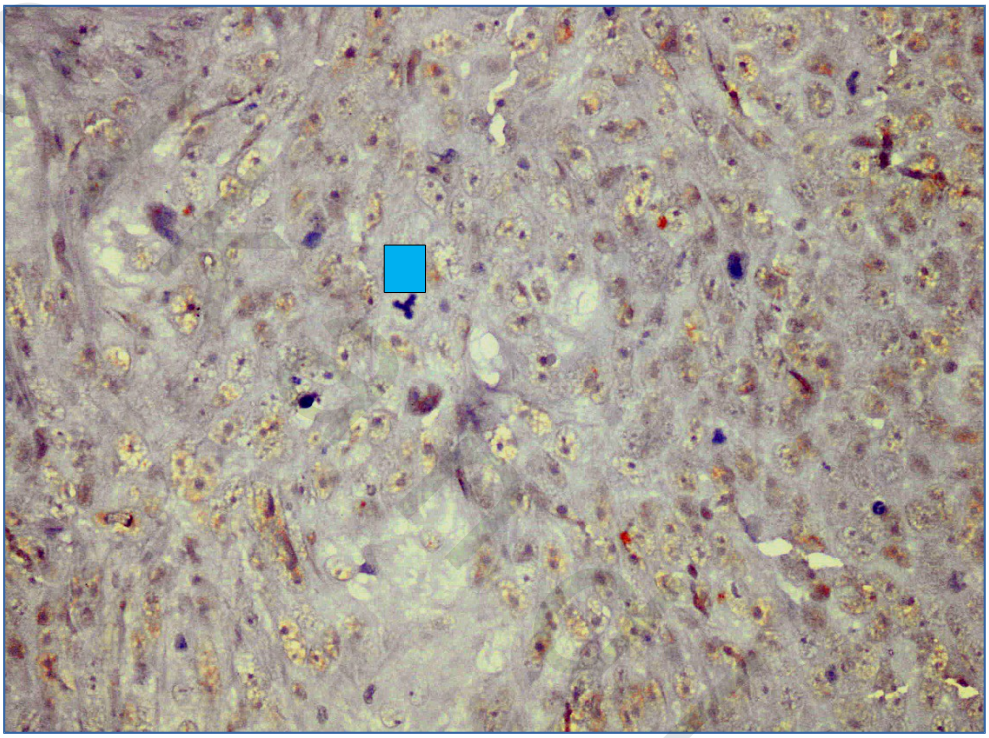


Figure 24: A case of osteoblastic OS, grade 2 showing weak Runx2 nuclear staining. Note the abnormal mitoic figure (arrow) (x 400).

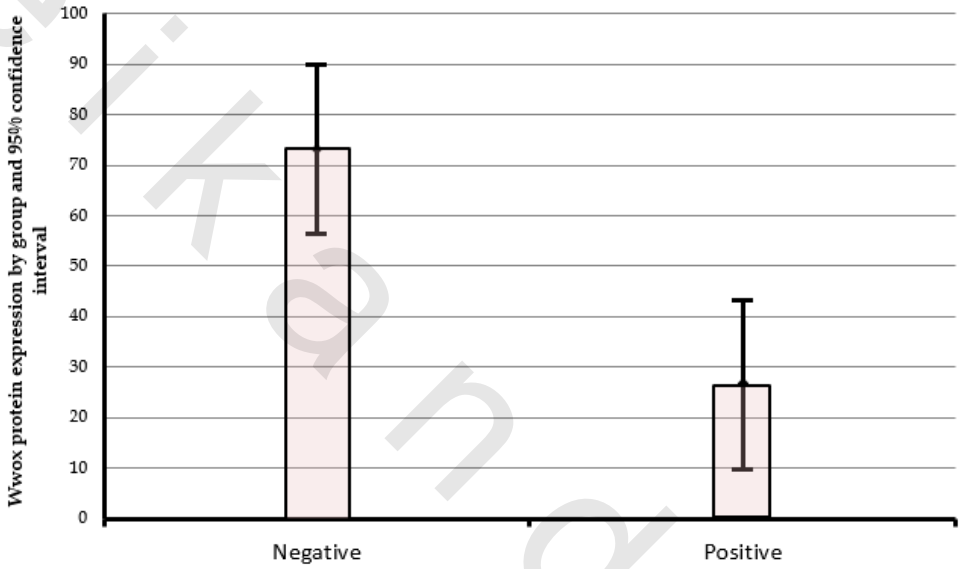


Figure 25: Bar chart showing the frequency of Wwox protein expression in OS cases according to groups.

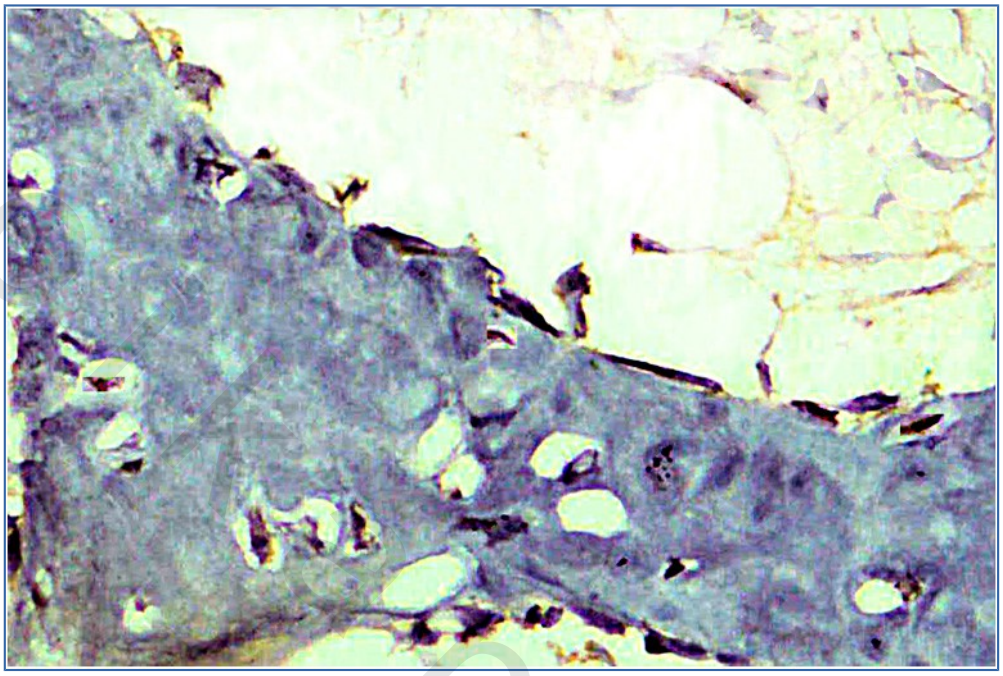


Figure 26: Positive Wwox expression in normal bone showing strong cytoplasmic staining (x 400).

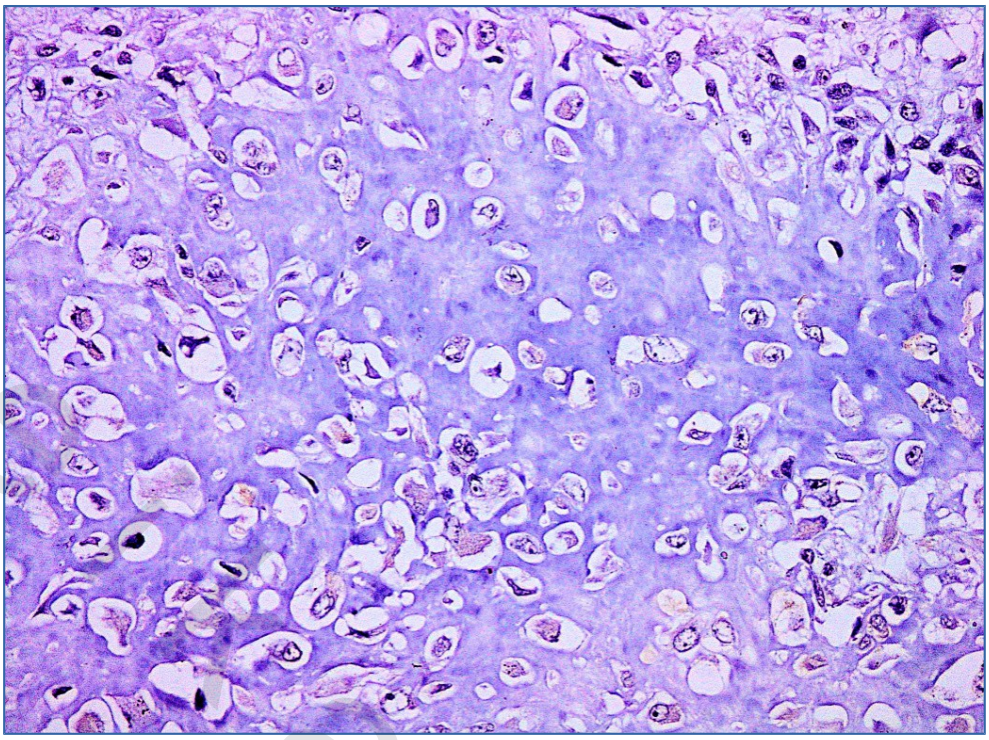


Figure 27: A case of osteoblastic OS, grade 2 showing negative Wwox cytoplasmic staining (x 400).

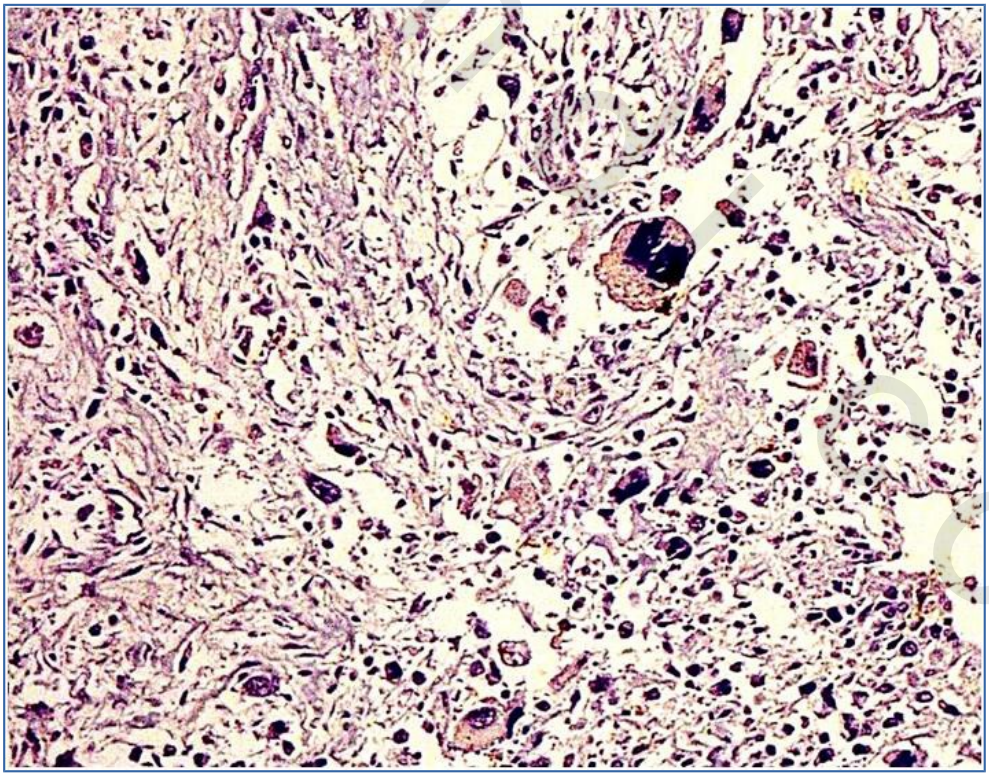


Figure 28: A case of osteoblastic OS, grade 3 showing moderate Wwox cytoplasmic staining (x 400).

There was a significant negative correlation between Runx2 and Wwox protein expressions by IHC (ρ=-.578, p=0.008, ρ=-.391, p=0.033 for the 20 and 30 cases respectively) (Figure 29).

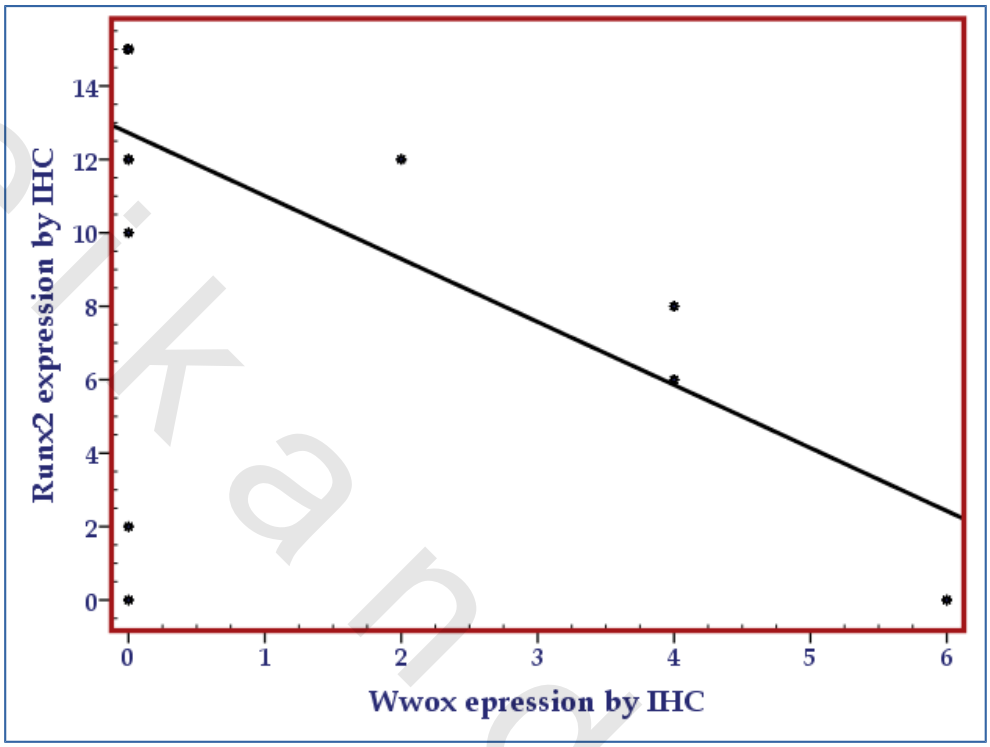


Figure 29: Scatter blot chart showing a negative correlation between Wwox and Runx2 protein expression in 30 OS cases (ρ=-.578, p=.008).

WWOX and *RUNX2* RNA expression:

In the molecular study, 20 cases only were included. In comparison to normal bone, ***RUNX2*** gene RNA expression was up-regulated in 15/20 (75%) cases and down-regulated in 5/20 (25%) cases (Figure 30).

In comparison to normal bone, ***WWOX*** gene RNA expression was down-regulated in 16/20 (80%) and up-regulated in 4/20 (20%) cases (Figure 31).

Figures 32 and 33 show the amplification curves of both ***RUNX2*** and ***WWOX*** RNA using SYBR green and TaqMan qRT-PCR respectively.

There was a significant negative correlation between ***RUNX2*** and ***WWOX*** genes RNA expressions by qRT-PCR (p=0.032).

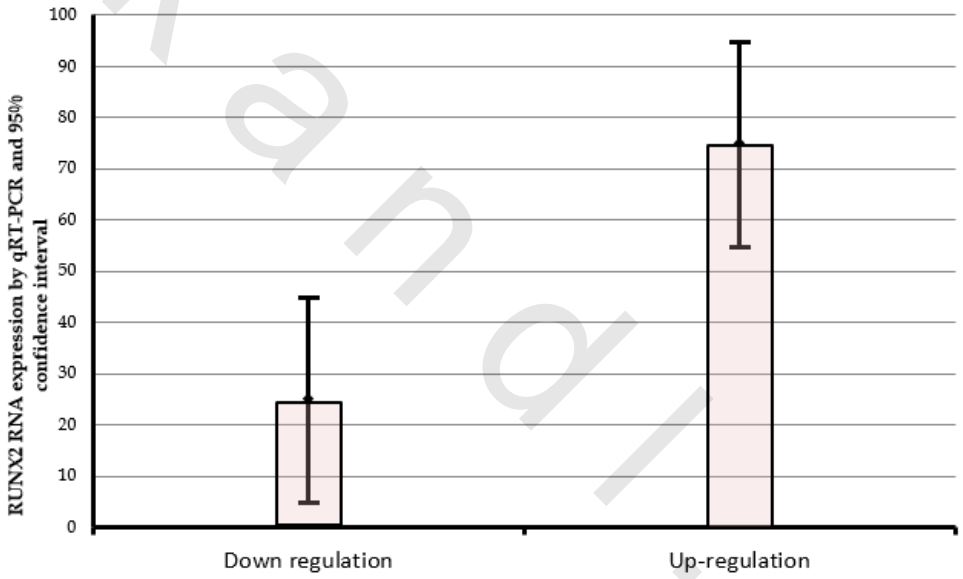


Figure 30: Bar chart showing *RUNX2* RNA expression in OS cases by qRT-PCR.

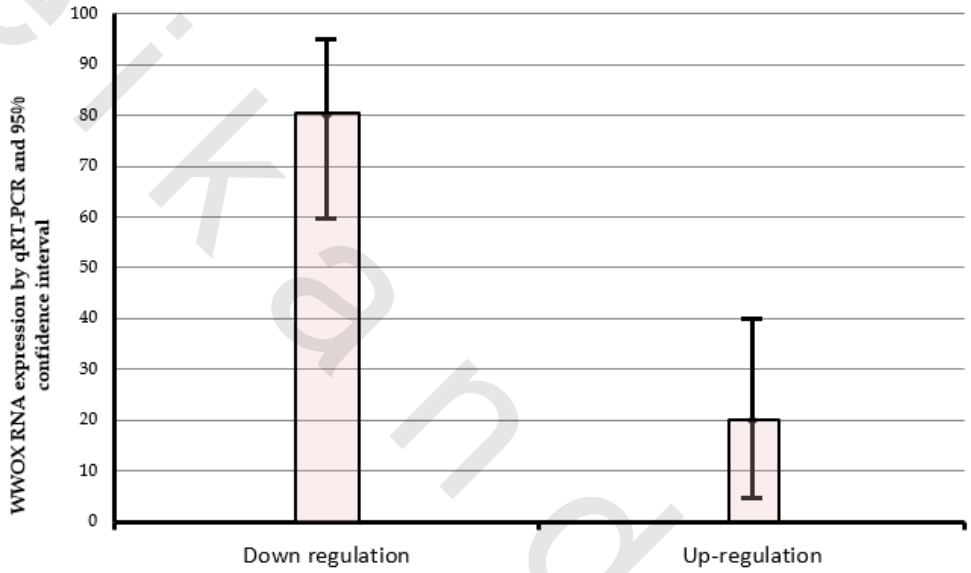


Figure 31: Bar chart showing *WWOX* RNA expression in OS cases by qRT-PCR.

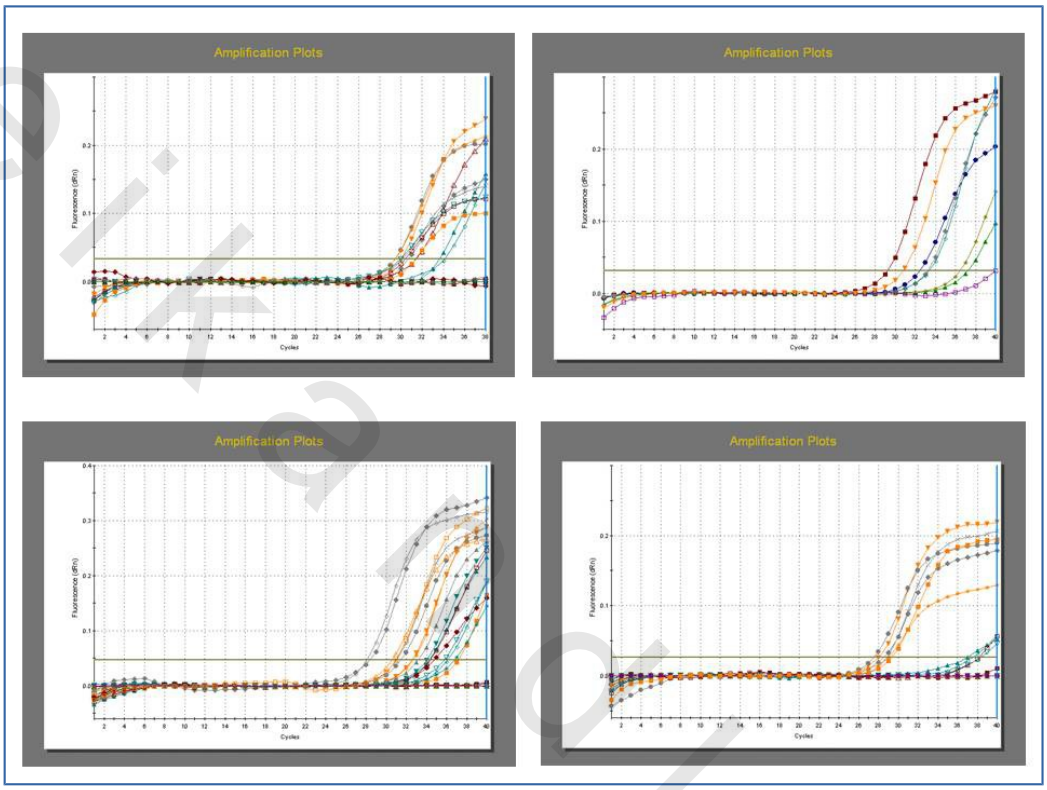
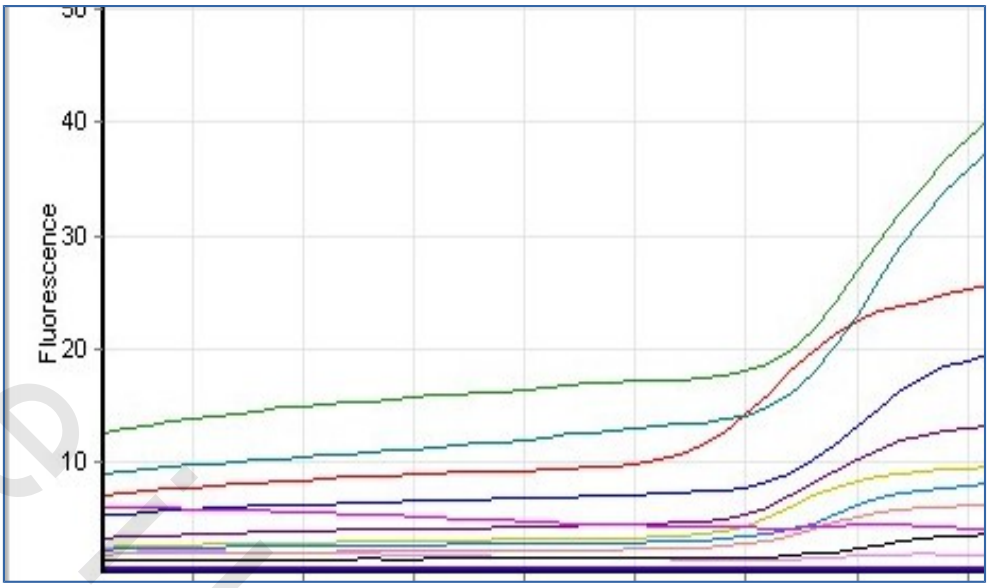


Figure 32: Amplification plots of *RUNX2*, SYBR green, MAX3000P (Applied Biosystems, Inc., Foster City, CA, USA).



Colour	Name
	Normal bone
	NTC
	Unknown
	Unknown
	Unknown
	Unknown
	Unknown

Figure 33: Amplification curves of *WWOX*, TaqMan, Rotor- gene Q (Qiagen, Hilden, Germany).

Association between RNA and protein expression of the studied genes/markers:

Statistical analysis of the 20 cases submitted to both IHC and qRT-PCR showed the following: Protein expressions of ***RUNX2*** and ***WWOX*** by IHC was significantly associated with their RNA expression by qRT-PCR (X^2_{trend} =19, p<.001 and X^2_{trend} =6.33; p=0.012 respectively) (Figures 34 and 35).

Increased ***RUNX2*** expression by qRT-PCR and positivity by IHC were present in 15/20 cases (75%) compared to 3/20 cases (15%) that were down-regulated by qRT-PCR and negative by IHC. On the other hand, 2/20 cases (10%) were down-regulated by qRT-PCR and positive by IHC.

WWOX up-regulation by qRT-PCR and positivity by IHC were shown in 4/20 cases (20%), compared to 16/20 cases (80%) that were down-regulated by qRT-PCR and negative by IHC.

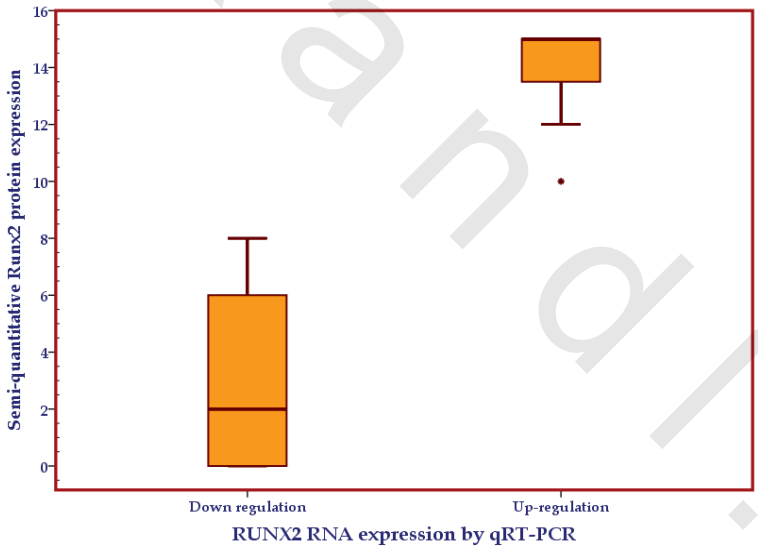


Figure 34: Box blot graph showing increased *RUNX2* gene and protein expressions in OS cases.

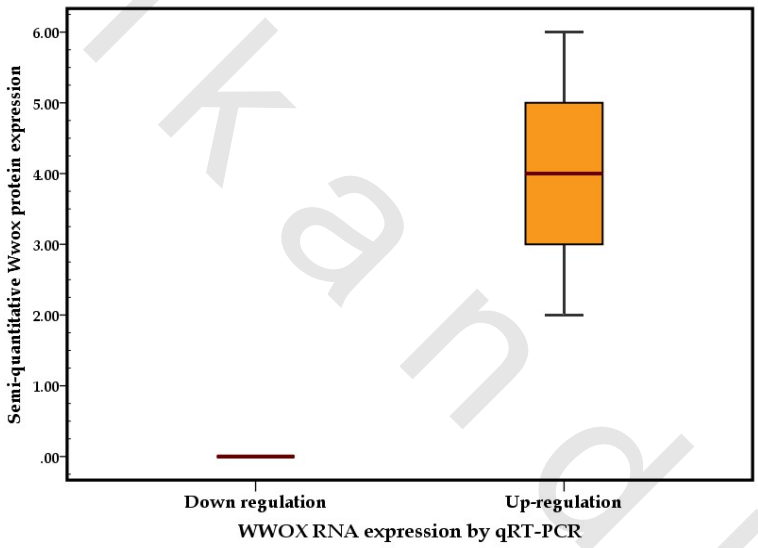


Figure 35: Box blot graph showing decreased *WWOX* gene and protein expressions in OS cases.

Correlation of the clinicopathological parameters of the studied cases with the genes'/markers' expressions:

Significant correlations were found between ***RUNX2*** RNA up-regulation and high tumor grade and TNM stage (p= 0.032 and p= 0.038 respectively) (Table 3) (Figures 36 and 37).

No statistically significant association was found between ***RUNX2*** RNA and any of the other clinicopathological features of the studied cases including age, sex, site, metastasis or survival (Table 3).

No statistically significant association was found between Runx2 protein and any of the clinicopathological data of the patients assessed (Tables 4 and 5).

No statistically significant association was found between ***WWOX*** RNA and protein expressions and any of the clinicopathological features of the patients assessed (Tables 6- 8).

Correlation between clinicopathological data and patients' outcome:

Statistically significant relation was found between tumor stage and occurrence of metastasis ($p<0.001$). A significant relation was also found between tumor stage, metastasis and patients'survival (p= 0.003 and <.001respectively). No other significant statistical relations were found between the other clinicopathological data and the patient's outcome.

Table 3: Correlations between *RUNX2* RNA expression by qRT-PCR and clinicopathological data of 20 human patients:

Clinical & pathological feature	*RUNX2* RNA expression		Test (p value)
	Downregulation n (%)	Up-regulation n (%)	
Gender			
Males	2 (15)	11 (85)	FET (.290)
females	3 (43)	4 (57)	
Age			
<20 years	2 (40)	8 (60)	FET=1
>20 years	3 (60)	7 (40)	
Site			
Femur	3(25)	9(75)	FET (.623)
Tibia	2(40)	3(60)	
Humerus	0(0)	3(100)	
Grade			
2	3 (75)	1 (25)	FET (.032)*
3	2 (12.5)	14 (87.5)	
Stage			
II	5 (50)	5 (50)	FET (.038)*
III	0 (0)	10 (100)	
Metastasis			
Present	0 (0)	5 (100)	FET (.266)
Absent	5 (33)	10 (67)	
Overall Survival			
Alive	5 (31)	11 (69)	FET (.530)
Dead	0 (0)	4 (100)	
Total		20 (100)	

**Statistically significant*

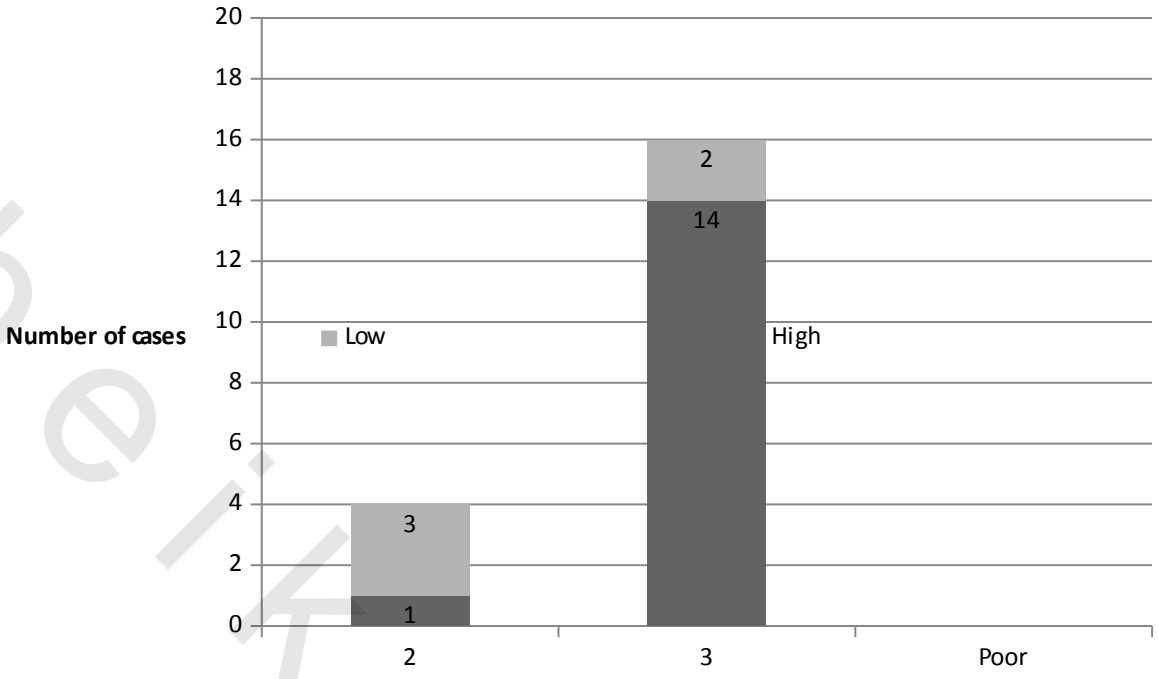


Figure 36: Stacked bar graph showing *RUNX2* RNA upregulation in high tumor grade.

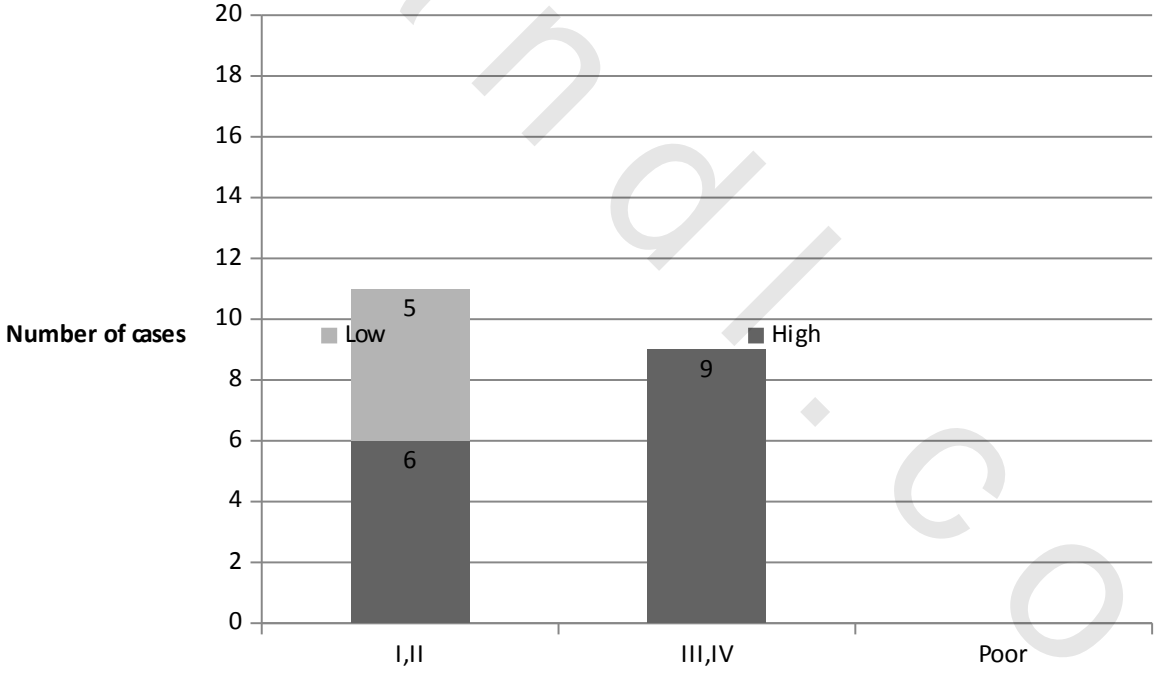


Figure 37: Stacked bar graph showing *RUNX2* RNA upregulation in high tumor stage.

- ***RT= qRT-PCR***

Table 4: Correlations between Runx2 protein expression by IHC (groups) and clinicopathological data of 30 human patients:

Clinical & pathological feature	Runx2 IHC group		Test (p value)
	Negative	Positive	
	n (%)	n (%)	
Gender			
Males	2 (9.5)	19 (90.5)	FET (1)
females	1 (11.1)	8 (88.9)	
Age			
<19 years	3(20)	12(80)	U=23.5
>19 years	8(53)	7(47)	P=(.654)
Site			
Femur	1 (8)	11 (92)	X^2_{MC}
Tibia	1 (20)	4 (80)	
Humerus	0 (0)	3 (100)	(1.000)
Grade			
2	2 (33.3)	4 (66.7)	
3	1 (4.2)	23 (95.8)	
Stage			
II	1 (10)	9 (90)	FET (.537)
III	2 (10)	18 (20)	
Recurrence			
Present	6 (20)	0	FET (1.5)
Absent	24(80)	0	
Metastasis			
Present	0 (0)	11(100)	FET (.126)
Absent	3(16)	16(84)	
Overall Survival			
Alive	3(14)	19(86)	FET (.282)
Dead	0 (0)	8(100)	
Total	30 (100)		

Table 5: Correlation between Runx2 protein expression (semiquantitative) by IHC and clinicopathological data of 30 human patients:

Clinical & pathological feature	Runx2 protein expression			Test (p value)
	Median	Minimum	Maximum	
Gender				
Males	12	0	15	U=20
females	12	0	15	(P=.470)
Age				
<19 years	12	0	15	U=20
>19 years	10	0	15	(P=.530)
Site				
Femur	10	0	15	U=12
Tibia	10	0	15	(P=.522)
Humerus	12	0	15	
Grade				
2	10	0	15	U=32
3	15	15	15	(P=.820)
Stage				
II	10	0	15	U=30
III	15	15	15	(P=.152)
Recurrence				
Present	12	0	15	U=20
Absent	15	15	15	(P=.822)
Metastasis				
Present	10	0	15	U=15
Absent	15	15	15	(P=.053)
Overall Survival				
Alive	12	0	15	U=14
Dead	15	15	15	(P=.099)
Total		30 (100)		

Table 6: Correlation between *WWOX* RNA expression by qRT-PCR and clinicopathological data of 20 human patients:

Clinical & pathological feature	*WWOX* RNA expression		Test (p value)
	Downregulation n (%)	Up-regulation n (%)	
Gender			
Males	12 (92)	1 (8)	FET (.101)
females	4 (57)	3 (43)	
Age			
<20 years	10 (66)	5 (34)	U=23.5
>20 years	10 (66)	5(34)	(P=.437)
Site			
Femur	10(83)	2(17)	FET(.571)
Tibia	3(60)	2(40)	
Humerus	3(100)	0(0)	
Grade			
2	2 (50)	2(50)	FET (.162)
3	14 (87.5)	2 (12.5)	
Stage			
II	8 (80)	2 (20)	FET (.591)
III	8 (80)	2 (20)	
Metastasis			
Present	5 (100)	0 (0)	FET (.530)
Absent	11 (73)	4 (27)	
Overall Survival			
Alive	12 (75)	4 (25)	FET (.538)
Dead	4 (100)	0 (0)	
Total	20 (100)		

Table 7: Correlation between Wwox protein expression by IHC (groups) and clinicopathological data of 30 human patients:

Clinical & pathological feature	Wwox IHC group		Test (p value)
	Negative n (%)	Positive n (%)	
Gender			
Males	18 (85%)	3 (15%)	FET (.332)
females	4 (45%)	5 (55%)	
Age			
<19 years	8 (40)	2 (10)	U=23.5
>19 years	8 (40)	2 (10)	(.437)
Site			
Femur	10 (8)	11 (92)	FET (3)
Tibia	2 (20)	4 (80)	
Humerus	0 (0)	3 (100)	
Grade			
2	3(50)	3(50)	
3	19(79)	5(21)	
Stage			
II	8 (80)	2(20)	FET (1)
III	15(75)	5(25)	
Recurrence			
Present	5(83)	1(17)	FET (.503)
Absent	17(71)	7(29)	
Metastasis			
Present	9 (82)	2 (18)	FET (.279)
Absent	13 (68)	6 (32)	
Overall Survival			
Alive	15(68)	7(32)	FET(.254)
Dead	7(87.5)	1(12.5)	
Total	30 (100)		

Table 8: Correlation between Wwox protein expression (semiquantitative) by IHC and clinicopathological data of 30 human patients:

Clinical & pathological feature	Wwox protein expression			Test (p value)
	Median	Minimum	Maximum	
Gender				
Males	0	0	2	U=41.5
females	0	0	6	(P=.552)
Age				
>19 years	0	0	4	U=48.5
<19 years	0	0	6	(.912)
Site				
Femur	0	0	1	U=48.5
Tibia	0	0	1	(P=.912)
Humerus	0	0	4	
Grade				
2	0	0	2	
3	0	0	6	
Stage				
II	0	0	2	U=40
III	0	0	6	(P=.503)
Recurrence				
Present	0	0	2	U=48
Absent	0	0	6	(P=.9)
Metastasis				
Present	0	0	2	U=27.5
Absent	0	0	6	(P=.395)
Overall Survival				
Alive	0	0	6	U=24
Dead	0	0	0	(P=.494)
Total		30 (100)		

DISCUSSION

The current study assessed aberrant ***RUNX2*** and ***WWOX*** genes' expression in a cohort of chemotherapeutic naïve Egyptian patients diagnosed as osteoblastic OS. Both genes contribute to bone metabolism and OS tumorigenesis.[54, 71] The study of the relation of both genes, their expressions, functions and interactions is mandatory for further understanding of bone biology. In addition, they could be considered as candidates for targeted therapy [19]. Few studies were conducted on human OS samples and to the best of our knowledge no previous research used IHC and qRT-PCR together to study both ***RUNX2*** and ***WWOX*** genes' expression on FFPE human OS samples.

In the current study, all the cases were primary OS without previous diseases. The age distribution of the patients was bimodal, ranging from 15 to 57 years old. Males were more affected than females. In the large study of Mirabello et al [1], they found that OS in the elderly was primary and suggested that misdiagnosis caused an overestimation of OS as a secondary lesion in this age group. They confirmed the bimodal incidence of OS with male predominance in elderly and that female predominance was mainly in the first peak which correlated with the adolescent growth spurt [1].

In the present study, 77% of the cases were high grade. This was in line with Thomas et al [72]and Hopyan et al [73]who demonstrated, in a large study, that 81% of OS were undifferentiated with undetectable osteocalcin, the marker of osteogenic differentiation, denoting an antagonistic role between oncogenesis and osteoblastic differentiation.

In the present study, a positive significant relation was found between high tumor stage and the poor outcome of the patient represented by the occurrence of metastasis and death. This was in agreement with the literature which considered high tumor stage as a poor prognostic factor in OS.[9, 13, 63]

In the current study, significant correlation was also found between tumor metastasis and poor patient survival. This was in agreement with many studies of OS which denoted that metastasis is an important indicator of low patient survival estimated to be 25%-30% in metastatic cases.[9, 74]

qRT-PCR and IHC were the techniques used in the present study. qRT- PCR is considered as a gold standard test for the detection of gene expression due to its high specificity and sensitivity, thus, it was chosen as a quantitative method to assess ***RUNX2*** and ***WWOX*** genes' expression from archived FFPE blocks. [75]

In the current study, the two most commonly used qRT-PCR methods SYBR green and TaqMan were utilized to detect ***RUNX2*** and ***WWOX*** RNA expressions respectively. In SYBR green method, PCR products were validated by dissociation curve immediately after PCR and hot start was used by a DNA polymerase which required heat activation to increase the specificity of the test. Arikawa et al [75] demonstrated in their study that SYBR green was a reliable qRT- PCR method with high inter-run and inter- laboratory reproducibility. They proved that the results of SYBR green were highly comparable to those of TaqMan for the same genes.[75] Tajadini et al [76] showed, by measuring same gene expressions profile, that the performance and quality of optimized SYBR green was comparable to that of TaqMan. Taken

together, optimization of SYBR green by the use of high performance protocol and materials make the results of this cost effective and easy method comparable to those of TaqMan.[75, 76]

IHC was used to detect the protein expression of both genes, as a reliable and applicable method in routine clinical practice.[62]IHC was used effectively on OS cell lines in the studies of Aqeilan et al[54] and Salah et al and on FFPE blocks of human OS and other malignancies as in the study of Yang et al.[5]

In the current study, significant Runx2 positive protein expression was detected by IHC and significant upregulation of ***RUNX2*** RNA was detected by qRT-PCR, compared to their expressions in normal bone. This was in agreement with the study of Yang et al study [65] which identified an increase in ***RUNX2*** gene and protein expressions in 55% and 48.1% of human OS cases using molecular and IHC techniques on paraffin blocks respectively. Sadikovic et al [11] reported a significant upregulation of ***RUNX2*** by qRT- PCR. Lu, et al [77] also demonstrated ***RUNX2*** RNA upregulation in all OS specimens derived from human tissue samples and cell lines. Van Der Deen et al [78] showed the lower expression of ***RUNX2*** in normal non-tumorigenic proliferating osteoblasts compared to OS cells. Andela et al[79] were the first to study Runx2 protein expression on 11 human OS samples, with different subtypes, by IHC and found significant positivity in all of them, the highest expression was in the chondroblastic variant of conventional OS.[79]

In the present study, a significant negative Wwox protein expression was detected by IHC. Significant downregulation of ***WWOX*** RNA was detected by qRT-PCR, compared to normal bone. This was in general agreement with many studies as that of Kurek et al [24] which found negative Wwox protein expression in 58% of human OS samples by IHC. Diniz et al [80] demonstrated downregulation of ***WWOX*** RNA in human OS using qRT-PCR. Yang et al [65] identified loss of ***WWOX*** gene and protein expressions in 30% and 61.1% of OS cases by molecular and IHC techniques on paraffin blocks respectively.

In the current study, ***WWOX*** RNA/ protein expressions were found in 4 cases of OS. The presence of ***WWOX*** in OS although being a tumor suppressor gene was attributed by Aqeilan et al [54] to the presence of other mechanisms of loss of function of ***WWOX*** gene as promoter hypermethylation or other genetic and epigenetic anomalies.

Our results suggest that ***WWOX*** and ***RUNX2*** are implicated in OS oncogenesis. In addition, a statistically significant inverse correlation was detected between ***WWOX*** and ***RUNX2*** RNA and protein expressions at the level of qRT-PCR and IHC, suggesting an inverse role of both genes in OS development. This was in agreement with many studies which discussed the role of both genes in bone development, differentiation as well as their role in the development of many cancers notably OS.[17, 55, 81] One of these studies was that of Aqeilan et al [55] which discussed the role of ***RUNX2*** in the development of OS in ***WWOX*** deficient mice. ***RUNX2*** protein and RNA were found to be elevated in the femurs of the ***WWOX*** deficient mice and in some OS cell lines. They attributed that finding to the physical association of ***WWOX*** to ***RUNX2***, via its first WW domain, causing functional suppression of the latter by inhibiting its transactivation in both osteoblastic and neoplastic cells and concluded that ***WWOX*** deficiency causes the up-regulation of ***RUNX2*** [55].

In the study of Kurek et al [24], an inverse correlation between ***RUNX2*** and ***WWOX*** in vitro, cell lines was found. On the other hand, this correlation was not detected in vivo by

Kurek et al as well as by Yang et al [24, 65] in their studies on human OS on the human tissue samples. This was referred, by Kurek et al [24], to the complicated pathways in vivo compared to the cell lines.

Taken together, our results together with those of previous studies further emphasized the reversed role of both ***RUNX2***, as an oncogene, and ***WWOX***, as a tumor suppressor gene, in OS development. Sadikovic et al [11] demonstrated that ***RUNX2*** was the only gene of 16 genes included in their study which had a significant overexpression in OS. Van Der Deen et al[38] suggested that the high expression of ***RUNX2*** in OS denoted its proliferative role.

Tandon et al[82] study stated that ***RUNX2*** played an important role in the activation of cancer related genes, the cooperation with oncogenes and the suppression of apoptosis. Thomas et al [72] assumed that ***RUNX2*** overexpression was related to loss of pRb in OS to which ***RUNX2*** binds with subsequent loss of pRb dependent growth arrest. Lengner et al [83] attributed the increase of ***RUNX2*** in human OS to the loss of p53. Del Mare et al [17] mentioned that the most important regulator of ***RUNX2*** was ***WWOX*** through both physical and functional association and that ***RUNX2-WWOX*** relation was necessary for the development of OS.

Aqeilan et al [81] showed that ***WWOX*** suppression of ***RUNX2*** activation was contributed to the tumor suppressor role of ***WWOX***. They also showed that ectopic expression of ***WWOX*** reduced the tumorigenicity of OS in ***WWOX*** knockout mice.[81] The same was proved by Kurek et al [24] in both WWOX-/- as well as OS metastatic cell lines, providing that ***WWOX*** expression is clinically and therapeutically significant in OS.

Diniz et al [80]demonstrated that the absence and low expression of ***WWOX*** gene in OS indicated its role as tumor suppressor gene. Salah et al [42] found that ***WWOX*** tumor suppressor is commonly deleted or altered in most human cancers. ***WWOX***, tumor suppressor gene, ablation in mice, in the study of Abdeen et al [45]ended by the deregulation of osteoprogenitor cells and the development of OS. Bednarek et al [84]showed that ectopic expression of ***WWOX*** gene decreased the tumorigenicity in breast cell lines, which developed the possibility of the use of ***WWOX*** tumor suppressor gene as targeted therapy.

In the present study, a significant relation was found between RNA and protein expressions of the studied markers. This result highlighted the role of IHC as a screening tool for the study of ***RUNX2*** and ***WWOX*** preceding the costly and sophisticated molecular studies. This result was in consistence with Aqeilan et al[55] and Salah et al[42] who found that OS samples with gene deletion lost their protein expression, suggesting that gene copy number alteration was an important mechanism in the aberration of protein expression.

Conversely, Kurek et al [24] and Yang et al [65] did not observe this correlation, regarding ***WWOX*** gene, in their studies. The absence of this positive association was attributed by the latter to two reasons: the presence of other factors affecting Wwox protein expression as missing exons, LOH and hypermethylation of the ***WWOX*** gene and the lack of sensitivity of IHC on paraffin blocks compared to qRT-PCR on cell lines [5].

In the current study, Wwox protein expression by IHC was dominantly cytoplasmic, with occasional nuclear staining. This was in agreement with the study of Yang et al [5] . Chang et al [43] traced, using light and electron microscopies, the subcellular localization of Wwox

protein. They found it present predominantly in the cytoplasm in the mitochondria and other organelles as Golgi apparatus, rough endoplasmic reticulum as well as in the nuclei [43]. Abu Odeh et al [44]assumed that ***WWOX*** could be present in the cytoplasm and nucleus and that the latter is related to its transcriptional activity.

In this study, a significant relation was found between ***RUNX2*** RNA expression and high tumor grade as well as high stage of OS, suggesting an important role of ***RUNX2*** in OS progression and its potentially poor prognostic impact in OS patients. This result was supported by many studies which demonstrated the role of ***RUNX2*** in the progression, metastasis and poor prognosis of other non-osseous tumor types as ovary, prostate, breast, lung and pituitary. Browne et al [85]demonstrated that ***RUNX2*** overexpression in prostatic carcinoma was correlated with high Gleason's score, rapid invasion and metastasis. Pratap et al[32] showed that ectopic expression of ***RUNX2*** in breast epithelial cells played a role in their malignant transformation and progression by its activation to anti-apoptotic factor Bcl2 [28, 82, 86, 87].

In the current research, ***RUNX2*** RNA/ protein expressions showed no significant association with metastasis or survival. This was in consistence with Yang et al [65]who did not find similar relations in his study on human OS. On the contrary, Won et al [74] showed that Runx2 protein expression was significantly related to metastasis and poor survival. Lee et al [88] demonstrated that ***RUNX2*** stimulated the promoter and protein expression of vascular endothelial growth factor (***VEGF***) which has an important role in tumor angiogenesis, migration and metastasis of neoplastic cells. Van der Deen et al [78] showed that ***RUNX2*** facilitated the pathways responsible of cell adhesions and motility of mobile OS cells.

Similarly, in other non-osseous tumors, Tandon et al [82] concluded that ***RUNX2*** increases the migration potential in lung cancer by its activation of transforming growth factor –β (TGF—β). Lamour et al[39] found that ***RUNX2*** activation of high bone sialoprotein (HBSP), a bone matrix protein, is responsible of bone metastasis in osteotropic malignancies. Neovascularization, production of proangiogenic factors and the secretion of metalloproteinase enzymes are necessary for malignant cells invasion and metastasis.[89] Pratap et al [32] found that ***RUNX2*** mediated angiogenesis by the activation of genes necessary for metastasis and invasion (***MMP2***, ***MMP9***, ***MMP13***), angiogenesis (***VEGF***, ***osteopontin***), and survival (***survivin***) in breast cancer cells.

On the contrary, Galindo et al [34] showed growth inhibitory properties of ***RUNX2*** in OS cell lines. ***RUNX2***-deficient cells exhibited accelerated proliferative potential which was inhibited after ***RUNX2*** restoration. They also mentioned that cell cycle regulation of ***RUNX2*** expression is disrupted in OS cells exhibiting loss of its cell growth control. They referred this to the regulatory role of ***RUNX2*** on the cell cycle to control cell proliferation. Eliseev et al [35]stated that ***RUNX2*** functions as growth suppressor and apoptosis inducer in OS.

In the current study, no significant correlation was found between ***WWOX*** protein/ gene expressions and any of the clinicopathological data including the age, sex, site, grade, TNM stage, metastasis and survival. This was in concordance with the results of Yang et al[65] who studied ***WWOX*** expression in human OS samples using both IHC and molecular techniques. Similarly, Bloomston et al [90]did not find any significant association between

WWOX and clinicopathological parameters of the patients with pancreaticobiliary neoplasms by IHC.

Conversely, Abdeen et al [45] mentioned that ***WWOX*** genetic and epigenetic alteration is associated with the aggressiveness of OS and that patient survival and ***WWOX*** loss denoted poor prognosis. Donati et al [91]demonstrated, by IHC study, that loss of ***WWOX*** expression was related to high tumor aggressiveness in non small cell lung cancer (NSCLC). Ramos et al [92]showed a significant correlation between loss of ***WWOX*** and clinicopathological parameters of the patients with urinary bladder malignancies considering ***WWOX*** as a potential factor of progressive disease. Yang et al [65]and Bloomston et al [90]attributed the lack of significant correlations between ***WWOX*** and the clinicopathological data to the involvement of ***WWOX*** by deletion in the early pathogenesis of cancer development. Bednark et al [84] also considered that the presence of ***WWOX*** in fragile site characterized by allelic loss and chromosomal anomalies clarified its role in the initiation of oncogenesis rather than tumor progression.

In the current study, the lack of statistical relation between ***WWOX*** RNA and protein expressions with the clinicopathological data of the patients could be attributed to the small sample size included in this study which warrants validation of the present results on a larger cohort of patients.

SUMMARY

Osteosarcoma (OS) is the most common primary malignant, non-hematopoietic, bone tumor worldwide with poor survival. OS shows a bimodal age distribution worldwide, it is rarely diagnosed before the age of five, but the incidence increases with age until around puberty during the period of active bone growth. This primary peak is followed by a decrease and plateau in incidence in individuals between 25 and 60 years of age. A second peak is observed during the seventh and eighth decades of life. Males' incidence is higher than females.

Personalized medical care and the development of biologically tailored therapy based on molecular diagnosis, within a multidisciplinary approach, remains the hope for further advancement in the treatment of OS with better survival. Complex genomic profile of OS makes it a candidate for study of new genes: runt-related transcription factor 2 (***RUNX2***) and WW domain containing oxidoreductase (***WWOX***) are two important genes recently implicated in normal osteogenesis, OS development and progression.

Molecular studies of OS are important for tissue engineering and gene therapy. Molecular techniques are important to investigate the role of different genes in tumorigenesis, tumor growth and metastasis. They are also important in the measurement of the prognostic and predictive values of each gene. One of these molecular techniques is the quantitative real time polymerase chain reaction (q RT-PCR). qRT-PCR is a quick, quantitative, and sensitive method to measure a target nucleic acid. It proved to be a biological tool which can be used as a routine instrument to gain knowledge about the biology and behavior of the tumors.

Immunohistochemistry (IHC) is a complementary diagnostic method with variable useful applications including the diagnosis of undifferentiated malignancies, the subtyping of tumors as in lymphomas, identification of the primary tumor in metastatic cases, and research of predictive factors and prognostic markers. IHC is a simple, relatively low cost and highly effective tool. The drawbacks of IHC are mainly the technical limitations and interpretative bias.

The current study included 30 cases of primary osteoblastic OS (q RT-PCR was performed on only 20 cases) obtained from chemotherapeutic-naïve Egyptian patients (range 15-57 years, median age=19 years, M: F ratio = 2:1). 20 tumors (67%) were located in the femur, 6 cases (20%) in the tibia and 4 cases (13%) in the humerus. In addition, 10 normal bone biopsies (range 15-50 years, median age= 20, M: F ratio = 2:1), obtained from non-pathologically fractured bones served as a control group.

Disease progression of the patients was followed regularly every 6 months to 1 year. During the follow up period (range=18-72 months, *M*= 45 months, *SD*= 38.18), 6 (20%) patients had local recurrence, 11 (37%) patients had distant metastasis, and 8 (27%) patients succumbed to the disease. The disease free survival (DFS) time ranged from 6 to 72 months with an estimated mean survival time of 56 months (95%CI=46.4, 65.4).

Paraffin blocks were used for IHC and quantitative real time PCR (qRT- PCR): SYBR green for ***RUNX2*** and TaqMan for ***WWOX*** RNA expressions' detection respectively.

In OS cases, ***RUNX2*** protein expression was detected in 27/30 (90%) and was negative in 3/30 (10%) cases. In normal bone, Runx2 protein expression was negative in 8/10 control cases (80%).

Median expression of Runx2 in OS was 13 (*Min.-Max.* = 0-15) which was significantly higher than normal bone 5 (*Min.-Max.* = 0-8) *(U*=3.5, p <.001)

In OS cases, Wwox protein expression was lost in 22 cases (73%) and was detected in the remaining 8 cases (27%). In normal bone, Wwox protein expression was detected in 10/10 control cases (100%) with strong cytoplasmic staining intensity in all cases (100%).

Median expression of Wwox in OS was 0 (*Min.-Max.* = 0- 6) which was significantly lower than normal bone 7 (*Min.-Max.* = 4-10) *(U*=3.5, p <.001)

There was a significant negative correlation between Runx2 and Wwox protein expressions by IHC (ρ=-.578, p=0.008; and ρ=-.391, p=0.033 for the 20 and 30 cases respectively).

In comparison to normal bone, ***RUNX2*** gene RNA expression was up-regulated in 15/20 (75%) cases and down-regulated in 5/20 (25%) cases, ***WWOX*** gene RNA expression was down-regulated in 16/20 (80%) and up-regulated in 4/20 (20%) cases.

There was a significant negative correlation between ***RUNX2*** and ***WWOX*** genes RNA expressions by qRT-PCR (p=0.032)

The current study of ***WWOX*** and ***RUNX2*** genes RNA and protein expressions on a cohort of human OS Egyptian patients suggests the important reverse role of both genes in OS oncogenesis.

RUNX2 gene might be implicated in OS progression and poor prognosis due to its significant association with high tumor grade and stage. Regarding the other clinicopathological data of the patients, no significant association was found between them with ***RUNX2*** and ***WWOX***.

The significant positive association between the results of IHC and qRT-PCR in each gene in the current study implies that IHC can be useful as a screening test for the assessment of ***WWOX*** and ***RUNX2*** before proceeding to further molecular diagnostic techniques.

We conclude that our study provides preliminary promising results that need to be validated on a larger cohort of patients

CONCLUSIONS

From the current study, the following were concluded:

1- ***RUNX2*** and ***WWOX*** play an important reverse role in OS oncogenesis.
2- In contrast to ***WWOX***, ***RUNX2*** might be implicated in OS progression and could contribute to the poor prognosis of OS patients.
3- The significant positive association between the results of IHC and qRT-PCR for each gene in the current study implies that IHC can be useful as a screening test for the assessment of ***WWOX*** and ***RUNX2*** abnormalities before proceeding to further molecular diagnostic techniques.
4- RNA extracted from FFPE blocks has adequate purity and concentration which highlights its possible use in molecular studies.

RECOMMENDATIONS

From the present study, the following are recommended:

1- Upregulation of ***RUNX2*** and downregulation of ***WWOX*** in OS could make them candidate genes for targeted therapy, however, further studies are recommended to detect other genetic anomalies of both genes.
2- Further studies are required to verify the relation of both genes with other genes implicated in OS pathogenesis.
3- A larger study including other cases of conventional and non-conventional OS is required to assess the expression of both genes in all types of OS with different prognosis and their relations with the clinicopathological data.

REFERENCES

1. Mirabello L, Troisi RJ, Savage SA. International osteosarcoma incidence patterns in children and adolescents, middle ages and elderly persons. International journal of cancer Journal international du cancer. 2009;125(1):229-34.
2. Lian JB, Javed A, Zaidi SK, Lengner C, Montecino M, van Wijnen AJ, et al. Regulatory controls for osteoblast growth and differentiation: role of Runx/Cbfa/AML factors. Critical reviews in eukaryotic gene expression. 2004;14(1-2):1-41.
3. Ando K, Heymann MF, Stresing V, Mori K, Redini F, Heymann D. Current therapeutic strategies and novel approaches in osteosarcoma. Cancers. 2013;5(2):591-616.
4. Kager L, Zoubek A, Potschger U, Kastner U, Flege S, Kempf-Bielack B, et al. Primary metastatic osteosarcoma: presentation and outcome of patients treated on neoadjuvant Cooperative Osteosarcoma Study Group protocols. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2003;21(10):2011-8.
5. Yang J, Cogdell D, Yang D, Hu L, Li H, Zheng H, et al. Deletion of the WWOX gene and frequent loss of its protein expression in human osteosarcoma. Cancer letters. 2010;291(1):31-8.
6. Martin JW, Zielenska M, Stein GS, van Wijnen AJ, Squire JA. The Role of RUNX2 in Osteosarcoma Oncogenesis. Sarcoma. 2011;2011:282745.
7. Bednarek AK, Laflin KJ, Daniel RL, Liao Q, Hawkins KA, Aldaz CM. WWOX, a novel WW domain-containing protein mapping to human chromosome 16q23.3-24.1, a region frequently affected in breast cancer. Cancer research. 2000;60(8):2140-5.
8. Del Mare S, Salah Z, Aqeilan RI. WWOX: its genomics, partners, and functions. Journal of cellular biochemistry. 2009;108(4):737-45.
9. Mirabello L, Troisi RJ, Savage SA. Osteosarcoma incidence and survival rates from 1973 to 2004: data from the Surveillance, Epidemiology, and End Results Program. Cancer. 2009;115(7):1531-43.
10. Mokhtar N GI, Adel I. . . Malignant bone tumors. editors: Cancer pathology registry 2003-2004 and timed trend analysis. 2007;1st edition. Cairo: NCI, Elsheraa Press (11):88-9.
11. Sadikovic B, Thorner P, Chilton-Macneill S, Martin JW, Cervigne NK, Squire J, et al. Expression analysis of genes associated with human osteosarcoma tumors shows correlation of RUNX2 overexpression with poor response to chemotherapy. BMC cancer. 2010;10:202.
12. Cripe TP. Pediatric Osteosarcoma Medscape.
13. Inwards. ALFaCY. Foundations in diagnostic pathology: Bone and soft tisue pathology. Saunders 2010.
14. Fletcher CDM BJ, Hogendoorn PCW, Mertens F, . . World Health Organization, classification of tumours: Pathology and genetics of tumors of soft tissue and bone. editors Lyon: IARC Press. 2013.
15. Edge SB BD, Compton CC, et al. . AJCC Cancer Staging Manual. Bone, New York, NY: Springer. 2010;7th ed:pp 281-90.
16. Yang J, Zhang W. New molecular insights into osteosarcoma targeted therapy. Current opinion in oncology. 2013;25(4):398-406.
17. Del Mare S, Kurek KC, Stein GS, Lian JB, Aqeilan RI. Role of the WWOX tumor suppressor gene in bone homeostasis and the pathogenesis of osteosarcoma. American journal of cancer research. 2011;1(5):585-94.

18. Cade S. Osteogenic sarcoma; a study based on 133 patients. Journal of the Royal College of Surgeons of Edinburgh. 1955;1(2):79-111.
19. Jaffe N, Puri A, Gelderblom H. Osteosarcoma: evolution of treatment paradigms. Sarcoma. 2013;2013:203531.
20. Datta HK, Ng WF, Walker JA, Tuck SP, Varanasi SS. The cell biology of bone metabolism. Journal of clinical pathology. 2008;61(5):577-87.
21. Komori T. Regulation of bone development and extracellular matrix protein genes by RUNX2. Cell and tissue research. 2010;339(1):189-95.
22. van Wijnen AJ, Stein GS, Gergen JP, Groner Y, Hiebert SW, Ito Y, et al. Nomenclature for Runt-related (RUNX) proteins. Oncogene. 2004;23(24):4209-10.
23. Miller J, Horner A, Stacy T, Lowrey C, Lian JB, Stein G, et al. The core-binding factor beta subunit is required for bone formation and hematopoietic maturation. Nature genetics. 2002;32(4):645-9.
24. Kurek KC, Del Mare S, Salah Z, Abdeen S, Sadiq H, Lee SH, et al. Frequent attenuation of the WWOX tumor suppressor in osteosarcoma is associated with increased tumorigenicity and aberrant RUNX2 expression. Cancer research. 2010;70(13):5577-86.
25. Lengner CJ, Hassan MQ, Serra RW, Lepper C, van Wijnen AJ, Stein JL, et al. Nkx3.2-mediated repression of Runx2 promotes chondrogenic differentiation. The Journal of biological chemistry. 2005;280(16):15872-9.
26. Javed A, Gutierrez S, Montecino M, van Wijnen AJ, Stein JL, Stein GS, et al. Multiple Cbfa/AML sites in the rat osteocalcin promoter are required for basal and vitamin D-responsive transcription and contribute to chromatin organization. Molecular and cellular biology. 1999;19(11):7491-500.
27. Jemtland R, Divieti P, Lee K, Segre GV. Hedgehog promotes primary osteoblast differentiation and increases PTHrP mRNA expression and iPTHrP secretion. Bone. 2003;32(6):611-20.
28. Li W, Xu S, Lin S, Zhao W. Overexpression of runt-related transcription factor-2 is associated with advanced tumor progression and poor prognosis in epithelial ovarian cancer. Journal of biomedicine & biotechnology. 2012;2012:456534.
29. Liu JC, Lengner CJ, Gaur T, Lou Y, Hussain S, Jones MD, et al. Runx2 protein expression utilizes the Runx2 P1 promoter to establish osteoprogenitor cell number for normal bone formation. The Journal of biological chemistry. 2011;286(34):30057-70.
30. Reinhold MI, Naski MC. Direct interactions of Runx2 and canonical Wnt signaling induce FGF18. The Journal of biological chemistry. 2007;282(6):3653-63.
31. Bais MV, Wigner N, Young M, Toholka R, Graves DT, Morgan EF, et al. BMP2 is essential for post natal osteogenesis but not for recruitment of osteogenic stem cells. Bone. 2009;45(2):254-66.
32. Pratap J, Wixted JJ, Gaur T, Zaidi SK, Dobson J, Gokul KD, et al. Runx2 transcriptional activation of Indian Hedgehog and a downstream bone metastatic pathway in breast cancer cells. Cancer research. 2008;68(19):7795-802.
33. Javed A, Bae JS, Afzal F, Gutierrez S, Pratap J, Zaidi SK, et al. Structural coupling of Smad and Runx2 for execution of the BMP2 osteogenic signal. The Journal of biological chemistry. 2008;283(13):8412-22.
34. Galindo M, Pratap J, Young DW, Hovhannisyan H, Im HJ, Choi JY, et al. The bone-specific expression of Runx2 oscillates during the cell cycle to support a G1-related antiproliferative function in osteoblasts. The Journal of biological chemistry. 2005;280(21):20274-85.

35. Eliseev RA, Dong YF, Sampson E, Zuscik MJ, Schwarz EM, O'Keefe RJ, et al. Runx2-mediated activation of the Bax gene increases osteosarcoma cell sensitivity to apoptosis. Oncogene. 2008;27(25):3605-14.
36. Leong DT, Lim J, Goh X, Pratap J, Pereira BP, Kwok HS, et al. Cancer-related ectopic expression of the bone-related transcription factor RUNX2 in non-osseous metastatic tumor cells is linked to cell proliferation and motility. Breast cancer research : BCR. 2010;12(5):R89.
37. Mak KK, Bi Y, Wan C, Chuang PT, Clemens T, Young M, et al. Hedgehog signaling in mature osteoblasts regulates bone formation and resorption by controlling PTHrP and RANKL expression. Developmental cell. 2008;14(5):674-88.
38. van der Deen M, Taipaleenmaki H, Zhang Y, Teplyuk NM, Gupta A, Cinghu S, et al. MicroRNA-34c inversely couples the biological functions of the runt-related transcription factor RUNX2 and the tumor suppressor p53 in osteosarcoma. The Journal of biological chemistry. 2013;288(29):21307-19.
39. Lamour V, Detry C, Sanchez C, Henrotin Y, Castronovo V, Bellahcene A. Runx2- and histone deacetylase 3-mediated repression is relieved in differentiating human osteoblast cells to allow high bone sialoprotein expression. The Journal of biological chemistry. 2007;282(50):36240-9.
40. Lucero CM, Vega OA, Osorio MM, Tapia JC, Antonelli M, Stein GS, et al. The cancer-related transcription factor Runx2 modulates cell proliferation in human osteosarcoma cell lines. Journal of cellular physiology. 2013;228(4):714-23.
41. Sutherland GR, Baker E, Richards RI. Fragile sites still breaking. Trends in genetics : TIG. 1998;14(12):501-6.
42. Salah Z, Aqeilan R, Huebner K. WWOX gene and gene product: tumor suppression through specific protein interactions. Future oncology (London, England). 2010;6(2):249-59.
43. Chang NS, Hsu LJ, Lin YS, Lai FJ, Sheu HM. WW domain-containing oxidoreductase: a candidate tumor suppressor. Trends in molecular medicine. 2007;13(1):12-22.
44. Abu-Odeh M, Bar-Mag T, Huang H, Kim T, Salah Z, Abdeen SK, et al. Characterizing WW Domain Interactions of Tumor Suppressor WWOX Reveals its Association with Multiprotein Networks. The Journal of biological chemistry. 2014.
45. Abdeen SK, Del Mare S, Hussain S, Abu-Remaileh M, Salah Z, Hagan J, et al. Conditional inactivation of the mouse Wwox tumor suppressor gene recapitulates the null phenotype. Journal of cellular physiology. 2013;228(7):1377-82.
46. Bouteille N, Driouch K, Hage PE, Sin S, Formstecher E, Camonis J, et al. Inhibition of the Wnt/beta-catenin pathway by the WWOX tumor suppressor protein. Oncogene. 2009;28(28):2569-80.
47. Aqeilan RI, Donati V, Gaudio E, Nicoloso MS, Sundvall M, Korhonen A, et al. Association of Wwox with ErbB4 in breast cancer. Cancer research. 2007;67(19):9330-6.
48. Ishii H, Mimori K, Inageta T, Murakumo Y, Vecchione A, Mori M, et al. Components of DNA damage checkpoint pathway regulate UV exposure-dependent alterations of gene expression of FHIT and WWOX at chromosome fragile sites. Molecular cancer research : MCR. 2005;3(3):130-8.
49. Gourley C, Paige AJ, Taylor KJ, Ward C, Kuske B, Zhang J, et al. WWOX gene expression abolishes ovarian cancer tumorigenicity in vivo and decreases attachment to fibronectin via integrin alpha3. Cancer research. 2009;69(11):4835-42.

50. Fais S. A role for ezrin in a neglected metastatic tumor function. Trends in molecular medicine. 2004;10(6):249-50.
51. Conforti F, Sayan AE, Sreekumar R, Sayan BS. Regulation of p73 activity by post-translational modifications. Cell death & disease. 2012;3:e285.
52. Sayan AE, Sayan BS, Gogvadze V, Dinsdale D, Nyman U, Hansen TM, et al. P73 and caspase-cleaved p73 fragments localize to mitochondria and augment TRAIL-induced apoptosis. Oncogene. 2008;27(31):4363-72.
53. Chang NS, Doherty J, Ensign A, Schultz L, Hsu LJ, Hong Q. WOX1 is essential for tumor necrosis factor-, UV light-, staurosporine-, and p53-mediated cell death, and its tyrosine 33-phosphorylated form binds and stabilizes serine 46-phosphorylated p53. The Journal of biological chemistry. 2005;280(52):43100-8.
54. Aqeilan RI, Croce CM. WWOX in biological control and tumorigenesis. Journal of cellular physiology. 2007;212(2):307-10.
55. Aqeilan RI, Hassan MQ, de Bruin A, Hagan JP, Volinia S, Palumbo T, et al. The WWOX tumor suppressor is essential for postnatal survival and normal bone metabolism. The Journal of biological chemistry. 2008;283(31):21629-39.
56. Chua CW, Chiu YT, Yuen HF, Chan KW, Man K, Wang X, et al. Suppression of androgen-independent prostate cancer cell aggressiveness by FTY720: validating Runx2 as a potential antimetastatic drug screening platform. Clinical cancer research : an official journal of the American Association for Cancer Research. 2009;15(13):4322-35.
57. Raymonds R Tubbs MHS. Cell and tissue based molecular pathology.Foundations in diagnostic pathology.
58. Morteza Seifi AG, Siamak Heidarzadeh, Mahmood Khosravi, Atefeh Namipashaki, Vahid Mehri Soofiany, Ali Alizadeh Khosroshahi and Nasim Danaei. Overview of Real-Time PCR Principles, Polymerase Chain Reaction, Dr Patricia Hernandez-Rodriguez (Ed.). InTech,

Available from: http://wwwintechopencom/books/polymerase-chain-reaction/overview-of-real-time-pcrprinciples. 2012;ISBN: 978-953-51-0612-8.
59. Andreasson H, Gyllensten U, Allen M. Real-time DNA quantification of nuclear and mitochondrial DNA in forensic analysis. BioTechniques. 2002;33(2):402-4, 7-11.
60. Elmaagacli AH. Real-time PCR for monitoring minimal residual disease and chimerism in patients after allogeneic transplantation. International journal of hematology. 2002;76 Suppl 2:204-5.
61. Philip S. Bernarda CTW. Real-Time PCR Technology for Cancer Diagnostics

Clinical Chemistry. 2002;48(8):1178 –85.
62. Leandro Luongo de Matos DCT, Maria Graciela Luongo de Matos, Maria Aparecida da Silva Pinhal. Immunohistochemistry as an Important Tool in Biomarkers Detection and Clinical Practice

Biomark Insights. 2010; 5:9-20.
63. www.nccn.org/professionals/physician_gls/PDF/bone.pdf.
64. Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society. 1981;29(4):577-80.

65. Yang J, Zhao L, Tian W, Liao Z, Zheng H, Wang G, et al. Correlation of WWOX, RUNX2 and VEGFA protein expression in human osteosarcoma. BMC medical genomics. 2013;6:56.
66. www.stratec.com/share/.../RNA/InviTrapSpinTissueRNAMiniKit.pdf.
67. www.lifetechnologies.com/.../superscript-iii-reverse-transcriptase.html.
68. www.qiagen.com/products/catalog/assay.../quantitect-primer-assays.
69. QuantiFast® Probe Assay Handbook. Qiagen, Sample & Assay Technologies. 05/2011 Sample & Assay Technologies.
70. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nature protocols. 2008;3(6):1101-8.
71. Nathan SS, Pereira BP, Zhou YF, Gupta A, Dombrowski C, Soong R, et al. Elevated expression of Runx2 as a key parameter in the etiology of osteosarcoma. Molecular biology reports. 2009;36(1):153-8.
72. Thomas DM, Johnson SA, Sims NA, Trivett MK, Slavin JL, Rubin BP, et al. Terminal osteoblast differentiation, mediated by runx2 and p27KIP1, is disrupted in osteosarcoma. The Journal of cell biology. 2004;167(5):925-34.
73. Hopyan S, Gokgoz N, Bell RS, Andrulis IL, Alman BA, Wunder JS. Expression of osteocalcin and its transcriptional regulators core-binding factor alpha 1 and MSX2 in osteoid-forming tumours. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 1999;17(5):633-8.
74. Won KY, Park HR, Park YK. Prognostic implication of immunohistochemical Runx2 expression in osteosarcoma. Tumori. 2009;95(3):311-6.
75. Arikawa E, Sun Y, Wang J, Zhou Q, Ning B, Dial SL, et al. Cross-platform comparison of SYBR Green real-time PCR with TaqMan PCR, microarrays and other gene expression measurement technologies evaluated in the MicroArray Quality Control (MAQC) study. BMC genomics. 2008;9:328.
76. Tajadini M, Panjehpour M, Javanmard SH. Comparison of SYBR Green and TaqMan methods in quantitative real-time polymerase chain reaction analysis of four adenosine receptor subtypes. Advanced biomedical research. 2014;3:85.
77. Lu XY, Lu Y, Zhao YJ, Jaeweon K, Kang J, Xiao-Nan L, et al. Cell cycle regulator gene CDC5L, a potential target for 6p12-p21 amplicon in osteosarcoma. Molecular cancer research : MCR. 2008;6(6):937-46.
78. van der Deen M, Akech J, Lapointe D, Gupta S, Young DW, Montecino MA, et al. Genomic promoter occupancy of runt-related transcription factor RUNX2 in Osteosarcoma cells identifies genes involved in cell adhesion and motility. The Journal of biological chemistry. 2012;287(7):4503-17.
79. V B Andela FS, 2 A Groman,3 and R N Rosier2. An immunohistochemical analysis to evaluate an inverse correlation between Runx2/Cbfa1 and NFκB in human osteosarcoma. J Clin Pathol. Mar 2005328–330. ;58(3):328–30.
80. Diniz MG, Borges ER, Pimenta FJ, De Mesquita Netto AC, De Marco L, Gomez RS, et al. Evidence of molecular alterations in the tumour suppressor gene WWOX in benign and malignant bone related lesions of the jaws. Oncology reports. 2011;25(2):499-502.
81. Aqeilan RI, Trapasso F, Hussain S, Costinean S, Marshall D, Pekarsky Y, et al. Targeted deletion of Wwox reveals a tumor suppressor function. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(10):3949-54.

82. Tandon M, Gokul K, Ali SA, Chen Z, Lian J, Stein GS, et al. Runx2 mediates epigenetic silencing of the bone morphogenetic protein-3B (BMP-3B/GDF10) in lung cancer cells. Molecular cancer. 2012;11:27.
83. Lengner CJ, Steinman HA, Gagnon J, Smith TW, Henderson JE, Kream BE, et al. Osteoblast differentiation and skeletal development are regulated by Mdm2-p53 signaling. The Journal of cell biology. 2006;172(6):909-21.
84. Bednarek AK, Keck-Waggoner CL, Daniel RL, Laflin KJ, Bergsagel PL, Kiguchi K, et al. WWOX, the FRA16D gene, behaves as a suppressor of tumor growth. Cancer research. 2001;61(22):8068-73.
85. Browne G, Nesbitt H, Ming L, Stein GS, Lian JB, McKeown SR, et al. Bicalutamide-induced hypoxia potentiates RUNX2-mediated Bcl-2 expression resulting in apoptosis resistance. British journal of cancer. 2012;107(10):1714-21.
86. Zhang HY, Jin L, Stilling GA, Ruebel KH, Coonse K, Tanizaki Y, et al. RUNX1 and RUNX2 upregulate Galectin-3 expression in human pituitary tumors. Endocrine. 2009;35(1):101-11.
87. Javed A, Barnes GL, Pratap J, Antkowiak T, Gerstenfeld LC, van Wijnen AJ, et al. Impaired intranuclear trafficking of Runx2 (AML3/CBFA1) transcription factors in breast cancer cells inhibits osteolysis in vivo. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(5):1454-9.
88. Lee SH, Che X, Jeong JH, Choi JY, Lee YJ, Lee YH, et al. Runx2 protein stabilizes hypoxia-inducible factor-1alpha through competition with von Hippel-Lindau protein (pVHL) and stimulates angiogenesis in growth plate hypertrophic chondrocytes. The Journal of biological chemistry. 2012;287(18):14760-71.
89. Ando K, Mori K, Verrecchia F, Marc B, Redini F, Heymann D. Molecular alterations associated with osteosarcoma development. Sarcoma. 2012;2012:523432.
90. Bloomston M, Kneile J, Butterfield M, Dillhoff M, Muscarella P, Ellison EC, et al. Coordinate loss of fragile gene expression in pancreatobiliary cancers: correlations among markers and clinical features. Annals of surgical oncology. 2009;16(8):2331-8.
91. Donati V FG, Dell'Omodarme M, Prati MC, Nuti S, Lucchi M, Mussi A, Fabbri M, Basolo F, Croce CM, Aqeilan RI. WWOXexpression in different histological types and subtypes of non-small cell lung cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2007;13:884-91.
92. Ramos D, Abba M, Lopez-Guerrero JA, Rubio J, Solsona E, Almenar S, et al. Low levels of WWOX protein immunoexpression correlate with tumour grade and a less favourable outcome in patients with urinary bladder tumours. Histopathology. 2008;52(7):831-9.

العربى الملخص

الساركومة العظمية (OS) هى أكثر ورم عظمى خبيث ،غير مكون للدم، شيوعا على مستوى العالم مع بقاء على قيد الحياة ضعيف. الساركومة العظمية تَظهر توزيع عمرى ذا نسقين على مستوى العالم، فهى نادرا ما تُشخَص قبل عمر الخامسة ، لكن نسبة الحدوث يزداد مع تقدم العمر حول سن البلوغ خلال مرحلة نمو العظام النشط. هذا الذروة الأولية تُتبَع بنقص و استقرار فى نسبة الحدوث بين عمرى 25 و ستين عاما. ذروة أخرى تُلاحَظ خلال العقدين السابع و الثامن من العمر. نسبة الحدوث فى الرجال أعلى منها عن النساء.

الرعاية الطبية الشخصية و تطور العلاج المُصَمَم بيولوجيا المُؤَسَس على التشخيص الجزيئى، من ضمن نهج متعدد التخصصات يظل الأمل للمزيد من التقدم فى علاج الساركومة العظمية مع امكانية بقاء على قيد الحياة افضل. اللمحة المختصرة الجينية المعقدة للساركومة العظمية تجعله مرشح لدراسة جينات جديدة: الرانت- المرتبط بعامل النسخ 2 و مجال WW المحتوى على المؤكسدة المختزلة (*WWOX*) يكونان جينين مهمين ثبت تورطهما حديثا فى التخلق العظمى، و نمو و تقدم الساركومة العظمية.

الدراسات الجزيئية للساركومة العظمية هامة للهندسة النسيجية و العلاج الجينى. التقنيات الجزيئية تكون هامة لتحرى دور الجينات المختلفة فى التخلق الورمى، النمو الورمى و النقيلات. أنها أيضا هامة فى قياس قيم تقدم و تنبؤ كل جين. واحد من هذه التقنيات الجزيئية هو تفاعل البوليميراز المتسلسل للوقت الحقيقى الكمى (q RT-PCR). لقد ثبت انه أداة بيولوجية يمكن أن تُستخدَم كأداة روتينية لاكتساب المعرفة عن بيولوجيا و سلوك الأورام. هو تفاعل البوليميراز المتسلسل للوقت الحقيقى الكمى هو طريقة سريعة، كمية، حساسة لقياس الحمض النووى المستهدَف.

كيمياء المناعة النسيجية هى طريقة تشخيص مكملة ذات تطبيقات مفيدة مختلفة تشمل تشخيص الأورام الخبيثة غير المتمايزة، التصنيف الفرعى للأورام مثل الأورام الليمفاوية، التعرف على الورم الأولى فى حالات النقليات و بحث عوامل التنبؤ و علامات التقدم. عيوب كيمياء المناعة النسيجية هى الانحيازات التقنية و التفسيرية.

الدراسة الحالية تشمل 30 حالة من الساركومة العظمية أولية لبانيات العظم (تفاعل البوليميراز المتسلسل للوقت الحقيقى الكمى قد نفذ فقط فى 20 حالة (تم الحصول عليها من مرضى مصريين غير حاصلين على علاج كيمائى (مدى العمر 15– 57 عاما، متوسط العمر = 19 عاما: نسبة الرجال الى النساء=2:1). 20 حالة (67%) حدثت فى عظمة الفخذ، 6 حالات (20%) فى عظمة الساق الأكبر، و 4 حالات (13%) فى العضد. بالاضافة، 6 عينات عظم طبيعية (مدى العمر 15-50 عاما، متوسط العمر=20، نسبة الرجال: النساء= 2:1)، تم الحصول عليها من عظام مكسورة غير مرضيا استُخدمت كمجموعة ضابطة.

تقدم المرض فى المرضى قد تم تتبعه بانتظام كل 6 أِهر الى سنة. تبعا للمبادىء التوجيهية للشبكة الوطنية الشاملة للسرطان (NCCN)، فترات المراقبة للمرضى، الموصى بها فى التجارب متعددة الجنسيات تكون كل ستة أسابيع الى ثلاثة أشهر فى السنة الأولى و الثانية بعد التشخيص، كل شهرين- أربعة أشهر فى السنتين الثالثة و الرابعة ، كل 6 أشهر فى السنوات 5-10 و كل 6-12 شهر بعد ذلك.

خلال مرحلة المتابعة (المدى=18-72 شهرا، المتوسط= 45 شهرا، الانحراف المعيارى= 38.18) ، 6 (20%) كان لديهم تكرار موضعى، 11(37%) كان لديهم نقليات بعيدةن و 8 (27%) توفوا بسبب المرض. فترة البقاء على قيد الحياة الخالية من المرض (DFS) تتراوح بين 6 الى 72 شهر مع متوسط وقت للبقاء على قيد الحياة مُقَدَر ب 56 شهر.

كتل البرافين استُخدمت لكيمياء المناعة النسيجية و تفاعل البوليميراز المتسلسل للوقت الحقيقى الكمى: سيبر الأخضر لتحديد تعبيرات RUNX2و تاك مان ل WWOX على التوالى.

فى حالات الساركومة العظمية، تعبير بروتين RUNX2 تم تحديده فى 27/30 (90%) و كان سلبيا فى 3/30 (10%) من الحالات . فى العظم الطبيعى ، تعبير بروتين RUNX2 كان سلبيا فى 8/10 من الحالات الضابطة (80%)

متوسط تعبير RUNX2 فى حالات الساركومة العظمية كان 13 (أدنى-أقصى=0-15) و كان أقل بشكل كبير من العظم الطبيعى 5 (أدنى-أقصى=0-8) (*U*=3.5, p <.001)

فى حالات الساركومة العظمية، تعبير بروتين WWOX فُقد فى 22 حالة (73%) . و تم اكتشافه فى الحالات الثمان النتبقية (27%) . فى العظم الطبيعى، تعبير بروتين WWOX تم اكتشافه فى 10/10 من الحالات (100%). مع كثافة تلطيخ حشوية قوية فى جميع الحالات(100%) .

متوسط التعبير عن WWOX فى الساركومة العظمية كان 0 (أدنى-أقصى=0-6) و كان أعلى بشكل كبير عن العظم الطبيعى 7 (أدنى-أقصى=4-10) (*U*=3.5, p <.001)

كان يوجد ترابط سلبى ذو مغزى بين تعبيرات بروتين RUNX2 و WWOX بواسطة كيمياء المناعة النسيجية (ρ=-.578, p=0.008, ρ=-.391, p=0.033 للحالات ال 20 و ال 30 على التوالى).

بالمقارنة بالعظم الطبيعى، تعبير رنا لجين RUNX2 كان استجابته أعلى للمحفزات فى 15/20 (75%) من الحالات و كانت استجابته أقل فى 5/20 (25%) من الحالات . تعبير رنا لجين WWOX كان استجابته أقل للمحفزات فى 16/20 (80%) من الحالات و كانت استجابته أقل فى 4/20 (20%) من الحالات .

الدراسة الحالية لتعبيرات بروتين و رنا الجينات RUNX2 و WWOXفى جماعة بشرية من المرضى المصريين تقترح الدور العكسى الهام لكلا الجيني نفى التخلق الورمى للساركومة العظمية.

جين RUNX2 يمكن أن يكون متورطا فى التقدم الزهيد لمرضى الساركومة العظمية. الارتباط الايجابى ذو المغزى بين نتائج كيمياء المناعة النسيجية و تفاعل البوليميراز المتسلسل للوقت الحقيقى الكمى فى كل جين فى الدراسة الحالية تفترض أن كيمياء المناعة النسيجية يمكن أن يكون مفيدا كاختبار فحص لتقدير WWOX و RUNX2قبل التقدم الى تقنيات تشخيصية جزيئية.