Ther Adv Med Oncol
2021, Vol. 13: 1–16
DOI: 10.1177/17588359211029125

Molecular profiling of soft-tissue sarcomas with FoundationOne® Heme identifies potential targets for sarcoma therapy: a single-centre experience

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Abstract

Background: Molecular diagnosis has become an established tool in the characterisation of adult soft-tissue sarcomas (STS). FoundationOne® Heme analyses somatic gene alterations in sarcomas *via* DNA and RNA-hotspot sequencing of tumour-associated genes.

Methods: We evaluated FoundationOne® Heme testing in 81 localised STS including 35 translocation-associated and 46 complex-karyotyped cases from a single institution.

Results: Although FoundationOne® Heme achieved broad patient coverage and identified at least five genetic alterations in each sample, the sensitivity for fusion detection was rather low, at 42.4%. Nevertheless, potential targets for STS treatment were detected using the FoundationOne® Heme assay: complex-karyotyped sarcomas frequently displayed copy-number alterations of common tumour-suppressor genes, particularly deletions in *TP53*, *NF1*, *ATRX*, and *CDKN2A*. A subset of myxofibrosarcomas (MFS) was amplified for *HGF* ($n = 3$) and *MET* ($n = 1$). *PIK3CA* was mutated in 7/15 cases of myxoid liposarcoma (MLS; 46.7%). Epigenetic regulators (e.g. *MLL2* and *MLL3*) were frequently mutated.

Conclusions: In summary, FoundationOne® Heme detected a broad range of genetic alterations and potential therapeutic targets in STS (e.g. *HGF/MET* in a subset of MFS, or *PIK3CA* in MLS). The assay's sensitivity for fusion detection was low in our sample and needs to be re-evaluated in a larger cohort.

Keywords: complex karyotype, FoundationOne® Heme, next-generation sequencing, soft-tissue sarcoma, targeted therapy, translocation-associated sarcoma

Received: 7 March 2021; revised manuscript accepted: 11 June 2021.

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Background

Adult soft-tissue sarcomas (STS) are rare mesenchymal malignancies which occur with a reported incidence of 50 per million per year and account for approximately 1% of solid adult cancers.[1] Sarcoma pathology comprises a diverse landscape of more than 100 entities that vary in their clinical behaviour and aggressiveness. These neoplasms are heterogeneous diseases classified based on the normal mesenchymal tissue type they closest resemble; however, based on next-generation sequencing (NGS), new tumour entities have been described, old ones reshaped, and others erased.[1,2] Even though surgery is the usual course of treatment, independent variables such as histological subtype, tumour size (>5 cm), and grading were shown to be significantly associated with overall survival (OS) and the risk for distant metastases.[1,3] Stringent classifications are required for exact diagnosis, prediction of behaviour, prediction of

OS, better treatment selection, and identification of potential treatment targets, as well as prediction of response. The World Health Organization (WHO)'s 2020 Classification of Soft Tissue and Bone Tumours[1] therefore underlines the significance of conventional morphology and immunohistochemistry in the diagnosis of STS, and integrates molecular genetics as a powerful diagnostic tool. Thus, efforts need to be undertaken to move forward with individualised treatment concepts considering the diversities of the disease that result in differences in individual prognoses. Even though histology-tailored chemotherapeutic regimens have not yielded results superior to those of standard chemotherapy in an international, randomised trial,[4] a growing body of evidence suggests that different therapeutic approaches must be undertaken for molecularly distinct sarcoma subtypes.[5] To compare data obtained from different study collectives, the histologic diagnosis of STS should be based on the recent WHO classification, and the diagnosis needs to be confirmed by molecular methods if the underlying genetic mechanism is known. The necessity of molecular subtyping of sarcomas is emphasised, in particular, by the recent recognition of *NTRK* fusions in a broader spectrum of soft-tissue tumours[6] and the achievement of phenomenal treatment responses with TRK inhibitors.[7] The improvement and increasing availability of NGS technologies, as well as novel trial designs such as basket trials, further nourish the current trend towards a molecular characterisation of mesenchymal neoplasms.[5] In view of these developments, reliable NGS technologies are desirable for routine clinical use, to support pathological diagnosis and to help assign patients with potentially druggable targets to biomarker-based therapies. To provide individualised treatment modalities for patients with STS in a clinical observation-driven approach, we analysed a sample of 81 primary localised STS of a single institution *via* FoundationOne® Heme DNA- and RNA-hotspot sequencing.

Materials and methods

Subjects

Eighty-one patients with histologically confirmed STS, who underwent surgery between October 1998 and June 2016 at the Department of Orthopaedics and Trauma, Medical University of Graz, were enrolled in this retrospective study. All patients were included in the routine follow-up programme until December. A total of 2018 follow-up examinations were conducted in regular intervals (3 months in years 1–3, 6 months in years 4–5, and 12 months in years 6–15 after diagnosis). Follow-up investigations included clinical check-ups and radiological analyses (computed tomography, magnetic resonance imaging, abdominal ultrasound and chest X-ray). Clinicopathological data, histopathological diagnosis, and tumour grade were retrospectively obtained from the patient's histories. Histological specimens were centrally re-reviewed by an experienced soft-tissue pathologist (BL-A) prior to their inclusion in this study. All sarcomas were diagnosed according to the WHO Classification of Tumours of Soft Tissue and Bone[8] and graded according to the French Federation of Cancer Centres Sarcoma Group (FNCLCC);[9] or tumour grade was defined by tumour entity. Malignant fibrous histiocytomas have been re-classified according to the current diagnostic criteria.[10] This study has been approved by the Institutional Review Board (IRB) of the Medical University of Graz, Graz, Austria (29-205 ex 16/17).

FoundationOne® Heme

FoundationOne® Heme is an integrated genomic test that analyses and interprets sequence information for somatically altered genes in human haematologic malignancies and sarcomas. Genes included in this assay encode known or likely targets of therapies, either approved or in clinical trials, or otherwise known drivers of oncogenesis. This assay analyses the complete coding DNA sequences of 406 genes, as well as selected introns of 31 genes involved in rearrangements. FoundationOne® Heme also interrogates the RNA sequence (complementary DNA, cDNA) of 265 commonly rearranged genes to better identify gene fusions (gene list used in this project: https://assets.ctfassets.net/vhribv12lmne/zBxaQC12cScqgsEk8seMO/1ef755665cc0dfa7134df9b158e2db4c/F1H_TechSpecs_v02-05_sph.pdf).

DNA and RNA were extracted from formalin-fixed and paraffin-embedded (FFPE) material. From each FFPE specimen, a 5 µm section was stained using haematoxylin and eosin (H&E) and was then reviewed by a pathologist to confirm ⩾20% tumour nuclei and a tissue volume of ⩾2 mm^3. If required, macro-dissection of samples was performed to enrich tumour content. DNA and RNA were extracted from 40 µm of unstained FFPE sections (16 unstained FFPE slides) for each sample as previously described.[11] All

analyses were performed by Foundation Medicine Inc., Cambridge, MA, USA.

Archer FusionPlex Sarcoma Panel

Archer FusionPlex Sarcoma Panel (AFPSP) is an alternative targeted RNA-sequencing NGS panel.[12] For the current analysis, the following genes were used for fusion/rearrangement/translocation detection, with the application of unidirectional gene-specific PCR and NGS-sequencing: *ALK, BCOR, BRAF, CAMTA1, CCNB3, CHMP2a, CIC, EPC1, EWSR1, FOSB, FOXO1, FUS, GLI1, HMGA2, JAZF1, MEAF6, MKL2, NCOA2, NTRK1, NTRK2, NTRK3, PAX3, PDGFB, PLAG1, RAB7a, ROS1, SS18 (SYT), STAT6, TAF15, TCF12, TFE3, TFG, USP6, VCP*, and *YWHAE*. For each sample, 5–8 × 10 µm FFPE sections were cut from a representative block, and macro-dissection was performed with a scalpel to enrich tumour content. According to the manufacturer's instructions, RNA was isolated using the Maxwell RSC RNA FFPE kit. RNA was quantified by ribogreen fluorescence, and 250 ng total RNA was used for the Archer Fusion Plex Sarcoma Kit. NGS libraries were sequenced on Ion Torrent Proton using the Ion PI Hi-Q Sequencing 200 kit (Thermo Fisher Scientific, Waltham, MA, USA). The analysis was performed with ArcherDX Analysis software version 5.1.3. All analyses were performed at the Institute of Pathology, Medical University of Graz, Graz, Austria. All tests for diagnostic purposes performed at the Institute of Pathology (including the AFPSP) were subject to *EN ISO 15189* validation. Tests were validated for performance characteristics, such as accuracy, sensitivity (true positive rate), specificity (true negative rate) and precision.

FISH, rt-PCR

Fluorescence *in situ* hybridisation (FISH) and real-time polymerase chain reaction (rt-PCR) were performed on FFPE tissue as described previously.[13,14] For rt-PCR detection of SYT-SSX as illustrated in Figure 4(c), 1 µg total RNA was reversely transcribed using the SuperScript™ III first-strand synthesis system (Thermo Fisher) according to the manufacturer's instructions. PCR was performed in a 20 µl reaction containing 2 µl of the reverse-transcription reaction, 1X HotStarTaq master mix (Qiagen, Hilden, Germany), and 0.4 µmol/l of each primer. The SYT-SSX2 product was amplified using primers for SYT (SYT-FP: 5′ CCA GCA GAG GCC TTA TGG ATA 3′) and SSX18 (SSX2-RP: 5′ GCA CAG CTC TTT CCC ATC 3′). After an initial incubation step at 95°C for 15 min, the samples were amplified by running 40 cycles at 94°C for 50 s, 58°C for 30 s, and 72°C for 1 min, followed by 1 cycle at 72°C for 10 min and cooling down to 4°C. The PCR products were analysed by gel electrophoresis on a 3% agarose gel. A control rt-PCR reaction for porphobilinogen deaminase (PBG-D) was used to check for the presence of amplifiable RNA in the samples (data not shown).

Plot generation

Plots were generated in R, version 3.5.1, and GraphPad Prism v9. We used the maftools R/Bioconductor package for visualisation and summarisation of mutation-annotation-file format files from this study.[15] For the analyses, synovial sarcomas (SS) with *SS18-SSX1* and *SS18-SSX2* rearrangements and myxoid liposarcomas (MLS) with *DDIT3-FUS* and *DDIT3-EWSR1* rearrangements were pooled together.

Statistical analysis of clinical data

Variables are described as measures of central tendencies (e.g. proportion, mean, median), as appropriate. OS was defined as the time from surgery date to death of any cause or last follow up. Recurrence-free survival (RFS) was defined as the time from surgery to the date of local recurrence, date of distant metastases or last follow up. The 5- and 10-year estimates of OS and RFS and the corresponding 95% confidence intervals are reported. Survival analysis was carried out with the statistical program Stata/MP 13.0 (StataCorp, College Station, TX, USA).

Results

Patients' characteristics

Our cohort included 81 patients (51 males, 30 females; Table 1, Figure 1) with a median age at the time of surgery of 63.1 years (range 19.7–94.7 years). A total of 46 patients (56.8%) were diagnosed with STS with a complex karyotype and 35 patients (43.2%) with translocation-associated STS. The median follow-up time was 50.3 months (range 3.7–218.0 months). During the follow-up period, 10 (12.4%) and 38 (46.9%) patients developed local recurrences and distant

Table 1. Patient characteristics and histotypes.

Variable	All ($n=81$)
Age in years	Median 63.1 (range 19.7–94.7)
Age <40 (%)	14 (17.3)
Age 40–59 (%)	23 (28.4)
Age ⩾60 (%)	44 (54.3)
Sex (%)	
Male	51 (63.0)
Female	30 (37.0)
Tumour location (%)	
Head/neck	3 (3.7)
Thoracic/trunk	5 (6.2)
Retro/intra-abdominal	1 (1.2)
Upper extremity	9 (11.1)
Lower extremity	63 (77.8)
Tumour size in cm	Median 9 (range 1–25)
Tumour size, categories (%)	
<5 cm	20 (24.7)
5–10 cm	33 (40.7)
>10 cm	28 (34.6)
Tumour depth (%)	
Superficial	24 (29.6)
Deep	51 (63.0)
Superficial and deep	6 (7.4)
Tumour grading (%)	
G1	9 (11.1)
G2	20 (24.7)
G3	52 (64.2)
Histological group (%)	
Specific translocation	35 (43.2)
Complex karyotype	46 (56.8)
Histological subtype (%)	
Myxofibrosarcoma (MFS)	25 (30.9)
Undifferentiated pleomorphic sarcoma (UPS)	11 (13.6)

(continued)

Table 1. (continued)

Variable	All ($n=81$)
Leiomyosarcoma (LMS)	10 (12.4)
Myxoid liposarcoma (MLS)	15 (18.5)
Synovial sarcoma (SS)	11 (13.6)
Dermatofibrosarcoma protuberans (DFSP)	4 (4.9)
Low-grade fibromyxoid sarcoma (LGFMS)	3 (3.7)
Extraskeletal myxoid chondrosarcoma (EMC)	1 (1.2)
Clear-cell sarcoma (CCS)	1 (1.2)
Resection margins (%)	
R0	80 (98.8)
R1	1 (1.2)
Radiation (RTX; %)	
Neoadjuvant	3 (3.7)
Adjuvant	48 (59.3)
No radiation	30 (37.0)
Chemotherapy (CTX; %)	
Neoadjuvant	4 (4.9)
Adjuvant	7 (8.6)
No chemotherapy	70 (86.4)

metastases, respectively. Both local recurrence and distant metastases developed in seven (8.6%) patients. The 5- and 10-year OS rates were 73.3% [95% confidence interval (CI) 61.3–82.3] and 60.5% (95% CI 44.0–73.5), respectively, and the 5- and 10-year RFS rate was 54.5% (95% CI 42.5–65.1) and 36.0% (95% CI 19.3–53.1), respectively. Of the 26 patients (32.1%) who died, 22 (27.2%) had deaths attributable to STS, and 4 (4.9%) died of other causes. The patients' characteristics are summarised in Table 1 and Figure 1.

Molecular profiling using the FoundationOne® Heme test

All samples were centrally analysed using the FoundationOne® Heme platform, an NGS-based test covering the entire coding region of 406 genes. Moreover, selected introns of 31 genes involved in the rearrangements and RNA sequences of 265 commonly rearranged genes were interrogated to identify translocations. FoundationOne® Heme detects all classes of genomic alterations, including base substitutions, insertion-and-deletions (indels) and copy-number alterations (CNAs). Taken together, FoundationOne® Heme revealed a total of 678 genetic alterations in 386 genes, of which 148 were mutated only in a single sample. Overall, 58 genes were affected by a genetic alteration in at least five samples (6% of the overall cohort) [Figure 2(a)]. A median of 13 (range 5–49) genetic alterations was detected in 46 STS with a complex karyotype [Figure 2(b) and (c)]. The median number of genetic alterations identified in 35 translocation-associated STS was significantly lower, with a count of 10 [range 5–29, Mann–Whitney U test $p<0.0001$; Figure 2(c)]. Consistent with previous reports, the most commonly affected genes for STS with a complex karyotype included *TP53*, *RB1* and *ATRX*, while

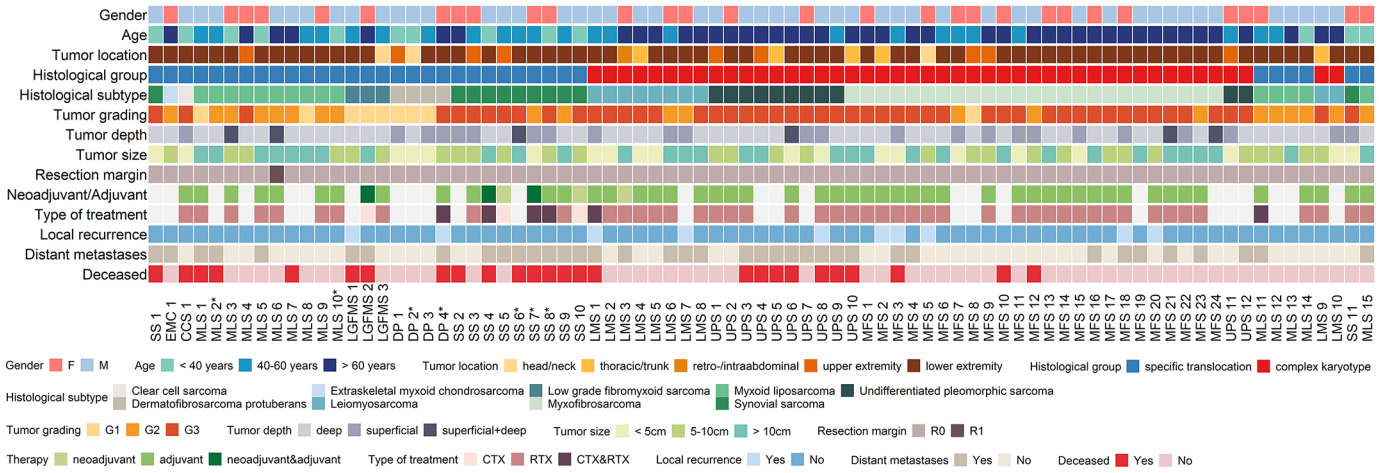

Figure 1. Heatmap summarising clinical data of the study cohort ($n = 81$).
Plotted are sex, age at diagnosis in three categories, classification into sarcomas with nonspecific complex karyotypes and translocation-associated sarcomas, tumour grading based on differentiation, mitotic count, and tumour necrosis, tumour size and depth, surgical resection margins (R0: microscopically margin-negative resection; R1: removal of all macroscopic disease, but with positive microscopic margins for tumour), treatment regimen [neoadjuvant *versus* adjuvant radiotherapy (RTX) and chemotherapy (CTX)], local recurrence, the presence of distant metastases and whether the patient was deceased. A detailed summary of clinical data and patient characteristics is given in Table 1.

gene fusions such as *DDIT3-FUS* or *DDIT3-EWSR1* in MLS, or *SS18-SSX* (including *SS18-SSX1* and *SS18-SSX2*) in SS were the main drivers for translocation-associated sarcomas (Figures 3 and 4, Supplemental Figure S1),[2,16,17]

FoundationOne® Heme detected translocations with low sensitivity in translocation-associated STS

Initially, 35 of 81 (43%) tumours were classified as translocation-associated sarcomas during routine workup based on morphology and immunohistochemistry (IHC). Translocations were confirmed in all tumours during routine workup by FISH ($n = 11$), rt-PCR ($n = 7$) or by the AFPSP ($n = 15$; Figure 4). The fusions detected by the AFPSP comprised three *SS18-SSX1* and one *SS18-SSX2* translocations in four cases of synovial sarcoma (SS), five *FUS-DDIT3* fusions and one *EWSR1-DDIT3* fusion in six myxoid liposarcomas (MLS), one *FUS-CREB3L1* and two *FUS-CREB3L2* fusions in three low-grade fibromyxoid sarcomas (LGFMS) and two *COL1A1-PDGFB* fusions in two cases of dermatofibrosarcoma protuberans (DFSP). However, in two other cases of DFSP (Table 2), diagnosis was solely based on H&E and IHC, as in one case, no translocation was detected by the AFPSP, and in the other case the material quality of the sample was not sufficient to conduct either FISH, rt-PCR or the AFPSP (Supplemental Figure S2). Therefore, the latter case was not considered for further sensitivity and specificity calculations. Taken together, fusions were identified in 33/34 (97%) cases with FISH, rt-PCR or the AFPSP.

Using the FoundationOne® Heme test, true positive fusions were detected in 14/33 (42.4%) cases. These included six *FUS-DDIT3* fusions, one *EWSR1-DDIT3* fusion and one *MYC* rearrangement in MLS, three *SS18-SSX1* translocations and one *SS18-SSX2* fusion in SS, one *EWSR1-CREB1* fusion in a clear-cell sarcoma (CCS) and one *EWSR1-NR4A3* fusion in an extraskeletal mesenchymal chondrosarcoma (EMS; Table 2). The case of DFSP, in which neither FoundationOne® Heme nor the AFPSP detected any translocation, was considered a truly negative test result. False-negative testing with the FoundationOne® Heme assay occurred in 19/33 (57.6%) cases in which translocations had been verified by other methods, as outlined above.

Thus, if only those $n = 33$ cases were considered, in which fusions were detected with other diagnostic methods, the detection rates for fusion events in STS by FoundationOne® Heme ($n = 14$) resulted in a specificity of 100% and a comparatively low sensitivity of 42.4% for this assay (Supplemental Table S2).

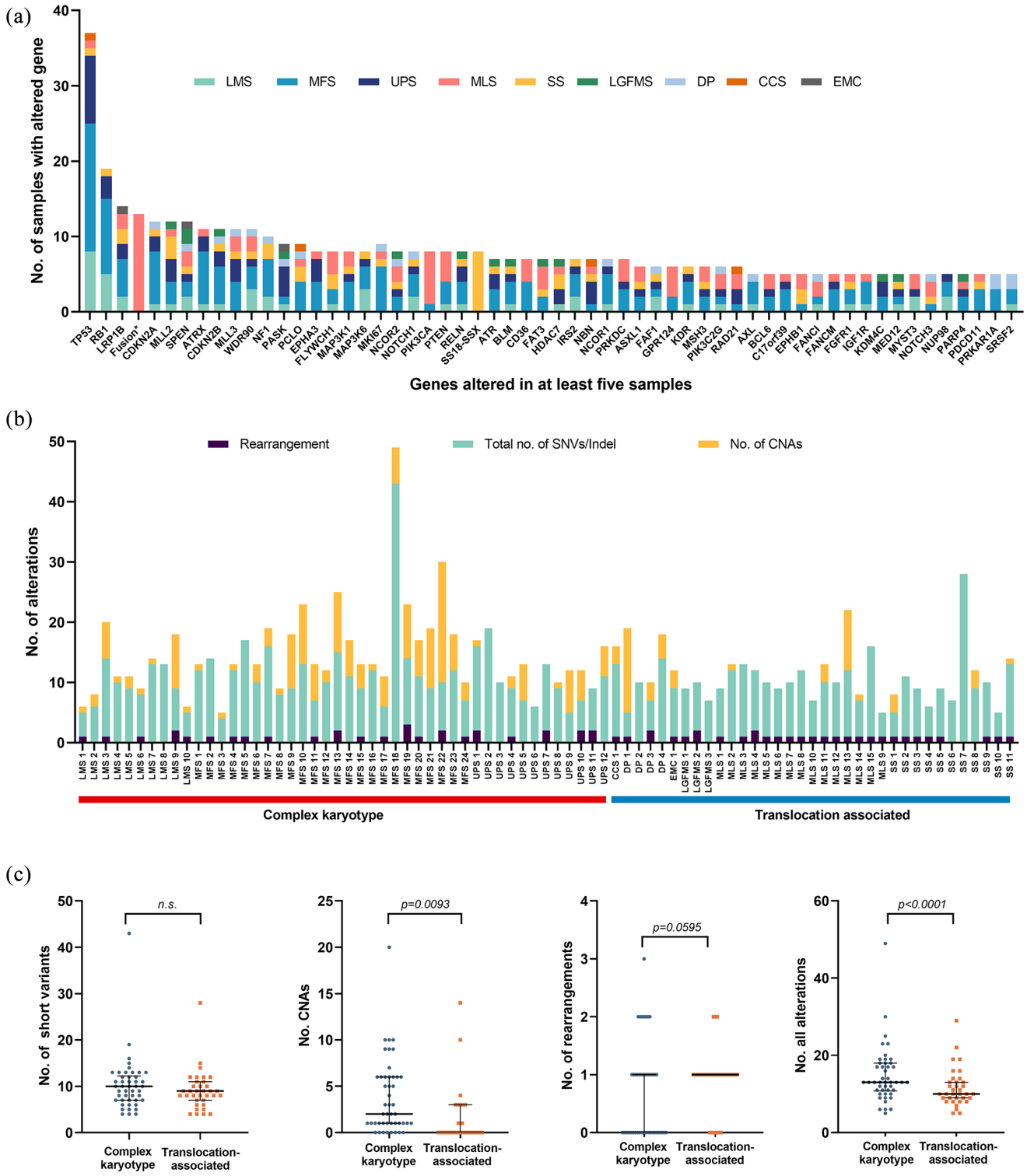

Figure 2. Comparison of genetic alterations in STS with a complex karyotype *versus* translocation-associated STS.
(a) Bar plot indicating genes that were altered in at least 5 out of 81 samples (6% of the overall cohort). Bars indicate the number (*X* axis) and subtype (different colour) of samples harbouring any type of alteration in the respective gene. Confirming previous reports, the most frequently mutated genes were *TP53*, *RB1* and *LRP1B*. (b) Summary of rearrangements, single-nucleotide variants (SNVs), insertion-and-deletions (indels) and copy-number alterations (CNAs) for each patient. (c) Distribution of short variants (SNVs and indels), CNAs and rearrangements in complex-karyotyped *versus* translocation-associated STS. While there was no significant difference in the number of short variants, and only borderline significance for rearrangements, CNAs were significantly more common in complex-karyotyped compared with translocation-associated sarcomas [77.6% (28/46) *versus* 22.5% (11/35), Mann–Whitney *U*, $p = 0.0093$]. Moreover, STS with a complex karyotype had a significantly higher number of genetic alterations (Mann–Whitney *U* test $p < 0.0001$).

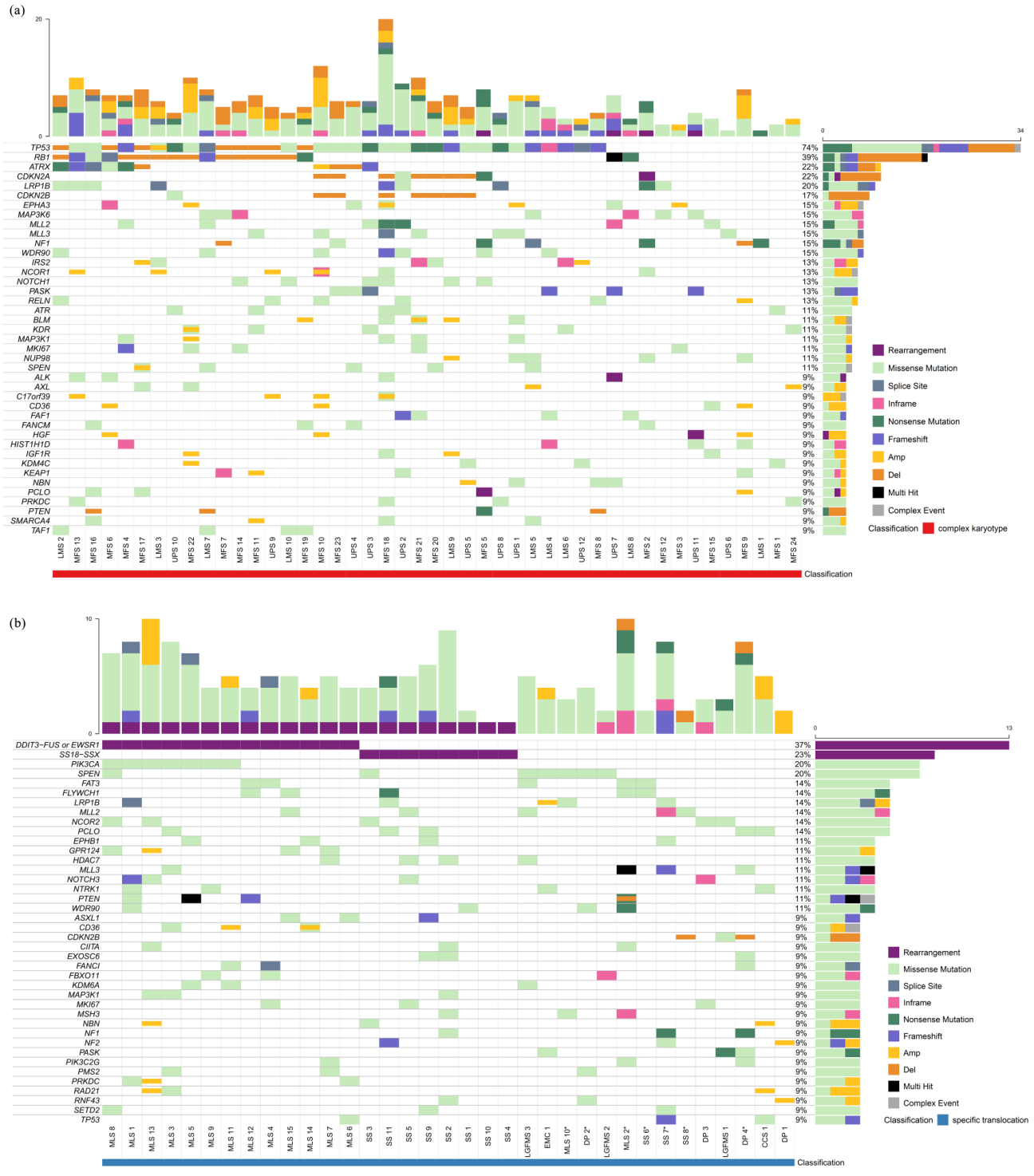

Figure 3. Oncoplot of genes altered in at least 9% of the analysed tumours.
The figure illustrates the mutational landscape of (a) sarcomas with nonspecific complex karyotypes and (b) translocation-associated sarcomas. Each column represents a sample, and each row, a different gene. Coloured squares show altered genes, while white squares indicate a wild-type status for the respective gene. Variant types are displayed in various colours. Genes annotated as 'Multi Hit' have more than one alteration in the same sample. The barplot at the top shows the number of mutated genes for each patient, coloured according to the mutation type. The barplot on the right reports the number of mutated patients for each gene, coloured according to the mutation type. The sample designation indicates the subtype: leiomyosarcoma (LMS), myxofibrosarcoma (MFS), undifferentiated pleomorphic sarcoma (UPS), myxoid liposarcoma (MLS), synovial sarcoma (SS), low-grade fibromyxoid sarcoma (LGFMS), extraskeletal myxoid chondrosarcoma (EMC), dermatofibrosarcoma protuberans (DFSP) and clear-cell sarcoma (CCS).

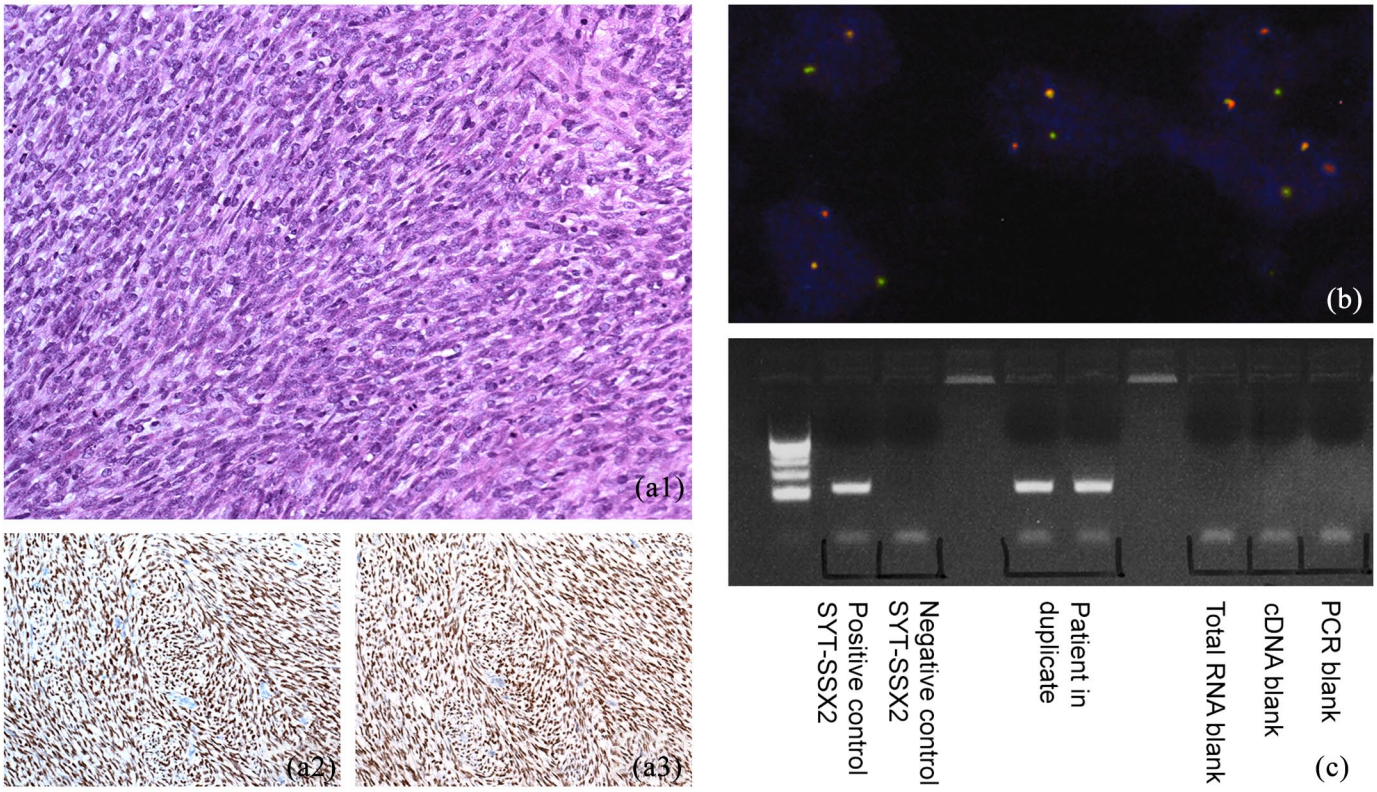

Figure 4. Validation of a SS18-SSX2 fusion demonstrated by the ArcherFusion Plex Sarcoma Panel using alternative methods for fusion detection.
(a1) H&E morphology of a monophasic synovial sarcoma with a SS18-SSX2 fusion detected by the AFPSP. (a2) IHC demonstrates a strong nuclear staining with a SS18-SSX fusion specific antibody (clone E9X9V; cat# 72364; Cell Signaling Technology, Danvers, MA, USA). (a3) IHC shows strong nuclear staining with an SSX-specific antibody (clone E5A2C; cat# 23855; Cell Signaling Technology). (b) FISH demonstrating a SS18 rearrangement using the Zyto Light SPEC SS18 Dual Color Break Apart Probe (Zytovision, Bremerhaven, Bremen, Germany). (c) Gel electrophoresis image illustrating the rt-PCR results with confirmation of the SYT-SSX2 fusion product.
AFPSP, ArcherFusion Plex Sarcoma Panel; FISH, fluorescence *in situ* hybridisation; H&E, haematoxylin and eosin; IHC, immunohistochemistry; rt-PCR, real-time polymerase chain reaction.

Mutations in translocation-associated STS: PIK3CA is frequently mutated in MLS

In addition to gene rearrangements in translocation-associated sarcomas, the most commonly mutated genes were *PIK3CA* and *SPEN*, which were affected by missense mutations in 20% of cases. The most common *PIK3CA* variants were known 'hotspot' mutations H1047R ($n=3$) and H1047L ($n=2$) localised in exon 20 [Figure 3(b), Supplemental Table S1]. Interestingly, *PIK3CA* mutations were restricted to MLS and detected in 7 of 15 MLS cases (46.7%), while *SPEN* was altered in a variety of fusion-driven sarcomas including MLS, DFSP, LGFMS, SS and EMC [Figure 3(b), Supplemental Table S1]. Other frequently mutated genes included *FAT3, FLYWCH1, LRP1B, NCOR* and *PCLO*. Moreover, mutations in genes involved in chromatin organisation such as lysine methyltransferases or histone deacetylases were commonly found. *MLL2* was altered in five sarcomas (14%; MLS: $n=3$; SS: $n=1$; LGFMS: $n=1$); *MLL3* showed five mutations in four sarcoma samples (11%; MLS: $n=3$; SS: $n=1$; DFS: $n=1$). Histone deacetylase 7 (HDAC7) was altered five times in four sarcomas (11%; SS: $n=3$; MLS: $n=1$; LGFMS: $n=1$). Interestingly, in two cases (LGFMS: $n=1$; SS: $n=1$), *MLL2* mutations coexisted with *HDAC7* alterations, whereas one patient with SS showed (coexisting) mutations in the genes *MLL2* and *MLL3*. In contrast, CNAs were less common. Deletions were detected in *CDKN2B* ($n=2$, DFSP, SS) and *PTEN* ($n=1$, MLS). Amplifications were present in *CD36* ($n=2$, MLS), *NBN* ($n=2$, MLS, CCS) and *RAD21* ($n=2$, MLS, CCS). Furthermore, individual cases were amplified for *LRP1B* ($n=1$, EMC), *GPR124* ($n=1$, MLS), *NF2* ($n=1$, DFSP), *PRKDC* ($n=1$, MLS) and *RNF43* ($n=1$, DFSP).

Table 2. Summary of all translocations that were detected with FISH, rt-PCR, Archer Fusion Plex Sarcoma Panel (AFPSP) and FoundationOne® Heme.

Histological subtype	FISH	rt-PCR	AFPSP	FoundationOne® Heme
Synovial sarcoma (SS; *n* = 11)		*SS18 SSX1* or *SSX2* (formerly *SYT-SSX; n* = 7)	*SS18 SSX1* or *SSX2* (*n* = 4)	*SS18 SSX1* or *SSX2* (*n* = 4)
Myxoid liposarcoma (MLS; *n* = 15)	*FUS-DDIT3* or *EWSR1* (formerly *CHOP; n* = 9)		*FUS-DDIT3* or *EWSR1* (*n* = 6)	*FUS-DDIT3* or *EWSR1* (*n* = 7); *MYC* rearrangement (*n* = 1)
Extraskeletal mesenchymal chondrosarcoma (EMS; *n* = 1)	*NR4A3* (*n* = 1)			*EWSR1-NR4A3* (*n* = 1)
Clear cell sarcoma (CCS; *n* = 1)	*EWSR1* (*n = 1)*			*EWSR1-CREB1* (*n* = 1)
Low grade fibromyxoid sarcoma (LGFMS; *n* = 3)			*FUS-CREB3L2* (*n* = 2); *FUS-CREB3L1* (*n* = 1)	
Dermatofibrosarcoma protuberans (DFSP; *n* = 4)			*COL1A1-PDGFB* (*n* = 2); insufficient material (*n* = 1)	

FISH, fluorescence *in situ* hybridisation; rt-PCR, real-time polymerase chain reaction.

Complex karyotype STS are mainly driven by alterations in recurrently mutated tumour suppressors and CNA

A total of 46 sarcomas of a complex karyotype, including 25 myxofibrosarcomas (MFS; 54%), 11 undifferentiated pleomorphic sarcomas (UPS; 24%) and 10 leiomyosarcomas (LMS; 22%), were profiled using the FoundationOne® Heme assay. In contrast to the translocation-associated sarcomas, these tumours were characterised by highly recurrently mutated genes [Figures 2(b) and (c), 3(a)]. Moreover, CNAs were significantly more common compared with translocation-associated sarcomas [77.6% (28/46) *versus* 22.5% (11/35), Fisher's exact test, $p = 0.0093$; Figure 2(c)]. The most commonly altered gene was *TP53*, which was affected by both mutations and CNAs in 74% of cases. Genetic alterations, mostly deletions, in *RB1* were detected in 39% of cases, followed by *ATRX* and *CDKN2A*, which were altered in 22% of cases [Figure 3(a)]. In general, deletions were mainly observed in MFS. Unsurprisingly, deletions were mostly observed in well-characterised tumour suppressors, such as *TP53*, *RB1*, *ATRX*, *CDKN2A*, *CDKN2B*, *NF1* and *PTEN*. Recurrent amplifications were detected in *EPHA3* (UPS: *n* = 1; MFS: *n* = 3), *NCOR1* (MFS: *n* = 2; LMS: *n* = 1; UPS: *n* = 1), *C17orf39* (MFS: *n* = 3; UPS: *n* = 1) and *CD36* (MFS: *n* = 3) [Figure 3(a), Supplemental Table S1].

The axis of hepatocyte growth factor (*HGF*) and its receptor (*HGF* receptor, *MET*) was affected in five cases (MFS: *n* = 4; UPS: *n* = 1), of which the majority displayed amplifications. *HGF* was amplified in three cases of MFS; one of these cases was additionally amplified for *MET*. Occasionally, amplifications were also seen in genes encoding for other cell-surface receptors, such as *KDR* (*n* = 1, MFS), *AXL* (*n* = 2, MFS and LMS) or *IGF1R* (*n* = 2, MFS and LMS). Apart from cell-surface receptors, relevant downstream effectors were also affected by amplifications in individual cases; these included *MAP3K1* (*n* = 1, MFS) and *ISR2* (*n* = 2, MFS and UPS).

Apart from *TP53*, mutations at the nucleotide level were mostly observed in *LRP1B* (*n* = 9; 20%), *MAP3K6* (*n* = 8; 15%) and the histone methyltransferases *MLL2* and *MLL3*, which were mutated in 15% of cases. *MLL2* and *MLL3* mutations mainly occurred in MFS (*n* = 7) and UPS (*n* = 6); LMS was affected only once (*MLL2*). Notably, mutations in these genes were also commonly observed in translocation-associated sarcomas.

Discussion

Genetic profiling of STS has become an essential part of the diagnostic routine as a confirmation of H&E and IHC.[2,18] Characteristic genetic alterations now enable better definition and distinction of different histological mesenchymal tumour (sub)types.[16] Several novel entities have recently entered the updated 2020 WHO Classification of

Bone and Soft-tissue Tumours, including *CIC*-, *BCOR*- and *NTRK*-rearranged sarcomas.[6,19–21] Tyrosine kinase fusions including *ALK, ROS2, RET, BRAF* and recently, *NTRK*, are useful actionable targets in STS.[22–24] *NRTK* fusion-rearranged tumours are a newly evolving molecularly defined tumour group showing very heterogenous morphologies (e.g. similar to infantile fibrosarcoma, inflammatory myofibroblastic tumour, solitary fibrous tumour or MPNST) and biologically range from benign to highly malignant.[7,25,26] *NTRK1-3* gene fusions have been identified as a diagnostic marker for treatment response with selective small-molecule inhibitors of the TRK kinases.[24] Targeted therapies with TRK inhibitors demonstrated phenomenal responses in the majority of patients with *NTRK1/2/3* fusion-positive tumours.[7,25,26] Considering that limited treatment options are available for most STS, it is crucial to detect tumours harbouring druggable fusions.[7,22–26] Despite the development of specific targeted approaches, however, the robust detection of structural variants remains a challenge, especially in FFPE specimens. Therefore, comprehensive validation of such assays is highly recommended.

Here, we performed molecular profiling of 81 STS using the commercially available FoundationOne® Heme assay. At least five genetic alterations with an average of 12 alterations could be detected per patient, indicating the assay has broad patient coverage. Surprisingly, FoundationOne® Heme did not detect characteristic fusion events in a large proportion ($n = 19$) of STS with otherwise confirmed fusion events ($n = 33$). As outlined above, characteristic translocations were detected either by FISH ($n = 11$), rt-PCR ($n = 7$) or by the AFPSP ($n = 15$) in 33/35 translocation-associated STS (Table 2). One case of DFSP (Supplemental Figure S2) was solely diagnosed based on H&E and IHC, as the material quality of this sample was neither sufficient to conduct FISH nor rt-PCR. However, the tumour showed the characteristic histopathologic features of a DFSP with uniform spindle-cell proliferations growing in a storiform pattern and a strong, diffuse immunoreactivity for CD34 (Supplemental Figure S2). In another case of a H&E- and IHC-diagnosed DFSP with transition into a fibrosarcomatous (high-grade) variant of DFSP, material quality was sufficient but nevertheless, no fusion could be detected by either the AFPSP or FoundationOne® Heme. After re-evaluation of this sample's H&E stains and IHC, a possible explanation for this lack of detection could be the presence of a recently described fusion involving the platelet-derived growth-factor-D gene, as this gene is neither covered by our AFPSP, nor by the FoundationOne Heme assay.[27] Of note, the AFPSP as an alternative NGS approach detected fusions in 15 cases, in which FoundationOne® Heme yielded fusion-negative results even though the quality of the provided material was sufficient. Based on detection rates in cases of good material quality and otherwise proven translocations ($n = 33$), this resulted in an overall sensitivity of less than 45% for FoundationOne® Heme fusion detection ($n = 14$) in our STS sample.

To date, we are not able to provide a satisfactory explanation for our observed failures in fusion detections with FoundationOne® Heme testing. However, false-negative results with FoundationOne® Heme testing were not solely associated with the age of the FFPE material or possibly poor RNA quality, as false-negative results occurred throughout our cohort. In contrast, the AFPSP particularly failed in samples older than 15 years in which the quality of extracted RNA was insufficient.

In general, the sensitivity of RNA-based analyses is affected by low expression of the fusion gene or highly degraded RNA (especially in FFPE samples), which impacts cDNA generation, bait hybridisation or PCR. Furthermore, FoundationOne® Heme and AFPSP are based on different enrichment technologies, which might explain the observed differences in fusion detection rates. The FoundationOne® Heme assay is a hybridisation-based approach relying on the efficient hybridisation of baits to cDNA molecules to extract the fusion gene product. If hybridisation is inefficient or the fusion gene is lowly expressed, the resulting library may contain only very few copies of the fusion product, which leads to an under-representation of this product in the sequencing data. In fact, we observed a wide range of sequence-read coverage of translocations in the FoundationOne® data, ranging from 500× down to 12×. Since no translocation was covered by less than 12 reads, is it likely that the FoundationOne® analysis pipeline applies an internal cut-off for fusion detection and does not report fusions supported by only a few reads. In contrast, PCR-based enrichment technologies such as the AFPSP are more robust, as they selectively amplify the fusion product alongside the wild-type molecule. This type of library generation process seems to have a higher efficiency in our samples, which are FFPE blocks of varying ages.

Since the content of the two assays varies, we furthermore checked whether target selectivity might be another reason for the discrepant results. While the AFPSP targets fusions of 35 genes associated with STS, the FoundationOne® kit enriches selected introns of 31 genes involved in rearrangements and RNA of 265 genes commonly rearranged in cancer. Although most of the genes included in the AFPSP are also covered by FoundationOne®, 10 genes are specific for the AFPSP (Supplemental Table S2). However, since no fusions involved in those genes were detected in our samples, this does not explain the superior performance of the AFPSP.

We are aware, though, that due to our small sample size, the findings regarding the assay's sensitivity must be interpreted with caution: these results need to be re-evaluated in a larger and ideally prospective STS patient cohort.

The translational potential of this article lies in FoundationOne® Heme's detection of putative therapeutic targets for STS treatment in our sample: consistent with previous reports,[2,16,17] our data indicate a higher abundance of genetic alterations in the STS of a complex karyotype, as compared with fusion-driven STS. Moreover, well-known tumour-suppressor genes, such as *TP53*, *RB1*, *ATRX*, *CDKN2A*, *CDKN2B*, *NF1* and *PTEN*, were recurrently affected by various alterations, including single-nucleotide variants and CNAs, with *TP53* and *ATRX* being the most frequently altered genes, affecting 22% of sarcomas with a complex karyotype. Previous reports have highlighted the contribution of mutations of the *TP53* gene in sarcomagenesis and tumour aggressiveness.[17–19] Furthermore, alterations in the *NF1* and *ATRX* genes have recently been associated with the tumourigenesis of various cancers, including mesenchymal and glial neoplasms.[28] Notably, Oppel *et al.*[28] have demonstrated that an additional loss of *ARTX* in *p53*/*nf1*-deficient zebrafish is implicated in the development of specific tumours, such as various types of STS. Further work is urgently needed to better understand the potential interaction between the well-characterised tumour-suppressor genes to promote the development of sarcomas.[28,29]

Based on genomic findings in STS, efforts are being made to compensate for lost or aberrant gene functions *via* various therapeutic approaches.[30] For example, cyclin-dependent kinase (CDK) pathway activation is recognised as an important driver of sarcomagenesis.[17,29,31] Losses of crucial regulators of this pathway, including *RB1*, *CDKN2A* and *CDKN2B* were common in our samples and have also been frequently observed in other mesenchymal neoplasms and sarcomas.[17,29] *CDKN2A* losses were furthermore correlated with a poorer prognosis in several subtypes of sarcoma.[17,29] For that reason, CDK inhibitors, namely the CDK4/6 inhibitor palbociclib (Ibrance®), are currently being evaluated in clinical phase II trials in CDK4 positive sarcomas [ClinicalTrials.gov identifier: NCT03242381] and chordomas [ClinicalTrials.gov identifier: NCT03110744].[29,32] In a phase II study on well-differentiated or dedifferentiated liposarcoma [ClinicalTrials.gov identifier: NCT01209598], palbociclib has already resulted in favourable progression-free survival (PFS) and occasional tumour responses.[29,33] Depending on the results of ongoing trials, CDK inhibitors could be further investigated as additional therapeutic tools in adult STS with proven genetic alterations of these crucial cell-cycle regulators.

Furthermore, a subset of sarcomas of a complex karyotype displayed gene amplifications. It is therefore interesting to speculate that selective inhibitors could serve as additional treatment options for these sarcomas, particularly if these amplifications affect receptor tyrosine kinases and other important regulators of intracellular pathways. Hence, several clinical trials are currently exploring targeted therapies alone or in combination with chemotherapies in sarcoma patients.[5] Our data indicate that the *HGF–MET* axis might be a relevant target. *MET* is a receptor tyrosine kinase shown to be involved in tumour-cell growth, invasion and drug resistance in many cancer types.[34,35] It is activated upon binding of its sole ligand, the HGF.[34] HGF is secreted by the tumour environment or in an autocrine manner by the tumour cells.[34] Indeed, *HGF*/*MET* was amplified in a subset of MFS. A total of 3 of 24 (12.5%) MFS displayed *HGF* amplifications; 1 of these cases was additionally amplified for *MET*. Several authors have previously reported polysomy of the *MET* gene locus on chromosome 7q in MFS and linked MET overexpression to an aggressive MFS biology.[36–38] Although MET inhibition showed promising results in solid cancers, such as non-small-cell lung cancer, gastrointestinal cancer or hepatocellular carcinoma,[39] the potential use of approved MET inhibitors, such as crizotinib in MFS, has been insufficiently investigated.[35]

Interestingly, a high proportion of MLS (7 of 15, 46.7%) carried mutations in *PIK3CA*. The *PIK3CA* gene (encoding the catalytic p110-alpha subunit of phosphatidylinositol-3-kinase, PI3K) is one of the most frequently mutated oncogenes in human cancers, with three 'hotspots' localised in exons 9 (codons 542 and 545) and 20 (codon 1047) being most commonly affected. Accordingly, the mutations we detected were also located in these hotspots. Our data support recent findings reporting the presence of activating mutations in *PIK3CA* in MLS and suggest an essential role of PI3K/Akt signalling in this entity.[40–42] In 2010, Barretina *et al.*[42] reported that *PIK3CA* mutations were associated with a worse prognosis of MLS. In postmenopausal women and men with hormone-receptor-positive, HER2-negative breast cancer, the PI3K inhibitor alpelisib was recently approved for patients with detected *PIK3CA* mutations for use in combination with fulvestrant, as it improved PFS and OS.[43] Moreover, this inhibitor class is under investigation in several cancer trials.[44] *In vitro* studies have indicated that inhibition of the PI3K pathway might also prove beneficial for MLS.[40] Therefore, PI3K inhibitors should be considered as additional treatment options in selected cases of MLS. In sum, the role of *PIK3CA* mutations should be further explored in larger MLS patient cohorts and multicentric approaches.

Finally, our sarcoma sample frequently displayed alterations in genes regulating DNA packaging, accessibility to transcription factors and other epigenetic modulators.[45] These recurrent mutations in epigenetic modulators indicate a role of epigenetic regulation in sarcoma pathogenesis.[2] Alongside *ATRX* deletions, which occurred in sarcomas of a complex karyotype, the histone lysine methyltransferases *KMT2D*/*MLL2* and *KMT2C*/*MLL3* were amongst the most frequently altered genes in both fusion-driven STS and sarcomas of a complex karyotype.[16] The latter, particularly MFS und UPS, displayed mutations in *MLL2* and *MLL3* in 15% of samples. In fusion-driven sarcomas, these genes were mutated in 14% and 11% of samples, respectively, particularly in MLS and SS. These lysine methyltransferases are putative tumour suppressors recently recognised as important in carcinogenesis;[45] they were shown to be mutated in a broad variety of blood and solid cancers.[45] Initial data obtained for other cancer types suggest that treatments with poly(ADP-ribose) polymerase and mitogen-activated protein-kinase inhibitors might be beneficial in treating *KMT2C* and *KMT2D*-mutated tumours, respectively.[45] However, the role of *KMT2* mutations in sarcoma pathogenesis and treatment remains to be explored.

Of note, the present analysis was exclusively performed on localised neoplasms at a single timepoint, thereby providing only a snapshot of the disease. However, to study tumour evolution and to identify progression from early-stage to advanced disease, or to detect early local recurrence, longitudinal studies are required.[46] Due to the invasive nature of biopsies, instigators face ethical, organisational and financial issues when performing serial sampling of tissue.[46] However, in the last decade, the analysis of tumour-derived circulating markers from blood ('liquid biopsy') has evolved as a minimal-invasive alternative to tissue biopsies.[47] Despite the broad variety of histological and genetic subtypes of STS and the lack of a common biomarker, several studies have already demonstrated promising results for circulating tumour DNA (ctDNA) as a marker for monitoring disease burden,[48–50] or to detect minimal residual disease and recurrence.[51,52] In general, ctDNA levels might be low in sarcomas but can be readily detected in aggressive or larger tumours such as advanced LMS or MLS.[48,49,52] Detecting a lower disease burden is a challenge but this might be overcome by developing patient-specific high-resolution assays.[47] Overall, validation of the diagnostic and prognostic relevance of ctDNA in large and longitudinal studies is required to enable routine clinical implementation.

Another major limitation of this study is its comparatively small sample size. Even though many of our results align with those of the published data, these data are also often derived from study cohorts of limited size.[36–38,40–42] Nevertheless, it is important to see these studies published and to report interesting findings that may ground further scientific hypotheses.

Conclusion

In conclusion, the most striking observation of our study was the low sensitivity of fusion detection of the FoundationOne® Heme assay in translocation-associated STS. The assay detected translocations in only 14 out of 33 cases, in which fusions were detected using other methods such as FISH, rt-PCR or the AFPSP. However, due to our small sample size, these findings must be interpreted with caution and need to be re-evaluated in a

larger and prospective patient cohort. Nevertheless, the molecular findings in our comparatively small cohort of STS support existing evidence for potential therapeutic targets for STS treatment, for instance, of a putative role of *CDKN2A* losses in sarcoma pathogenesis. Furthermore, mainly due to amplification, an upregulation of *HGF*/*MET* signalling was observed in a subset of MFS. Moreover, MLS frequently harboured *PI3KCA* mutations, supporting the putative role of the PI3K/Akt pathway in this entity. Finally, recurrent mutations in epigenetic modulators such as *MLL2* and *MLL3* in translocation-associated and complex-karyotyped STS indicate a role for epigenetic regulation in sarcoma pathogenesis. Identification of molecular alterations in STS, for example, by FoundationOne® Heme sequencing, paves the way for personalised treatment strategies in STS, making these approaches highly translationally relevant in the clinical setting.

Acknowledgements

The authors would like to thank the teams of the immunohistochemistry and molecular pathology laboratories of the Diagnostic and Research Institute of Pathology, Medical University of Graz, Graz, Austria, for their help with tissue preparation and shipment. We would furthermore like to thank Dr Karl Kashofer for his advice and help with the revisions of this manuscript. We are grateful to all clinicians who care for these patients, and for the patients who generously donated their tissue for this research.

Authors' contributions

S Scheipl: investigation, methodology, writing: original draft preparation, writing: review and editing; I Brcic: data curation, investigation, writing: review and editing; T Moser: formal analysis, investigation, visualisation, writing: review and editing; S Fischerauer: formal analysis, investigation, writing: review and editing; J Riedl: data curation, investigation, writing: review and editing; M Bergovec: data curation, investigation, writing: review and editing; M Smolle: data curation, investigation, writing: review and editing; F Posch: data curation, investigation, writing: review and editing; A Gerger: data curation, investigation, writing: review and editing; M Pichler: data curation, investigation, writing: review and editing; H Stoeger: data curation, investigation, funding acquisition, writing: review and editing; A Leithner: data curation, investigation, writing: review and editing; E Heitzer: formal analysis, methodology, validation, visualisation, writing: review and editing; B Liegl-Atzwanger: conceptualisation, methodology, project administration, supervision, validation, writing: original draft preparation; J Szkandera: conceptualisation, methodology, project administration, supervision, validation, funding acquisition, writing: original draft preparation. All authors critically reviewed the manuscript and approved its final version.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Funding

The authors disclosed receipt of the following financial support for the research, authorship and/or publication of this article: the study was funded by Roche Austria (Vienna, Austria). The funding source had no role in the design of this study: neither in its execution, data analyses, nor interpretation; writing of the manuscript; nor decision to submit the article for publication.

Ethics approval

This study has been approved by the IRB of the Medical University of Graz, Graz, Austria (29-205 ex 16/17). Patients provided their informed consent. The study was performed in accordance with the Declaration of Helsinki.

Data availability

A detailed summary of the sequencing data of each individual case reported in this manuscript is provided in Supplementary Table 1 (Table S1).

Supplemental material

Supplemental material for this article is available online.

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