

## Editorial

# Investigation of osteosarcoma genomics and its impact on targeted therapy: an international collaboration to conquer human osteosarcoma

Ji-Long Yang<sup>1,2,3</sup>

## Abstract

Osteosarcoma is a genetically unstable malignancy that most frequently occurs in children and young adults. The lack of progress in managing this devastating disease in the clinic has prompted international researchers to collaborate to profile key genomic alterations that define osteosarcoma. A team of researchers and clinicians from China, Finland, and the United States investigated human osteosarcoma by integrating transcriptome sequencing (RNA-seq), high-density genome-wide array comparative genomic hybridization (aCGH), fluorescence *in situ* hybridization (FISH), reverse transcription-polymerase chain reaction (RT-PCR), Sanger sequencing, cell culture, and molecular biological approaches. Systematic analysis of genetic/genomic alterations and further functional studies have led to several important findings, including novel rearrangement hotspots, osteosarcoma-specific *LRP1-SNRNP25* and *KCNMB4-CCND3* fusion genes, VEGF and Wnt signaling pathway alterations, deletion of the *WWOX* gene, and amplification of the *APEX1* and *RUNX2* genes. Importantly, these genetic events associate significantly with pathogenesis, prognosis, progression, and therapeutic activity in osteosarcoma, suggesting their potential impact on improved managements of human osteosarcoma. This international initiative provides opportunities for developing new treatment modalities to conquer osteosarcoma.

**Key words** Osteosarcoma, transcriptome sequencing, aCGH, *LRP1-SNRNP25* fusion gene, *KCNMB4-CCND3* fusion gene, the VEGF pathway, the Wnt pathway, *WWOX* gene, *RUNX2* gene, *APEX1* gene

## International Collaboration to Conquer Human Osteosarcoma

Osteosarcoma is the most common primary tumor of the bone, affecting approximately 1,500 individuals per year in the United States (Surveillance, Epidemiology, and End Results database, National Cancer Institute)<sup>[1]</sup>. According to the latest statistics from the Tianjin Cancer Registry Center, there were 56 patients with osteosarcoma among the population of 9,488,400 in year 2006 in Tianjin, the third largest city in China<sup>[1]</sup>. Osteosarcoma primarily affects individuals

in the second decade of life but the incidence decreases gradually thereafter. This cancer has been found to harbor complex karyotypes and a highly unstable genome, exhibiting both numerical and structural chromosomal instability<sup>[2]</sup>. Survival rates in patients with metastatic osteosarcoma have not improved significantly in recent years. To develop new therapies with the potential to improve patient outcomes, identifying the key genetic and molecular events for osteosarcoma is critical.

One of the major challenges in investigating osteosarcoma is the difficulty in obtaining fresh untreated tumor tissues. Since its inception in 2008, the United States Chinese Anti-Cancer Association (USCACA) has been actively facilitating and promoting international collaboration in cancer research. Together with collaborative partners that include the Chinese Anti-Cancer Association (CACA), Chinese Society of Oncology (CSO), American Cancer Research Association (AACR), National Foundation for Cancer Research (NFCR), American Cancer Society (ACS), and Chinese Society of Clinical Oncology (CSCO)<sup>[3-5]</sup>, the USCACA defined a vision to elevate Chinese translational and clinical research to the leading global level, requiring close partnership among academia, industry sponsors, and

**Author's Affiliations:** <sup>1</sup>Departments of Bone and Soft Tissue Tumor, Tianjin Medical University Cancer Institute & Hospital, Tianjin 300060, P. R. China; <sup>2</sup>National Clinical Research Center of Cancer, Tianjin Medical University Cancer Institute & Hospital, Tianjin 300060, P. R. China; <sup>3</sup>Key Laboratory of Cancer Prevention and Therapy, Tianjin Medical University Cancer Institute & Hospital, Tianjin 300060, P. R. China.

**Corresponding Author:** Ji-Long Yang, Department of Bone and Soft Tissue Tumor, Tianjin Medical University Cancer Institute & Hospital, Tianjin 300060, P. R. China. Tel: +86-22-23340123-1081; Email: yangjilong@tjmuch.com.

**doi:** 10.5732/cjc.014.10209

regulatory agencies internationally<sup>[3-5]</sup>. Through the concerted effort of tissue banking during the last 10 years, our sarcoma center has amassed a collection of Chinese osteosarcoma samples with clinical follow-up data.

In collaboration with scientists at the Tianjin Medical University Cancer Institute & Hospital (TMUCIH), Tampere University of Technology, University of Texas MD Anderson Cancer Center, Sylvester Comprehensive Cancer Center, Ohio State University, and Chinese National Clinical Research Center of Cancer, whole-genome sequencing, transcriptome sequencing (RNA-seq), high-density genome-wide array comparative genomic hybridization (aCGH), fluorescence *in situ* hybridization (FISH), reverse transcription-polymerase chain reaction (RT-PCR), Sanger sequencing, cell culture, and molecular approaches were recently used to explore the genomic alterations in human osteosarcoma. This work revealed several important genetic aberrations such as low-density lipoprotein receptor-related protein 1-small nuclear ribonucleoprotein 25 kDa (U11/U12) (*LRP1-SNRNP25*) and potassium large conductance calcium-activated channel, subfamily M, beta member 4-cyclin D3 (*KCNMB4-CCND3*) fusion genes, vascular endothelial growth factor (VEGF) and wingless-type MMTV integration site family (Wnt) signaling pathway alterations, deletion of *WW* domain-containing oxidoreductase gene (*WWOX*), and amplification of *APEX* nuclease (multifunctional DNA repair enzyme) 1 (*APEX1*) and runt-related transcription factor 2 (*RUNX2*) genes<sup>[1,2,6-13]</sup>.

Our RNA-seq study represents the first whole transcriptome sequencing analysis of human osteosarcoma<sup>[13]</sup>, and the results on the VEGF signaling pathway were cited by the 2013 WHO Classification of Tumors of Soft Tissue and Bone<sup>[14]</sup>. Our discovery of novel osteosarcoma-specific fusion genes and rearrangement hotspots provides insight that gene fusion is an important mechanism by which to inactivate the TP53 tumor suppression pathway and to reduce cell motility<sup>[13]</sup>. Our findings on genetic aberrations in the VEGF and Wnt signaling pathway, as well as alterations in copy number and expression of the *RUNX2*, *APEX1*, and *WWOX* genes may supply potential targets for osteosarcoma. Collectively, our work highlights the heterogeneity of osteosarcoma and provides opportunities for developing new treatment modalities to conquer osteosarcoma. Indeed, in the era of molecularly targeted therapy, investigations like these are essential to understand the mechanism of targeted therapies and to achieve the ultimate goal of personalized medicine for patients with osteosarcoma<sup>[15]</sup>. Here we summarize the knowledge we have gained about human osteosarcoma through this collaborative effort.

## Identification of Two Fusion Gene Hotspots in Human Osteosarcoma: 17p Associated with *TP53*-disrupting Rearrangements and 12q Associated with 12q Amplification

For the studies at TMUCIH, osteosarcoma tissues and patient information were collected per the protocol approved by the TMUCIH Institutional Review Board (IRB). Patient consent was also obtained.

We acquired from the Tumor Tissue Bank (TTB) at TMUCIH primary tumor tissue from 31 untreated osteosarcomas obtained during biopsy. DNA from 10 of 31 samples was labeled and hybridized to a human genome comparative genomic hybridization microarray (4 × 44 k) (Agilent Technologies, Palo Alto, CA, USA) to investigate gene copy number alterations in human osteosarcomas by aCGH<sup>[1,9,10]</sup>. In addition, a sufficient quantity of high quality RNA was obtained from 11 of 31 samples (10 conventional subtype and 1 parosteal subtype). The extracted whole RNA from the 11 samples was sequenced using IlluminaHiSeq™ 2000 instruments at Beijing Genetic Institute (BGI). Sequence quality was high in all samples, with 30% of coding regions covered by 10x or higher coverage. All Spearman correlations between sample gene expression profiles were above 0.85<sup>[13]</sup>.

Based on transcriptome sequencing, we sought to detect fusion genes and identified a total of 16 fusion genes. Seven of 11 osteosarcomas harbored at least 1 fusion gene. We identified a pattern of interchromosomal gene fusions clustered at 2 hotspots in the genome: one in 17p, associated with *TP53*-disrupting rearrangements, and the other in 12q, associated with 12q amplification<sup>[13]</sup>. Further analysis of exon frames at the 17p hotspot suggested that none of the *TP53* rearrangements produces a chimeric protein. We also observed a strong, localized gene dosage effect in 3 of the 12q rearrangements, suggesting that fusion genes at this locus often arise as a byproduct of *MDM2/CDK4* co-amplification<sup>[13]</sup>. Many of the 12q fusion genes produced chimeric proteins or disrupted cancer-associated genes such as *RUNX2*, *CCND3*, and *LRP1*, indicating that some of the fusions may contribute to cancer progression in a manner independent of *MDM2/CDK4* co-amplification<sup>[13,16,17]</sup>.

## Widespread Alterations of the p53 and RB1 Pathways in Human Osteosarcoma

Based on the observed rearrangements in hotspots at the *TP53* and *MDM2/CDK4* loci in 11 human osteosarcoma samples, we analyzed the p53 and RB1 pathways for further alterations. We looked for oncogenic mutations in expressed regions of the genome and performed a gene dosage analysis to identify signs of copy number alterations. We found several alterations including *TP53* rearrangement, *TP53* mutation with loss of heterozygosity, and *TP53* deletions in 6 of the 12 osteosarcoma samples. Of the remaining 5 samples in which *TP53* was intact, there were *MDM2/CDK4* co-amplification, loss of *CDKN2A* expression, and loss of *RB1* gene. Taken together, all 11 osteosarcomas in our cohort had lost either p53 or RB1 pathway function through one of these mechanisms<sup>[13]</sup>.

## Osteosarcoma-specific, Recurrent *LRP1-SNRNP25* and *KCNMB4-CCND3* Fusion Genes Are Associated With Cell Motility in Human Osteosarcoma

The identification of fusion genes such as *SYT-SSX1/SSX2*, *PAX3-FOXO1*, *TPM3/4-ALK*, *BCOR-CCNB3*, and *EWS-FLI1* in human sarcomas has provided important insight into diagnosis

and targeted therapy<sup>[18-23]</sup>. A *EWSR1-CREB3L1* fusion transcript in a single case of small cell osteosarcoma has been reported<sup>[20]</sup>; however, there are no reports of recurrent fusions in osteosarcoma. By contrast, osteosarcoma is known to exhibit frequent numerical and structural chromosomal aberrations, including *TP53* mutations and deletions, *MDM2* amplification, *CDKN2A* deletion, and hemi- or homozygous loss of *Rb1*<sup>[24-26]</sup>.

According to the 16 fusion genes identified by transcriptome sequencing, we selected the fusion genes *LRP1-SNRNP25*, *KCNMB4-CCND3*, *MDM2-RUNX2*, *TP53-CCNB1*, *DPM1-CD63*, and *ZFC3H1-MDM2* for further validation, as these genes play recognized roles in cancer progression. Only the *LRP1-SNRNP25* and *KCNMB4-CCND3* fusion genes were found to be recurrent and somatic, occurring in 6.5% (2 of 31) of our osteosarcoma samples. RT-PCR analysis on 240 other sarcoma subtypes revealed that none of the 240 other sarcomas were positive for the *LRP1-SNRNP25* or *KCNMB4-CCND3* fusions, indicating these 2 fusions were specific to osteosarcomas. Expression of the *LRP1-SNRNP25* and *KCNMB4-CCND3* fusion genes in SAOS-2 osteosarcoma cells promoted cellular motility, suggesting an oncogenic contribution independent of 12q amplification<sup>[13]</sup>.

Our study represents the first whole transcriptome analysis of untreated human osteosarcoma<sup>[13]</sup>. The discovery of novel osteosarcoma-specific fusion genes and rearrangement hotspots provides important insight that gene fusion is an important mechanism for inactivating the p53 tumor suppression pathway and for enhancing cell motility in osteosarcoma cells<sup>[13]</sup>. Future functional studies with an expanded cohort will determine the frequency of these events in osteosarcoma and the strength of their association with osteosarcoma progression, thus providing insight into their potential as therapeutic targets.

## Amplification of VEGF Pathway Genes in Human Osteosarcoma

A number of preclinical and clinical studies have provided evidence that antiangiogenic therapies such as antibodies and small-molecule inhibitors that target the VEGF-VEGFR axis are promising strategies in the treatment of osteosarcoma. However, despite numerous studies reporting chromosomal and gene aberrations in human osteosarcoma, no genetic aberrations of the VEGF pathway have been reported in this tumor type<sup>[27]</sup>. Using aCGH, FISH, and immunohistochemistry analyses, we discovered genetic amplification of VEGF pathway genes and validated *VEGFA* gene amplification<sup>[1]</sup>. Amplification of *VEGFA* gene and elevated expression of the VEGFA protein were significantly associated with microvascular density and poor tumor-free survival in patients with osteosarcoma<sup>[1,14]</sup>.

The anti-VEGF antibody bevacizumab (Avastin, Genentech, Inc.), when used in combination with chemotherapy, has been shown to significantly improve survival rates and response rates in patients with metastatic colorectal cancer. Anti-VEGF treatment has not been commonly used to treat osteosarcoma. If such a treatment were studied in osteosarcoma, it would be critical to know the relationship between *VEGFA* gene amplification and VEGFA protein

levels and their collective association with prognosis and response to anti-vascular therapy. Our data suggest that amplification of the *VEGFA* gene is a poor prognostic factor for tumor-free survival time. Furthermore, *VEGFA* gene amplification and elevated VEGFA protein expression level, if validated in future studies, may be useful molecular features for stratifying and selecting patients for anti-vascular therapy<sup>[1]</sup>.

## Genetic Inactivation of Wnt Signaling Pathway in Human Osteosarcoma

The Wnt pathway is clearly important in many types of human cancer, particularly in epithelial cancer types wherein gain- or loss-of-function events appear to contribute to both inherited cancer risk and somatic carcinogenesis. Most previous studies on human osteosarcoma have suggested that active Wnt signaling contributes to osteosarcoma development, as evidenced by cytoplasmic and/or membranous  $\beta$ -catenin staining or detection of Wnt pathway components. However, Cai *et al.*<sup>[28,29]</sup> recently reported that the Wnt pathway is inactivated in osteosarcomas. These contradictory findings provoke debate and strengthen the rationale for further research into the role of Wnt signaling in osteosarcoma.

In contrast to the observed amplification of VEGF and mTOR pathway genes<sup>[1]</sup>, we determined that there were a significant number of deleted genes in several signaling pathways<sup>[1,12]</sup>. Among these pathways, the Wnt signaling pathway was most highly affected. We further investigated copy number alterations of individual genes in the Wnt signaling pathway. In the canonical Wnt signaling pathway, the following genes were significantly deleted across the osteosarcoma dataset: *WNT*, *FRP*, *Frizzled*, *GBP*, *GSK-3 $\beta$* , *TCF/LET*, *TAK1*, *CK1*, *CtBP*, and *B-TrCP*. Specifically, the *WNT1* gene was deleted with a frequency of 50%<sup>[12]</sup>. At the mRNA level, transcriptome sequencing revealed reduced levels of Wnt pathway transcripts. At the protein level, WNT1 protein was detected with immunohistochemistry in 69.6% of osteosarcoma samples, but no  $\beta$ -catenin protein was detected in the nucleus. C-myc protein was detected in only 47.8% of osteosarcoma samples, and cyclin D1 protein was detected in 52.2%. Kaplan-Meier survival analysis showed WNT1-negative patients who were also cyclin D1-negative had significantly longer disease-free survival time than those who were cyclin D1-positive<sup>[12]</sup>. Hence, we provided the first evidence of significant deletions of Wnt signaling pathway genes in human osteosarcoma, findings which demonstrate that this important signaling pathway is genetically inactivated in osteosarcoma. Nevertheless, the conflicting results about the Wnt signaling pathway highlight its complexity and suggest it is still poorly understood in human osteosarcoma.

## Loss of WWOX at the Gene and Protein Levels in Human Osteosarcoma

Supporting evidence for *WWOX* as a tumor suppressor gene was obtained in a genetically engineered mouse model. In this model, targeted homozygous deletion of *WWOX* gene resulted in the development of osteosarcomas in juvenile mice and lung

papillary carcinomas in adult mice<sup>[30]</sup>. Although these experiments linked *WWOX* gene to osteosarcoma in mice, there is no information regarding the status of *WWOX* gene in human osteosarcoma.

We investigated the *WWOX* gene in the pathogenesis of human osteosarcoma by examining this gene's copy number status and *WWOX* protein expression level in primary osteosarcoma tissues. Global genomic profiling by aCGH revealed that the *WWOX* gene was deleted in 30% of osteosarcomas in both our dataset and an independent aCGH dataset published by Squire *et al.*<sup>[27]</sup>. These findings were consistent with reported data, primarily collected in various cancer cell lines<sup>[30]</sup>. A paper published later also confirmed the occurrence and frequency of *WWOX* deletion, further deepening the evidence of a tumor suppressor role for *WWOX* in osteosarcoma and highlighting the prognostic and therapeutic significance of *WWOX* in this disease<sup>[31]</sup>. Normal cutaneous, muscular, and skeletal tissues exhibited strong *WWOX*-positive staining in the cytoplasm. *WWOX* protein staining was negative in 61.8% of cases, positive in 38.2% of cases, weakly positive in 18.2% of cases, and moderately positive in 10.9%. Furthermore, the *WWOX* protein was not detected in cases with *WWOX* gene deletions. In contrast to previous reports in some cancer types such as breast cancer, *WWOX* protein expression in osteosarcoma in our study had no association with prognosis or with other clinical characteristics<sup>[10]</sup>.

### ***APEX1*: gene amplification, frequent protein expression, and role in target therapy in human osteosarcoma**

The expression of apurinic/apyrimidinic exonuclease 1 (*APEX1*) protein in tumors has been linked to chemoresistance, radioresistance, and shorter patient survival times. We sought to gain insight into the role of *APEX1* in human osteosarcoma by evaluating gene copy number alterations and protein expression levels in patients with osteosarcoma. *APEX1* gene amplification was observed in 50% of the studied osteosarcoma samples. Overexpression of *APEX1* protein was detected in 64.9% of osteosarcoma samples. More specifically, the following expression patterns were observed: negative, 35.1%; weakly positive, 35.1%; moderately positive, 14%; and strongly positive, 15.8%. *APEX1* expression was significantly associated with local recurrence and/or metastasis of osteosarcoma. Moreover, multivariate analysis showed that *APEX1* expression was an independent predictor of disease-free survival for patients with osteosarcomas<sup>[9]</sup>. An *APEX1* shRNA expression plasmid was successfully constructed and transfected into U2-OS cells. As a result of *APEX1* silencing, U2-OS cell proliferation was inhibited via down-regulation of nuclear NF- $\kappa$ B protein<sup>[11]</sup>. Thus, *APEX1* may serve as a prognostic marker and potential therapeutic target for osteosarcoma.

### ***RUNX2*: gene amplification, protein overexpression, and association with *WWOX* and *VEGFA* in human osteosarcoma**

*RUNX2* was previously reported as a critical factor in stimulating *VEGFA* transcription during bone organogenesis. In

our study, *RUNX2* gene amplification was observed in 30% of osteosarcoma specimens. The positive rate of *RUNX2* protein expression in 59 cases of osteosarcoma was 50.8% (30 of 59). *RUNX2* protein expression level was not significantly associated with clinicopathological features or survival rate in patients with osteosarcoma. The gene amplification, elevated protein expression, and lack of association with clinical characteristics or prognosis indicate that *RUNX2* gene amplification and protein overexpression might be early events in osteosarcoma pathogenesis<sup>[6,32]</sup>.

*WWOX* was physically and functionally associated with *RUNX2* and can suppress *RUNX2* transactivation by interaction between the first WW domain and *RUNX2*<sup>[33]</sup>. Kurek *et al.*<sup>[30]</sup> reported an inverse correlation between *WWOX* and *RUNX2* expression in *WWOX*-deficient mice and osteosarcoma cell lines. Additionally, *RUNX2* is a critical element for *VEGF* mRNA transcription and protein expression during tumorigenesis. Aqeilan *et al.*<sup>[33]</sup> found that ectopic expression of *WWOX* in MDA-MB-231 breast cancer cells reduced expression of *RUNX2* and its target genes, including *VEGF*. In our study, we found genetic and molecular alterations and key associations between *WWOX*, *RUNX2*, and *VEGFA* expression in human osteosarcoma<sup>[6]</sup>. Our data show a positive correlation between *RUNX2* and *VEGFA*, suggesting that increased *VEGFA* gene copy number and *RUNX2* overexpression both facilitate increased level of *VEGFA*, a key factor in tumor angiogenesis. Functional cross-talk between *WWOX*, *RUNX2*, and *VEGFA* may be essential for osteosarcoma pathogenesis and angiogenesis, and this pathway might provide a new molecular basis for targeted *RUNX2*-*VEGFA* therapy in patients with osteosarcoma<sup>[6]</sup>.

## **Conclusions**

We have formed an international collaboration of scientists across 3 continents to advance the understanding of human osteosarcoma and progress toward effective therapies for this disease. Using high-throughput technologies, we have explored genomic alterations in human osteosarcoma and identified several important events, including recurrent *LRP1-SNRNP25* and *KCNMB4-CCND3* fusion genes, *VEGF* and Wnt signaling pathway alterations, deletion of *WWOX* gene, and amplification of *APEX1* and *RUNX2* genes. Both continued studies and research collaborations are essential to achieve the goal of targeted therapy and personalized medicine for patients with osteosarcoma. The ultimate goal of this work is to conquer human osteosarcoma.

## **Acknowledgments**

These works were partly supported by grants from the National Natural Science Foundation of China (81372872 to JY, 81402215 to XD, and 81320108022 to KC), the University Cancer Foundation via the Sister Institution Network Fund (SINF) (to JY and WZ), the Program for Changjiang Scholars and Innovative Research Team in University (PCSIRT) in China (IRT1076 to JY and KC), the Liddy Shriver Sarcoma Initiative (to JY and WZ), and the National Key Scientific and Technological Project (2011ZX09307-001-04 to KC).

The Tissue Bank of Tianjin Medical University Cancer Institute & Hospital is partially supported by the National Foundation for Cancer Research (US).

We are indebted to Wei Zhang, Ke-Xin Chen, Xi-Shan Hao, Matti Annala, Matti Nykter, Jonathan Trent, Raphael E. Pollock, Alexander J. F. Lazar, Kelly K. Hunt, Antti Ylipää, Dina C. Lev, DeJka M. Araujo, Nancy Ratner, Wei Tian, Lin-Ru Zhao, Yan-Xue Liu, Yan-Rui Zhao,

Hong Zheng, Hai-Xin Li, Jia-Yong Liu, Jing Chen, Yan Zhang, Hui-Xiang Li, Yi Pan, Yan Sun, Ping Ji, Bao-Cun Sun, Li-Mei Hu, David Codgelland, Guo-Wen Wang, Yun Yang, Jun Zhao, Zhi-Chao Liao, Sheng Teng, Jian Duo, and Xiao-Ling Du from China, Finland, and USA for their contributions.

Received: 2014-10-08; accepted: 2014-11-14.

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