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Original contribution

RUNX2 (6p21.1) amplification in osteosarcoma☆,☆☆

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Summary Prior cytogenetic profiling of osteosarcomas has suggested that amplifications at the 6p12-21 locus are relatively common alterations in these tumors. However, these studies have been limited by variable testing methodologies used as well as by the relatively small numbers of cases that have been analyzed. To better define the frequency of this alteration, 111 osteosarcomas were profiled using hybridization capture-based next-generation sequencing (NGS) platform (Memorial Sloan Kettering Integrated Mutation Profiling of Actionable Cancer Targets) as part of an institutional clinical cancer genomics initiative. Using this platform, amplification at the 6p12-21 locus was determined by copy number assessment of the *VEGFA* and *CCND3* genes. In addition, fluorescence in situ hybridization was used to assess copy number status for *RUNX2*, a known transcriptional regulator of osteoblastic differentiation which has previously been reported to be dysregulated in osteosarcomas. 6p12-21 amplification using NGS-based copy number assessment was confirmed in more than a fifth of all cases tested (24 of 111, 21.6%). Most of these cases, when tested using fluorescence in situ hybridization, were found to include *RUNX2* within the amplified locus (17 of 18, 94.4%). Whereas many laboratories lack access to large-panel NGS assays, the use of fluorescence in situ hybridization to identify 6p12-21 amplification events by targeting *RUNX2* represents a widely available diagnostic modality for the identification of such cases. This could help better define the role of *RUNX2* in osteoblastic differentiation and serve as a surrogate for the identification of potentially targetable alterations such as *VEGFA* amplification at this locus.

1. Introduction

Initial functional studies of *Runt-related transcription factor 2* (*RUNX2*) showed that transgenic mice with loss-of-function alterations of this gene showed a complete absence of bone formation [1,2]. Currently, *RUNX2* is regarded

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as a master transcriptional regulator of osteoblastic differentiation [3,4]. Specifically, *RUNX2* is thought to play a crucial role in the commitment of mesenchymal stem cells to an osteoblastic lineage and promotes osteoblastic differentiation through the expression of downstream genes that play a key role in bone matrix formation such as *collagen type I alpha 1 chain* (*COL1A1*), *collagen type I alpha 2 chain* (*COL1A2*), *osteopontin/ secreted phosphoprotein 1* (*OPN/SPP1*), *integrin binding sialoprotein* (*IBSP/BSP*), and *osteocalcin/ bone gamma-carboxyglutamate protein* (*OCN/BGLAP*) [5-7]. This is supported by in vitro studies where, for instance, the induction of *RUNX2* expression has been found to reproducibly promote osteogenesis [8].

It may therefore be hypothesized that dysregulation of *RUNX2* may contribute to osteosarcoma pathogenesis. In fact, a few reports have documented chromosomal alterations of the 6p12-21 locus in osteosarcomas, which includes *RUNX2* [6,9-11]. In a study conducted by Lau et al, at least 7 cases of osteosarcoma (7/25, 28%) were found to harbor both amplifications and rearrangements at the 6p12-21 locus [9]. A follow-up study by Lu et al documented amplifications at the same locus in 12 cases (12/48, 25%) with confirmatory gene expression profiling using quantitative reverse-transcription polymerase chain reaction. This study also revealed the presence of 3 candidate oncogenes at this locus: *RUNX2*, *CDC5L*, and *CCND3* [10]. Subsequently, Yang et al reported *VEGFA* amplification at this locus in 32 of 50 cases tested (64%) [12]. In addition, our initial studies had documented *VEGFA* and/or *CCND3* amplification at this locus in 17 of 72 (23.6%) specimens tested [11].

Few studies have suggested that *RUNX2* expression may predict response to chemotherapy and outcomes [6]. For instance, a study by Sadikovic et al assessed *RUNX2* mRNA expression in a cohort of 22 cases of osteosarcoma with differential response to chemotherapy and concluded that osteosarcomas showed higher expression of this gene compared to normal osteoblasts [13]. In this study, tumors that lacked significant response to chemotherapy (defined as <90% necrosis) had on average a 3.3-fold higher expression of *RUNX2* [13]. In addition, 2 separate studies have shown that either higher incidence of RUNX2 protein expression in metastases compared to primary tumors or higher RUNX2 protein expression correlated with poorer outcomes [14,15]. Finally, our preliminary studies had shown that osteosarcomas with 6p12-21 gain showed a trend toward increased disease recurrence and/or metastasis within 5 years compared to cases that lacked this genomic amplification (32.1% versus 12.8%, $P = .05$) [11]. At the molecular level, *RUNX2* is thought to have a synergistic effect on cell cycle progression

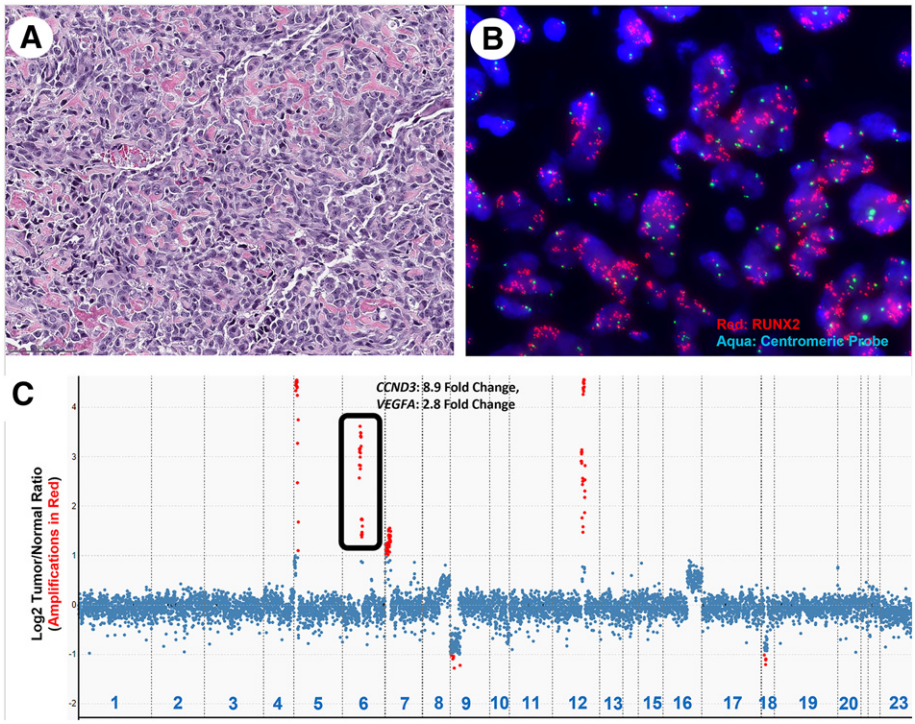

Figure Histopathology, FISH, and copy number alterations. A representative hematoxylin and eosin–stained image (A), with corresponding FISH results demonstrating amplification of *RUNX2* (B) and next-generation sequencing–based results of copy number analysis have been depicted (C). Specifically, an osteosarcoma profiled using MSK-IMPACT is shown, with relative ($\log_2$) tumor/normal ratios on the y-axis and corresponding chromosomes on the x-axis. Amplifications at 6p21 (*VEGFA/CCND3*) have been depicted. Additional copy number changes include amplification of *TERT* (5p15), *CARD11/PMS2/RAC1* (7p22), and *CDK4/MDM2* (12q14-15) and losses of *JAK2/CD274/PDCD1LG2/PTPRD* (9p24) and *PIK3C3* (18q12). Each blue dot represents an individual probe region, and amplified regions (6p21.1) are shown in red.

in the presence of *TP53* and *RB1* alterations, both of which are commonly seen in high-grade osteosarcomas [6,16,17].

2. Materials and methods

2.1. Patient specimens

This study was approved by the Memorial Sloan Kettering Cancer Center institutional review board. Herein, we have profiled 111 osteosarcomas at the molecular level as part of an institutional clinical cancer genomics initiative [11].

2.2. Next-generation sequencing–based copy number assessment

This was done using a next-generation sequencing assay that involves hybridization capture-based library preparation where the capture probes target approximately 1.5 megabases of the human genome, and this is followed by deep sequencing of select noncoding and coding regions (Memorial Sloan Kettering Cancer Center Integrated Mutation Profiling of Actionable Cancer Targets, MSK-IMPACT). Details of this assay have been previously reported [18]. Accurate genome-wide copy number assessment in this assay is facilitated by homogenous

Table *RUNX2* amplification in osteosarcoma

Case no.	Age (y)	Sex	Primary/ metastasis	Histologic subtype	Treatment status	Copy number: *VEGFA*	Copy number: *CCND3*	FISH: *RUNX2*
1	36	Male	Metastasis	Telangiectatic	Post treatment	2.8	8.9	Amplified
2	22	Male	Primary	Osteoblastic	-	7.4	5.5	Amplified
3	27	Male	Metastasis	Telangiectatic	Post treatment	4.1	7.3	-
4	12	Male	Metastasis	Telangiectatic	Post treatment	2.4	6.3	Amplified
5	14	Female	-	NOS	-	2.5	6.0	-
6	19	Male	Primary	Osteoblastic	-	4.4	4.4	-
7	17	Male	Metastasis	Osteoblastic with giant cell rich component	-	3.6	3.6	Amplified
8	18	Female	Metastasis	Chondroblastic	Post treatment	3.5	3.5	Amplified
9	18	Female	Metastasis	Osteoblastic	Post treatment	3.3	No gain	Amplified
10	22	Male	Primary	Osteoblastic, focally chondroblastic	Post treatment	3.0	No gain	Amplified
11	59	Male	Primary	Giant cell rich with telangiectatic features	-	2.9	2.9	Amplified
12	14	Female	Metastasis	Osteoblastic and chondroblastic	-	2.9	2.9	Amplified
13	19	Male	Metastasis	Osteoblastic	-	2.5	2.5	Amplified
14	11	Male	Primary	Osteoblastic	-	2.1	2.5	-
15	26	Female	Primary	Osteoblastic	-	2.3	2.3	Amplified
16	11	Male	Metastasis	Osteoblastic	-	2.3	2.3	-
17	17	Male	Primary	Fibroblastic	-	2.2	2.2	Amplified
18	8	Female	Primary	Osteoblastic	-	No gain	2.1	-
19	15	Female	Metastasis	Osteoblastic and chondroblastic	Post treatment	1.8	1.8	Amplified
20	19	Male	Metastasis	Osteoblastic	Post treatment	1.7	1.7	Amplified
21	18	Female	Metastasis	Osteoblastic	-	1.7	1.7	Amplified
22	13	Male	Primary	Telangiectatic	-	1.7	1.4	Amplified
23	11	Male	Primary	Osteoblastic	Post treatment	1.6	1.6	Amplified
24	19	Male	Metastasis	Osteoblastic	Post treatment	1.8	1.1	Not Amplified
Summarized data								
24 Cases	Mean: 19.4 (range: 8-59)	Male/ female 16:8	Primary/ metastasis 10:13	Osteoblastic: 12; chondroblastic: 1; telangiectatic: 4; others: 7	Post treatment: 10 cases	Mean: 2.8	Mean: 3.4	Amplified: 17/18

NOTE. Seventeen of 72 cases previously reported to have 6p21.1 amplifications without assessment of *RUNX2* status (Suehara et al [11]).
Abbreviation: NOS, not otherwise specified.

distribution of single nucleotide polymorphism tiling probes across the genome, and based on previously reported criteria, amplifications at 6p12-21 were defined as a fold change ≥2.0 and borderline amplifications/gains were defined as a fold change ≥1.5 and <2.0 [19,20]. This assay is currently approved by the United States Food and Drug Administration as a class II in vitro diagnostic test. As part of routine 468-gene panel MSK-IMPACT test, osteosarcomas were profiled for 6p12-21 copy number alterations using targeted probes for *VEGFA* and *CCND3* (Figure A and C).

2.3. Fluorescence in situ hybridization–based copy number assessment

We hypothesized that the *RUNX2* gene (not included in the MSK-IMPACT panel) was included in the amplified locus. *RUNX2* amplification status was therefore assessed using fluorescence in situ hybridization (FISH). FISH was performed as previously described using probes for *RUNX2* (labeled with a red fluorophore; Empire Genomics, Buffalo, NY) and a centromere probe for chromosome 6 (labeled with an aqua fluorophore; CEP6, Abbott Molecular, Des Plaines, IL) [19]. Signal analysis was performed in combination with morphology correlation, and at least 100 interphase cells within marked tumor areas were evaluated and imaged using a Zeiss fluorescence microscope coupled with Metasystems ISIS software (Newton, MA). A positive result was determined if more than 10% of cells showed amplification and >10:1 ratio of *RUNX2* signals to the reference centromeric probe.

3. Results

Of 111 osteosarcomas profiled using next-generation sequencing–based copy number assessment, amplification at 6p12-21 was documented for 24 of 111 cases using probes for *VEGFA/CCND3* (21.6%; Table). Of these 24 cases, 18 had material available for confirmatory downstream analysis, and amplification of *RUNX2* was confirmed in 17 (of 18) cases using FISH (Figure B, Table). No amplification was seen in a single case (case 24) which had a 1.8-fold copy number gain of *VEGFA* without a concurrent amplification of *CCND3*, and it is possible that *RUNX2* was not a part of the amplified locus in this case. Five of 24 specimens (20.8%) had alterations of *TP53* (cases 3, 6, and 17) and *RB1* (cases 16 and 22), and only 2 of these cases had confirmatory testing for *RUNX2* amplification using FISH.

RUNX2 (6p21.1) amplifications were observed in a wide range of clinical specimens including primary and metastatic tumors and included multiple (n = 10) postchemotherapy specimens (Table). Of note, these amplifications were noted twice as frequently in females compared to males (16:8), and this suggests a sex predilection which needs to be confirmed in future studies.

4. Discussion

There is a paucity of functional studies regarding the identification of driver alterations at the 6p12-21 locus. Although there is interest in the presence of *VEGFA* amplification at this locus due to the availability of targeted therapies, it is important to identify other oncogenes at this locus [11]. Although prior studies by Lu et al had suggested *RUNX2*, *CDC5L*, and *CCND3* as important oncogenic drivers at this locus, there have been limited systematic follow-up studies [10]. Given the vast body of literature pertaining to the role of *RUNX2* in osteoblastic differentiation, the identification of potentially dysregulated *RUNX2* expression in these cases is significant because it helps us understand the underlying biology of these tumors [1-4]. For instance, as *RUNX2* may directly induce vascular endothelial growth factor gene expression and angiogenesis, the documentation of combined *RUNX2/VEGFA* amplifications may help us better understand the pathogenic alterations that drive tumor biology in such cases [11,21-23].

Overall, the estimated frequency of genomic amplification events at the 6p12-21 locus, after taking into consideration both the current study (24 of 111 cases, 21.6%) as well as previous studies by Lau et al (7 of 25 cases, 28%), Lu et al (12 of 48 cases, 25%), and Yang et al (32 of 50 cases, 64%), is likely to be approximately 32.1% (75 of 234 cases) [9,10,12]. From our results, it could be hypothesized that most of these cases with 6p12-21 amplification have dysregulated expression of the *RUNX2* gene which contributes to osteosarcoma pathogenesis. Oncogenes that have been implicated in other tumor types with 6p12-21 amplifications include *TFEB/VEGFA* in renal cell carcinomas and *DEK/E2F3* in retinoblastomas, whereas the pathogenic role of genes such as *CDC5L* and *CCND3* in osteosarcomas needs to be further defined [10,24-27].

In addition, it is possible that poorly defined structural rearrangement events at the 6p12-21 locus have a significant role in pathogenesis [9,24]. Of note, recent studies have suggested that neoantigens generated secondary to gene fusions may be positively correlated with response to immunotherapy [28-30]. A recent study demonstrated the presence of *RUNX2* fusions in osteosarcoma in the background of high levels of RUNX2 mRNA transcript expression [28]. On the basis of our current results, we hypothesize that the high levels of *RUNX2* expression in these cases may represent osteosarcoma with 6p12-21 amplification. Therefore, the presence of poorly defined structural rearrangement events at the 6p12-21 locus which may drive response to immunotherapy is an area that needs to be further investigated in future studies.

Of note, *RUNX2* amplifications were identified in both primary and postchemotherapy specimens, supporting this event being a primary/de novo alteration rather than a

secondary alteration. Similarly, the presence of these amplifications in both primary and metastatic specimens argues against these amplifications being a late event in tumor progression. *RUNX2* is thought to have a synergistic effect on cell cycle progression in the presence of *TP53* and *RB1* alterations [6,16,17]. Five of 24 specimens (20.8%) had alterations of *TP53* and *RB1*, and these cases included primary and metastatic tumors as well as postchemotherapy specimens. Because of the limited number of 6p12-21 amplified cases with co-alterations involving the *TP53* and *RB1* genes, no definitive conclusions can be drawn regarding their prognostic impact.

Identification of 6p12-21 amplifications as well as candidate oncogenes such as *RUNX2* will aid in understanding the biology of osteoblastic differentiation, and future studies with larger cohorts are needed to better define the prognostic impact of these genomic alterations. FISH for *RUNX2* therefore represents a rapid and widely available diagnostic modality to identify such cases, whereas more comprehensive molecular profiling including global copy number analysis is likely to help in better defining the individual genes and alterations present at this amplicon. At present, *VEGFA* amplifications at this locus may represent an immediate avenue for targeted therapy using antiangiogenic agents as has been recently proposed [11].

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