Correlation of WWOX, RUNX2 and VEGFA protein expression in human osteosarcoma

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Abstract

Background: To investigate associations between WW domain-containing oxidoreductase (WWOX), runt-related transcription factor 2 (RUNX2) and vascular endothelial growth factor alpha (VEGFA) in human osteosarcoma (OS).

Methods: Copy number aberrations of WWOX, RUNX2 and VEGFA genes were detected by microarray comparative genomic hybridization (aCGH) in 10 fresh OS tissue samples. VEGFA gene alterations were also investigated and validated by fluorescence in situ hybridization (FISH) in 54 formalin-fixed and paraffin-embedded (FFPE) OS samples. Protein expression of WWOX, RUNX2 and VEGFA were examined in 54 FFPE OS samples by immunohistochemistry (IHC).

Results: Analysis of previously published OS aCGH data (GSE9654) and aCGH data from this study (GSE19180) identified significant deletion of WWOX in 30% (6/20) of OS samples, whilst significant increase in both RUNX2 and VEGFA gene copy numbers were detected in 55% (11/20) and 60% (12/20) of OS samples, respectively. FISH demonstrated increased VEGFA gene copy number in 65.9% (31/47) of evaluable samples, in either focal or large fragment forms. Compared with positive expression of WWOX in 38.9% of the OS samples, positive expression of RUNX2 and VEGFA protein was found in 48.1 and 75.9% of samples. Although there was no significant association between gene copy number aberration and protein expression for WWOX and RUNX2, significant positive correlation between increased VEGFA gene copy number and VEGFA protein expression was observed. Although there was no significant reverse association between WWOX and RUNX2 expression, a significantly positive relationship was observed between RUNX2 and VEGFA protein expression.

Conclusions: Our data show increased RUNX2 and VEGFA gene copy numbers and elevation of their respective proteins in human OS. Positive correlation of RUNX2 and VEGFA suggests that both increased VEGFA gene copy number and RUNX2 overexpression facilitate increased expression of VEGFA.

Keywords: Osteosarcoma, WWOX, RUNX2, VEGFA, aCGH, FISH, Gene amplification

Background

Osteosarcoma (OS) is the most common, primary, malignant bone tumor within the non-hematopoietic system. OS frequently occurs in the metaphysis of actively growing long bones and is characteristic of short and rapid progression. It has high incidence of pulmonary metastasis and poor prognosis, and mainly affects children and adolescents [1]. Because of a complexity of karyotypes and a highly unstable genome, OS usually exhibits both numerical and structural chromosomal alterations [2].

As a putative tumor suppressor gene, WWOX is located at chromosome 16q23.3-q24.1, spanning common fragile site FRA16D [3]. WWOX is detected as functional loss or frequent attenuation of protein expression in combination with poor prognosis. This often results from abnormal mRNA splicing of WWOX, missing exons, loss of heterozygosity (LOH) and hypermethylation in numerous carcinomas [4-9]. WWOX might play tumor-suppressor function through interaction with TNF, p53, Bcl-2, ErbB-4 and c-Jun [3,10-12]. A recent study indicated that WWOX was physically and functionally associated with RUNX2 and can suppress RUNX2 transactivation by interaction between the first WW domain and RUNX2 [13]. In addition, absence of WWOX seems to...
contribute to increased RUNX2 expression, further affecting bone growth and metabolism, initiating OS tumorigenesis [14].

The RUNX2 region (6p12-21) is often detected in OS using gene amplification and protein overexpression, suggesting upregulation of this gene and its protein is associated with tumorigenesis, progression, metastasis and unfavorable outcome [15-21]. Importantly, Kyle and colleagues observed an inverse relationship between WWOX and RUNX2 expression in WWOX-deficient mice and OS cell lines [14]. Additionally, RUNX2 is a critical element for VEGF mRNA transcription and protein expression in tumorigenesis [22]. Aqeilan and colleagues found that ectopic expression of WWOX in MDAMB231 breast cancer reduced expression of RUNX2 and its target genes, including VEGF [13]. However, little is known about the correlation of WWOX, RUNX2 and VEGF in human OS tissues. In this study, we observed WWOX, RUNX2 and VEGFA gene copy number status and protein expression levels using microarray comparative genomic hybridization (aCGH), immunohistochemistry (IHC) staining and fluorescence in situ hybridization (FISH), in order to investigate correlations between these components.

Methods

Clinical information for OS tissues

Ten fresh OS tissue biopsies were obtained for aCGH analysis. Fifty-four formalin-fixed and paraffin-embedded (FFPE) OS tissues were obtained for FISH and IHC analysis (including nine aCGH analysis samples. One case which had aCGH data was excluded from further assay because of no enough sample). Clinicopathological characteristics comprised age, sex, pTNM stage, recurrence and metastasis (Table 1). Disease-free and overall survival rate ranged from 0 to 94 months, with medians of 7.5 and 12 months, respectively. All samples and clinical data were obtained and analyzed at Tianjin Medical University Cancer Institute and Hospital, China. The study and all procedures were approved by the Institutional Review Board (IRB) at Tianjin Medical University Cancer Institute & Hospital (TMUCIH).

| Characteristics       | RUNX2 | VEGFA |
|-----------------------|-------|-------|
|                       | -     | +     | ++    | +++   | P value | -     | +     | ++    | +++   | P value |
| Sex                   |       |       |       |       |         |       |       |       |       |         |
| Male (26)             | 15    | 4     | 1     | 6     | 0.435   | 10    | 5     | 4     | 7     | 0.012*  |
| Female (28)           | 13    | 9     | 2     | 4     |         | 3     | 17    | 2     | 6     |         |
| Age groups            |       |       |       |       |         |       |       |       |       |         |
| ≤15 y (14)            | 8     | 3     | 0     | 3     | 0.027*  | 4     | 6     | 0     | 4     | 0.730   |
| 15–20 y (22)          | 11    | 7     | 2     | 2     |         | 5     | 9     | 2     | 6     |         |
| 21–30 y (9)           | 5     | 2     | 0     | 2     |         | 1     | 4     | 2     | 2     |         |
| 31–40 y (1)           | 0     | 0     | 1     | 0     |         | 1     | 0     | 0     | 0     |         |
| >40 y (8)             | 4     | 1     | 0     | 3     |         | 2     | 3     | 2     | 1     |         |
| PTNM stage            |       |       |       |       |         |       |       |       |       |         |
| I stage (4)           | 2     | 1     | 0     | 1     | 0.006   | 0     | 3     | 1     | 0     | 0.168   |
| II stage (18)         | 8     | 4     | 3     | 3     |         | 5     | 5     | 1     | 7     |         |
| III stage (2)         | 2     | 0     | 0     | 0     |         | 0     | 1     | 1     | 0     |         |
| IV stage (1)          | 1     | 0     | 0     | 0     |         | 1     | 0     | 0     | 0     |         |
| Recurrence            |       |       |       |       |         |       |       |       |       |         |
| No (47)               | 26    | 11    | 2     | 8     | 0.484   | 13    | 20    | 4     | 10    | 0.132   |
| Yes (7)               | 2     | 2     | 1     | 2     |         | 0     | 2     | 2     | 3     |         |
| Metastasis            |       |       |       |       |         |       |       |       |       |         |
| No (35)               | 19    | 8     | 2     | 6     | 0.951   | 11    | 14    | 3     | 7     | 0.351   |
| Yes (11)              | 5     | 3     | 1     | 2     |         | 1     | 4     | 2     | 4     |         |

Disease free survival (mean months ± sd) Log Rankχ² = 1.331 (60.238 ± 7.881) 0.722 Log Rankχ² = 6.277 (60.238 ± 7.881) 0.099 Overall survival (mean months ± sd) Log Rankχ² = 0.984 (70.903 ± 6.737) 0.925

*Significantly different P < 0.05.
aCGH investigation and data analysis

aCGH was performed as previously described [23]. Labeled genomic DNAs were hybridized using the Agilent Human Genome CGH Microarray (4×44 k) (Agilent Technologies, Palo Alto, CA). Genomic DNA was isolated according to standard procedure. These arrays represent over 43,000 coding and non-coding human sequences, yielding an average 35kbp oligonucleotide probe spatial resolution. At least one target sequence was measured for every characterized gene, and known cancer genes were measured using a minimum of two probes. Probes were designed based on the University of California Santa Cruz hg17 human genome (National Center for Biotechnology build 35, May 2004). aCGH analysis was also carried out as previously described [23]. Briefly, ratio of intensity values from tumor and normal tissues was transformed to log2-space. Log ratio data were then subjected to a circular binary segmentation (CBS) algorithm to reduce the effect of noise. Following this, a CGHcall algorithm was used to give each segment an aberration label: normal, deletion, or amplification. All our aCGH data can be accessed through the GEO ID GSE19180. Previously published aCGH data (GSE9654) was downloaded to perform the analysis [20].

FISH detection and data analysis

FISH detection and analysis was performed in 54 FFPE OS samples as previously described [24]. FISH was performed using the VEGFA probe(Empire Genomics, Buffalo, NY) for detection and the CEP 6 probe(Abbott Molecular, Abbott Park, IL) as the reference. The VEGFA probe was hybridized to the short arm of chromosome 6 (6p12), producing an orange signal, the intensity of which represented the VEGFA gene copy number. The CEP 6 probe was hybridized to the chromosome 6 centromere (6p11.1-q11.1), producing a green signal.

FISH results were interpreted independently and blindly by two pathologists [25]. Copy number alterations in which >90% of nuclei showed hybridization signals were considered informative. An informative case was considered VEGFA amplification if the ratio of orange to green signals was greater than 1 and there were more than two orange and green signals in each single tumor cell. If the ratio was equal to 1 and there were more than two green and orange signals in each single tumor cell, the case was considered to have increased VEGFA gene copy number. A case with a ratio lower than 1 or with only two green and orange signals in each single tumor cell was considered to have no VEGFA amplification [24].

IHC analysis

Fifty-four representative FFPE sections were obtained for IHC staining as previously described [14,23,24]. Antibodies for WWOX, RUNX2 and VEGFA were purchased from Abcam (Abcam company, Cambridge, UK) with dilutions of 1:500, 1:100 and 1:100, respectively. Skin tissue, fetal cartilage from abortion tissue and breast carcinoma tissue served as positive controls for WWOX, RUNX2 and VEGFA staining, respectively. For negative controls, primary antibodies were substituted by PBS.

Two pathologists, blinded to the clinical information, evaluated and scored the IHC staining. Scoring of cytoplasmic WWOX and VEGFA staining, and of nuclear RUNX2 staining, was based on the staining intensity and extent. Microscopically, each section was observed randomly within 10 high-power fields(40×), each of which included 100 cells. First, staining extent was evaluated according to the proportion of positive tumor cells: 0% (score 0), <10% (score 1), 11–25% (score 2), 26–50% (score 3), 51–75% (score 4) and >75% (score 5). Then staining intensity was scored: no cell stain (score 0), yellow (score 1), tan (score 2) and brown (score 3). Final scores were calculated by multiplying intensity and extent scores and the results were divided as follows: negative ("-", score 0–1), weak positive (" +", score 2–4), moderate positive ("++", score 5–9) and strong positive ("+++", score 10–15). For further study, staining results were also grouped into negative and positive (including weak positive, moderate positive and strong positive).

Statistical analysis

We adopted SPSS version 16.0 for Windows to analyze the data. Student’s t-test or ANOVA was used to compare means, and frequencies were compared by means of the Chi-Square test. Survival analysis was carried out to inspect relevance between survival rate and expression using the Kaplan–Meier method and log-rank test. Correlations between the WWOX, RUNX2 and VEGFA genes and protein expression were assessed using Spearman’s test. Two-tailed P values less than 0.05 were considered to be statistically significant.

Results

Deletion of WWOX and frequent amplification of RUNX2 and VEGFA genes in human OS

Analysis of gene copy number alteration in 10 OS samples (GSE19180) detected several chromosome genetic aberrations, including amplification of 1p35,1q23.1–1q21.1, 6p22.1–6p21.31, and 19p13.11–p13.2 and deletion of 5q12.3–5q13.2, 5q14.3–5q22.2, and 13q13.2–13p14.3 [23]. These findings were validated by analyzing genetic aberrations in previously published aCGH data (GSE9654, 10 human OS samples) [20]. The overall recurrent gene copy alteration patterns of these two independent populations in two different countries (China and Canada) were very similar, suggesting that OS from diverse populations shares common genetic alterations at the gene copy level.
We therefore pooled the two aCGH data sets (total 20 OS) to perform further analysis (Figure 1A). Notably, the 12q13-15 amplification reported in low-grade OS was not significant in either dataset, nor were the MDM2 and CDK4 genes located in this region [26,27].

Analysis of the two aCGH data sets, GSE19180 and GSE 9654, identified a 30% (6/20) deletion rate of the WWOX gene in 20 human OS patients (3 cases from GSE19180 and 3 cases from GSE 9654) [23]. Furthermore, significant amplification of the RUNX2 gene copy number was observed in 11 OS patients with an amplification frequency of 55% (11/20; 3 cases from GSE19180 and 8 cases from GSE 9654) (Figure 1B). For the VEGFA gene, we identified a 60% (12/20) amplification frequency (6 cases from GSE19180 and 6 cases from GSE 9654) in 20 OS patients [24].

To validate the aCGH analysis, FISH was used to detect VEGFA gene status in 54 human FFPE OS tissues. We detected increased VEGFA gene copy number in 31 of 47 evaluable samples (65.9%) in either focal or large fragment forms (FISH results were not available for seven samples because there was insufficient tissue or loss of tissue during the pretreatment process) (Figure 2) [24]. From six samples showing increased VEGFA gene copy numbers based on the aCGH method (GSE 19180), FISH detected five samples showing increased VEGFA gene copy number. These two methods showed a good level of concordance (P = 0.048, r = 0.816).

To analyze possible interaction between WWOX, RUNX2 and VEGFA gene copy number aberrations, we applied Spearman’s correlation test. However, there was no significant correlation between any two genes.

**Loss of WWOX protein expression, elevated RUNX2 and VEGFA protein expression in osteosarcoma tissues and their correlation**

WWOX and VEGFA protein expression was predominantly located in the cytoplasmic compartment with RUNX2
expression located in the cell nuclei (Figure 3). Frequent loss of WWOX protein expression (Figure 3A) was recorded in 61.1% (33/54) of samples with a positive WWOX expression rate in 38.9% (21/54) of samples (Figure 3B) [23]. RUNX2 protein expression was detected in 48.1% (26/54) of samples, including RUNX2 (+): 24.1% (13/54), (++): 5.6% (3/54) and (+++): 18.5% (10/54) (Figure 3C). VEGFA protein expression was detected in 75.9% (41/54) of samples, including VEGFA(+) 40.7% (22/54), (++): 11.1% (6/54) and (+++): 24.1% (13/54) (Figure 3D).

Although no statistically significant association was found between WWOX expression and clinical pathologic factors, including sex, age, pTNM stage, recurrence, metastasis and survival, we found increased RUNX2 and VEGFA expression was significantly associated with age ($P = 0.027$) and sex($P = 0.012$), respectively (Table 1).

Compared with increased VEGFA gene copy number, detected using FISH analysis, we identified a significant positive association between increased VEGFA gene copy number (65.9%, 31/47) and increased VEGFA protein expression (75.9%, 41/54) ($P = 0.022$) (Table 2). However, no significant correlation was detected between gene copy number (GSE 19180, 10 OS samples) and protein expression for WWOX (n = 10) and RUNX2 (n = 8) (IHC assay of RUNX2 protein expression was performed in 9 cases in which fresh samples were used in the aCGH analysis. One case which had aCGH data was excluded from IHC assay because of the sample was insufficient. For WWOX
protein expression, only 8 cases could be used in the IHC assay. Therefore, we analyzed copy number alteration of the WWOX gene and its protein expression in 8 cases only (Table 2). Negative RUNX2 protein expression was observed in all 3 OS samples with RUNX2 amplification identified using aCGH detection (GSE 19180) (Table 2). Interestingly, the FFPE samples used for the IHC assay in these 3 cases were obtained from post-chemotherapy tissues, therefore this likely reflects the influence of chemotherapy on RUNX2 expression [14].

In 39.4% (13/33) of OS samples with negative WWOX expression, RUNX2 was overexpressed, whereas 60.6% of samples with WWOX expression loss remained RUNX2 negative. However, statistical analysis failed to identify any significant inverse association between WWOX and RUNX2 protein expression ($P = 0.073$, $r = 0.246$) (Table 3). IHC analysis revealed 40.7% (22/54) concurrent positive expression between RUNX2 and VEGFA in OS samples (Table 3). Furthermore, Spearman’s correlation coefficient test showed a significant concordant relationship between RUNX2 and VEGFA ($P = 0.008$, $r = 0.359$) (Table 3).

### Discussion
OS is a malignant tumor of bone tissue with unknown etiology, and the survival rate has failed to improve since the introduction of chemotherapy [28]. In this study, we present genetic and molecular alterations and key associations between WWOX, RUNX2 and VEGFA expression in human OS. Our data show increased RUNX2 and VEGFA gene copy number and protein elevation in human OS. Although significant reverse correlation was not observed between WWOX and RUNX2, we identified positive correlation of RUNX2 and VEGFA, suggesting both increased VEGFA gene copy number and RUNX2 overexpression facilitate increased expression of VEGFA, a key factor in tumor angiogenesis.

In the present study, we found no significant positive correlation between WWOX gene deletion (by aCGH) and reduced WWOX protein expression (by IHC) in OS. The result could stem from the low sample number in present study. Furthermore, there are several other factors involved the aberrant WWOX protein expression. According to previous reports, loss or attenuation of WWOX protein expression frequently results following abnormal mRNA splicing, missing exons, LOH and hypermethylation in the WWOX gene [4-9]. Furthermore, treatment such as chemotherapy might affect WWOX protein expression [14,29]. Aqeilan and colleagues showed that WWOX levels frequently increase in tumors resected following chemotherapy when compared with their primary biopsies. For these tumors, chemotherapy appears to induce tumor cell normalization rather than death.
accompanied by restoration of WWOX expression [14,29]. The present study shows that OS samples with WWOX gene deletion loose protein expression, suggesting that WWOX gene copy number alteration also remains an important mechanism in the aberration of WWOX protein expression.

Consistent with previous reports, our data found RUNX2 gene amplification and overexpression of its protein to be common in OS [21]. However, we found negative RUNX2 protein expression in three samples with positive RUNX2 gene amplification. Closer assessment of these samples revealed they were obtained from post-chemotherapy tissues, suggesting chemotherapy may affect RUNX2 expression. Further evidence for this was found by Aqeilan and colleagues [14]. In their study, IHC analysis of 56 OS cases revealed 60% (12/20) pre-treatment biopsies were positive for RUNX2. However, only 16% (4/25) post-treatment resections were positive for RUNX2. Paired pre-treatment biopsy and post-treatment resections were available for 12 OS patients. Eight biopsies were RUNX2 positive and in all 8 cases (100%) these were RUNX2 negative post-treatment [14]. We therefore hypothesize that negative expression of RUNX2 protein in samples with RUNX2 gene amplification might due to post-treatment. Pre-treatment tissues would be required to perform such analysis and support this hypothesis in future studies.

WWOX is known to suppress transactivation of RUNX2 by association with the first WW-domain, therefore expression of RUNX2 can be promoted in the absence of WWOX protein [13]. Kyle and colleagues report an inverse correlation between WWOX and RUNX2 expression in WWOX-deficient mice and OS cell lines [14]. In the present study, no significant correlation was observed between WWOX and RUNX2 expression. In fact, the inverse relationship between these factors observed by Kyle and colleagues was not evident when performing paired comparisons, similarly to our study results [14]. Thus, the relationship between WWOX and RUNX2 expression in OS remains unclear and warrants further investigation.

RUNX2 was previously reported as an essential component for the stimulation of VEGFA transcription during bone organogenesis [22]. In the present study, IHC analysis revealed a significant positive relationship between RUNX2 and VEGFA protein expression. These data suggest RUNX2 overexpression can induce increased VEGFA expression. VEGFA is known as a target of RUNX2 and VEGFA functional crosstalk may be essential for the stimulation of VEGFA transcription during the process of angiogenesis, which is in accordance with a previous report [22]. At the same time, the significant relation between increased VEGFA gene copy number and increased VEGFA expression suggests that increased VEGFA gene copy number is also important in VEGFA protein expression. These data provide powerful evidence that increased VEGFA gene copy number and RUNX2 overexpression facilitate increased expression of VEGFA, a key factor in tumor angiogenesis.

Conclusions
The present study found no significant correlation between WWOX, RUNX2, and VEGFA genes with respect to gene copy number aberration. Significant association was observed between increased VEGFA gene copy number and protein expression, whilst WWOX and RUNX2 genes failed to show such association. This may be due to the regulation of other factors in addition to the effect of pre-treatment. No significant association was observed between WWOX and RUNX2 protein expression, but a significantly positive relationship was observed between RUNX2 and VEGFA protein expression. RUNX2 and VEGFA functional crosstalk may be essential for the pathogenesis and angiogenesis of OS, and this pathway might provide a new molecular basis for targeted RUNX2-VEGFA therapy in OS patients.
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