Analysis of FUS-CHOP Fusion Transcripts in Different Types of Soft Tissue Liposarcoma and Their Diagnostic Implications

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ABSTRACT

In myxoid and round cell liposarcomas, a specific chromosomal translocation [(12;16)(q13;p11)] results in the expression of chimeric fusion transcripts encompassing parts of the FUS gene (16p11) at their 5′ ends and the CHOP gene (12q13) at their 3′ ends. Using a reverse transcription-PCR protocol, we determined the prevalence of FUS-CHOP fusion transcripts in a series of liposarcoma samples. Fusion transcripts were detected in 13 of 30 biopsy samples from soft tissue liposarcomas. Expression of fusion transcripts was not restricted to myxoid and round cell liposarcomas, as suggested previously; it was also detected in 1 of 3 well-differentiated and 4 of 14 pleomorphic liposarcomas. Sequence analysis revealed four different FUS-CHOP fusion transcript variants, two of which have not been described before. Furthermore, using FUS-CHOP fusion transcripts as targets in reverse transcription-PCR assays, we detected disseminated tumor cells in peripheral blood or bone marrow in 3 of 5 patients undergoing surgery for soft tissue liposarcoma.

INTRODUCTION

Specific chromosomal translocations resulting in gene fusions have long been recognized as important events in the oncogenesis of hematopoietic malignancies, particularly chronic myeloid leukemia (1–3). In solid tumors, however, stable chromosomal translocations appear to be mainly restricted to various soft tissue sarcomas (4). Cytogenetically, a stable translocation [(12;16)(q13;p11)] was frequently observed in myxoid and round cell liposarcomas. A more detailed analysis of the fusion locus revealed that the gene which encodes the transcription factor CHOP was fused to an, at that time, unknown gene which was later referred to as FUS (fused in liposarcoma, also known as TLS; Ref. 5). Subsequently, two groups independently identified clones that included CHOP sequences in cDNA libraries derived from liposarcoma samples. The sequence analysis of these clones revealed that the 5′ part of the FUS gene (exons 1–7) is fused to CHOP sequences (exons 2–4; Refs. 6 and 7). The CHOP gene, which is also referred to as GADD153, encodes a transcriptional regulator of the CCAAT/enhancer binding protein family (8). As a member of the leucine zipper transcription factor family, CHOP is implicated in adipocyte differentiation and growth arrest (9, 10). The physiological function of the FUS gene in normal cells is not yet clear. However, recent work suggests that FUS sequences expressed in fusion transcripts in liposarcomas display DNA-binding activities and might act as transcriptional activator (11).

In myxoid and round cell liposarcomas, FUS-CHOP fusion transcripts are expressed in different structural variants (Fig. 1). These are generated through splicing of exon 5, 7, or 8 of the FUS gene to exon 2 of the CHOP gene (5–7, 11–15). Translation of wild-type CHOP transcripts starts in exon 3, whereas in the fusion gene, translation of the fusion transcript results in chimeric proteins, including peptide fragments encoded by CHOP exon 2 (6, 13–15; Fig. 1). Several lines of evidence point to a possible oncogenic activity of the fusion gene product. Expression of the FUS-CHOP fusion gene (Fig. 1, transcripts I and II) in NIH 3T3 cells confers release from contact inhibition, anchorage-independent growth in soft agar, and tumor formation in nude mice (16, 17). The wild-type CHOP gene product interferes with G1-S cell cycle progression (18). In contrast, the FUS-CHOP gene product does not induce growth arrest at the G1-S checkpoint in NIH 3T3 cells, but it negatively interferes with the cell cycle regulatory activity of the wild-type CHOP gene product (18). Taken together, these observations suggest that the generation of FUS-CHOP fusion transcripts represents a critical event in the oncogenesis of an abundant subset of liposarcomas.

RT-PCR amplification of fusion transcripts generated through specific chromosomal translocations has been used for the detection of MRD in hematopoietic malignancies. Various studies suggest a direct correlation between the amount of residual tumor cells and prognosis for these patients (2, 3). Dissemination of tumor cells in the PB or BM has also been

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3 The abbreviations used are: RT-PCR, reverse transcription-PCR; MRD, minimal residual disease; PB, peripheral blood; BM, bone marrow.
observed in patients with solid cancers, even in the absence of clinical detectable metastatic disease (19–21). A considerable number of soft tissue sarcoma patients develop local recurrence and/or distant metastases even after radical surgical resection, indicating that tumor cell dissemination occurred either before or during surgical extirpation of the primary tumor. Detection of disseminated tumor cells before, during, or after surgery, therefore, might have an important prognostic impact for patients.

Here, we analyzed the prevalence of different \textit{FUS-CHOP} fusion transcripts in tumor specimens of patients with all histological subtypes of soft tissue liposarcoma. Furthermore, we evaluated the feasibility of \textit{FUS-CHOP} fusion transcripts as markers for MRD in PB or BM samples. We found that the expression of fusion transcripts is not restricted to myxoid and round cell subtypes and indicate that disseminated sarcoma cells can be detected in PB and BM of patients with localized and those with metastatic disease.

\section*{MATERIALS AND METHODS}

\textbf{Patients and Tissue Samples.} Tissue samples were obtained from 30 patients treated for liposarcoma and from 22 patients with nonlipomatous soft tissue sarcomas (leiomyosarcoma, stromal sarcoma, malignant schwannoma, and malignant fibrous histiocytoma) between 1990 and 1997 at the Department of Surgery, University of Heidelberg (Heidelberg, Germany). All tumors were staged according to the recent Union International Contre Cancer classification (22). The histological sections were examined by one of us (G. M.). The tumors were classified according to Enzinger and Weiss (23) and Weiss (24). All patients with liposarcomas treated since October 1996 ($n = 9$) gave informed consent for the parallel examination of pre-, intra-, and postoperative blood samples and BM aspirates. Twenty blood samples from healthy donors and BM aspirates from 10 patients with colon cancer served as negative controls. The study protocol was approved by the local ethics committee.

Tumor samples collected during surgery were immediately shock-frozen in liquid nitrogen and stored at $-80^\circ$C. RNA was extracted from 12–15 consecutive cryosections (thickness, 20 \mu m). Serial control cryostat sections (5 \mu m) were prepared to confirm the histopathology of the analyzed tumor samples and to ensure a relative amount of at least 70% tumor cells in each sample. The liposarcoma cell line 1955-91 (6), which carries a (12;16) translocation, resulting in the expression of a \textit{FUS-CHOP} fusion transcript, served as a positive control and was used for dilution experiments.

\textbf{Blood and BM Samples.} PB (10 ml) was drawn through a central venous catheter (placed in the superior vena cava) pre-, intra-, and postoperatively (24 h after surgery) and diluted 1:2 with PBS. After density centrifugation (30 min at 400 $\times$ g) through Ficoll-Paque (Pharmacia Biotech, Freiburg, Germany), mononuclear PB cells were harvested from the interphase and washed twice in PBS. The cell pellet was subsequently snap-frozen in liquid nitrogen and stored until further use at $-80^\circ$C. Ten ml of BM aspirate were collected from both sides of the superior iliac crest under general anesthesia prior to tumor resection. One half of the sample was used for cytological analysis. The second charge was diluted with 10 ml of PBS, and nucleated cells were harvested through Ficoll-Paque density centrifugation, as described above.

\textbf{RNA Extraction.} Total RNA of tumor samples, peripheral mononuclear blood samples, BM aspirates, and the liposarcoma cell line 1955-91 was isolated using a commercially available RNA isolation kit (Glassmax; Life Technologies, Inc., Eggenstein, Germany). To eliminate contaminating DNA within RNA preparations, samples were digested with RNase free DNase I, as recommended by the supplier (Life Technologies, Inc.; 15 min at 25$^\circ$C).

\textbf{RT-PCR and Oligonucleotide Primers.} One \mu g of total RNA was reverse transcribed (Superscript II; Life Technologies, Inc.) in 20 \mu l of the reaction mixture using either random hexanucleotide primers (40 ng) for the analysis of tumor tissues or oligonucleotide primer FC 2 for MRD analysis. To control for RNA integrity and reverse transcription efficacy, PCRs using glyceraldehyde phosphate dehydrogenase-specific primers served as an internal control (25). Subsequently, 2.5 \mu l (7.5 \mu l for MRD analysis) of the cDNA preparation were used for amplification in a PCR containing 25 pmol of each primer, 200 \mu M of each dNTP, 2.5 units of Taq DNA polymerase (Life Technologies, Inc.), and 1.25 mm MgCl$_2$ in a final volume of 50 \mu l in an Omnipene ThermoCycler (Hybaid; Teddington, United Kingdom). After initial template denaturation (3 min at 95$^\circ$C), amplification was performed using primers FC 1 and FC 2 (Fig. 1).
1) for 20 cycles at 95°C for 30 s, 54°C for 45 s, and 72°C for 1 min and a final extension at 72°C for 5 min. One μl of PCR products (15 μl for MRD analysis) was further amplified in a nested PCR with primers FC3 and FC4 (Fig. 1) under the following conditions: denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 64°C for 45 s, and extension at 72°C for 1 min. Oligonucleotide primers for the amplification of the FUS-CHOP fusion gene were identified based on the FUS-CHOP sequence deposited in GenBank (accession no. S62138). Primer sequences were: FC 1

<table>
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<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
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<td></td>
<td></td>
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<td>+</td>
<td></td>
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</tr>
<tr>
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<tr>
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<td>9</td>
<td>Round cell</td>
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<td>10</td>
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<tr>
<td>11</td>
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<td>PT</td>
<td>+</td>
<td>(+)</td>
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<td>+</td>
<td></td>
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</tbody>
</table>

1955-91

* PT, primary tumor; LR, local recurrence; DM, distant metastasis; +, dominant amplicon; (+), less abundant amplicon.

Table 1 Detection of fusion transcripts in all histological subtypes of liposarcoma

FUS-CHOP fusion transcripts were detected in all histological subtypes of liposarcoma. Liposarcoma cell line 1955-91, harboring t((12;16)(q13;p11)) (6), was used as a control.

RESULTS

Detection of Different FUS-CHOP Fusion Transcripts in Various Types of Liposarcoma. FUS-CHOP fusion transcripts were amplified by a nested RT-PCR protocol. Oligonucleotide primers were selected to permit the amplification of all three previously reported chimeric transcripts (Fig. 1, transcripts I–III; Refs. 13–15). In this study, we analyzed tumor samples displaying all major histological subtypes of liposarcoma. FUS-CHOP transcripts were detected in tumor samples from 13 of 30 patients (43%). Apparently, the expression of FUS-CHOP fusion transcripts was not restricted to myxoid (7 of 12) and round cell liposarcomas (1 of 1). One of 3 analyzed well-differentiated liposarcomas, as well as 4 of 14 pleomorphic liposarcomas, expressed FUS-CHOP transcripts (Table 1). In two patients with recurrent disease, identical fusion transcripts were detected in the primary tumors and in up to three local or distant recurrences, emphasizing the clonal origin of these lesions. All non-lipomatous soft tissue sarcomas (n = 22), including leiomyosarcoma, stromal sarcoma, malignant schwannoma, and malignant fibrous histiocytoma, tested negative for FUS-CHOP transcripts.

Nucleotide sequencing of all RT-PCR products revealed two FUS-CHOP transcript variants (referred to as types IV and V, respectively) that have not been described before. These variants are comparable to the already known FUS-CHOP transcripts of types I and III in comprising either exon 7 (transcripts I and V) or both exon 7 and exon 8 (transcripts III and IV) of the FUS gene (Fig. 1). However, these new variants commonly lack a domain of 210 bp within exons 5 and 6 of FUS. Detailed analyses of the nucleotide sequences surrounding the deleted domain revealed that a precise determination of the potential fusion point is not possible, due to the existence of an identical nucleotide repeat of 6 bp (5'-TGGTGG-3') in both nucleotide positions 576–581 and 786–791 (Fig. 2).

In three of the analyzed tumor samples, more than a single transcript was detected by RT-PCR (Table 1). Similar to the liposarcoma cell line 1955-91 (6), showing three different FUS-CHOP transcripts, the relative abundance of the individual types of transcripts varied in these samples. Furthermore, in 10 tumor samples harboring FUS-CHOP transcripts, transcript II (3 of 10), transcript IV (6 of 10), or transcript V (1 of 10), respectively, was observed as the sole type of fusion transcript (Table 1).

Analysis of MRD in Liposarcoma Patients. The sensitivity of the RT-PCR assay that was specific for FUS-CHOP was assessed in dilution experiments using the liposarcoma cell line 1955-91, which was previously shown to express the fusion transcript (6, 7). Ten, 10², or 10³ 1955-91 liposarcoma cells were added to 10 ml of PB samples of healthy volunteers. Subsequently, mononuclear cells were isolated, and total RNA was extracted from the blood samples and subjected to RT-PCR analysis. A FUS-CHOP transcript was amplified from samples spiked with tumor cells up to a dilution of 10 sarcoma cells in 10 ml of blood (Fig. 3), corresponding to a 5 × 10⁻⁶ fold dilution of the tumor cells in PB cells. No fusion transcript was detected in blood samples from healthy volunteers (n = 20) or...
in BM aspirates from patients with colon cancer (n = 10). Since we started this analysis of MRD, five of nine tumors from liposarcoma patients were shown to express a fusion transcript, and therefore, these patients were eligible for this examination. Total RNA was extracted from blood samples drawn before, during, and after the surgical resection of the respective liposarcoma, as well as from BM aspirates collected directly before tumor extirpation. In two of five patients, FUS-CHOP fusion transcripts were detected in PB samples (Table 2). One patient treated for abdominal metastasis was shown to be positive in either the pre- and intraoperatively drawn sample (first operation) or solely in the intraoperatively drawn sample during the resection of recurrent disease. In another patient with a stage IIIB myxoid liposarcoma of the thigh, the blood sample drawn postoperatively revealed a fusion transcript, whereas analysis of pre- and intraoperative blood samples tested negative (Fig. 4; Table 2). In addition, BM analysis by RT-PCR revealed the existence of fusion transcripts in two patients with localized disease (Fig. 4; Table 2).

DISCUSSION

Myxoid and round cell liposarcomas have been cytogenetically characterized by (12;16) chromosomal translocations, which result in the expression of FUS-CHOP fusion transcripts. Here, we have analyzed the prevalence of chimeric FUS-CHOP transcripts in all histological subtypes of soft tissue liposarcomas. Our analysis revealed that the frequency of myxoid and round cell liposarcomas expressing a FUS-CHOP fusion transcript (8 of 13; 61%) is lower, as compared to previous studies (13–15). This observation may be partially due to the fact that, in most studies reported thus far, panels of myxoid and round cell liposarcomas were analyzed that had been cytogenetically preselected to contain a (12;16) translocation (13, 14). Furthermore, we cannot exclude the possibility that translocations other than (12;16) are involved in the pathogenesis of liposarcomas clearly showing myxoid morphology but lacking FUS-CHOP fusion transcripts (26). We have extended the search for FUS-CHOP fusion transcripts to all histological subtypes of soft tissue liposarcoma. Surprisingly, specific fusion transcripts were also identified in 1 of 3 analyzed well-differentiated and in 4 of 14 pleomorphic subtypes. Until now, fusion transcripts have been exclusively detected in myxoid and round cell liposarcomas (6, 7, 13–15, 27). Only two tumors each of well-differentiated and pleomorphic liposarcomas had been tested thus far and were shown to be negative in FUS-CHOP RT-PCR analysis (15, 27).

After the initial description of a single defined FUS-CHOP fusion transcript in two cell lines (6, 7), the subsequent analysis of several liposarcoma samples revealed the existence of a total of three variants (Fig. 1, transcripts I–III; Refs. 13–15, 27). The detection of two further variants in our liposarcoma samples demonstrates the variability of the 3′-terminal region of the FUS part in the chimeric transcripts. This variability may be explained by alternative splicing of the FUS gene (13). Either FUS exon 5 (transcript II), exon 7 (transcripts I and V), or exon 8 (transcripts III and IV) are fused to exon 2 of the CHOP gene (Fig. 1; Refs. 6, 13, 15). This assumption is further supported by the observation that (a) the different fusion transcripts appear with variant frequencies in the liposarcoma samples in the five studies reported thus far (13–15) and (b) more than a single type of transcript is detected in several tumor samples (Table 1; Ref. 13).

The two newly identified types of transcripts (transcripts IV and V) differ from the formerly reported transcripts of types
Postoperative blood sample (Lane Fig. 4 FUS-CHOP fusion not determined. 785 might serve as a cryptic splice acceptor site (Fig. 2).

The clinical significance of the detection of fusion transcripts in various clinical samples of a patient (Table 2, patient 5) with a myxoid liposarcoma of the proximal thigh and resection of a local recurrence in the groin. Fusion transcript type IV was detected in the primary tumor (Lane T), the BM aspirate (Lane BM), and the postoperative blood sample (Lane PB III). Lane e, negative control; Lane M, molecular weight standard; Lane T, patient's tumor; Lanes PB I, PB II, and PB III, blood samples taken pre-, intra-, and postoperatively, respectively.

Table 2 Analysis of MRD

<table>
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<tr>
<th>Patient no.</th>
<th>Indication</th>
<th>Fusion transcript</th>
<th>BM Preoperative</th>
<th>Intraoperative</th>
<th>Postoperative</th>
<th>UICC* stage</th>
<th>DM</th>
<th>Follow-up</th>
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<tbody>
<tr>
<td>3</td>
<td>Abdominal metastases II</td>
<td>II</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>IV B +</td>
<td></td>
<td></td>
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<tr>
<td>4</td>
<td>Suspected recurrence II</td>
<td>II</td>
<td>--</td>
<td>--</td>
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<td>IV B +</td>
<td></td>
<td></td>
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<tr>
<td>5</td>
<td>PT</td>
<td>IV</td>
<td>n.d.</td>
<td>--</td>
<td>--</td>
<td>II B --</td>
<td>NED, 12 months</td>
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<tr>
<td>6</td>
<td>LR</td>
<td>IV</td>
<td>+</td>
<td>--</td>
<td>+</td>
<td>II B --</td>
<td>NED, 10 months</td>
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<tr>
<td>9</td>
<td>Abdominal metastases IV</td>
<td>IV</td>
<td>n.d.</td>
<td>+</td>
<td>n.d.</td>
<td>IV B +</td>
<td>LR, 6 months</td>
<td></td>
</tr>
<tr>
<td>9</td>
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<td>--</td>
<td>--</td>
<td>+</td>
<td>IV B +</td>
<td>DM, 14 months</td>
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</table>

* UICC, Union International Contre Cancer; PT, primary tumor; LR, local recurrence; DM, distant metastasis; NED, no evidence of disease; n.d., not determined.

I–III in showing a 210-bp in-frame deletion. The authenticity of these transcripts was further confirmed by a control PCR using a 5'-primer located within the deleted 210-bp region (nucleotide positions 611–632). Specifically, those tumor samples harboring only transcript IV or V failed to demonstrate the respective control PCR fragment (data not shown). Sequence analyses of transcripts IV and V revealed the lack of genuine splice donor and acceptor consensus sequences at the junctions of the deleted 210-bp fragments. However, as there is an identical repetitive sequence at the putative junction sites, which prevents a precise definition of the respective breakpoints, it can only be speculated whether the CAG nucleotide sequence in positions 783–785 might serve as a cryptic splice acceptor site (Fig. 2). The exact mechanism by which these transcripts are generated remains unclear. The transforming activity of these new variant transcripts has yet to be evaluated. However, the occurrence of transcript IV and V as the single fusion transcript in a variety of tumors (Table 1) suggests a comparable oncogenic activity, as has been demonstrated for transcript I and II using in vitro transformation assays (16, 17).

Solid tumors such as liposarcomas are primarily treated as localized malignancies. However, even after radical tumor resection, local recurrences or distant metastases develop in as many as 40% of patients (23), suggesting that tumor cells were released from the primary tumor either before or during surgery. Recently, the PCR-based amplification of the chromosomal translocation breakpoints using genomic DNA instead of RNA preparations was reported (28). In 4 of 20 patients carrying myxoid liposarcomas with cytogenetically proven translocations, PCR fragments comprising the individual translocation breaking points were detected in PB samples. The lack of prognostic significance in this retrospective study might be related, in part, to the rather low sensitivity of the long distance genomic PCR protocol. The detection limit reported for the genomic PCR protocol allows the identification of 1 tumor cell within 10⁴ blood cells (28), whereas the amplification of the fusion transcripts by RT-PCR shown in this study is capable to detect a single liposarcoma cell within 5 × 10⁸ blood cells. Applying this sensitive RT-PCR protocol, liposarcoma cells were detected in PB or BM in three of five patients, suggesting the frequent presence of residual disease in liposarcoma patients undergoing surgery. The prognostic impact of this finding remains to be established. However, in the early follow-up period (median, 10 months), two patients developed local recurrence, one of whom demonstrated perioperative tumor cells in PB (patient 9), whereas the other patient tested negative in RT-PCR in all analyzed compartments (Table 2, patient 3). The clinical significance of the detection of fusion transcripts in the tumor and of MRD at the time of therapeutic intervention now must be evaluated in prospective studies. Depending on the prognostic significance, additional chemo- or
immunotherapies might contribute to an improved outcome of selected patients.

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REFERENCES


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