Monoclonality of Multifocal Myxoid Liposarcoma: Confirmation by Analysis of $\text{TLS-CHOP}$ or $\text{EWS-CHOP}$ Rearrangements


ABSTRACT

Multifocal presentation, defined as the presence of tumor at two or more anatomically separate sites, before the manifestation of disease in sites where sarcomas usually metastasize (e.g., lungs) occurs in about 1% of extremity soft tissue sarcomas (STSs). Debate still persists whether multifocal STSs represent an unusual pattern of metastasis or multiple separate primary tumors. Among STSs with multifocal presentation, myxoid liposarcoma is the predominant histological type. This subtype of liposarcoma contains the specific t(12;16) chromosomal translocation, which results in rearrangement of the $\text{TLS}$ and $\text{CHOP}$ genes that is clone specific at the DNA level. We, therefore, sought to address the question of clonality by molecular analysis in six patients who presented with either synchronous or metachronous multifocal myxoid liposarcoma. In all six cases, adequate frozen tumor was available for DNA extraction from at least two distinct anatomical sites. Southern blot analysis using $\text{CHOP}$, $\text{TLS}$, and $\text{EWS}$ cDNA probes was performed on genomic DNA. Five cases contained a $\text{TLS-CHOP}$ rearrangement, and one case had the variant $\text{EWS-CHOP}$ fusion (seen in $<5\%$ of cases). The size of the rearranged $\text{CHOP}$ fragment differed among the six patients, as expected, but was identical in all anatomically separate tumor samples from each patient. Likewise, the sizes of the rearranged bands observed with either the $\text{TLS}$ or $\text{EWS}$ probes supported the monoclonality of all cases. Our results confirm the monoclonal origin of multifocal myxoid liposarcoma, establishing the metastatic nature of distant soft tissue lesions in these cases. It remains unclear whether this unusual pattern of metastasis represents an intrinsic property of this subset of myxoid liposarcoma or merely a rare chance occurrence. The clinical outcomes observed in this small series suggest that the prognosis of multifocal myxoid liposarcoma is poor, regardless of its often bland or “low-grade” histological appearance.

INTRODUCTION

Multifocal STS is a rare and controversial entity, accounting for about 1% of extremity STS (1). As a general rule, multicentricity in STS is defined as the presence of sarcoma at two or more anatomically separate sites, before the manifestation of disease in sites where STSs most commonly metastasize, such as the lungs (1, 2). On the basis of clinical or pathological data alone, it is difficult to determine in any specific case whether a tumor arising in a site unusual for metastasis, but common for a primary tumor, is a metastasis or a second primary tumor of the same histological type. The first reported case of multifocal or multicentric STS dates to 1934, when Siegmund (3) described a patient with multiple fatty tumors, which was interpreted as “Lipoblastische Sarkomatose” or a systemic malignant disease of the soft tissue. Since then, fewer than 50 cases have been reported in the literature, and debate still persists as to whether this entity represents separate primary tumors or is simply an unusual pattern of metastasis (4–10).

A recent study from our institution investigating the prevalence of multifocal sarcoma in a large cohort of extremity STSs identified liposarcoma as the predominant histological type (9 of 16 cases; Ref. 1). Although not stated in the report (1), these were all myxoid liposarcomas. Myxoid liposarcoma is the most common subtype of liposarcoma, accounting for more than half of all cases (2). It has a tendency to recur locally, and about one-third of patients develop distant metastases (11). A proportion of cases shows histological progression to round cell histology, significantly associated with a poor prognosis (12). The cytogenetic hallmark of myxoid liposarcoma is the t(12;16)(q13;p11), which appears highly specific for this tumor type and is present by conventional cytogenetics in >85% of the cases. The translocation leads to the fusion of the $\text{CHOP}$ and $\text{TLS}$ (also called $\text{FUS}$) genes at 12q13 and 16p11, respectively, and the generation of a $\text{TLS-CHOP}$ hybrid gene encoding an aberrant transcriptional regulator (13, 14). We have recently confirmed the strong specificity of the $\text{TLS-CHOP}$ rearrangement for the entity of myxoid/round cell liposarcoma (15). In four cases of myxoid liposarcoma, a variant chromosomal translocation has been described, t(12;22), resulting in an $\text{EWS-CHOP}$ fusion (16, 17).

As in other rearrangements producing specific gene fu-
TLS probes were partial cDNAs, derived by restriction enzyme digestion in specific introns of the genomic breakpoints of the t(12;16) are widely dispersed in one tumor to the next. Using the Southern blot pattern of genomic rearrangements in TLS (or EWS) and CHOP as clone-specific markers, we studied multiple tumors from six patients with multifocal myxoid liposarcoma to distinguish true multifocal (multiclonal) sarcoma from a metastatic (monoclonal) sarcoma. As part of this study, we also report the fifth case of myxoid liposarcoma with the variant EWS-CHOP gene fusion.

**MATERIALS AND METHODS**

**Study Group and Demographic Data.** Among 43 consecutive cases of myxoid liposarcoma operated at Memorial Sloan-Kettering Cancer Center that had confirmatory molecular evidence of CHOP gene rearrangement, we identified seven patients (16%) who presented with either synchronous or metachronous multifocal myxoid liposarcoma. Adequate frozen tumor for DNA extraction was available from at least two distinct anatomical sites in six of these seven cases. The seventh case had material only from a single site and was, therefore, uninformative for the present analysis. Among the six patients further analyzed, there were four male and two female patients, and their ages ranged from 25–68 years (mean, 53).

**Southern Blot Analysis.** Sixteen tumor samples from different anatomical sites were available for DNA extraction from these six multifocal myxoid liposarcoma cases (two samples in four cases, three samples in one case, and five samples in one case). DNA was isolated from snap-frozen tissue stored at −70°C using a standard organic extraction protocol. In 15 of 16 tumor samples the extracted DNA was adequate for analysis. Genomic DNA was digested with appropriate restriction enzymes and separated by 0.7% agarose gel electrophoresis, transferred onto nylon membranes, and hybridized with radiolabeled probes. BamHI and SacI restriction enzymes were used for the hybridization with CHOP probe; BglII and PstI for TLS probe; and EcoRI, BamHI, and HindIII for EWS probe. The CHOP and TLS probes were partial cDNAs, derived by restriction enzyme digestion of a full-length TLS-CHOP cDNA clone (LPS41; a gift from D. Ron, New York University Medical Center, New York, NY; Ref. 13). The CHOP probe consisted of a 753-bp PstI-XhoI fragment corresponding to exons 3 and 4. This probe detects essentially all CHOP rearrangements in genomic DNA digested with BamHI or SacI (18, 19). The TLS cDNA probe was a 780-bp XbaI-BglII fragment including exons 1–6 of TLS. According to restriction enzyme site analysis of the complete genomic sequence of TLS (GenBank accession no. AF071213; Ref. 20), this probe covers the entire TLS break point region in BclI-digested DNA. The EWS probe was a 741-bp PCR-generated partial cDNA probe that hybridizes to exons 6–12 of EWS, covering in EcoRI and HindIII-digested DNA the entire genomic break point cluster region, as described in detail elsewhere (21).

**RT-PCR Analysis.** The case showing EWS rearrangement was further tested for the presence of an EWS-CHOP chimeric transcript by RT-PCR. Three micrograms of total RNA was reverse transcribed (Superscript II; Life Technologies, Inc.) using random hexamers, and the cDNA was subjected to PCR using a forward primer in exon 7 of EWS (5′-CTGGATCTTAACGCCAACGTCCAAG-3′) and a reverse primer in exon 3 of CHOP (5′-TGTCGGAAGAAAGGCAATG-3'). The RT-PCR product was identified by agarose gel electrophoresis and confirmed by direct automated sequencing.

**RESULTS**

**Histopathological Data and Clinical Course.** Among the six patients with multifocal myxoid liposarcoma, three patients presented with synchronous multifocal disease and three presented with metachronous lesions (Table 1 and Fig. 1). The histopathological findings revealed low-grade myxoid liposarcoma in all anatomical locations examined in two cases, and a high-grade, round cell-type myxoid liposarcoma in at least one of the sites in the remaining four cases (Fig. 2). In five of six patients, the first site to be operated (site 1) revealed low-grade myxoid liposarcoma (Table 1). The number of distinct tumor foci that were clinically evident and surgically removed, excluding local recurrences or lung metastases, ranged from two to six (mean, 4). In one case, the multiple tumors were restricted to the extremities; in the remaining five cases there was also involve-

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age/sex</th>
<th>Site 1</th>
<th>Gene fusion</th>
<th>Other sites</th>
<th>Chronology of multifocal disease</th>
<th>Local recurrence at site 1</th>
<th>Lung mets after diagnosis</th>
<th>Follow-up status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>68/M</td>
<td>Intra-abdominal*</td>
<td>Low</td>
<td>TLS-CHOP</td>
<td>R’ thigh, chest wall,* neck, abd wall,* buttoc</td>
<td>Mc - 14 months</td>
<td>10 months</td>
<td>46 months</td>
</tr>
<tr>
<td>2</td>
<td>28/M</td>
<td>Thigh</td>
<td>Low</td>
<td>TLS-CHOP</td>
<td>Chest wall,* neck, abd wall,* buttoc</td>
<td>Mc - 14 months</td>
<td>10 months</td>
<td>46 months</td>
</tr>
<tr>
<td>3</td>
<td>60/M</td>
<td>Thigh*</td>
<td>Low</td>
<td>TLS-CHOP</td>
<td>Retropertioneum*</td>
<td>Mc - 67 months</td>
<td>42 months</td>
<td>DOD 120 months</td>
</tr>
<tr>
<td>4</td>
<td>51/M</td>
<td>R thigh/R femur</td>
<td>Low</td>
<td>TLS-CHOP</td>
<td>L femur*</td>
<td>Sy</td>
<td>7 months</td>
<td>DOD 51 months</td>
</tr>
<tr>
<td>5</td>
<td>33/F</td>
<td>R axilla*</td>
<td>Low</td>
<td>EWS-CHOP</td>
<td>R thigh,* L arm,* sternum, intra-abdominal*</td>
<td>Mc - 6 months</td>
<td>Sy</td>
<td>DOD 6 months</td>
</tr>
<tr>
<td>6</td>
<td>25/F</td>
<td>L axilla*/L breast</td>
<td>High</td>
<td>TLS-CHOP</td>
<td>R flank, L thigh,* R thigh, abd. wall, intra-abdominal*/ small bowel</td>
<td>Sy</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Samples analyzed for TLS-CHOP or EWS-CHOP rearrangements.
* Right, L, left; Mc, metachronous; Sy, synchronous; DOD, dead of disease; AWD, alive with disease.
ment of the trunk and intra-abdominal sites (Table 1). Three patients developed local recurrences at the primary site (the anatomical site at which the sarcoma was initially diagnosed).

Five patients died of disease, and one (patient 4) was alive with disease 12 months after diagnosis. As per the definition of multifocal STS and the study criteria, none of the patients had lung metastases at diagnosis. Indeed, only one patient (2) developed pulmonary metastases, which became evident 46 months after diagnosis. After his initial presentation, but prior to the lung metastases, patient 2 developed myxoid liposarcoma in multiple additional foci, including chest wall, at which times imaging studies also failed to detect lung metastases. In four patients, lung metastases were detected neither at presentation, nor at the time of disease-related death. The remaining patient (4) was alive with nonpulmonary disease 12 months after diagnosis.

Analysis of \textit{CHOP}, \textit{TLS}, and \textit{EWS} Rearrangements.

In all 15 tumor samples with adequate DNA, available from these six patients with multifocal myxoid liposarcoma, \textit{CHOP} rearrangement was detected by Southern blotting, using either \textit{Bam}H\textsubscript{I} or \textit{Sac}I digestion. The size of the rearranged bands from multifocal tumors in each individual patient was identical (Fig. 3). Rearranged bands were identified with the \textit{TLS} probe in 11 tumor samples from five patients, using \textit{Bcl}I digestion, and the size of these bands was also constant in different samples from each patient (Fig. 4). Tumor samples from patient 5 showed only germline configuration of \textit{TLS}. Because \textit{EWS} has been reported as an alternative translocation partner for \textit{CHOP} in rare cases of myxoid liposarcoma, we examined \textit{EWS} in this case (four samples), using an \textit{EWS} cDNA probe. All four tumor samples from patient 5 showed a rearranged \textit{EWS} band of equal size, in \textit{Hind}III-digested tumor DNA (Fig. 5).

\textbf{DISCUSSION}

The \textit{CHOP} gene, a member of leucine zipper transcription factor family, is implicated in adipocyte differentiation and growth arrest (reviewed in Ref. 22). In myxoid liposarcoma, the 3’ end of the \textit{CHOP} gene is fused to the 5’ portion of \textit{TLS} (for translocated in liposarcoma; Ref. 14), also known as \textit{FUS} (for fusion; Ref. 13). The hybrid gene encodes a protein that consists of the 5’ portion of \textit{TLS} fused to the entire coding region of \textit{CHOP}. The TLS-CHOP protein is thought to function primarily
Fig. 3 Monoclonality of CHOP rearrangements by Southern blot analysis. The normal germline band pattern seen in the control lane containing genomic placental DNA (Lane PL), consists of two bands, of 2.1 and 9.4 kb, in SauI-digested DNA. Each lane contains DNA from a separate site in each patient (case 4: right thigh and left femur; case 5: right axilla, right thigh, left arm, and intra-abdominal; case 2: abdominal wall and chest wall). The rearranged CHOP bands are different in each patient but identical in all sites from each case.

Fig. 4 Monoclonality of TLS rearrangements in case 6. The normal germline band pattern seen in the control lane containing DNA from a well-differentiated (nonmyxoid) liposarcoma (Lane C) consists of a single 9.0-kb band in BstIII-digested DNA. Three DNA samples from separate sites in case 6 (intra-abdominal, left axilla, and left thigh) all showed the same rearranged TLS band.

Fig. 5 Monoclonality of EWS rearrangements in case 5. The normal germline band pattern seen in the control lane containing genomic placental DNA (Lane PL) consists of three bands in HindIII-digested DNA. Case 5 contained an EWS rearrangement, absent in three other liposarcomas (left). Four DNA samples from separate sites in case 5 (right axilla, right thigh, left arm, and intra-abdominal) all showed the same rearranged EWS band (right). RT-PCR and sequencing confirmed an EWS-CHOP fusion transcript in this case (see “Results”).

Numerous studies using cytogenetic or RT-PCR-based detection of the t(12;16) have confirmed the concept that round cell liposarcoma represents the high-grade form of myxoid liposarcoma (26–29). In the present study, the initial histopathology (at site 1) was low-grade myxoid liposarcoma in five patients, three of whom showed high-grade (round cell) histology at other synchronous or metachronous sites (Table 1). Anecdotally, the clinical outcome of these five patients seems as poor as that of patients with exclusively high-grade myxoid or round cell liposarcoma, suggesting that the behavior of “low-grade” myxoid liposarcomas with TLS-CHOP may be closer to that of “high-grade” myxoid/round cell liposarcomas than to other types of low-grade liposarcoma. Older clinical studies of liposarcoma have found both grade and histological type to be prognostically significant (11), but a systematic analysis of the prognostic significance of tumor grade in myxoid/round cell liposarcoma in a uniformly managed series of cases defined by the presence of TLS-CHOP remains to be done.

In our study group, we found that 16% of patients with myxoid liposarcoma presented with either synchronous or metachronous multifocal tumors. This prevalence may be a slight overestimate because patients with multiple tumors may have been more likely to have tumor available for molecular studies. Other authors place the prevalence of apparent multifocal disease in liposarcoma at approximately 10% (2). As discussed above, in patients with multifocal myxoid liposarcoma, tumor foci from different anatomical locations may have different histological grades (e.g., low-grade myxoid versus high-grade round cell). Therefore, the clonality of multifocal disease cannot be deduced from histological features alone. In addition, the clinical details of our group of cases, with only one patient developing pulmonary metastases, and this only after 4 years from presentation with multifocal disease, were also not helpful in resolving the issue of monoclonal metastatic versus multiclonal multifocal disease. Blair et al. (1) attempted to address this question by comparing the survival of the patients present-
ing with multifocal STS with patients having solitary tumors with lung metastases. The authors found that the two groups of patients have a similar clinical outcome and consequently concluded that multifocal STS should be treated as advanced-stage disease (1).

Clonality has been investigated by molecular approaches in various synchronous or metachronous multifocal solid tumors, including, among others, carcinomas of the breast (30), lung (31), liver (32), bladder, head and neck (33, 34), ovary and endometrium (35), ovary and appendix (36, 37), and contralateral ovaries (35), as well as in leiomyosarcomas arising in immunocompromised individuals (38, 39), gastrointestinal and contralateral ovaries (35), as well as in leiomyosarcomas (40), and Kaposi’s sarcomas (41). The genetic markers used have included analysis of X inactivation (30, 34, 40, 41), specific point mutations (e.g., in p53, K-ras, and MEN1; Refs. 31, 35–37, and 40), viral integration (32), viral episome structure (38, 39), patterns of allelic loss (34, 40), and numerical or structural chromosome abnormalities (33). Certain clonal markers, due to their limited number of possible values or states (e.g., X inactivation, allelic loss), may lack the statistical power to convincingly confirm or exclude clonal relationships between separate tumors. In contrast, chromosomal translocations resulting in fusion products provide, at the genomic level, perhaps the most powerful idiotypic clonal marker. In these translocations, the genomic breaks occur within introns separating more proximal and distal exons encoding the functional domains of the respective genes, the expression and juxtaposition of which is oncogenic in a specific cell type. Within these introns, the distribution of breakpoints from different cases seems either random or shows loose clustering at one or more putative recombiningogenic elements. This accounts for the essentially idiotypic specificity of these rearrangements, especially when both translocation partners are examined. A detailed analysis of 11 myxoid liposarcomas with the TLS-CHOP rearrangement found a wide distribution of genomic breakpoints across the involved introns, with minimal clustering at one ALU site in TLS intron 5 (42). Genomic breakpoints in EWS, thus far studied only in Ewing’s sarcomas with EWS-FLI1, seem essentially random (43). This provides formal support for the use of these rearrangements to establish clonal relationships in multifocal myxoid liposarcoma.

Only one previous study has attempted to address the issue of multicentric myxoid liposarcoma using a molecular approach. Schneider-Stock et al. (44) reported a patient with multicentric myxoid liposarcoma, in whom they detected by RT-PCR the same uncommon type of TLS-CHOP fusion transcript (i.e., “type III”: fusion of TLS exon 8 to CHOP exon 2) in a local soft tissue recurrence and in one distant site (44). Another, more speculative hypothesis of the origin of multifocal myxoid liposarcoma should be mentioned. If the TLS-CHOP rearrangement is necessary but not sufficient for the development of this tumor, it would be conceivable that the progeny of an adipose precursor cell in which the rearrangement had occurred could populate multiple areas and result, after independent putative second genetic “hits,” in the formation of anatomically independent tumors containing the same TLS-CHOP rearrangement. This hypothesis would also suggest the presence of residual nonneoplastic fat cells bearing the TLS-CHOP rearrangement in the vicinity of the tumors. We find no evidence for this model of myxoid liposarcoma, insofar as a Southern blot analysis of CHOP in peritumoral normal adipose tissue in three cases (two “unifocal” and one multifocal, not included in the present series) is negative.5

Our present analysis of the genomic rearrangements of TLS, CHOP, or EWS in six patients confirms the monoclonal origin of multifocal myxoid liposarcoma. This unusual clinical phenomenon most likely represents a pattern of presumably hematogenous metastasis to other soft tissue sites, by tumor cells seemingly incompetent to seed the lungs. It remains unclear whether this pattern of early metastasis to other soft tissue sites (and late or absent lung metastases) represents an intrinsic property of this subset of myxoid liposarcomas or merely a rare chance occurrence.

ACKNOWLEDGMENTS

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