Gene amplification and tumor grading in parosteal osteosarcoma

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Abstract

Background: Parosteal osteosarcoma (POS) is a unique low grade osteosarcoma. Two separate oncogenes, MDM2 and CDK4, are specifically amplified in POS. Its clinical behavior is usually indolent. In some occasions, it may progress to high grade and become fatal. Malignant transformation with high grade differentiation is the most reliable indicator to predict its aggressiveness and metastatic potential. This study is to discover the relationship between gene amplification and grading.

Methods: Retrospective analysis of MDM2/CDK4 expression/amplification using immunostaining, multiplex quantitative polymerase chain reaction (MQPCR) and fluorescence in situ hybridization (FISH) were studied on 14 patients with recurrent POS.

Results: Forty tumor specimens in formalin-fixed paraffin-embedded blocks from 14 patients of POS were included in this study. Twenty-seven tumors are low-grade, 13 are high-grade. All POS showed increased expression of both MDM2 and CDK4 proteins, but not those from conventional osteosarcoma. Except some tumors were non-informative (poor DNA quality), the rest of POS had a marked increase of MDM2 and CDK4 genes copies by MQPCR, and confirmed by MDM2 FISH. Moreover, the folds of amplification increase as tumors progress. And, the amplification folds in high-grade POS are consistently higher than those of conventional ones.

Conclusion: FISH and MQPCR are both useful assays for estimating oncogene amplification status in bone tumors. Amplification levels of MDM2 and CDK4 are related to tumor grading and progression. Molecular determination of gene amplification status can be a reliable alternative for predicting clinical behavior of POS at small biopsies.

Keywords: Amplification; Dedifferentiation; Parosteal osteosarcoma

1. INTRODUCTION

Parosteal osteosarcoma (POS) is a less common but distinctive variant of osteosarcoma (4%–7%). It often occurs in long bones (mostly distal femur and proximal tibia) of young adults, with slight female predominance.1 It grows on bone surface (juxta-cortical/parosteal) with a broad base and protruding cauliflower pattern. Intramedullary involvement can happen later. Clinically, it is a low-grade bone sarcoma. Patients with POS often show an indolent phase with minor discomfort and live for years, even without surgical intervention. POS seldom metastasizes unless progressing into a high-grade dedifferentiated sarcoma (HDS), which has a fair prognosis as that of conventional osteosarcoma (COS).2,3

Pathologically, POS is characterized by hypocellular and mild atypical fibroblast-like tumor cells in desmoplastic stroma, intermixed with parallel arrays of bland-looking woven bones. Foci of cellular matrix, including those with osteoblast-rimming osteoid and atypical chondroid cells, are commonly observed. Occasionally, areas of high-grade transformation and HDS, resembling those of COS or undifferentiated pleomorphic sarcoma, can be detected. It is difficult to differentiate HDS from COS based only on morphology, except in case of the rare presence of low-grade components in HDS. The incidence rates of POS are estimated – 4% in all osteosarcomas, whereas those of HDS are less.4

Cytogenetic and molecular genetic studies have revealed a unique genetic background of POS. It harbors a consistent minimal amplification of chromosome 12q13–15, which is rarely observed in other bone tumors or sarcomas.5–7 Genomic mapping analyses in this region identified two consistent but separate genes, cyclin-dependent kinase 4 gene (CDK4) and murine double-minute type 2 gene (MDM2).8,9 Different studies have reported that a significant increase in both CDK4 and MDM2 protein expression is present in 87%–89% and 70%–89% of POS, respectively.10,11 Antibodies against these proteins are now routinely used as diagnostic markers for POS at our institute. In this study, we performed a retrospective study on archived POS specimens at Taipei Veterans General Hospital from 2004 to 2014. Their clinical information and follow-up, with either recurrence...
or metastasis, were included in the study. The archived materials were analyzed by immunohistochemistry, multiplex quantitative polymerase chain reaction (MQPCR), and fluorescence in situ hybridization (FISH) to elucidate the relationships between oncogene amplification and tumor grading.

2. METHODS

Formalin-fixed paraffin-embedded (FFPE) blocks of POS and six COS were included in this study. Patient information, tumor size, initial clinical stage, clinical outcome, and long-term follow-up data were obtained from orthopedic surgeon (W.-M. Chen) and pediatrician (G.-Y. Hung). These cases had been completely reviewed at surgical-pathologic-clinical conferences. The bone tumors were graded (four-grade system) and staged according to the American Joint Committee on Cancer (AJCC) TNM staging system.

This study was approved by the institutional review board at Taipei Veterans General Hospital (IRB #: 2013-04-009A, Taipei, Taiwan). All specimens were collected and analyzed anonymously.

2.1. Immunohistochemistry

Immunohistochemical detection of MDM2/CDK4 protein expressions on FFPE tissue sections was performed on an automatic machine Bond-Max (Leica, Germany) with Bond Polymer Refine Detection DS9800 system (Leica) as previously described. The staining intensity ranged from 0 to 3. Scores 0 and 1 were considered negative, while scores 2 and 3 were considered positive. All sections were stained at least twice.

2.2. Fluorescence in situ hybridization analysis

MDM2 /CEP12 FISH probe kit was purchased from Vysis (Abbott, Abbott Park, IL, USA) and used on FFPE sections as described previously. MDM2 amplified ratio was calculated by the number of red dots divided by that of green dots in nuclei, at an average of at least 20 tumor nuclei per slide. The red dots obtained from high-grade tumors could not be exactly calculated because of tiny, nested, or overlapped signals. If a tumor cell showed a few separate red dots or a tiny cluster of red dots in a nucleus (<10), it was denoted as +. If a cell showed a few nests of amplified red dots (>10 in total), it was denoted as ++.

2.3. DNA extraction

The tumor portion (>3 mm in diameter) on the unstained slides was deparaffinized, dried, and extracted using Arcturus PicoPure DNA extraction kit (Cat#11815-00, Applied Biosystems, Foster City, CA, USA), following manufacturer’s protocol. DNA concentration (range: 100–400 µg/mL) was determined using Thermo NanoDrop 2000 (ThermoFisher Scientific, USA). Approximately 100 ng of template DNA extract was added to each MQPCR reaction that could ensure a successful PCR reaction with a Ct (cycles of threshold) value between 20 and 30 cycles.

2.4. Multiplex quantitative polymerase chain reaction

The reactions were performed using an ABI StepOnePlus real-time PCR detection system (Applied Biosystems). The primers of target genes and TaqMan fluorescent probes (with black hole quencher [Supplementary Table 1]) were designed using Beacon Designer 7.01 (Palo Alto, CA, USA). Each experiment was performed in 20 µL of reaction volume, including 10 µL of 2× QIAGEN Multiplex PCR master mix (Qiagen, Sussex, United Kingdom), 1 µL of template DNA, 2 µL of each 2.5 µmol/L primer-probe mixtures (Table 1), and 7 µL of distilled, deionized water. The thermal cycling program was as follows: initial denaturation in 1 cycle of 15 minutes at 95°C, followed by 40 cycles of 15 seconds at 94°C and 45 seconds at 60°C. Each sample was run in triplicate. An additional run was performed for samples with equivocal amplification.

Fold changes in target genes after MQPCR were calculated as ΔΔCt = (ΔCt_target − ΔCt_normal)CANCER − (ΔCt_target − ΔCt_normal)NORMAL. Relative copy ratio (RCR) of CDK4 and MDM2 compared with ASNS was presented as RCR = 2^{ΔΔCt}. The RCRs of control MDM2 (0.91 ± 0.25) and CDK4 (1.07 ± 0.22) were determined in triplicate in nontumor control tissue. Ratios over 97.5th percentile (RCR Max) of the control were regarded as amplified, and ratios under 2.5th percentile (RCR Min) were regarded as unsatisfactory. RCRs of MDM2 and CDK4 were categorized in 4 levels: <0.5 (unsatisfactory PCR), 0.5–1.5 (no amplification of gene), 1.5–10 (low amplification), and >10 (high amplification). Ct was chosen at the beginning of log phase amplification.

Separated bar graphs were prepared using Prism 5 for Windows (GraphPad software, Inc., CA, USA). Standard deviation (SD) and probability (p) were calculated by diagnostic tests. Probability (p) in unpaired and non-parametric comparison of immunostaining was calculated using Mann-Whitney test. Probability (p) in unpaired and parametric comparison of MQPCR was calculated by t-test.

Table 1.
Clinical information of patients with POS.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (y/o)</th>
<th>Site</th>
<th>Size (cm)</th>
<th>Recur (month)</th>
<th>Lung (month)</th>
<th>F/U (month)</th>
<th>DOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>F</td>
<td>40</td>
<td>Distal humerus</td>
<td>4</td>
<td>192</td>
<td>–</td>
<td>216</td>
<td>–</td>
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<tr>
<td>B</td>
<td>M</td>
<td>13</td>
<td>Distal femur</td>
<td>5</td>
<td>–</td>
<td>–</td>
<td>180</td>
<td>–</td>
</tr>
<tr>
<td>C</td>
<td>M</td>
<td>32</td>
<td>Tibia, proximal</td>
<td>14</td>
<td>–</td>
<td>5</td>
<td>36</td>
<td>+</td>
</tr>
<tr>
<td>D</td>
<td>F</td>
<td>13</td>
<td>Femur, proximal</td>
<td>5</td>
<td>30,129,141,173</td>
<td>–</td>
<td>200</td>
<td>+</td>
</tr>
<tr>
<td>E</td>
<td>M</td>
<td>37</td>
<td>Femur, distal</td>
<td>12</td>
<td>64, 92</td>
<td>–</td>
<td>133</td>
<td>–</td>
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<td>F</td>
<td>F</td>
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<td>45</td>
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<td>180</td>
<td>–</td>
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<td>G</td>
<td>F</td>
<td>23</td>
<td>Femur, distal</td>
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<td>–</td>
<td>130</td>
<td>–</td>
</tr>
<tr>
<td>H</td>
<td>F</td>
<td>17</td>
<td>Proximal tibia</td>
<td>4.7</td>
<td>24</td>
<td>–</td>
<td>94</td>
<td>–</td>
</tr>
<tr>
<td>I</td>
<td>M</td>
<td>40</td>
<td>Proximal tibia</td>
<td>5</td>
<td>14, 25, 65, 116</td>
<td>–</td>
<td>144</td>
<td>–</td>
</tr>
<tr>
<td>J</td>
<td>F</td>
<td>33</td>
<td>Distal femur</td>
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<td>30, 43, 48, 62</td>
<td>–</td>
<td>69</td>
<td>–</td>
</tr>
<tr>
<td>K</td>
<td>F</td>
<td>29</td>
<td>Tibia, proximal</td>
<td>11</td>
<td>36, 173, 183, 197</td>
<td>198</td>
<td>212</td>
<td>+</td>
</tr>
<tr>
<td>L</td>
<td>F</td>
<td>25</td>
<td>Distal humerus</td>
<td>8</td>
<td>48, 57, 65</td>
<td>60</td>
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<td>+</td>
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<tr>
<td>M</td>
<td>F</td>
<td>29</td>
<td>Distal femur</td>
<td>3</td>
<td>13</td>
<td>–</td>
<td>43</td>
<td>–</td>
</tr>
<tr>
<td>N</td>
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<td>6</td>
<td>Distal femur</td>
<td>6.4</td>
<td>–</td>
<td>–</td>
<td>34</td>
<td>–</td>
</tr>
</tbody>
</table>

Recurrence, presence of tumor recurrence (months) after initial diagnosis. DOD = die of disease; F/U = total follow-up interval (months); Lung = presence of lung metastasis (months) after initial diagnosis; POS = parosteal osteosarcoma.
3. RESULTS

3.1. Cases selection
Fourteen patients, who had at least one biopsy-proven diagnosis of POS in extremities and follow-up information (range, 34–216 months; mean, 124.4 months) at Taipei-Veterans General Hospital, were selected in this study. The patients comprised of 5 men and 9 women, and their ages were in the first 4 decades (mean, 27.9 years old; range, 13–40 years old). The primary affected sites were femur in eight cases, tibia in four cases, and humerus in two cases. Maximal tumor axis ranged from 5 to 14 cm (median, 7.1 cm). Twelve patients had experienced recurrent tumors at least once; eight of them experienced high-grade progression in recurrences; three had lung metastasis, and four of them died of the disease (Table 1).

Forty tumor blocks from the POS group and six blocks from the COS group were retrieved for histological grading. The H&E slides were reviewed by two pathologists (P.C.-H. Chen and C.-C. Pan) with consensus. The six tumors in COS group were all high-grade, either with osteoblastic or/and chondroblastic differentiations (Fig. 1A). In the POS group, 27 tumors were low grade (Grade 1–2, POS, Fig. 1B), 13 had high-grade components (Grade 3–4, HDS, Fig. 1C), with or without residual low-grade components. Among those with high-grade dedifferentiation, seven samples showed a major component of fibrosarcoma-like fascicular arrangement of neoplastic spindle tumor cells. Three of them showed sheets of giant cell rich MFH-like pattern, consistent with undifferentiated sarcoma. Three samples showed both chondroblastic and osteoblastic differentiations, similar to chondroblastic osteosarcoma.

3.2. MDM2 and CDK4 Protein Expression
All sections in POS showed at least one of the MDM2 (Fig. 1E) or CDK4 (Fig. 1H) positive staining (score 1 and above [Supplementary Table 2]). Double positives of MDM2 and CDK4 were observed in 10 tumors (Fig. 1J–L).

Fig. 1 A–C, Histological features of COS, POS, and high-grade dedifferentiated POS. D–F, MDM2 immunostains of the osteosarcomas, respectively. G–I, CDK4 immunostains of the osteosarcomas, respectively. J–L, MDM2 FISH analysis of the osteosarcomas, respectively. J, tumor nucleus contains two green dots (chromosome 12 centromere control probe) and two red dots (MDM2 gene probe). K, tumor nucleus contains two green dots and several separated red dots (>5). L, tumor nucleus contains two green dots and >20 red dots (most of them are in clusters and hard to be counted separately). COS = conventional osteosarcoma; FISH = fluorescence in situ hybridization; POS = parosteal osteosarcoma.
CDK4 were observed in most POS (25/27). Furthermore, sections in the HDS group showed even a stronger staining of both MDM2 and CDK4 (score 2 and above) than that of low-grade tumors (Fig. 1F and 1I). The mean staining intensities of MDM2 and CDK4 in the POS group were 1.3±0.8 and 1.8±0.9, while those in the HDS group were 2.1±0.8 and 2.5±0.7, respectively. These differences were significant (p = 0.015 for MDM2 and p = 0.036 for CDK4; Fig. 2). No COS sample was strongly reactive for MDM2 or CDK4 immunostainings, except for two samples that were focally weak reactive for CDK4 (score 1).

Non-specific staining of MDM2 was often noted at benign histiocytes and osteoclasts. Our results were consistent with previous reports which demonstrated that both MDM2 and CDK4 antibodies were specific and can be used as differential diagnostic markers of POS. We also noted that these two markers were more specific, if not the same, for HDS. Interestingly, a similar phenomenon has been reported for dedifferentiated liposarcoma, in which high levels of oncogenes amplification often indicates poor tumor outcomes (see Discussion). However, the amplification status of the oncogenes has not been well characterized in HDS, and its clinical significance may be underestimated.

3.3. MDM2 Amplification Detected by fluorescence in situ hybridization

FISH analysis on bone tumor was difficult to perform. Most of the bone tumor specimens suffered from low or undetectable fluorescent signals, which might be due to genomic DNA detrimental effect exerted from the acid decalcification procedure (15% hydrochloric acid, Leica Biosystems). Only eight specimens from POS, five from HDS, and three from COS were analyzable by MQPCR. Among them, four patients had two additional recurrent/metastatic tumor specimens (patients A, C, D, and I), and five patients had three additional recurrent/metastatic specimens (patients E, F, G, K, and L). The magnitudes of amplification in the recurrent/metastatic tumors were often larger than (A, C, D, F, I, and K), if not similar to (E, G, and L), but not less than those of the primary tumors (Fig. 4). This phenomenon reflects the progressive changes of amplification during recurrence, malignant transformation, or metastasis. The average RCRs of MDM2 and CDK4 in the first biopsy tumors were 5.1±3.0 and 7.3±3.6, respectively, while those of MDM2 and CDK4 from the last recurrent or metastatic tumors were 16.2±2.1 and 24.3±5.5, respectively, in four patients who died of the disease. Both differences were statistically significant (both p < 0.001). This result implies that these amplified oncogenes are further amplified during uncontrolled tumor progression, metastasis, and disease-related death.

3.4. Multiplex quantitative polymerase chain reaction

PCR analysis was also affected by genomic disruption and low template DNA yield after decalcification procedure. Consistent PCR amplification was possible only in 30 FFPE (70%) tumor specimens which had sufficient DNA integrity preserved. Two specimens having low percent tumor volumes (<50% after slides review) were discarded (Supplementary Table 2). Among these specimens, 15 were POS, 10 were HDS, and 3 were COS. None of the COS samples showed amplification of MDM2 (RCR, 1.05±0.5) and CDK4 (RCR, 1.17±0.6). The POS group had an average MDM2 RCR of 1.7±0.6 and an average CDK4 RCR of 7.2±3.5, while the HDS group had an average MDM2 RCR of 16.8±2.3 and an average CDK4 RCR of 22.7±8.3 (Fig. 3). The RCRs of both MDM2 and CDK4 in the HDS group were higher than those in the POS group. The differences were statistically significant (both p < 0.001). Our results indicate that genetic amplification levels are related to tumor grading and differentiation. The optimal cutoff values were both ~10.

In longitudinal study, among the 14 patients with POS and/or HDS, 12 had recurrent and/or metastatic tumor specimens analyzable by MQPCR. Among them, four patients had two additional recurrent/metastatic tumor specimens (patients A, C, D, and I), and five patients had three additional recurrent/metastatic specimens (patients E, F, G, K, and L). The magnitudes of amplification in the recurrent/metastatic tumors were often larger than (A, C, D, F, I, and K), if not similar to (E, G, and L), but not less than those of the primary tumors (Fig. 4). This phenomenon reflects the progressive changes of amplification during recurrence, malignant transformation, or metastasis. The average RCRs of MDM2 and CDK4 in the first biopsy tumors were 5.1±3.0 and 7.3±3.6, respectively, while those of MDM2 and CDK4 from the last recurrent or metastatic tumors were 16.2±2.1 and 24.3±5.5, respectively, in four patients who died of the disease. Both differences were statistically significant (both p < 0.001). This result implies that these amplified oncogenes are further amplified during uncontrolled tumor progression, metastasis, and disease-related death.

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Fig. 2 Comparison of mean staining intensity scores between COS, low-grade POS, and high grade POS. Black column, MDM2 staining intensity; column with squares = CDK4 staining intensity; COS = conventional osteosarcoma; HDS = high-grade dedifferentiated POS; POS = parosteal osteosarcoma.

Fig. 3 Comparison of mean gene amplification folds between low-grade POS and high-grade dedifferentiated POS. Low = low-grade; High = high-grade; Vertical line and bracket = mean with SD; POS = parosteal osteosarcoma.
In addition to the recent discovery of genetic amplification, POS has been known to be a distinct bone tumor with its bland-looking morphological features and indolent clinical presentation. Although its clinical behavior has been proven to be related with morphology (tumor grading), the underlying relationships between genetic amplification and morphology or clinical behavior are largely unknown. The reported gene amplification levels are quite variable (Table 2). The following reasons may explain these discrepancies: (1) liable and disrupted genomic DNA under different decalcification and preservation protocols; (2) different techniques of amplification measurement may not be comparable; (3) reported amplification statuses are not related to tumor grading. Our results showed that different levels of MDM2 and CDK4 amplification were related to tumor grading, clinical progression, and metastasis. Their amplification statuses were also consistent with protein expressions.

An important drawback of FISH and MQPCR techniques in this study was the success rates (32.5% and 70%, respectively), as a significant portion of data were non-informative. Several studies using either calcium ion chelating agent (10% unbuffered ethylenediaminetetraacetic acid [EDTA]) or weak acids (formic acid) and different incubation conditions (increased temperature and microwave) in decalcification procedures have shown better genomic DNA preservation results. Further validation of these decalcification solutions/protocols for better success rates of FISH and MQPCR analyses is mandatory.

Malignant transformation and dedifferentiation have been described in liposarcoma, chondrosarcoma, and POS. Pathologically, they are characterized by both a loss of normal histological arrangement (dedifferentiation) and an increase in nuclear grading and pleomorphism (malignant transformation). Although these two features are often seen together, they may not be synchronized. A similar phenomenon has been observed in retroperitoneal liposarcoma, in which a low-grade myxofibrosarcoma-like pattern has been proposed as an early sign of malignant dedifferentiation. However, none of the morphological features of low-grade dedifferentiation has been characterized in POS. This may be because of the rarity of dedifferentiated POS and its unknown clinical significance. Semi-quantitative or quantitative measurement of oncogene amplification in this study circumscribes the morphological ambiguity in a transformed POS. Either low-grade or high-grade dedifferentiation in POS can be correlated with amplification status and expression status of MDM2 and CDK4. This indicates that these two genes are not only required for low-grade tumor initiation at lesser copies but also drive tumor progression and dedifferentiation in additional gene copies. Similar oncogene amplifications have been observed in other tumors. Amplification of oncogenes such as MYC and HER2 is considered as prognosis indicators of neuroblastoma, breast, and gastric carcinoma.

In addition to POS, other distinct sarcomas, including liposarcoma and intimal sarcoma, share common molecular cytogenetic abnormalities such as supernumerary ring chromosome, giant marker chromosome, and amplified fragments of chromosome 12q13-15, in which extra copies of MDM2 and CDK4 loci have been discovered. Several studies have shown that high levels of CDK4 and MDM2 amplification correlate with poor outcome of patients with liposarcoma (well-differentiated and dedifferentiated), similar to what we observed in this study. Interestingly in one report, although the methodologies used are not identical (quantitative PCR and multiplex ligation-dependent probe amplification analysis vs MQPCR), and the tumors are different (soft tissue tumor vs bone tumor), MDM2-high and CDK4-high cutoffs (both ≥ 10) are the same as those of our study (see Materials and Methods). This implies that these two sarcomas, although histologically distinct, are genetically similar in tumor initiation and progression. By using MDM2 and CDK4 antagonists, several clinical trials against liposarcoma have been undertaken. Since POS is refractory to chemotherapy and radiation therapy, these treatment alternatives may be beneficial for recurrent and intractable bone tumors.

In conclusion, we demonstrated that immunohistochemistry, FISH, and MQPCR were reliable and specific assays for POS diagnosis. They are practical, non-labor intensive tools in pathological laboratory, although they have certain limitations. We also demonstrated that the histological grades in POS could be correlated with amplified copies of MDM2 and CDK4. Furthermore, both MDM2 and CDK4 amplification levels may be useful for prediction of tumor behavior and for treatment stratification, in patients undergoing either targeted or aggressive therapies. Further research using improved decalciﬁed procedures and more POS patients with clinical follow-up will be needed for elucidating the underlying relationships between oncogenes anomalies, nuclear grades, and survival outcomes in POS.

ACKNOWLEDGMENTS

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://doi.org/10.1097/jcma.0000000000000211.

REFERENCES