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BRIEF REPORT

Genomic evolution of high-risk soft tissue sarcomas under thermo- and chemotherapeutic selection pressure

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ABSTRACT

Background: The integration of regional hyperthermia into the multimodal treatment of patients with localized high-risk soft tissue sarcomas has been shown to improve overall survival. However, specific effects on the tumors' genetic makeup and biology are largely unknown. Since clonal selection of malignant cells capable of thermoresistance might contribute to disease progression, a better understanding of the induced tumor evolution could inform strategies for improving treatment efficacy.

Methods: We performed whole exome sequencing on paired sarcoma samples obtained before and after treatment with chemotherapy combined with regional hyperthermia ($n=12$ patients; $n=9$ paired samples, $n=3$ post-treatment only).

Results: Tumor evolution was evident in all paired samples, with shifts in small- and large-scale genomic alterations. Overall, these alterations appeared tumor-specific, but recurrent mutations in histone H3-modifying genes were found exclusively in post-therapeutic samples.

Conclusion: Our findings showcase the diversity of genomic evolution patterns in sarcomas emerging under combined thermo- and chemotherapeutic selection pressure, indicating that treatment response may vary according to the specific tumor composition. With previous studies linking histone functions to heat stress response, the alterations found in genes modifying H3 in our post-therapeutic cohort provide biological evidence for synergy of chemotherapy combined with regional hyperthermia in soft tissue sarcomas.

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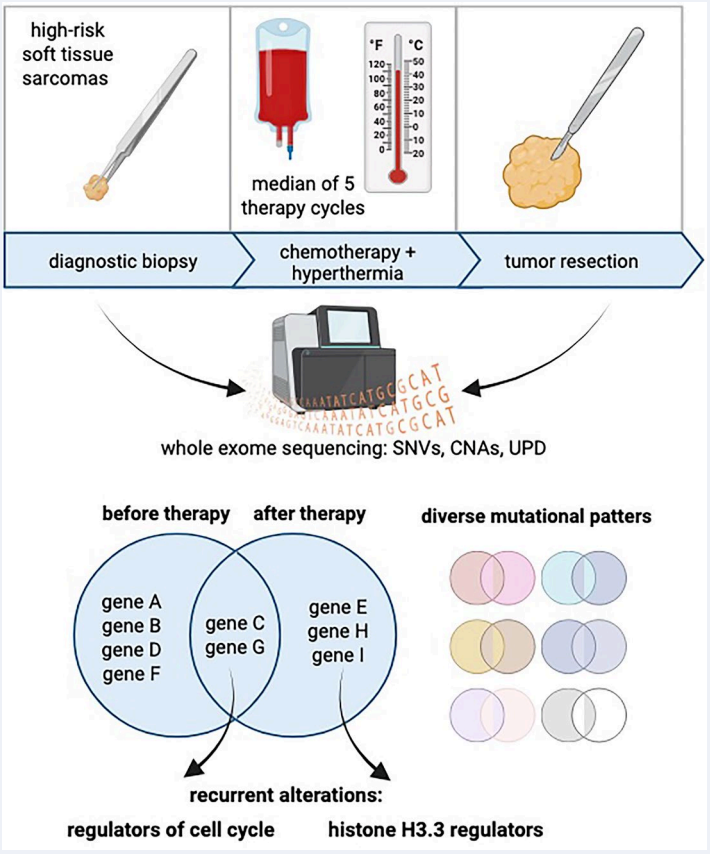
GRAPHICAL ABTSRACT



Introduction

Soft tissue sarcomas (STS) are mesenchymal malignancies that account for only ~1% of all cancer subtypes in adults. They are genomically and biologically diverse, with over 100 histologic subtypes recognized by the World Health Organization [1,2]. On a genomic level, STS can be classified as translocation-associated tumors, in which sarcomagenesis is driven by defined oncogenic fusion proteins, and complex-karyotype sarcomas, which are more genetically unstable and harbor large-scale genomic alterations such as increased mutational burden, somatic copy number alterations (SCNAs) and uniparental disomy (UPD) [3]. The use of broad genomic profiling is expanding in both research and clinical practice as it can reveal actionable targets for individualized therapy. Therefore, comprehensive genomic profiling is increasingly used in precision oncology programs [4–6].

The rarity and complexity of STS presents challenges to research aimed at improving outcomes, and despite recent advancements in treatment options, nearly 40% of newly diagnosed patients eventually die of the disease [7]. The specific clinical behavior and prognosis of STS is heavily determined by tumor (histological grading and subtype, size, location) and patient (age, concomitant diseases) characteristics. Treatment of STSs usually includes multiple therapeutic modalities such as systemic (chemo)therapy, surgery, radiotherapy and/or regional hyperthermia and is best managed at specialized sarcoma centers [8].

For patients with localized STS, complete resection is essential to achieving a cure [9]. Even then, factors that define a high risk for local recurrence or distant metastases are tumor size > 5 cm, histological grade 2–3 (according to the Fédération Nationale des Centers de Lutte Contre Le Cancer, FNCLCC) and localization beneath the investing fascia [10,11]. For this group, tumor control can be improved by combining neoadjuvant chemotherapy with regional hyperthermia (CT/RHT). This therapeutic approach is associated with better response rates, longer progression-free and disease-free survival and an overall survival advantage compared to chemotherapy alone [12,13].

Synergistic effects of heat exposure (39–44 °C) and other treatment modalities have been reported in various human cancers. The therapy-enhancing mechanisms of hyperthermia are ascribed to a variety of effects: On a macroscopic level, increased perfusion and oxygenation is believed to drive chemosensitization. On a molecular level, alterations in cell cycle regulation, apoptosis, reactive oxygen species production, DNA damage response and functions of heat shock proteins and histones likely contribute to therapeutic efficacy. Heat stress therefore constitutes a selection pressure, and exposed cells can activate an ample network of processes to protect themselves from damage. In the context of cancer treatment, this translates into therapy resistance [14,15]. A recent meta-analysis by Scutigliani et al. examined hyperthermia-induced transcriptome alterations in various human cancer lines. They identified a "core set" of 72 genes found to be differentially expressed across most datasets, but noted the absence of a truly universal gene expression signature, underscoring both the need for more controlled experiments and suggesting that the heat stress response varies between tumor subtypes [16].

Specific mechanisms underlying both the beneficial effects of hyperthermia treatment and acquired resistance to this therapy are poorly understood in STS. Since this knowledge will likely help in optimizing the integration of RHT into treatment regimens, we set out to characterize genomic evolutionary patterns under thermo- and chemotherapeutic selection pressure in these cancers.

Results

Patient characteristics

We identified $n = 12$ STS patients treated with CT/RHT at Charité – Universitätsmedizin Berlin between 2015 and 2018 where tumor and matched normal tissue were available for molecular studies. All but one pre-therapeutic samples were obtained from patients with therapy-naïve tumors. One patient had previously undergone surgery and radiation for the primary tumor, later relapsed, and was subsequently included in this study. The median age of patients in our cohort was 61 years (range = 34–73) at initial diagnosis. Histologic subtypes included relatively common STSs, such as liposarcomas ($n = 5$) and leiomyosarcomas ($n = 3$), as well as rare sarcoma subtypes. In line with current clinical practice guidelines, nine sarcomas treated with CT/RHT were classified as high-risk and thereby met the indication for RHT treatment [9]. In addition, three tumors were treated as individualized therapeutic approaches despite not meeting high-risk criteria (one > 5 cm G1 leiomyosarcoma, one < 5 cm undifferentiated pleomorphic sarcoma and one superficial myxofibrosarcoma). We included these samples to increase sample size for analysis of underlying biological mechanisms, which are not expected to be influenced by clinical risk classifications. A median of five CT/RHT (range = 1–6) cycles were administered. Variation in cycle number reflected clinical circumstances: One patient received only one cycle because of treatment-related sepsis, which led to expedited surgery. We chose to retain this patient in the analysis as the genomic alterations observed were consistent with findings in other patients. Radiological treatment response data during CT/RHT therapy was available for eleven patients, with three patients exhibiting a partial response, five patients maintaining a stable disease, and three patients experiencing tumor progression. Seven patients achieved long-term survival and five suffered relapses within five years of initial diagnosis, with one patient dying of disease. All baseline and clinical characteristics are shown in Table 1.

Sarcomas exhibit extensive evolution of somatic variants and retain a low mutational burden after CT/RHT

Sufficient DNA for analysis could be obtained from all post-therapeutically resected tumors and from nine paired diagnostic biopsies obtained before exposure to CT/RHT. The unpaired samples were included in the analyses to provide additional exploratory insights into tumor biology after therapy. In accordance with previous reports [5,6,17–20], whole-exome sequencing (WES) revealed a low overall somatic mutational burden, with an average of 0.45 mutations per megabase (range = 0.16–1.71) found across all samples. Before and after exposure to CT/RHT, samples exhibited an average of 17.4 (range = 8–30) and 16.8 (range = 6–63) genes affected by somatic sequence-level variants per tumor, respectively. Temporal comparison revealed tumor evolution in all paired samples, with an average of 24% (range = 0–80%) of

Table 1. Patient characteristics.

Baseline characteristics		N
age (years, median (range))		61 (34–73)
BMI (mean ± SD)*		26.6 ± 3.2
Charlson Comorbidity Index (MW ± SD)		1.3 ± 1.2
sex	female	4
	male	8
histology	liposarcoma	5
	pleomorphic liposarcoma	2
	dedifferentiated liposarcoma	2
	myxoid liposarcoma	1
	leiomyosarcoma	3
	myxofibrosarcoma	1
	chordoma	1
	pleomorphic sarcoma	1
	synovial sarcoma	1
tumor site	lower extremity	6
	retroperitoneum	4
	trunk	2
tumor depth	deep	11
	subcutaneous	1
tumor size	>5 cm	10
	<5 cm	2
FNCLCC grading	G1	1
	G2	6
	G3	5
Clinical characteristics		**N**
chemotherapy regimen	doxorubicin + ifosfamide	8
	doxorubicin + DTIC	2
	doxorubicin + olaratumab	2
cycles of chemotherapy administered (median (range))		5 (1–6)
cycles of RHT administered (median (range))		5 (1–6)
therapy response*	partial response	3
	stable disease	5
	progressive disease	3
morphological regression grade Salzer-Kuntschik (mean ± SD)		5.1 ± 0.7
Outcome parameters		**N**
status	long-term remission	7
	early relapse**	5
	died of disease	1

*data available for 11 patients.
**within 5 years of diagnosis.

altered genes shared between the pre- and post-therapeutic sample. On average, 39% (range = 10–61%) of altered genes were found exclusively prior to and 37% (range = 0–63%) after CT/RHT (Figure 1, Supplemental Table 1).

Somatic variants affect common cancer driver genes and point to disrupted chromatin biology after CT/RHT

Next, we evaluated our cohort for potential cancer driver mutations, i.e. mutations that increase cancer cell fitness [21]. In line with previous studies of STS, sequence-level somatic variants most frequently affected regulators of the cell cycle, namely *RB1* and *TP53*[17]. Earlier research has also demonstrated that genes related to genome integrity, transcription regulation and chromatin and histone modification are frequently altered in STS [5,6,17–20], and we found similar cellular functions to be altered both before and after CT/RHT (Figure 1).

Prior to neoadjuvant CT/RHT, eight of nine tumors harbored at least one somatic variant in a known cancer-related driver gene (Figure 1). Within this subgroup, the genes *RB1* and *SMARCAL1* were each recurrently mutated ($n = 2$). The latter encodes for a chromatin remodeling factor and other closely related genes have been found altered in previous sarcoma studies [5,6,19,20,22]. After CT/RHT, ten of twelve tumors presented with at least one oncogenic driver mutation. Recurrent mutations affected *RB1* ($n = 3$), *TP53* ($n = 2$), *BRWD3* ($n = 2$), *MS4A14* ($n = 2$) and *SMARCAL1* ($n = 2$).

In tumors with matched pre- and post-therapeutic samples available, we evaluated for recurrent mutations present exclusively after exposure to CT/RHT, as these could potentially be linked to

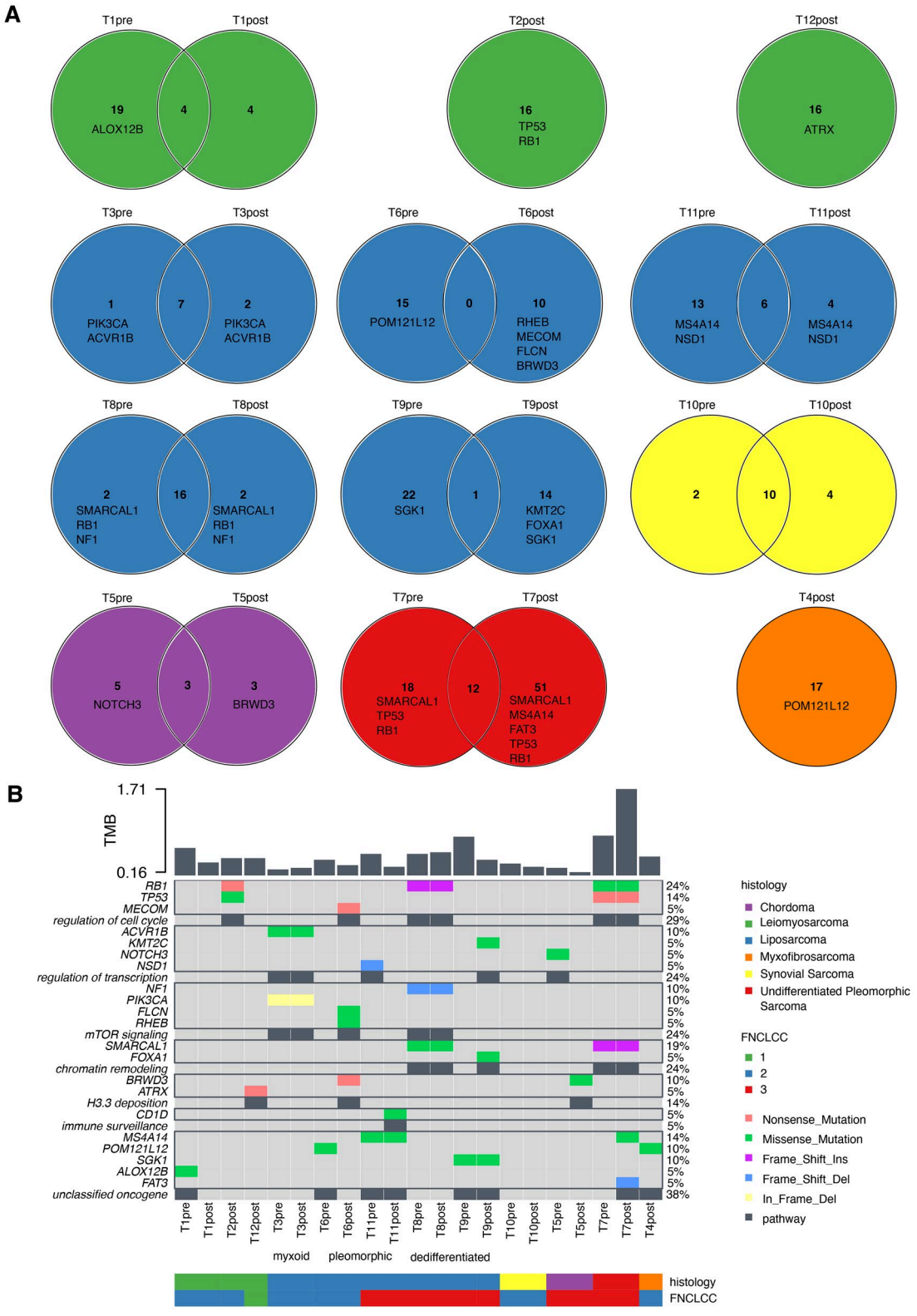


Figure 1. Somatic variants in STSs before and after CT/RHT.

A) Venn diagrams for all available tumor samples before and after CT/RHT shaded and ordered according to histology. The numbers indicate the total number of genes affected by sequence-level somatic variants present in samples obtained before or after exposure to CT/RHT, or both. Altered cancer driver genes found before and after CT/RHT are named. B) Oncoplot depicting the mutational landscape of STSs before and after CT/RHT. From top to bottom the panels indicate: Recurrent sequence-level somatic variants and/or somatic variants in selected cancer driver genes; sarcoma histology; sarcoma grade (FNCLCC). TMB: Tumor mutational burden calculated as sequence-level somatic variant/megabase. Percentages on the right indicate the fraction of samples with alterations in the corresponding gene.. Sample labels indicate patient ID and timepoint: "Txpre" refers to the sample acquired from patient x before CT/RHT, and "Txpost" refers to the matched sample collected after CT/RHT.

thermoresistance of malignant clones capable of surviving hyperthermia. The observed *BRWD3* alterations fit this criterion and are likely to impair protein function: We found one p.E467X variant that introduces a premature stop codon and a p.N714S substitution that is predicted to be deleterious by SIFT (Sorting Intolerant From Tolerant) in silico analysis [23] (score = 0.00). BRWD3 regulates gene expression via histone H3 modification [24–26]. *ATRX*, another gene involved in H3 regulation[27–29], harbored a stopgain mutation (p.K1001X) in one STS sample obtained after CT/RHT.

Sarcomas display prominent copy number alterations with temporal shifts after CT/RHT

Next, we performed an exome-wide evaluation for SCNAs. SCNAs are predominant genomic aberrations in STSs and were foreseeably frequent in our cohort (Figure 2(A)) [5,6,17,18,20,30].

Prior to CT/RHT, only the one synovial sarcoma in our cohort did not harbor SCNAs changes. Aptly, this prototypical translocation-associated STS is known to be copy number quiet [3]. In the other STS, recurrent SCNAs (defined as present in at least $n = 3$ of 8 analyzed samples) affected chromosomes 10q, 11q, 12p, 13q, 14q, 19p.

After neoadjuvant treatment, again only the synovial sarcoma showed no unbalanced segments. In the rest of the cohort, recurrent SCNAs (defined as present in at least $n = 3$ of 9 analyzed samples) affected regions of chromosomes 1p, 10q, 12p, 13q, 14q, 19p. Overall, SCNAs were linked to numerous known oncogenes, impacting FoxO, PI3K-Akt, MAPK and Ras signaling pathways (Supplemental Tables 3 and 4). Changes in copy number profiles of patients with samples before and after CT/RHT were observed (Figure 2(B)), once more suggesting tumor evolution by selection pressure.

Since gene expression levels correlate with SCNAs in many cancers[31,32], we assessed whether a recently identified set of genes found to be differentially expressed in cancers upon heat exposure [16] was affected by copy number changes in our cohort. SCNAs indeed encompassed individual genes from the set in all paired tumor samples both before and after heat exposure, but no enrichment or pattern emerged when comparing pre- and post-therapeutic samples (Supplemental Table 2).

CT/RHT does not impact the incidence of acquired uniparental disomy

Somatically acquired UPD occurs after lost chromosomes or chromosomal regions are replaced by a copy of the remaining chromosome, resulting in copy neutral loss of heterozygosity events. Preexisting (epi-) genetic alterations, including mutations, aberrant DNA methylation and histone modifications, thus become homozygous and can contribute to cancerogenesis [33]. These events were much less common in our cohort than SCNAs, and no bias for pre- or post-therapeutic occurrence could be elucidated. In total, five of 12 tumors harbored segmental UPD before and/or after CT/RHT (Figure 2(B)), with a range of $n = 1$–8 affected chromosomal regions. Three tumors harbored UPD before and after exposure to CT/RHT, with distinct shifts of the affected regions, paralleling our previous observations regarding temporal tumor evolution. UPD in the other two tumors was found in the pre- or post-therapeutic sample only, respectively. All UPD events found in our study encompassed multiple cancer driver genes.

Discussion

Sequential biopsies before and after treatment are rarely analyzed in sarcoma research, yet offer a powerful means to capture dynamic, treatment-induced tumor changes and potential correlates of therapy response. Using this design, a recent immunohistochemical study showed that higher baseline and post-treatment infiltration of STS by T cells is associated with improved response to CT/RHT [34]. Our study used a similar strategy to provide the first longitudinal genomic analysis of paired STS samples obtained before and after CT/RHT.

We unveil diverse genomic variations across different sarcoma subtypes both before and after exposure to thermo- and chemotherapeutic pressure. Across all analyzed samples and timepoints, we find a low mutational burden and a high incidence of somatic copy number alterations. High-throughput sequencing approaches used to characterize primary STS' genomic landscape have revealed a similar mutational pattern: Gains or losses of genetic material are the predominant alterations in these tumors,

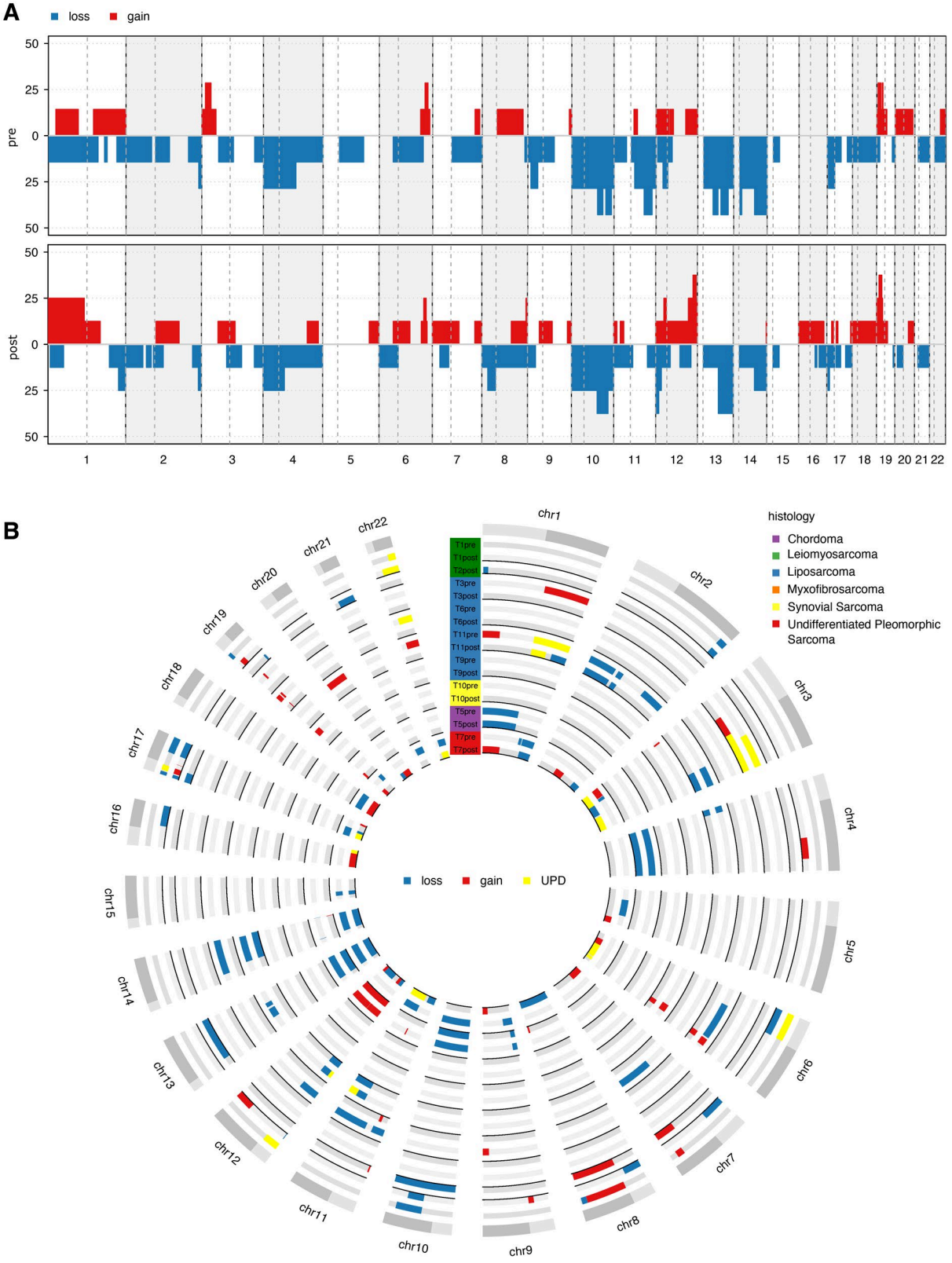


Figure 2. Somatic copy number alterations (SCNAs) and uniparental disomy (UPD) in STS samples before and after CT/RHT.

A) Frequency plot of SCNAs across all paired tumors before (top) and after (bottom) CT/RHT.

B) Circos-style plot showing SCNAs and UPD events in individual STS samples. Each row represents a single tumor sample and each column corresponds to a chromosome. Regions harboring copy number gains are shown in red, losses in blue, and UPD events in yellow. Histologic subtypes are indicated by the shaded background behind sample labels. Sample labels indicate patient ID and timepoint: "Txpre" refers to the sample acquired from patient x before CT/RHT, and "Txpost" refers to the matched sample collected after CT/RHT. This visualization allows direct comparison of chromosomal alterations and UPD events across samples and between pre- and post-treatment states.

and very few recurrent, "pan-sarcoma" mutations exist. The Cancer Genome Atlas sarcoma analysis identified only *RB1*, *TP53* and *ATRX* as significantly mutated genes across a large cohort of STS, and we found these genes to be recurrently altered, too. In addition, we, like others, found significant genomic heterogeneity across different sarcoma subtypes [5,6,17].

We characterized the genomic evolution of STS after CT/RHT to better understand how hyperthermia affects these cancers, comparing the mutational landscape of the therapy-naïve tumor to the residual disease after treatment. All analyzed paired tumors were treated with both chemotherapy and regional hyperthermia, but chemotherapy exposure likely does not confound our findings: It is well established that anthracyclines, the agents most commonly used in STS treatment regimens and received by all patients in our cohort, are not linked to specific mutational signatures across various malignancies [35,36]. In addition, a large, recent study by Christensen et al. found no significant differences comparing the genetic landscape of 666 primary, therapy-naïve and 463 metastatic, chemotherapy-treated STS, indicating that chemotherapy alone indeed leaves no distinctive mutational mark [37]. The absence of a specific mutational signature associated with chemotherapy in such a large group of STS patients therefore suggests that recurrent alterations present in our post-CT/RHT cohort could indeed be the result of selection pressure imposed by hyperthermia.

The SCNAs and UPD events observed in our cohort are in line with previous reports: SCNAs partially overlapped with known chromosomal regions frequently impacted by numerical alterations in STS [6,17]. The observed frequency of UPD mirrors that of previous studies. For example, Mille et al. identified UPD in 47.9% of STSs, while also delineating distinct alteration patterns for specific histologic subtypes. In leiomyosarcoma, they observed frequent UPD at chromosome 17p13.3-13.1 [38], a region also affected in the respective histologic subtype in our cohort. We found that all analyzed tumors harbored shifts in SCNAs and UPD events when comparing pre- and post-therapeutic samples, indicative of genomic instability and cancer evolution [39]. However, no unifying pattern was evident across samples. Generally, these structural variations can confer therapy resistance by altering gene transcription levels, but a recent meta-analysis of studies examining the effect of heat stress on the human transcriptome determined that a universal gene expression signature associated with hyperthermia treatment likely does not exist [16]. This is in line with our data but should be confirmed in larger, more controlled studies.

When analyzing somatic variants, we found recurrent alterations in the cancer-associated gene *BRWD3*. The encoded protein is evolutionarily conserved and belongs to the family of bromodomains, which regulate gene expression through post-translational histone modifications: methylation, acetylation, and phosphorylation of histone N-terminal tails alter chromatin structure, affecting transcription factor and regulatory protein binding. They therefore exert context-dependent effects on gene expression, leading to either transcriptional activation or repression, and influencing cell and disease states [21,40]. Imbalances in histone levels and modifications can impact tumor traits and therapy efficacy; for example, cisplatin resistance has been linked to altered histone acetylation in cervical and liver cancers [41–44]. While BRWD3's role in human epigenetic maintenance remains incompletely understood, studies in *Drosophila* show that dBRWD3 depletion impacts both H3 methylation and acetylation levels [24,25]. *dBRWD3* mutants also show increased chromatic deposition of the non-canonical H3 variant H3.3, a protein that promotes viability upon exposure to heat stress [26,45]. The loss-of-function *BRWD3* mutations observed in our cohort may therefore similarly modulate H3 modification. Given that heat stress induces heterochromatin-associated H3 methylation linked to transcription silencing in human cells [46], we hypothesize that STS cells acquiring *BRWD3* mutations may gain a survival advantage by altering chromatin states in a way that protects against hyperthermia-induced transcriptional repression.

Notably, *ATRX*, a member of the H3.3-specific histone chaperone complex ATRX/DAXX, was also mutated in one STS sample after CT/RHT; however, no pretreatment sample was available to determine whether this mutation emerged post-therapy. ATRX regulates H3.3 levels and contributes to the maintenance of heterochromatic modifications [27–29]. Consistent with our *BRWD3* model, the *ATRX* stopgain detected after CT/RHT may therefore alter transcriptional capacity under thermal stress and confer a survival advantage.

Strikingly, all alterations in H3 modifiers were exclusive to post-therapeutic samples, suggesting that CT/RHT may exert selective pressure on STS cells via chromatin-based mechanisms. The co-occurrence of *BRWD3* and *ATRX* mutations post-treatment highlights a potential link between epigenetic remodeling

and therapeutic stress resistance, providing a possible mechanistic explanation for the emergence of STS cells with altered chromatin states following therapy. Importantly, these observations come from a small cohort and will require validation in larger studies, as well as further investigation to confirm the proposed mechanism experimentally.

In summary, we uncover tumor evolution between pre- and post-therapeutic samples by delineating shifts in somatic variant, SCNA and UPD patterns, many of which appear to be tumor-specific. The heterogeneity observed in the genetic landscape of the examined STS cohort likely reflects important limitations of our study, i.e. a small cohort size and diversity regarding histology and clinical history. Future studies with larger, subtype-specific cohorts and control groups treated with only chemotherapy will be important to determine whether these findings are consistent within individual sarcoma subtypes and to explore potential subtype-specific mechanisms of CT/RHT resistance. Nevertheless, the emergence of recurrent alterations in H3-modifying genes such as *BRWD3* and *ATRX* highlights possible chromatin-based mechanisms of therapy-induced stress resistance. Since these mutations emerged only after treatment, they cannot serve as predictive biomarkers but may provide a basis for future studies exploring molecular changes that indicate tumor response to CT/RHT. Our results are indicative of a substantial selective pressure imposed upon STS cells when combining chemotherapy and regional hyperthermia, supporting this treatment strategy.

Methods

Study design and sample collection

Archived formaldehyde-fixed paraffin-embedded tumor and matched normal tissue from $n = 12$ patients with localized STS treated with neoadjuvant CT/RHT and subsequent resection at Charité-Universitätsmedizin Berlin between 2015 and 2018 were analyzed in this study. For all patients, pre-therapeutic analyses were performed on diagnostic biopsy material, and resected tumor tissue was used for post-therapeutic analyses. The samples were obtained per institutional clinical practice standard. Informed consent following institutional guidelines was obtained from all patients. Clinical data was retrospectively extracted from archived patient records with approval of the local ethical review committee of Charité-Universitätsmedizin Berlin (EA2/190/21) and in accordance with the Declaration of Helsinki.

Whole exome sequencing

DNA extraction from formalin-fixed paraffin-embedded tissue was performed using Maxwell 16 FFPE Plus LEV DNA Purification Kit (Promega). WES libraries were prepared using the Sure Select Low Input All Exon V7 Kit (Agilent) and sequenced on the Illumina HiSeq 4000 platform at the Genomics Core Facility of the German Cancer Research Center (DKFZ). Sequencing coverage and quality statistics for all samples are provided in the Supplementary Materials. WES data were processed as described previously [47]. We defined the following filter criteria for variants: ≥ 10 reads supporting the variant allele, variant allele frequency in tumor ≥ 7% due to the sensitivity of the method and the DNA quality from FFPE tissue, ≥2 tumor variant supporting reads in the forward and reverse direction, excluding variants located in segmental duplication areas and variants located in polymer-repeat regions and variants with entry in database of single nucleotide polymorphisms (dbSNP Build ID151, 1000 Genomes Project) [48,49], but not in COSMIC v92 [50].

Copy number data analysis

Bcftools mpileup (v1.10.2) [51] was used to pile up reads at positions of known SNPs from the dbSNP database (v151) for tumor and normal samples for each patient. These piled up reads were used for copy number calling with ASCAT (v2.5.2) [52] and subsequent refphase (v0.2.0) [53] according to the example workflow (https://bitbucket.org/schwarzlab/refphase/src/master/). Additionally, only positions on autosomes, with read depth > 10 reads and that passed a sliding-window filter with max 3 positions within 100 bp were used as an input for copy number calling. All R packages were used with R v4.0.5.

Reported SCNAs were manually filtered based on the following criteria: 1) Samples with poor quality log ratio data (e.g. excessive noise, genomic waves) were excluded. 2) SCNA calls were included if an adequate read depth and clear separation of peak B allele frequencies (BAF) was evident in BAF profiles. 3) Very small SCNAs (< 500 kb) were excluded.

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Disclosure statement

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Data availability statement

Processed data are available in Supplemental Data. Raw data are available upon request from the corresponding authors.

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