Amplification of FRS2 and Activation of FGFR/FRS2 Signaling Pathway in High-Grade Liposarcoma

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Abstract

Fibroblast growth factor (FGF) receptor (FGFR) substrate 2 (FRS2) is an adaptor protein that plays a critical role in FGFR signaling. FRS2 is located on chromosome 12q13-15 that is frequently amplified in liposarcomas. The significance of FRS2 and FGFR signaling in high-grade liposarcomas is unknown. Herein, we first comparatively examined the amplification and expression of FRS2 with CDK4 and MDM2 in dedifferentiated liposarcoma (DDLS) and undifferentiated high-grade pleomorphic sarcoma (UHGPS). Amplification and expression of the three genes were identified in 90% to 100% (9–11 of 11) of DDLS, whereas that of FRS2, CDK4, and MDM2 were observed in 55% (41 of 75), 48% (36 of 75), and 44% (33/75) of clinically diagnosed UHGPS, suggesting that these "UHGPS" may represent DDLS despite lacking histologic evidence of lipoblasts. Immunohistochemical analysis of phosphorylated FRS2 protein indicated that the FGFR/FRS2 signaling axis was generally activated in about 75% of FRS2-positive high-grade liposarcomas. Moreover, we found that FRS2 and FGFRs proteins are highly expressed and functional in three high-grade liposarcoma cell lines: FU-DDLS-1, LiSa-2, and SW872. Importantly, the FGFR selective inhibitor NVP-BGJ-398 significantly inhibited the growth of FU-DDLS-1 and LiSa-2 cells with a concomitant suppression of FGFR signal transduction. Attenuation of FRS2 protein in FU-DDLS-1 and LiSa-2 cell lines decreased the phosphorylated extracellular signal–regulated kinase 1/2 and AKT and repressed cell proliferation. These findings indicate that analysis of FRS2 in combination with CDK4 and MDM2 will more accurately characterize pathologic features of high-grade liposarcomas. Activated FGFR/FRS2 signaling may play a functional role in the development of high-grade liposarcomas, therefore, serve as a potential therapeutic target.

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Introduction

Fibroblast growth factor (FGF) receptor (FGFR) substrate 2 (FRS2) is a member of the adaptor/scaffold protein family that binds to receptor tyrosine kinases (RTK) including FGFR, the neurotrophin receptor, RET, and ALK, and is required for signal transduction from FGF Rs (1). The FGFR family of 4 RTKs, FGFR1/2/3/4, mediate numerous physiologic processes including cell migration, proliferation, survival, and differentiation (2). Deregulation of FGFR signaling is associated with many developmental syndromes (3). Aberrant activation of FGFR signaling has been associated with tumor formation, angiogenesis, and metastasis in multiple cancer types including breast cancer, bladder cancer, multiple myeloma, hepatocellular, and renal cell carcinoma. Clinical studies have also revealed that aberrant FGFR signaling is associated with poor outcome of different human cancers (4–7). These observations make FGFRs signaling increasingly attractive as target for therapeutic intervention in various human cancers (2).

Liposarcomas are a heterogeneous group of mesenchymal tumors, and on the basis of morphologic features and cytogenetic aberrations, liposarcoma is classified into 3 biologic types encompassing 5 subtypes: well-differentiated liposarcoma/dedifferentiated liposarcoma (WDLS/DDLS), myxoid/round cell liposarcoma, and undifferentiated high-grade pleomorphic sarcoma (UHGPS; refs. 8–10). Surgery serves as the mainstay of therapy for localized liposarcoma. However, for locally advanced and disseminated disease, there are few effective treatment options (11). Liposarcomas with similar morphologic appearance can follow different clinical courses and show divergent responses to systemic therapy (12, 13). DDLS and UHGPS share similar histologic features, but compared with DDLS, UHGPS displays more aggressive local behavior and strong resistance to systemic ifosfamide-based chemotherapy, resulting in a 5-year survival rate of about 50% (10). DDLS tends to have a lower metastatic rate (15%–20%) than UHGPS (30%–50%; ref. 12). However, the accurate discrimination of pleomorphic from DDLS based on morphology...
alone is often a challenge even for an experienced soft-tissue pathologist. Recent reports indicate that "UHGPS" has been misused for several better classifiable, poorly differentiated soft-tissue sarcomas (14). For example, a study reported that 23% of 163 high-grade liposarcomas in the Netherlands Cancer Institute were reclassified on the basis of molecular characteristics of liposarcoma (15). Similarly, another study re-evaluated 159 cases of previously diagnosed UHGPS and found that a total of 97 of these cases could be reclassified as other sarcomas and 20 were proven to be nonmesenchymal neoplasms (16).

During the past decade, with the insight afforded by immunohistochemistry as well as improved cytogenetic and molecular diagnostics, the term UHGPS has been restricted to a group of soft-tissue malignancies without a specific line of differentiation (17). Therefore, it is critical to better classify and target various high-grade liposarcomas based on their specific pathologic features.

Amplification of MDM2 and CDK4 genes on chromosome 12q13-15 is noted to frequently occur in WDLS/DDLS and may also serve as genetic characteristics of DDLS from UHGPS. For example, Taylor and colleagues, using high-resolution genomic analysis, proved that there was no CDK4 amplification in UHGPS (18). The chromosome 12q13-15 amplicon is discontinuous and spans several megabases of DNA and contains multiple genes (19). The discontinuous amplification may lead to the patterns for overexpressed proteins that may provide growth advantages to tumors quite different between patients. Among genes on chromosome 12q13-15, CDK4 gene, a cyclin-dependent kinase promoting G1-phase cell-cycle progression, and MDM2 gene, a transcriptional target of p53, which mediates both ubiquitin-dependent pro-apoptotic degradation of p53 and ubiquitin-independent pro-apoptosomal degradation of p21, causing unchecked cell-cycle progression and proliferation, have been well-studied because of their critical function in oncogenesis of liposarcoma (20, 21). The FRS2 gene is located close to CDK4 and MDM2 on chromosome 12q13-15. Most recently, a study showed that the FRS2 gene is also frequently amplified in WDLS, and the FRS2 protein was overexpressed in WDLS, but not in normal fat or preadipocytes, which then raises the possibility that FRS2 could be a useful target in malignant liposarcomas (22).

In the present study, we first comparatively analyzed the amplification and overexpression of FRS2 with that of CDK4 and MDM2 in clinically diagnosed DDLS/UHGPS. We discovered a high frequency of amplification and overexpression of FRS2 in these high-grade liposarcomas. Considering that FRS2 acts as "a conning center" in FGFR signaling (1, 2), we further investigated the activation of FGR signaling in these malignant liposarcomas. We found that FGFR signaling was activated in 75% of high-grade liposarcomas expressing FRS2 protein. The biologic significance of FRS2 and FGFR signaling in the development of these malignant liposarcomas has not previously been evaluated, to the best of our knowledge. We examined expression of FRS2 and FGFRs genes in three human high-grade liposarcoma cell lines: FU-DDLS-1, LiSa-2 and SW872 and found that FRS2 and FGFRs are ubiquitously expressed and functional in these cell lines. Furthermore, we improved that targeting FGFR-FRS2 signaling may inhibit the in vitro growth of FU-DDLS-1 and LiSa-2 cells with a concomitant suppression of FGFR signal transduction. Our findings for the first time indicate that activated FGFR/FRS2 signaling may contribute to the progression of high-grade liposarcomas, therefore representing a potential therapeutic target that deserves further extensive study. Recent advances in molecular characterization of liposarcomas have shown that aberrant amplification of MDM2 and CDK4 genes within chromosome 12q13-15 distinguishes WDLS/DDLS from UHGPS (16–18). Accordingly, we also validated that a subset of clinically diagnosed "UHGPS" of current study may represent DDLS. Importantly, we observed that 7% of these "UHGPS" were immunopositive for FRS2 only. Our study also suggests that analysis of FRS2 in combination with CDK4 and MDM2 will even more accurately classify high-grade liposarcomas and lead to better prognostication.

Materials and Methods

Cellular proliferation and FRS2 siRNA knockdown assays
FU-DDLS-1 DDLS cell line was a kind gift from Dr. Hiroshi Iwasaki (Fukuoka University, School of Medicine, Fukuoka Japan; ref. 23) and was maintained in Dulbecco’s Modified Eagle's Medium (DMEM):F12 medium with 10% FBS. SW872 DDLS cell line was purchased from the American Type Culture Collection and was cultured in DMEM with 10% FBS. LiSa-2 pleomorphic liposarcoma cell line was a kind gift from Dr. Silke Bruderlein (University of Ulm, Ulm, Germany; ref. 24) and was maintained in Iscove’s modified Dulbecco’s medium/RPMI in a 4:1 ratio supplemented with 10% FBS, 2 mmol/L L-glutamine, and 0.1 mg/mL gentamicin. All cells were newly DNA fingerprinted to confirm identity as previously described (25).

Human adipose tissue total RNA survey Lot #07505959A and #0903009 were purchased from Ambion Inc. Human FRS2 gene–specific siRNA and scrambled siRNA (Life Technologies Corporation) were transfected into cells with Lipofectamine siRNA Maximum (Life Science Inc.,) according to the user manual. FRS2 siRNA–mediated inhibition of FU-DDLS-1 and LiSa-2 cells proliferation was monitored by using a W200 real-time cell electronic sensing analyzer (RT-CES) 16X workstation (Acea Biosciences) (26). A total of 2,500 cells were seeded into each well of RT-CES device in multiple duplicate of 8 and transfected with siRNAs. Twenty-four hours later, the index and curve of cell proliferation were monitored and plotted every half hour. At 72 hours after siRNA transfection, cells were also collected for Western blot analysis.

FGFR-specific inhibitor NVP-BGJ-398 treatment and MTS assay
FGFR in FU-DDLS-1 and LiSa-2 cells was specifically inhibited using NVP-BGJ-398 (BGJ-398) purchased from Novartis Oncology Inc (27). BGJ-398 concentration ranged from 0.125 to 4 μmol/L, dimethyl sulfoxide (DMSO) was served as vehicle controls. MTS assays were done according to manufacturer’s instructions (CellTiter 96 AQueous Assay reagent; Promega) to quantify the number of viable cells. Briefly, a total of 2,500 cells were seeded in a 96-well plate in multiples of 10 wells treated with NVP-BGJ-398. At 72 hours after treatment, cells were subjected to MTS assays. A function of the logarithm of the
inhibitor concentration was used to produce logistic fit of percentage cell viability. The IC_{50} value was determined as the concentration of compound needed to reduce cell viability to 50% of a DMSO control.

**Case selection**

The diagnosis of DDLS and UHGPS was done by pathologist P. Chu. The criteria for UHGPS were as follows: showing marked cytologic and nuclear pleomorphism, admixed with spindle cells, without histologic (lipogenic) or immunohistochemical (IHC; epithelial, neuromelanocytic, lymphohematopoietic, and muscular) evidence of specific lineage differentiation (28). Seventy-five such cases arising in various anatomic locations were selected from the surgical pathology files at City of Hope National Medical Center from 1990 to 2010 (Supplementary Table S1). Twenty-six of 75 UHGPS from retroperitoneum and thigh were previously reported (28). The mean age was 57 years with 1.2:1 male predominance and all 75 cases were resection specimens: 37 cases from lower extremity, mean age was 57 years with 1.2:1 male predominance and all 75 cases were resection specimens: 37 cases from lower extremity, 8 from chest/trunk, 2 from head/neck from upper extremity, 11 from abdomen/retroperitoneum, 8 cases were resection specimens: 37 cases from lower extremity, 8 from chest/trunk, 2 from head/neck from upper extremity, 11 from abdomen/retroperitoneum, 8 cases were resection specimens: 37 cases from lower extremity, 8 from chest/trunk, 2 from head/neck from upper extremity, 11 from abdomen/retroperitoneum, 8.

**Illumina Genome Analyzer (Solexa) whole-exome sequencing, sequence alignment, various calling, and annotation**

Three micrograms of genomic DNA was sheared and end- repaired. Illumina adaptor oligonucleotides were ligated to the ends. Ligation products were purified and enriched with a 12-cycle PCR. The enriched PCR products were subjected to the exon capture procedure using the SureSelect Human All Exon Kit. The captured products were then used for sequencing and cluster generation by synthesis using the Illumina Genome Analyzer IIx. Image Analysis and base calling, which was conducted by using Illumina default pipeline. The sequences were aligned to the human genome reference sequence (NCBI36) using Bowtie 0.12.7 (29). All subsequent analysis was done using customized R scripts. The log2 coverage ratio of each exon was calculated for tumor versus normal tissue and median-centered. Log2 ratios were smoothed by DNAcopy 25 using default values and copy number variation was detected by DNAcopy's circular binary segmentation algorithm (30).

**gDNA preparation and q-PCR**

The gDNAs of liposarcoma and the paired adjacent normal fat tissue were isolated from FFPE tissue sections using the QiAamp FFPE DNA Mini Kit (Qiagen) according to the user manual. To characterize the amplification of the chromosome 12q13-15 region in UHGPS, we selected FRAS2 and CDK4 and MDM2 genes of the region to measure their copy number by q-PCR analysis. For each gene, 2 primer pairs for 2 exons were designed. The primer sequences of 2 exons of CDK4, MDM2, and FRAS2 genes are listed in Supplementary Table 8. q-PCR analysis was conducted in 32 randomly selected UHGPS cases (Supplementary Table S4) and 15 DDLS cases (Supplementary Table S5) using ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). β-Globin was used as reference gene. Relative gene quantification method was used to calculate the fold change of gene copy number in a liposarcoma tissue (based on average of 2 exons of each gene) to gDNA extracted from the paired adjacent fat tissue (26).

**Immunohistochemistry**

Commercially available monoclonal antibodies against CDK4 (Clone DCS-31, 1:50, Invitrogen), MDM2 (Clone IF-2, 1:75, Zymed Laboratories), and FRAS2 (Clone H-91, 1:100, Santa Cruz Biotechnology Inc.) and a FRAS2-rabbit polyclonal (1:50, Proteintech) were used. Anti-total FGFR3 (DZG7E), phospho- FGFR receptor (Tyrr653/654), and phospho-FRAS2 (Tyrr436) antibody were purchased from Cell Signaling, Anti-FRAS2 antibodies for Western blotting were purchased from R&D Systems (Clone 462910) and GeneTex (N1N3/GTX103288). All UHGPS and DDLS cases were stained for CDK4, MDM2, and FRAS2 by immunohistochemistry. Only FRAS2-positive cases were subjected to phospho-FRAS2 staining. Sections were deparaffinized and rehydrated in graded alcohol. For heat-induced epitope retrieval (HIER), the sections were subjected to DIVA retrieval buffer (pH 6.0) in a Pressure Cooker (Biocare Medical) at 98°C for 60 minutes. The sections were then brought to an automated stain (DAKO) following the vendor's protocol. EnVision Plus and peroxidase detection methods were used and counterstained in 50% Mayer's hematoxylin for 1 minute. Nuclear CDK4 immunoreactivity, nuclear and cytoplasmic MDM2 immunoreactivity, and cytoplasmic FRAS2 immunoreactivity were assessed. Those cases with more than 1% of tumor cells showing positive staining were assessed as positive. The staining was graded as ++ (1%–5% tumor cells positive), + (5%–24% tumor cells positive), and +++ (>25% tumor cells positive; ref. 11).

**FISH**

To further validate FRAS2 gene amplification, dual color FISH was conducted using the FISH-mapped confirmed BAC probe, RP11-1130G21 (12q15) for FRAS2 labeled in Spectrum Orange (Abbott Laboratories, Inc.) and RP11-433j6 (12p13) for chromosome 12 centromere labeled in Spectrum Green (Abbott Laboratories, Inc.) were selected for FISH assays. After the unstained positron emission tomographic (PET) slides were hybridized with FISH probes, cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma). An Applied Imaging System was used to record images of representative cells. Two hundred interphase cells were scored per sample. All sections were scorable. Images were captured with the BioView Imaging System (BioView). Two red and 2 green signals (2R2G) are considered normal in a diploid genome. Because of truncation and overlapping cells,
at least 20% of cells must show an abnormal pattern to be considered abnormal.

Statistics

Data were collected using an MS Excel spreadsheet. Data were analyzed using the JMP Statistical Discovery Software version 6.0 (SAS Institute). Group comparisons for continuous data were done with the Student t test for independent means or 2-way ANOVA. Statistical significance was set at \( P < 0.05 \).

Results

**FRS2 and FGFR expression and activation of the FGFR/FRS2 axis in DDLS/UHGPS cell lines**

We first measured the copy numbers of *CDK4, MDM2,* and *FRS2* genes in 3 human high-grade liposarcoma cell lines: FU-DDLS-1 (DDLS), LiSa-2 (UHGPS), and SW872 (DDLS). Quantitative PCR analysis showed that the ratios of copy numbers of *CDK4, MDM2,* and *FRS2* to β-globin were about 7 to 10 in FU-DDLS-1, whereas the ratios of these 3 genes were less than 2 in LiSa-2 and SW872, indicating that the amplification of these 3 genes was only present in FU-DDLS-1 cells (Fig. 1A). FGFR/FRS2 signaling has not been studied previously in high-grade liposarcoma cell lines. We then examined the mRNA expression of both FRS2 and 4 FGFR genes, including isoforms of FGFR1, FGFR2, and FGFR3 in these 3 cell lines in comparison with that of human normal adipose tissues by quantitative reverse transcription PCR (qRT-PCR) analysis. The qRT-PCR analyses indicated that these cells expressed relatively high level of FGFR1 and various levels of FGFR2, FGFR3, and FGFR4 mRNA (Fig. 1B). The levels of FRS2, FGFR1, FGFR3, and FGFR4 mRNA were substantially higher in these cell lines than that of normal adipose tissues; especially in FU-DDLS-1 and LiSa-2 cells. Western blot analysis showed the relatively high expression of FGFR1 and FGFR3 proteins in these 3 liposarcoma cell lines (Fig. 1C). Although the copy number and mRNA of FRS2 gene were higher in FU-DDLS-1, the level of FRS2 protein was slightly higher in FU-DDLS-1 cells than that in other 2 cells, which may indicate the presence of posttranscriptional regulation of FRS2 expression. Coincidently, a previous article reported that for the distinction of liposarcomas, genomic profiling appears to be more useful than RNA expression analysis (19). We found that phosphorylated FRS2 protein was generally present in these 3 liposarcoma cell lines, and the levels of phosphorylated FRS2 protein in FU-DDLS-1 and LiSa-2 cells in serum were relatively higher (Fig. 1C). Consistently, a most recent study showed that high level of phosphorylated FRS2 protein was only present in liposarcoma but not in normal fat or preadipocytes (22). To determine the cellular responses to bFGF2 stimulation, these 3 cell lines were serum starved for about 72 hours and then stimulated with 10 ng/mL of basic FGF2 (bFGF2) for 30 minutes. As shown in Fig. 1D, bFGF2 stimulation promptly increased phosphorylation of total FGFR (p-FGFR), FRS2 (p-FRS2), extracellular signal–regulated kinase (ERK)1/2 (p-ERK1/2), and AKT (p-AKT) in all 3 high-grade liposarcoma cell lines. FRS2 and FGFR Signaling Pathway in High-Grade Liposarcoma

Figure 1. Expression and activation of FGFR/FRS2 in DDLS/UHGPS cell lines. A, q-PCR analysis showed relative copy numbers of *CDK4, MDM2,* and *FRS2* in 3 high-grade liposarcoma cell lines: FU-DDLS-1 (DDLS), LiSa-2 (UHGPS), and SW872 (DDLS). Data for gene copy number are presented as ratio to β-globin. B, q-PCR analysis of FRS2 and FGFR1–4 mRNA expression in FU-DDLS-1, LiSa-2, and SW872 cells. Shown are expression levels relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal loading control. Adipose tissue cells (Adipose) were used as normal control. C, Western blot analysis showed the expression of total and phosphorylated FRS2, as well as FGFR1/3 proteins in FU-DDLS-1 (Fu), LiSa-2 (Li), and SW872 (Sw) cells. D, bFGF2 stimulation activated phosphorylation of total FGFR, FRS2, AKT, and ERK1/2 in the 3 cells. FU-DDLS-1 was serum-starved for 48 hours; LiSa-2 and SW872 cells were starved for 72 hours before being stimulated with 10 ng/mL bFGF2 for 30 minutes.
liposarcoma cell lines, especially in FU-DDLS-1 and LiSa-2, showing the presence of a functional FGFR/FRS2 signaling after bFGF2 stimulation.

Effects of FGFR inhibitor BGJ-398 on FU-DDLS-1 and LiSa-2 cell proliferation

The FGFR pathway is an important stimulus to cancer development and progression in many epithelial tumors, and targeted inhibition of FGFR signaling has shown promise in in vitro studies (2, 31). As FGFR signaling was highly activated in FU-DDLS-1 and LiSa-2 in serum, we tested the effects of an FGFR inhibitor on the proliferation and activation of AKT and ERK signaling in these 2 cell lines. As shown in Fig. 2A and B, the selective FGFR inhibitor BGJ-398 significantly decreased FU-DDLS-1 and LiSa-2 cell proliferation in a dose-dependent manner. The IC_{50} of BGJ-398 was about 0.25 to 0.50 μmol/L for FU-DDLS-1 and LiSa-2 cells, respectively. After cells were exposed to BGJ-398 for 1 to 8 hours, Western blot analysis showed that p-FRS2, p-AKT, and p-ERK1/2 proteins rapidly decreased in a dosage-dependent manner in FU-DDLS-1 (Fig. 2C) and LiSa-2 cells (Fig. 2D). However, p-ERK1/2 and p-AKT in FU-DDLS-1 (Fig. 2C) and LiSa-2 cells (Fig. 2D) started to rebound after 24 to 48 hours of exposure to BGJ-398, which indicated that undefined feedback mechanisms might reactivate these pathways. In consistent with the low level of p-FRS2 in SW872 cells in serum, the same concentration of BGJ-398 barely inhibited the proliferation of SW872 cells, suggesting its proliferation in serum may be less dependent on FGFR signaling (data not shown). Overall, above data indicated that BGJ-398 may suppress cellular proliferation and survival through blocking FGFR-mediated signaling.

Effect of knockdown of FRS2 on cell proliferation and response to BGJ-398 treatment

FRS2 is an adaptor protein absolutely necessary for transfer of signal from activated FGFR. Knockdown of FRS2 blocks FGFR signaling and suppresses cancer cells proliferation (32, 33). Therefore, we further asked whether knockdown of FRS2 can also disrupt FGFR signaling and then attenuate cellular proliferation of these high-grade liposarcoma cells. Western blot analysis showed that knockdown of FRS2 substantially decreased the p-ERK1/2 and p-AKT in both FU-DDLS-1 (Fig. 3A) and LiSa-2 cells (Fig. 3B) in serum and dramatically reduced bFGF2-induced p-AKT and p-ERK1/2 in both serum-starved FU-DDLS-1 (Fig. 3A) and LiSa-2 cells (Fig. 3B), which suggested that knockdown of FRS2 may suppress the bFGF2-stimulated, FGFR-mediated activation of p-AKT and p-ERK1/2. We further examined the impact of knockdown of FRS2 on cellular proliferation of both FU-DDLS-1and LiSa-2 cells. Knockdown of FRS2 significantly decreased the proliferation of FU-DDLS-1 (Fig. 3C) and LiSa-2 cells (Fig. 3D) monitored in a real-time growth assay. In all, these data strongly indicate that knockdown of FRS2 may specifically block FGFR signaling in these high-grade liposarcoma cell lines.

Amplification of FRS2, CDK4, and MDM2 genes in high-grade liposarcoma

We initiated a pilot study to explore genomic abnormalities of UHGPS by conducting whole-exome sequencing on 7 cases of UHGPS. Sequencing results unveiled 2 large and discontinuous amplified regions of chromosome 12q13-15 and 12q22-24 in one "UHGPS" (Fig. 4A). q-PCR analysis showed that 3 selected genes CDK4, MDM2, and FRS2 on chromosome
12q13-15 were amplified 10-fold compared with saliva DNA of the same patient (Fig. 4B), suggesting the "UHGPS" may represent a DDLS. The chromosome 12q13-15 amplicon contains several important genes such as CDK4 and MDM2, which may play important roles in the development of liposarcoma, and has been well studied in WDLS/AL/DDLS (34, 35). We selected 8 genes (DDIT3, TSPAN31, CDK4, HMGA2, DYRK2, MDM2, YEATS4, and FRS2) mapping to chromosome 12q13-15 to compare their amplification patterns in 15 cases of DDLS and 32 cases of UHGPS using q-PCR assays. The results of q-PCR analysis indicated that the frequency for amplification of DDIT3 and DYRK2 was much lower than others (Supplementary Table S3), indicating that the amplicon was discontinuous. Details for gene amplification in individual sample of UHGPS and DDLS are listed in Supplementary Tables S4 and S5. The frequency of amplification of CDK4, MDM2, and FRS2 in DDLS was 100% (15 of 15), 93% (14 of 15), and 93% (14 of 15), respectively, whereas the frequency of amplification of CDK4, MDM2, and FRS2 in UHGPS was 44% (14 of 32), 25% (8 of 32), and 34% (11 of 32), respectively, indicating that a subgroup of clinically diagnosed "UHGPS" may represent DDLS despite lacking histologic evidence of lipoblasts (14, 16, 17). Figure 4C and D show the copy number distribution of CDK4, MDM2, and FRS2 genes in DDLS and clinically diagnosed UHGPS respectively, indicating the presence of co-amplification of FRS2 with CDK4 and MDM2 on chromosome 12q13-15.

Immunohistochemical analysis of expression FRS2, CDK4, and MDM2 proteins in high-grade liposarcoma

Furthermore, we measured the amplification and overexpression of FRS2 in high-grade liposarcomas by FISH and immunohistochemistry. The IHC staining results for each case of clinically diagnosed high-grade liposarcomas are listed in Supplementary Tables S1 and S2. All 11 of 11 cases of DDLS were positive for CDK4 (100%), 10 of 11 for MDM2 (91%), and 9 of 11 for FRS2 (82%). Accordingly, these 44 of 75 of "UHGPS" with positive IHC staining for one of CDK4, MDM2, and FRS2 proteins should be reclassified as DDLS (14, 16, 17). Of note, 5 of 75 cases UHGPS (7%) were positive for FRS2 protein only. Therefore, we concluded that analysis of FRS2 in combination with CDK4 and MDM2 will more accurately characterize pathologic features of high-grade liposarcomas. Table 1 summarized semiquantitative analysis of immunostaining of these 3 proteins in these high-grade liposarcomas. Hematoxylin and eosin (H&E) and IHC staining for 4 typical example cases are presented in Fig. 5. Dual-color FISH analysis showed that FRS2...
gene amplification was present in case 1 (originally diagnosed as DDLS) or case 2/3 (originally diagnosed as "UHGPS"), whereas immunostaining analysis revealed that these cases were FRS2-positive. FISH of case 4 (originally diagnosed as "UHGPS") displayed a normal pattern of 2 red signals of FRS2 gene with 2 green signals of centromere in the diploid genome, suggesting no FRS2 gene amplification was present in the liposarcoma tissue, which corresponded to the negative FRS2 IHC staining.

**Phosphorylated FRS2 protein in high-grade liposarcoma**

Activation of FGFR results in phosphorylation of FRS2 tyrosine residues and subsequent sustained levels of ERK activation and also activates the phosphoinositide 3-kinase (PI3K)/AKT pathway (36). Phosphorylated FRS2 protein may therefore serve as a marker for activated FGFR signaling. To further assess whether FGFR/FRS2 signaling was activated in these high-grade liposarcoma tissues, we investigated the phosphorylated FRS2 protein by immunohistochemistry. We found that 75% of 48 cases of high-grade liposarcoma with FRS2-positive IHC staining were immunopositive for phosphorylated FRS2 protein, which strongly suggests that the FGFR signaling was activated and might function in these liposarcoma tissues. The morphology of a high-grade liposarcoma was revealed by H&E staining (Fig. 6A), whereas IHC analysis showed strong staining for FRS2 protein (Fig. 6B) and moderately positive staining for phosphorylated FRS2 protein (Fig. 6C). Quantitative analysis showed that 37.5% of phosphorylated FRS2-positive high-grade liposarcomas were graded as + (1%–5% tumor cells positive), whereas 35.4% of them were graded as ++ (5%–24% tumor cells positive), and 2.1% of them were graded as +++ (>25% tumor cells positive). The IHC staining grade of p-FRS2 for each high-grade liposarcoma is also listed in Supplementary Tables S1 and S2.

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*aTotal of 55 cases of high-grade liposarcoma included 11 cases of DDLS and 44 cases of clinically diagnosed "UHGPS". The immunostaining was graded as +, (1%–5% tumor cells positive), ++ (5%–24% tumor cells positive), and +++ (>25% tumor cells positive).
Discussion

FISH analysis or immunohistochemistry analysis of amplification and overexpression of MDM2 and CDK4 on chromosome 12q13-15 have been proven useful adjuncts in diagnosing WDLS/DDLS (11, 35, 37) and may also represent genetic characteristics of DDLS from UHGPS. For example, Taylor and colleagues proved that there was no CDK4 gene amplification in UHGPS (18). Accordingly, in the present study, we also found that 44 of 75 cases of "UHGPS" should be reclassified as DDLS. The FRS2 gene is located close to CDK4 and MDM2 on chromosome 12q13-15 but has not been well-studied in high-grade liposarcoma. The amplification of chromosome 12q13-15 is typically large and discontinuous (20), which may lead to the profiles for overexpressed proteins that may provide growth advantages to tumors quite different between patients. Consistently, we also observed that the patterns for amplification and overexpression of genes on chromosome 12q13-15 are quite heterogeneous among high-grade liposarcoma tissues. Of note, 7% of UHGPS were immunopositive for FRS2 only, suggesting molecular and/or IHC analysis of FRS2 gene, in combination with MDM2 and CDK4 genes, may more precisely characterize and stratify high-grade liposarcomas.

The FGFR signaling pathways play a pivotal role in normal embryonic development. FRS2 gene knockout in mice causes lethality due to the interruption of the FGFR signaling pathway (36). FRS2 acts as "a conning center" in FGF signaling mainly because it induces sustained levels of activation of ERK (31, 38, 39). FGF ligands and their receptors are overexpressed

Figure 5. IHC staining of CDK4, MDM2, and FRS2 proteins in high-grade liposarcoma tissues. H&E staining (×200) shows the morphologic characters of 4 cases of high-grade liposarcomas. IHC staining of CDK4, MDM2, and FRS2 protein showed variable expression patterns in these high-grade liposarcoma tissues. Dual-color FISH assay validated amplification of FRS2 gene in 3 cases, and IHC analysis showed that FRS2 stain was positive in these tissues.

Figure 6. IHC staining of phosphorylated FRS2 protein in high-grade liposarcoma. A, H&E staining shows the morphologic features of a high-grade liposarcoma. B, IHC staining shows strongly positive FRS2 protein in the high-grade liposarcoma tissue. C, IHC staining shows moderately positive phosphorylated FRS2 protein in the high-grade liposarcoma tissue.
in a variety of cancers, including breast, stomach, prostate, pancreas, bladder, and colon (4–7). Aberrant activation of FGFR signaling has been associated with tumor formation, angiogenesis, and metastasis of these human cancers. A recent study has proven that amplification of FGFR1 was the strongest independent predictor of poor outcome in a cohort of unselected breast carcinomas (4). Increasing studies have uncovered solid evidences that activated FGFRs are driving oncogenes in certain cancers and act in a cell-autonomous fashion to maintain the malignant properties of tumor cells. These observations make FGFRs increasingly attractive as targets for therapeutic intervention in cancer (27, 31, 36). Many small compounds that inhibit RTKs and humanized antibodies against RTKs have been developed, and a number of potent inhibitors of the FGFR receptors are in early-phase clinical trials (2, 27, 40). Most recently, Wang and colleagues showed that the FRS2 gene was co-amplified, overexpressed, and activated in WDLS, but not in benign lipomas (22). To further investigate the biologic significance of FGFR-FRS2 signaling in high-grade liposarcomas, we examined phosphorylation of FRS2 protein: the functional form of FRS2, in these tissues expressing FRS2 protein. Interestingly, we identified a very high frequency of phosphorylation of FRS2 in these high-grade liposarcomas, indicating the FGFR signaling was generally activated.

Furthermore, we hypothesized that activated FGFR-FRS2 signaling might have a positive contribution to the development of high-grade liposarcoma and serve as a potential therapeutic target. To test the hypothesis indirectly, we first measured FRS2 and FGFR expression in human high-grade liposarcoma cell lines, whereas we found that FRS2 and FGFR1/3 transcripts and proteins are expressed in all 2 liposarcoma cell lines, whereas we found that FRS2 and FGFR1/3 transcripts and proteins are expressed in all 2 liposarcoma cell lines. The phosphorylated FRS2 in these cells was significantly upregulated by bFGF2 stimulation. Activated FGFR signaling in FU-DDLS-1 and LiSa-2 cells was indicated by the strong phosphorylated FRS2 signal in the presence of serum, whereas phosphorylated FRS2 was very low in the SW872 cell line. We next investigated the effects of an FGFR inhibitor on the proliferation and activation of AKT and ERK signaling of these 3 cell lines. Consistent with the differences observed for phosphorylated FRS2 between these cell lines, we found that an FGFR-selective inhibitor significantly suppressed the proliferation of FU-DDLS-1 and LiSa-2 cells, which have highly activated FGFR signaling, but not in SW872 cells, which do not. We also found that FGFR inhibitors suppressed the activation of ERK and PI3K signaling pathways. Similarly, studies on breast cancer cells showed that PD173074 selectively inhibits FGFR tyrosine kinase activity, mitogen-activated protein kinase (MAPK), and PI3K signaling pathways (41, 42). FRS2 itself is also a potentially attractive target for disruption of the mitogenic and tumorigenic effects of multiple FGFs. For example, studies in prostate cancer showed that knockdown of FRS2 might block global FGFR signaling in vitro and in vivo (32, 33). Our finding that knockdown of FRS2 in a UHGPS cell line inhibited cell growth and activation of ERK and AKT are consistent with this. FGFR signaling can be constitutively activated by upregulation of FGFs or by amplification or mutation of FGFRs (4–7). Currently, the underlying mechanisms responsible for activation of the FGFR/FRS2 signaling axis in these liposarcomas remain unknown, therefore, is deserving of further intensive study. However, our findings open a door that leads to dissecting and targeting FGFR/FRS2 signaling in high-grade liposarcoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Amplification of FRS2 and Activation of FGFR/FRS2 Signaling Pathway in High-Grade Liposarcoma

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