Immunohistochemical and Biogenetic Features of Diffuse-Type Tenosynovial Giant Cell Tumors: The Potential Roles of Cyclin A, P53, and Deletion of 15q in Sarcomatous Transformation

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Abstract

Purpose: Diffuse-type tenosynovial giant cell tumor (D-TSGCT) is an aggressive proliferation of synovial-like mononuclear cells with inflammatory infiltrates. Despite the COLEA3-CSF1 gene fusion discovered in benign lesions, molecular aberrations of malignant D-TSGCTs remain unidentified.

Experimental Design: We used fluorescent in situ hybridization and in situ hybridization to evaluate CSF1 translocation and mRNA expression in six malignant D-TSGCTs, which were further immunohistochemically compared with 24 benign cases for cell cycle regulators involving G1 phase and G1-S transition. Comparative genomic hybridization, real-time reverse transcription-PCR, and a combination of laser microdissection and sequencing were adopted to assess chromosomal imbalances, cyclin A expression, and TP53 gene, respectively.

Results: Five of six malignant D-TSGCTs displayed CSF1 mRNA expression by in situ hybridization, despite only one having CSF1 translocation. Cyclin A (P = 0.008) and P53 (P ≤ 0.001) could distinguish malignant from benign lesions without overlaps in labeling indices. Cyclin A transcripts were more abundant in malignant D-TSGCTs (P ≤ 0.001). All malignant cases revealed a wild-type TP53 gene, which was validated by an antibody specifically against wild-type P53 protein. Chromosomal imbalances were only detected in malignant D-TSGCTs, with DNA losses predominating over gains. Notably, 15q was recurrently identified in five malignant D-TSGCTs, four of which showed a minimal overlapping deletion at 15q22-24.

Conclusions: Deregulated CSF1 overexpression is frequent in malignant D-TSGCTs. The sarcomatous transformation involves aberrations of cyclin A, P53, and chromosome arm 15q. Cyclin A mRNA is up-regulated in malignant D-TSGCTs. Non-random losses at 15q22-24 suggest candidate tumor suppressor gene(s) in this region. However, P53 overexpression is likely caused by alternative mechanisms rather than mutations in hotspot exons.

Tenosynovial giant cell tumors (TSGCT) are unique mesenchymal lesions that arise from the synovial lining of articular spaces, bursal sacs, and tendon sheaths (1, 2). The neoplastic property of TSGCTs has been supported by the identification of DNA aneuploidy and clonal karyotypic aberrations in these tumors, such as trisomies 7 and 5 and/or translocations involving chromosomal regions 1p11-13, 2q35-37, or 16q22-24 (2-6). Given the difference in clinical behavior, TSGCTs are further divided by growth patterns into localized and diffuse types and by the predominant location of occurrence into extra-articular and intra-articular forms (1-4). Histologically, diffuse-type TSGCT (D-TSGCT), i.e., pigmented villonodular synovitis if located intra-articularly, is an infiltrative proliferation of synovial-like mononuclear cells accompanied by heterogeneous inflammatory infiltrates among varying degrees of collagenous stroma (1, 2, 7). It frequently develops multiple local recurrences that are sometimes difficult to control by surgical excision and can severely compromise joint function (1, 2).

In the absence of sarcomatous transformation, it is extremely rare for D-TSGCT to develop distant metastasis (1, 2, 7, 8). However, malignant D-TSGCT is characterized by an apparently...
Translational Relevance

Similar to benign lesions, deregulated CSF1 mRNA overexpression, as detected by in situ hybridization, is also frequent in malignant D-TSGCT. This suggests a central pathogenetic role for CSF1 deregulation in the early stage of TSGCTs. However, the separation of benign from malignant D-TSGCTs can be challenging on a purely morphologic basis. This study showed that alterations in cyclin A, P53, and chromosome arm 15q are present in the majority of malignant D-TSGCTs but not in benign lesions, which represent critical events in sarcomatous transformation. Combined evaluation of CSF1 expression status and aberrations of cyclin A, P53, and chromosome arm 15q may aid in the differential diagnosis and prediction of sarcomatous transformation in D-TSGCTs.

higher metastatic propensity with considerable tumor-related mortality, based on the clinical outcomes of 33 such cases reported to date (5, 7, 9, 10). In this context, malignant D-TSGCT merits recognition as an exceptionally rare but distinct tumor entity. Histologically, it contains a frank sarcoma associated with a preceding or concurrent typical benign D-TSGCT (7, 8), as initially set forth by Enzinger and Weiss (8). Intriguingly, some authors previously implied that D-TSGCTs should also be considered to be “malignant” due to its aggressive nature, although the morphologic appearance may remain unaltered during the course of the disease (8). Making the issues of nomenclature and diagnosis more complicated, others accepted all giant cell–containing sarcomas that originated within or adjacent to tenosynovial structure as malignant D-TSGCTs. The latter lumping approach might result in erroneous inclusion of a variety of mimicking lesions in the past, such as clear cell sarcoma or malignant fibrous histiocytoma of giant cell type, etc. (8). These diagnostic difficulties reflect considerable morphologic variation in the associated sarcomatous component of malignant D-TSGCTs, thereby posing a great challenge in prognostication and treatment decision.

Benign TSGCTs have recently been characterized by the discovery of COL6A3-CSF1 gene fusion derived from a recurrent chromosomal translocation, t(1; 2)(p13;q37) (ref. 11). This chimeric fusion in a minority of tumor cells results in the activation of CSF1 expression, which creates a “landscape” effect to increase neoplastic cells through an autocrine loop with CSF1R (11). In addition, CSF1 may recruit the more abundant, CSF1R-expressing inflammatory cells (11). To our knowledge, pathogenetic mechanisms of malignant D-TSGCTs remain, thus far, unknown. Therefore, it is highly desirable to identify critical molecular alterations implicated in the malignant transformation of benign lesions. In translocation-associated tumors, secondary deregulation of cell cycle regulators is thought to promote tumor progression, thereby conferring an adverse prognostic effect (12, 13). According to previous studies, chromosomal abnormalities in this type of neoplasm tend to be relatively few but apparently contribute to clinical aggressiveness (14). In this regard, comparative genomic hybridization (CGH) can serve as genomewide screening in these tumors to search for crucial secondary genomic gains or losses implicated in tumor progression (15, 16), although it is unable to detect the initiating reciprocal translocations per se (16).

Accordingly, the aims of this study on the pathogenesis of malignant D-TSGCTs were 2-fold: first, by fluorescent in situ hybridization (FISH) and in situ hybridization (ISH), we investigated whether translocation and mRNA overexpression of the CSF1 gene also occur in malignant D-TSGCTs, as reported previously in benign TSGCTs (11, 17). Second, we assessed whether malignant phenotypes of D-TSGCTs are attributed to the deregulated early G1 and G1-S transition checkpoints and/or non–random chromosomal aberrations, like other tumors with a specific chimeric oncogene (e.g., Ewing sarcoma; ref. 13). For the second issue, a panel of cell cycle regulators was evaluated by immunohistochemistry for both
malignant and benign D-TSGCTs to better characterize immunophenotypic and potential biogenetic alterations related to sarcomatous transformation. We found that overexpression of cyclin A and P53 proteins could robustly distinguish between these two groups without overlap in labeling indices. Accordingly, we did additional real-time reverse transcription-PCR assays for cyclin A to compare the difference in mRNA expression levels between malignant and benign cases. In addition, the genomic status in hotspot exons of TP53 gene were determined for the sarcomatous component of malignant D-TSGCTs by coupling laser capture microdissection (LCM) with bidirectional sequencing as well as immunostaining with a wild-type P53-specific antibody. Lastly, chromosomal alterations were analyzed by CGH for all malignant D-TSGCTs and selected benign control cases to compare the differences in the pattern of imbalances and to search critical regions related to sarcomatous transformation.

Materials and Methods

Inclusion criteria, case selection, and tissue specimens. The criteria for case selection have been recently described in a separate article addressing the clinicopathologic features and outcomes of malignant D-TSGCTs (7). In brief, we diagnosed a malignant D-TSGCT when it arose from or near the large joint (Fig. 1A) and displayed frankly sarcomatous histology at the same site of either a concurrent (de novo) or previous (metachronous) benign D-TSGCT (7, 8). After histologic and radiological reviews, paraffin tissue blocks of six surgically treated malignant D-TSGCTs, including four de novo and two metachronous cases (cases 1-4, 6, and 7 in Li et al.’s article; ref. 7), were available for immunohistochemical and molecular studies.

FISH and ISH for translocation and expression of CSF1. To evaluate the status of CSF1 translocation, FISH was done in six malignant D-TSGCTs. Chromogenic RNA ISH was also used in these cases to assess CSF1 mRNA expression. The protocols for FISH and ISH have been previously described (11, 17). The results of CSF1 break-apart FISH were determined by analyzing a minimum of 25 lesional cells, based on nuclear size and location within the tumor. Lesional cells were classified according to the distance between the break-apart probe pairs or the loss of one of the break-apart probes. A locus alteration was called if a consistent change was seen in at least 50% of the lesional cells.

Immunohistochemistry. Immunohistochemical studies were done on 3-μm-thick sections from paraffin blocks available in 6 malignant and 24 randomly selected benign D-TSGCTs, using the following antibodies and dilution folds: cyclin A (666, 1:50; Novocastra), cyclin E (13A3, 1:40; Novocastra), cyclin D1 (SP4, 1:100; LabVision), P53 (DO-7, 1:1,000; Serotec), P16 (6H12, 1:20; Novocastra), and P27 (1B4, 1:20; Novocastra). The DO-7 P53 antibody was previously considered uninterpretable because of poor RNA quality (21). Special attention was paid to the tissue sectioning step by changing the microtome blade between each block to avoid potential contamination. To extract total RNA from paraffin-embedded tissues for measuring cyclin A mRNA expression, eight 10-μm whole tissue sections were cut for each specimen from six malignant and eight benign D-TSGCTs. In parallel, the synovial tissues from five cases with degenerative tenosynovial or joint disorders were cut and extracted to serve as calibrator controls. Total RNA was extracted using RecoverAll total nucleic acid isolation kit (Ambion) following the manufacturer’s protocol. Briefly, tissues were dewaxed with ethanol, air-dried, incubated with protease at 50°C for 3 h, and extracted with the mixture of isolation additive and ethanol. The suspension was purified using a filter cartridge, digested with DNase for 30 min to remove residual DNA, and then washed thrice with provided buffers to acquire 32 μL of RNA eluant. By using Improm-II reverse transcription system (Promega), RNA samples were reverse-transcribed in a final volume of 40 μL under the following conditions: 0.5 mmol/L deoxynucleotide triphosphates, 25 units of RNase inhibitor, 16 μL of RNA eluant, and 4 μL of random primers. The reactions were done at 42°C for 60 min, followed by inactivation of the enzyme at 70°C for 15 min. Real-time reverse transcription-PCR assay for cyclin A mRNA quantification was done using the LightCycler instrument 2.0 (Roche Molecular Diagnostics). Intron-spanning primers and prevalidated LON probes for transcripts of cyclin A (CCNA2) and housekeeping POLR2A gene (RNA polymerase II, polypeptide A a.k.a. RPⅠ) were designed online with the ProbeFinder software7 and ordered from Universal ProbesLibrary (Roche Molecular Diagnostics). POLR2A was selected as the endogenous reference because it proved to be the gene with the most constant expression in a broad range of tissues (19). The amplification size of cyclin A and POLR2A cDNAs, and the corresponding sequences of specific PCR primer pairs and probes were listed in Supplementary Table S1. Amplification was conducted with LightCycler TaqMan MasterMix (Roche Applied Science) using 10 μL of CDNA, 100 nmol/L of the probes, and 200 nmol/L of the primers in a final 20 μL of reaction mixture. After 2 min incubation at 40°C to allow for uracil-N-glycosylase cleavage, Taq DNA polymerase was activated by incubation for 10 min at 95°C. Each reaction of the 45 PCR cycles consisted of 10 s of denaturation at 95°C and hybridization of the probe and primers for 30 s at 60°C and was done in duplicate. Fluorescence curves were plotted and analyzed by LightCycler software version 4.0 to determine the values of crossing points (Cp), defined as the maximum of the second derivative of the fluorescence curves. Relative expression level of cyclin A mRNA was calculated using the comparative Cp (Cp) method. The amount of cyclin A, after normalization to POLR2A, was then given by 2^-ΔΔCp, where ΔΔCp = ΔCp (sample) - ΔCp (calibrator: the mean of five degenerative synovial tissue specimens), and ΔCp represented the Cp of cyclin A subtracted from the Cp of POLR2A (20). Only samples with consistent amplification of POLR2A, i.e., Cp < 32, were included in the final analyses, whereas those with higher Cp values for POLR2A were considered uninterpretable because of poor RNA quality (21).

Mutation analysis of TP53 gene by LCM coupled with PCR/bidirectional sequencing. To circumvent the contaminating artifacts of numerous surrounding inflammatory and stromal cells, we adopted LCM technology to isolate pure sarcoma cells for mutation analysis of TP53 gene. One representative paraffin block in each case of six malignant D-TSGCTs was recut, stained with Histogene LCM Staining Kit (Arcturus Engineering, Inc.), and placed onto a PEN-membrane capture matrix (Arcturus Engineering, Inc.). Approximately 2,500 cells were collected on the Capsure Macro cap, extracted by Picopure total nucleic acid isolation kit (Ambion) at 65°C overnight with 50 μL of provided buffer, and then desalted by microspin column (Amersham Biosciences). By using primers from published sequences at National Center for Biotechnology Information, polypeptide sequences of TP53 gene were amplified using bidirectional sequencing.
**Table 1.** Clinicopathological features of six malignant D-TSGCTs and results of translocation and expression of CSF1

<table>
<thead>
<tr>
<th>Cases</th>
<th>Age (y)/sex*</th>
<th>Location</th>
<th>Size (cm) †</th>
<th>Type</th>
<th>Nature</th>
<th>Histology ‡</th>
<th>Mitotic count †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>45/M</td>
<td>Ankle</td>
<td>8</td>
<td>I</td>
<td>Metachronous (7 mo) ‡</td>
<td>FS-like</td>
<td>6/10 HPFs</td>
</tr>
<tr>
<td>Case 2</td>
<td>78/F</td>
<td>Knee</td>
<td>8</td>
<td>E</td>
<td>De novo</td>
<td>MFH-like</td>
<td>18/10 HPFs</td>
</tr>
<tr>
<td>Case 3</td>
<td>39/F</td>
<td>Forearm</td>
<td>12</td>
<td>E</td>
<td>Metachronous (31 y) ‡</td>
<td>MS-like</td>
<td>2/10 HPFs</td>
</tr>
<tr>
<td>Case 4</td>
<td>52/M</td>
<td>Suprapopliteal</td>
<td>10</td>
<td>E</td>
<td>De novo</td>
<td>MFH-like</td>
<td>3/10 HPFs</td>
</tr>
<tr>
<td>Case 5</td>
<td>67/F</td>
<td>Lower leg</td>
<td>17</td>
<td>E</td>
<td>De novo</td>
<td>GCT-like</td>
<td>56/10 HPFs</td>
</tr>
<tr>
<td>Case 6</td>
<td>46/F</td>
<td>Thigh</td>
<td>5</td>
<td>E</td>
<td>De novo</td>
<td>MFH-like</td>
<td>34/10 HPFs</td>
</tr>
</tbody>
</table>

Abbreviations: AWD, alive with disease; DOD, dead of disease; E, extra-articular; F, female; FS, fibrosarcoma; GCT, giant cell tumor; HPF, high-power field; I, intra-articular; M, male; MFH, malignant fibrous histiocytoma; MS, myxosarcoma; NED, no evidence of disease; R/T, radiation therapy.

*Age at initial diagnosis of either the primary malignant TSGCT or the preceding benign tumor of metachronous malignant D-TSGCT.
† Specified for malignant tumors, either de novo or metachronous.
‡ Time interval in diagnoses between primary benign and metachronous malignant TSGCTs.

Center for Biotechnology Information web site, the hotspots of somatic TP53 mutation, i.e., exons 5 to 9, were amplified by PCR as follows: an amount of 5 μL of DNA was subjected to 40 cycles of PCR in a final reaction volume of 50 μL, which contained 5 μmol/L of each oligonucleotide primer, 2.5 units of Platinum Taq DNA polymerase (Invitrogen), 4 μL of deoxynucleotide triphosphate mixture at 10 mmol/L, 33.5 μL of double-distilled water, 2 mmol/L of MgCl₂, and 5 μL of 1× PCR buffer. Except for exon 5, 3 μL of 50-fold diluted first PCR products was used as the DNA template of nested PCR for exons 6 to 9. PCR conditions were 94°C for 25 s, annealing temperature of each primer set (see Supplementary Table S1) for 25 s, and 72°C for 72°C for 3 min. Nested PCR products were electrophoresed on 2% agarose gel, and the amplified DNA fragments were purified and then bidirectionally sequenced using an ABI prism 3730 Sequencer (Applied Biosystems). The procedure of CGH was based on Kallioniemi’s method with minor modifications (15, 16). For each specimen with available paraffin blocks, four 30-μm tissue sections were cut from the same six malignant and eight benign D-TSGCTs subjected to real-time reverse transcription-PCR assay. Tissue sections were dewaxed in xylene, washed with absolute ethanol and allowed to air-dry, and then digested overnight at 55°C with 0.5 mg/mL of proteinase K solution (Sigma). The probe mixture was denatured at 70°C for 5 min and hybridized to metaphase slides at 37°C for 2 days. Hyridization and dehybridization were repeated twice with intermediate washes at 45°C in a humid chamber. The probe mixture was then washed in 4°C for 5 min, 7°C for 7°C with 2× SSC solution at 45°C and room temperature for 10 min each, and then in PN buffer at room temperature for 10 min. After air-drying, the slides were counterstained with 4′,6-diamidino-2-phenylindole in an antifading solution (200 ng/mL in 2× SS, H-1000; Vector Laboratories). Images from representative metaphase spreads were acquired by an Olympus fluorescence microscope (BX51) adapted to a Sensys CCD camera (Kodak KAF 1400 chip; Photometrics) and digitalized using a CytoVision imaging system (Applied Imaging). Karyotypes from 12 to 15 metaphases were combined to generate a mean CGH ratio profile for each test sample. Chromosomally imbalanced alterations were determined based on the calculation of standard reference intervals using the software package Cytovision High-Resolution CGH software, by which we could define DNA losses or gains as significant whenever the tumor profile and the standard reference interval profile at 99.5% confidence did not overlap (22). However, short chromosomal segments with a test-to-reference fluorescence ratio of >1.5 were considered as showing high-level amplification.

**Results**

**Clinicopathologic findings and follow-up.** The salient clinicopathologic features and follow-up information of six malignant D-TSGCTs are summarized in Table 1. The age at diagnosis of malignant D-TSGCTs, including one intra-articular and five extra-articular lesions, ranged from 46 to 78 years (mean, 59.7) in two male and four female patients. Six cases were 5 to 17 cm (mean, 10 cm) in size and located either within or near large joints of the extremities. The detailed radiological, histomorphological, and follow-up data of these six tumors were elaborated elsewhere (7). In the four de novo malignant D-TSGCTs, typical benign areas (Fig. 1B) were...
concurrent with frank sarcomas of various morphologic types, including the giant cell tumor–like histology in one case (Fig. 1C) and the malignant fibrous histiocytoma–like (Fig. 1D) pattern in three cases. Two malignant D-TSGCTs were metachronous in tumor evolution, and fibrosarcomatous (Fig. 1E) and multinodular myxosarcomatous (Fig. 1F) areas appeared only in the first (case no. 1) and fourth (case no. 3) recurrences. After the diagnosis of malignancy, one patient subsequently developed local recurrences twice and was treated with surgical re-excision. Metastatic disease was noted in three patients, one with lumbar vertebrae involvement, another with both multiple axillary lymph node and lung metastases, and a third with inguinal lymph node involvement. The patient with distant metastasis to the spine eventually died of disease.

**Findings of FISH and ISH.** Distinct CSF1 RNA expression was present in a subset of pleomorphic and/or spindle sarcoma cells (Fig. 1G; Table 1) in five of six malignant D-TSGCTs tested for ISH assay. One case (case no.3) also harbored rearranged CSF1 gene (Fig. 1G; Table 1) as detected by the locus-specific, split-apart FISH probe. In addition, ISH also showed expression of CSF1 receptor mRNA in the majority of both sarcomatous and heterogeneous inflammatory cells (data not shown).

**Immunohistochemical expression of cell cycle regulators.** The immunohistochemical findings of cell cycle regulators are shown in Fig. 2 and tabulated in Supplementary Table S2. When compared with 24 benign controls, cyclin A (Fig. 2A-C, P = 0.002), P53 (Fig. 2D-F, P = 0.029), and cyclin E (Fig. 2G-I, P = 0.043) showed significantly higher expression in the six malignant D-TSGCTs, although only cyclin A and P53 could robustly distinguish malignant from benign cases without overlaps in the labeling indices. In contrast, there was no significant difference in the expression of cyclin D1 (Fig. 2J-L), P27 (Fig. 2M-O), and P16 (Fig. 2P-R) between the two groups. Based on these findings, we further specifically explored the potential molecular abnormalities underlying overexpression of both cyclin A and P53.

**Molecular assays.** Real-time reverse transcription-PCR measurement of cyclin A mRNA could be successfully determined with sufficient RNA yields in five of six malignant D-TSGCTs and in seven of eight benign lesions tested. As shown in Fig. 3, the normalized relative expression copies of cyclin A mRNA were 3.25-fold more abundant (P < 0.001) in malignant D-TSGCTs (mean, 11.1649; range, 6.6576-14.3452) compared with those detected in the benign counterparts (mean, 3.4409; range, 0.9138-7.2854). This result suggested that up-regulated transcriptional activity could translate into overexpression of cyclin A protein, whereas the possibility of CCNA2 gene amplification as a more upstream oncogenic alteration could not be excluded.

Using LCM to isolate pure sarcoma cells (Fig. 4A-C), we found that there was no intragenic mutation in exons 5 to 9 of the TP53 gene in any of the six malignant D-TSGCTs sequenced bidirectionally. Furthermore, distinct overexpression of wild-type P53 protein was also substantiated in these malignant D-TSGCTs by immunohistochemistry with a specific Ab-5 antibody that does not cross-react to mutant P53 (Fig. 4A-D). These findings indicated that alternative mechanisms, instead of hotspot mutations, might operate in malignant D-TSGCTs to result in accumulation of wild-type P53 protein.

Among cases subjected to CGH, chromosomal imbalanced alterations were identified in six malignant D-TSGCTs but not in any of the eight benign lesions. Chromosomal losses were predominant over gains in these six malignant cases as illustrated in Fig. 5. The mean value of changes per tumor was 10.67 (losses, 9.83; gains, 0.83), whereas no whole chromosome aberration was discerned. DNA gains were detected in three cases and involved only three chromosomal regions. Of note, high-level amplifications of 3p12 were seen in two cases. As for DNA losses, the frequently affected chromosome arms were 15q in five cases and 2p, 9q, 16p, and 22q in three cases each. In all five malignant cases showing aberrations of 15q, 15q22-24 was found to be the minimal overlapping region of chromosomal deletion in four cases.

**Discussion**

Great histomorphologic variation can be present in the sarcomatous areas of malignant D-TSGCT, which is a distinct sarcoma entity with considerable mortality and metastatic potential, including a preference of spreading to regional lymph nodes (7). However, there have been no studies thus far reporting specific molecular aberrations to portend malignant transformation of benign D-TSGCTs. Given that the separation of benign from malignant D-TSGCTs can be challenging on a purely morphologic basis (1, 3, 5, 7–9), our goals were to identify molecular determinants whose genetic

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**Table 1. Clinicopathologic features of six malignant D-TSGCTs and results of translocation and expression of CSF1 (Cont’d)**

<table>
<thead>
<tr>
<th>Necrosis</th>
<th>Treatment</th>
<th>Recurrence or metastasis (duration)</th>
<th>Follow-up/status</th>
<th>CSF1 rearrangement (ISH)</th>
<th>CSF1 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>Tumor excision</td>
<td>Spinal metastases (9 mo)</td>
<td>10 mo/DOD</td>
<td>Absent</td>
<td>Positive</td>
</tr>
<tr>
<td>Present</td>
<td>Tumor excision</td>
<td>Local recurrences, twice (12, 27 mo)</td>
<td>36 mo/NED</td>
<td>Absent</td>
<td>Positive</td>
</tr>
<tr>
<td>Present</td>
<td>Amputation and R/T</td>
<td>Axillary nodal metastases (11 mo); pulmonary metastasis (22 mo)</td>
<td>17 mo/AWD</td>
<td>Present</td>
<td>Positive</td>
</tr>
<tr>
<td>Present</td>
<td>Wide tumor excision</td>
<td>None</td>
<td>12 mo/NED</td>
<td>Absent</td>
<td>Positive</td>
</tr>
<tr>
<td>Present</td>
<td>Tumor excision</td>
<td>None</td>
<td>8 mo/NED</td>
<td>Absent</td>
<td>Positive</td>
</tr>
<tr>
<td>Present</td>
<td>Wide tumor excision and R/T</td>
<td>Inguinal nodal metastases (9 mo)</td>
<td>10 mo/NED</td>
<td>Absent</td>
<td>Negative</td>
</tr>
</tbody>
</table>
changes and/or expression may aid in differential diagnosis, prognostication, and perhaps, treatment decision. The present study shows that similar to its benign counterpart, the involvement of CSF1 is frequently detected in malignant D-TSGCTs. The alterations in the expression of P53 and cyclin A as well as the deletion at 15q22-24 are uniquely implicated in the sarcomatous transformation of D-TSGCTs.

The finding that five of our six malignant D-TSGCTs exhibited CSF1 mRNA expression was generally in keeping with the frequency seen in benign TSGCTs from prior reports (11, 17). This suggests a pathogenetic role of deregulated CSF1 overexpression in the early stage of TSGCTs that may progress to malignant tumors if additional molecular aberrations were superimposed on the sustained CSF1-CSF1R tyrosine kinase pathway activity. The high levels of CSF1 expression are known to link with the chimeric fusion between COL6A3 and CSF1 genes (11), although this translocation does not seem to be universally present in each case (11, 17). Nevertheless, we are not yet clear about whether the lower frequency (1/6) of CSF1 translocation in malignant TSGCTs was ascribed to the small sample size or whether it might reflect a more frequent occurrence of unrecognized alternative mechanisms leading to CSF1 overexpression (23).
Deregulated cell growth is the most fundamental attribute of tumor development and progression. As a cyclin species with multiple roles in the cell cycle, the expression of cyclin A is not only involved in S phase progression, G2-M phase transition, and initiation of mitosis but is also closely linked to the cell proliferation rate (24). Cyclin A overexpression has been proven to correlate with tumor progression and/or adverse outcomes in a variety of cancers, including soft tissue sarcomas (24, 25). In this study, we have shown the overexpression of cyclin A protein in malignant D-TSGCTs with an average labeling index that was significantly higher and showed no overlap in the distribution range compared with benign lesions. Moreover, the abundance of cyclin A mRNA was also significantly increased in malignant D-TSGCTs, suggesting that cyclin A expression is primarily regulated at the transcriptional and/or genomic level, rather than through a posttranscriptional mechanism mediating protein degradation. Specifically for translocation-associated sarcoma, increased expression of cyclin A in synovial sarcomas was found to be associated with the SYT-SSX1 fusion type, a variant subtype conferring higher proliferative activity (26). It seems tempting to speculate that the COL6A3-CSF1 gene fusion, or some yet unknown but functionally similar genetic variation, could differentially affect the cell cycle machinery, thereby modulating the transcription of cyclin A in D-TSGCTs.

The expression of P53 protein is mainly regulated in the posttranscriptional stage and maintained at a low level with a short half-life in unperturbed cells (27, 28). In response to various cellular stresses, such as DNA damage and mitogenic signaling, a prompt increase in the intracellular level of functional P53 protein is required for the prevention of genomic instability and oncogenic transformation (27, 28). In this series, the apparent P53 protein overexpression detected by DO-7 antibody suggested that stabilization of either wild-type or mutant protein preferentially occurs in malignant D-TSGCTs but not in benign lesions. Nevertheless, we could not detect intragenic mutations in hotspot exons 5 to 9 of the TP53 gene in pure sarcoma cells of any malignant D-TSGCT.

Recently, Das et al. showed that soft tissue sarcomas might have a higher prevalence of TP53 exon 4 mutations than previously thought (29). Accordingly, the possibility of mutations outside the classical core-binding domain of TP53 could not be completely excluded in malignant D-TSGCTs (27, 28, 30). However, the strong staining with the wild-type–specific Ab-5 antibody in pleomorphic sarcoma cells corroborates our sequencing results, indicating that the P53 protein in malignant D-TSGCTs is most likely wild-type and possibly accumulates via alternative mechanisms leading to protein stabilization (18). Indeed, there are many human cancers without mutations in

Fig. 3. Representative curves of real-time PCR quantification for cyclin A mRNA expression in benign (A) and malignant (B) TSGCTs. The normalized relative abundance of cyclin A mRNA (C) were significantly higher (P < 0.001) in malignant TSGCTs than in the benign counterparts.

Fig. 4. LCM was applied to isolate pure sarcoma cells for mutation analysis of exons 5 to 9 of TP53 gene. The representative images of a malignant D-TSGCT (case no. 5) before (A) and after (B) capture were shown, together with bizarre sarcoma cells of interest in the collection cap (C). All five malignant cases tested showed wild-type TP53 gene by sequencing, which was also immunohistochemically validated by distinct overexpression of wild-type P53 protein using the specific Ab-5 antibody (D).
Fig. 5. Chromosomal imbalanced aberrations as detected by CGH in a representative malignant TSGCT (A, case no. 6) and the summarizing illustration (B) of DNA gains and losses in six malignant TSGCTs tested. Bars, one tumor, with gains on the right and losses on the left of the ideogram of each chromosome.
TP53 genes per se, whereas functionally disabled P53 protein is accumulated because of alterations in other proteins modulating its turnover and activity. Despite the complexity of regulatory networks, this process is best understood with regard to the deregulation of the upstream effectors in the P53 tumor suppressor pathway, such as inhibition of MDM2 and/or P14ARF activation (18, 31). The latter two events can consequently confer more aggressive behavior in transformed cells, as exemplified in both animal models and human cancer specimens (18, 31).

Chromosomal abnormalities point toward genes implicated in malignant transformation and disease progression (15, 16, 32). In this series, no imbalances were detected in any benign D-TSGCT by CGH, which, in keeping with the previous report (6), was most likely overshadowed by the diluted artifact of abundant inflammatory cells. In contrast, the sarcomatous areas of all six malignant lesions displayed varying numbers of chromosomal aberrations, predominantly in the form of chromosomal losses. The deletion at 15q, as detected in five cases, represented the most frequent non–random chromosomal alteration in malignant D-TSGCTs. Unlike hematologic malignancies, this aberration was only rarely documented by CGH in a few types of sarcomas, such as gastrointestinal intestinal tumors (33), endometrial stromal sarcomas (34), and osteosarcomas (35). Of special note, there were several P53-interacting genes located within the 19.3-Mb region of a common deletion at 15q22-24, such as RPS27L (15q22.2; ref. 36), PIAS1 (15q22.3; ref. 37), PML (15q22; ref. 38), and SIN3A (15q24.2; ref. 39). Among these, PML is an established tumor suppressor gene critical in the formation of the PML nuclear body by recruiting P53 (38, 40, 41), whereas RPS27L, encoding a novel ribosomal protein, may represent another candidate (36). More intriguingly, sequences in the intron 1 region of both genes can be bound by P53 to mediate its proapoptotic and/or antiproliferative functions upon cellular stresses (36, 40, 41). In this context, we postulate a model wherein oncogenic signaling abnormality (e.g., COL6A3-CSF1 gene fusion) may initially drive the development of benign D-TSGCT and elicit cellular responses mediated by wild-type P53. Nevertheless, sarcomatous transformation may arise once one or more P53-interacting tumor suppressor gene(s) within 15q22-24 is deleted, thereby compromising the tumor-suppressing function of P53.

In summary, we have, for the first time, provided evidence of CSF1 involvement in malignant D-TSGCTs and described potential molecular markers to help differentiate malignant from benign lesions. As compared with benign lesions, alterations in cyclin A, P53, and chromosome arm 15q are present in the majority of malignant D-TSGCTs, representative of critical events in sarcomatous transformation. Cyclin A overexpression in malignant D-TSGCTs can be ascribed to high levels of cyclin A transcripts. Instead of intragenic mutations in hotspot exons, P53 overexpression is likely to be caused by alternative mechanisms leading to the stabilization of wild-type P53 protein. Furthermore, frequent deletions at 15q22-24 suggest the presence of candidate tumor suppressor gene(s) in this region, functional abrogation of which may render malignant phenotypes of this rare entity. To test this hypothetical model of malignant progression, additional studies are required with a large series of benign and malignant D-TSGCTs to further delineate the deleted tumor suppressor gene(s) of pathogenetic relevance within 15q22-24.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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