Original Article

Comprehensive Molecular Characterization of Soft Tissue Sarcoma for Prediction of Pazopanib-Based Treatment Response

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Abstract

Purpose

Even though pazopanib, a multitargeted tyrosine kinase inhibitor, has been approved for refractory soft tissue sarcoma (STS), little is known about the molecular determinants of the response to pazopanib. We performed integrative molecular characterization to identify potential predictors of pazopanib efficacy.

Materials and Methods

We obtained fresh pre-treatment tumor tissue from 35 patients with advanced STS receiving pazopanib-based treatment. Among those, 18 (51.4%) received pazopanib monotherapy, and the remaining 17 (48.6%) received pazopanib in combination with durvalumab, PD-L1 blockade. Whole-exome and transcriptome sequencing were performed for each tumor and patient germline DNA.

Results

Of the 35 patients receiving pazopanib-based treatment, 9 achieved a partial response (PR), resulting in an objective response rate (ORR) of 27.3%, and the median progression-free survival (PFS) was 6.0 months. Patients with CDK4 amplification (copy ratio tumor to normal > 2) exhibited shorter PFS (3.7 vs 7.9 months, p=2.09 x10^{-4}) and a poorer response (ORR; 0% vs 33.3%) compared to those without a gene amplification (copy ratio ≤ 2). Moreover, non-responders demonstrated transcriptional activation of CDK4 via DNA amplification, resulting in cell cycle activation. In the durvalumab combination cohort, 7 (41.2%) of the 17 patients achieved a PR, and gene expression analysis revealed that durvalumab responders exhibited high immune/stromal cell infiltration, mainly comprising NK cells, compared to non-responders as well as increased expression of CD19, a B cell marker.
Conclusion

Despite the limitation of heterogeneity in the study population and treatment, we identified possible molecular predictors of pazopanib efficacy that can be employed in future clinical trials aimed at evaluating therapeutic strategies.

Key words

Soft tissue sarcoma, Pazopanib, Immune Checkpoint Inhibitor, Whole Exome Sequencings, Whole Transcriptome Sequencing
Introduction

Soft tissue sarcoma (STS) comprises diverse histological subtypes with distinct clinical and molecular features. Despite the heterogeneity, patients with advanced STS are generally treated in the same manner, mainly using doxorubicin- or ifosfamide-based regimens [1,2]. Gemcitabine, docetaxel, as well as taxane are the available treatment options after failure of the first-line treatment. However, despite several treatment possibilities, the prognosis of STS remains poor with a median overall survival of 12 months [3].

Pazopanib is multitargeted tyrosine kinase inhibitor active against vascular endothelial growth factors, platelet-derived growth factors, fibroblast growth factor receptors, and c-Kit. Given the histologic heterogeneity of STS, pazopanib has exhibited therapeutic activity against various subtypes except for liposarcoma in a stratified phase II trial [4]. A subsequent phase III PALETTE trial was designed for patients with non-adipocytic STS who had not benefited from standard chemotherapy. Progression-free survival (PFS) was improved by 3 months relative to placebo [5]. Beside tyrosine kinase inhibitors, immune checkpoint inhibitors have also demonstrated promising efficacy with respect to various STS subtypes. For advanced bone and soft tissue sarcomas, monotherapy with pembrolizumab, an anti-PD-1 antibody, was associated with clinically meaningful efficacy. However, the observed efficacy was mostly limited to specific tumor subtypes, including undifferentiated pleomorphic sarcoma (UPS), dedifferentiated liposarcoma, and synovial sarcoma (SS) [6]. Nivolumab—another anti-PD-1 monoclonal antibody—in combination with CTLA-4 inhibitor ipilimumab has also exhibited therapeutic activity in advanced STS [6]. These results led to the regulatory approval of pazopanib and immune checkpoint inhibitors as standard treatments for refractory STS.

The Cancer Genome Atlas (TCGA) Research Network has provided molecular insights into the major subtypes of STS [7]. The multi-platform genomic profiles revealed a high
frequency of copy number alterations and low mutational burdens for sarcoma, as well as other subtype-specific genomic features. However, even though pazopanib is the only approved targeted agent for this disease, little is known about the genetic feature for response discrimination in STS.

In the current study, we performed integrated molecular profiling of advanced STS in response to pazopanib-based treatment. All patients were subjected to tissue biopsies followed by whole-exome and transcriptome sequencing. We also conducted integrative analysis to explore specific genomic markers correlated with response to pazopanib-based treatment in an attempt to identify biomarkers that could aid the therapeutic strategies.

Patients and Methods

1. Patient and study procedure

We reviewed and included patients with histologically confirmed metastatic and/or recurrent STS as per the following inclusion criteria: (1) eligible for pazopanib treatment, and (2) willingness to undergo a procedure for fresh-frozen tissue collection for clinical sequencing. Clinical information including age, sex, etiology, Eastern Cooperative Oncology Group (ECOG) performance status, Fédération Nationale des Centres de Lutte Contre le Cancer (FNCLCC) system, staging, and previous treatment data were extracted from hospital records. The trial protocol was approved by the Institutional Review Board of each center, and all patients provided written informed consent before enrolment in accordance with the Declaration of Helsinki and the Guidelines for Good Clinical Practice.

2. Tumor sample collection for whole-exome and transcriptome sequencing

Biopsies were performed prior to initiation of pazopanib-based treatment. If tumor
content was estimated to be ≥ 40% after pathological review, tumor DNA and RNA were extracted from freshly obtained tissues using a QIAamp Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For DNA isolation, we used RNaseA (cat. #19101; Qiagen). We determined the concentrations and absorbance ratios, \( \text{OD}_{260}/\text{OD}_{280} \) and \( \text{OD}_{260}/\text{OD}_{230} \), on an ND1000 spectrophotometer (NanoDrop Technologies, Thermo-Fisher Scientific, MA) and quantified DNA/RNA using a Qubit fluorometer (Life Technologies, CA). Analysis pipeline details for sequencing are available online in the supplementary methods.

3. Statistical analysis

PFS was defined as the time from the start of pazopanib-based treatment until the date of disease progression or death resulting from any cause. Overall survival (OS) was measured from the start of treatment to the date of death due to any cause. Survival difference was assessed using the log-rank test. Objective response rate (ORR) was calculated as the percentage of patients experiencing a confirmed complete response or partial response (PR) as per the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 guidelines. The significance of multiple predictors of survival was assessed by Cox regression analysis. \( p < 0.05 \) was considered to indicate a significant difference. All statistical analyses were performed using R3.5.3 and RStudio v1.2.

Results

1. Clinical and pathological characteristics of sarcoma patients

Between September 2014 and December 2019, patients with unresectable or metastatic STS (n=199) received pazopanib-based treatment. Among those, fresh tumor and peripheral blood samples were obtained from a total of 35 patients and were subjected to whole-exome
and transcriptome sequencing (CONSORT, S1 Fig). Samples were obtained in patients with primary tumors (n=16) and metastases (n=19). Of these patients, 18 (51.4%) received pazopanib monotherapy, and the remaining 17 (48.6%) received pazopanib in combination with durvalumab after the biopsies had been collected for sequencing. Commonly observed histologies included leiomyosarcoma, (n=8), UPS (n=7), malignant peripheral nerve sheath tumors (MPNST, n=5), and dedifferentiated liposarcoma (DDLPS, n=5) as shown in Table 1. The majority were of high pathological grade (FNCLCC grade 3, n=23, 65.7%), and 45.7% had developed in the upper or lower abdomen. All patients had previously received at least 1 line of chemotherapy, mainly doxorubicin- and/or ifosfamide-based treatment.

2. Molecular characterization and response to pazopanib-based treatment

Data collection proceeded until April 31, 2020 with a median follow-up of 40.9 months (95% CI: 38.2-55.9). Of the 33 evaluable patients, 9 achieved a PR, 17 had stable disease, and 7 had progressive disease, resulting in an ORR of 27.3 % (Fig. 1). In the monotherapy group, 2 (12.5 %) of the 16 evaluable patients achieved a PR, 2.0 and 3.6 months after initiating treatment, and this lasted for 18.1 and 15.9 months for soft tissue leiomyosarcoma (ST-LMS) and SS, respectively (Fig. 2A, 2B). In the combination group, 7 (41.2%) out of the 17 patients achieved a PR, including patients with UPS (n=2), SS (n=2), MPNST (n=1), a desmoplastic small round cell tumor (n=1), and endometrial stromal cell sarcoma (n=1). Response was first detected 1.1-2.7 months after treatment initiation and lasted for a median of 7.8 months (range: 2.5 to 17.1 months; Fig. 2B, 2C).

Twenty-nine patients (75.0%) had progressive disease, and the median PFS was 5.9 months (95% CI 3.3-8.6) in the monotherapy group and 6.9 months (95% CI 3.5-10.3) in the combination group. Median OS was 19.5 months (95% CI 16.7-22.3) in the monotherapy
group, and was not reached in the combination group.

3. Genomic landscape and correlates of pazopanib efficacy

We first evaluated the genomic alterations in the somatic mutation spectrum, somatic copy number alterations (SCNAs), and fusion transcripts in all 35 patients (Fig. 1, S2 Table). Mutations in genes involved in telomere stabilization and double-strand repair, including TP53 (34%), NF1 (9%), ATRX (6%), and PRKDC (6%) were most prevalent in LMS, UPS, and MPNST. The overall somatic mutational burden was similar to that of the TCGA sarcoma (SARC) dataset with a relatively low tumor mutational burden compared to other cancer types (median: 40.5 non-synonymous mutations, S2 Fig). However, there was one case of clear cell sarcoma (YCC#18) that was hypermutated (856 non-synonymous mutations), including an MLH1 splice site mutation (Fig. 1). Mutational signature analysis revealed that DNA mismatch repair deficiency with microsatellite instability contributed to genomic instability in the YCC#18 sample (S3 Fig A, B).

Frequently observed SCNAs included 12q13-15 copy number gains or amplifications of genes CDK4, MDM2, FRS2, as well as HMGA2 (Fig. 1). Interestingly, 4 cases initially diagnosed as SS (n=1, YCC#4), MPNST (n=1, SMC#6), UPS (n=1, SMC#14), and LMS (n=1, YCC#9) were revised as DDLPS (n=4) based on molecular results with CDK4 and MDM2 co-amplification. Patients with CDK4 amplification exhibited a poor response to pazopanib treatment, in which none of the 9 partial responders had CDK4 amplification (Fig. 3A). A univariate cox regression analysis revealed that CDK4 amplifications was predictive factors of poor PFS [hazard ratio (HR)=0.35, 95% CI 0.14-0.86, Fig. 3B]. Cases with CDK4 amplification (n=6, copy ratio tumor to normal > 2.0) had significantly shorter PFS when compared to non-amplified (n=9, copy ratio tumor to normal ≤ 2.0) cases (Fig. 3C; CDK4: 3.7 vs 7.9 months,
p=2.09 \times 10^{-4}). Among the 6 cases with CDK4 amplifications, 5 (83.3\%) were DDLPS, and those showed poorer PFS than any other subtypes (Fig. 3D). The other UPS with CDK4 amplification (SMC#15) showed 2.3 months of PFS, suggesting that CDK4 may play a role as a poor predictor for pazopanib treatment.

Similarly, cases with FRS2 gain or amplification (n=9, copy ratio tumor to normal > 1.5) had significantly shorter PFS when compared to non-amplified (n=26, copy ratio tumor to normal ≤ 1.5) cases (S4 Fig A; 3.7 vs 7.9 months, p=0.038). On the other hand, patients with copy loss or deletion of the HDLBP gene (n=8, copy ratio tumor to normal < 0.75), located on chromosome 2q37.3, exhibited poor PFS (p=0.036) in both univariate and multivariate cox regression survival analysis, suggesting that HDLBP may play a role as a tumor suppressor in STS (S4 Fig B and C, HR=0.39, 95\% CI 0.16-0.99). However, tumor response to treatment was not significantly different (ORR 12.5\% vs 32.0\%, p=0.39 by chi-square test). However, in covariate cox regression analysis with the histologic variants, CDK4 and FRS2 amplifications were not associated with PFS (S4 Fig C).

We further analyze the clinical outcomes of all patients and pazopanib monotherapy patients according to the TP53 mutational status. However, tumor response to treatment was not significantly different between mutant and wild-type in all patients (ORR 16.7\% vs 33.3\%, p=0.43 by Fisher’s exact test) and pazopanib monotherapy patients (ORR 12.5\% vs 12.5\%, p=1.00 by Fisher’s exact test). We could not find significant differences in PFS in all patients and pazopanib monotherapy patients (S5 Fig). We also investigated the VEGF expression according to TP53 mutational status but could not find a statistically significant difference in the VEGF expression (S6 Fig).
4. Identification of transcriptional determinants dictating clinical response to pazopanib-based treatment

To assess distinct transcriptional features that dictate the clinical response to pazopanib, we analyzed the transcriptomic profiles of STS patients depending on their response to pazopanib. Gene Set Enrichment Analysis (GSEA) was performed to identify significantly enriched molecular pathways between responders and non-responders. GSEA revealed that gene sets associated with cell proliferation/cell cycle, hypoxia, and glycolysis were upregulated in non-responders while immune-associated gene sets were enriched in responders (Fig. 4A). Enrichment of cell cycle gene sets was observed in non-responders, which was in line with the observation that CDK4 was significantly amplified in non-responders, especially as it plays a major role in the cell cycle [8] (Fig. 4B). Additionally, we performed genome-wide analysis of differentially expressed genes (DEGs) between pazopanib-based treatment responders and non-responders in order to identify genes that correlate with a response to pazopanib in STS. As a result, the expression of 123 and 106 genes was significantly up/downregulated (q-value < 0.05 and absolute log2 fold change > 1) in responders (Responder DEGs) and non-responders (Non-Responder DEGs), respectively (S3 Table). CDK4 was among Non-Responder DEGs, concordant with the results of genomic and pathway analyses, which indicated that non-responders exhibited upregulation of CDK4 via DNA amplification, particular in DDLPS, resulting in cell cycle activation. We investigated the association between gene copy number variations (CNV) and mRNA expression and found that CDK4 gene expression levels of CDK4-amplified tumors were significantly higher than those of tumors with non-amplified (Wilcoxon rank sum, $p=1.5 \times 10^{-6}$, Fig. 4C). The FRS2 amplification also showed consistent results (Wilcoxon rank sum, $p=0.0018$) but HDLBP-deletion was not significant (Wilcoxon rank sum, $p=1.1 \times 10^{-6}$, S7 Fig).
5. Predictors of treatment response to pazopanib and PD-L1 blockade combination

Next, we sought to determine the potential correlates of PD-L1 blockade response using pre-treatment STS biopsies from 17 patients enrolled in the clinical trial (ClinicalTrials.gov Identifier: NCT03798106), which evaluated the efficacy of durvalumab in combination with pazopanib. Treatment consisted of pazopanib 800 mg orally, once a day, continuously, and durvalumab 1500 mg via 60 min intravenous infusion once every 3 weeks.

When we examined PD-1/PD-L1 gene expression via whole transcriptome sequencing, no association between pazopanib/durvalumab response and PD-1/PD-L1 expression was observed (S8 Fig). Therefore, in order to characterize STS samples responding to the pazopanib and durvalumab combination using immunological features other than PD1/PD-L1 expression, we evaluated immune cell profiles in STS samples in silico by applying the Microenvironment Cell Populations-counter (MCPcounter) method to gene expression profiles in STS (Fig. 5A) [9]. For each cell type, the Student’s t-test between responders and non-responders were performed and revealed that the MCPcounter scores for NK cells were higher in the responders (p=0.047 by Fig. 5B, p-value was not adjusted for multiple testing). In agreement with the results of tumor microenvironment cell count estimation, GSEA revealed that gene sets associated with the immune response were upregulated in responders, and, in particular, NK cell pathway enrichment was confirmed (Fig. 5C-E). DEG analysis between responders and non-responders revealed that CD19 was overexpressed in responders (Fig. 5F and S4 Table). Importantly, CD19 expression levels were not different between pazopanib-responders and non-responders (log2 fold change = 0.18, FDR = 0.95), indicating that the differential CD19 expression between responders and non-responders may be the result of durvalumab treatment.
Discussion

Using whole-exome and transcriptome sequencing, we performed integrative molecular characterization of STS in patients receiving pazopanib-based treatment. To our knowledge, this is the first study to characterize molecular determinants of the response to pazopanib in STS.

Despite the improved PFS in the phase III trial, the response was modest, and OS was not significant, implying that only a minority of the patients benefit from pazopanib treatment. The lack of understanding of the molecular background underlying the pazopanib response represents a major challenge in STS. While previous studies have indicated clinicopathological parameters, including circulating angiogenic factors or neutrophil to lymphocyte ratio, their predictive role remains elusive [10,11]. Furthermore, the biological basis for any association with pazopanib response is still very limited. In order to identify predictive markers of the response to pazopanib, we performed integrative genomic and transcriptomic analysis of STS patients who received pazopanib-based treatment in hopes of improving the treatment choices within the clinical framework.

To date, our genome-wide analysis provided the most comprehensive dataset of STS with pazopanib-based treatment. Concordant with TCGA SARC data [12], copy number alterations and gene fusions were more prominent than activating point mutations and a low mutational burden. Furthermore, we observed that tumors with CDK4 amplification were less responsive to pazopanib. In a phase II trial, liposarcoma was identified as a non-sensitive histological subtype. The subsequent phase III PALETTE trial was conducted to investigate responses in non-adipocytic STS [4]. Consistent with our results, both well differentiated and dedifferentiated liposarcomas were characterized by chromosome 12q13-15 amplifications of oncogenes MDM2, CDK4, and HMG2 [13] and were relatively insensitive to pazopanib [14].
Furthermore, in parallel to CDK4 overexpression, hypoxia- and glycolysis-associated genes were enriched in non-responders in our study. Importantly, these genes are known to be related to tumor cell proliferation, apoptosis inhibition, and resistance to angiogenesis inhibitors [15,16]. Aside from the initial diagnosis, 4 cases with non-adipocytic sarcoma revised as DDLPS based on molecular pathology in our study. Therefore, we hypothesize that alterations in CDK4 leading to its overexpression are associated with pazopanib resistance, and additional analyses are required to confirm and identify the underlying molecular mechanism. CDK4 amplification is present in 1.95% of the cases of AACR Project GENIE Consortium, with lung adenocarcinoma, dedifferentiated liposarcoma, conventional glioblastoma multiforme, glioblastoma, and well differentiated liposarcoma having the greatest prevalence.[17] So, further studies are needed which investigate CDK4 amplification as a predictive biomarker for anti-VEGF treatment in other cancer types.

Recently, TP53 alterations have been suggested as a biomarker of response to anti-VEGF treatment.[18,19] However, we could not find significant differences in the outcomes (both response and progression survival) according to TP53 mutational status (S5 Fig). Moreover, there was no statistically significant difference in the VEGF expression according to TP53 mutational status (S6 Fig). These results may be due to the small number of patients and the heterogeneity of the study population.

Previous integrated analyses have described the following immune phenotype classification based on the composition of the tumor microenvironment in STS: immune-low, immune-high, and vascularized groups [20]. In this study, the immune-high group exhibited the highest levels of tertiary lymphoid structure (TLS), including T cells, B cells, and NK cells. Among those, B cell infiltration was a key discriminative factor for longer survival and a favorable response to PD-1 blockade through pembrolizumab. Likewise, in our study, high
expression of CD19, a B cell marker, and the NK cell pathway were enriched in responders to the durvalumab combination, but not in those receiving pazopanib monotherapy. Therefore, high infiltration of B cell and TLS-rich features are a hallmark of better efficacy of anti-PD-L1 therapy in STS.

Based on the central role played by the vascular endothelial growth factor receptor (VEGFR) in immunosuppression, combination treatment strategies have been widely studied in various solid tumors. Addition of anti-angiogenic therapy to anti-PD-L1 regimens was found to induce high endothelial venules (HEVs) surrounding the tumor, resulting in enhanced cytolytic activity by recruiting active lymphocytes into the tumor [21]. Induction of HEVs by combination with a VEGFR inhibitor may transform immune-low tumors to immune-high tumors. Given the remarkable clinical response in renal cell carcinoma [22], the combination of axitinib and pembrolizumab has also demonstrated promising therapeutic activity (26.7% of ORR) in STS, particularly with respect to alveolar soft-part sarcoma [23]. Although these preliminary data and cross-study comparisons are speculative, we believe that high response rate in the combination of pazopanib and PD-L1 blockade is promising activity. Further investigation with ongoing clinical trial will help to elucidate these findings.

Our study has some limitations. This study included patients with heterogeneous subtypes of sarcoma and heterogeneous tumor samples (primary tumors and metastases). The study population also received heterogeneous treatment (pazopanib monotherapy and pazopanib+durvalumab).

While pazopanib is currently approved for STS treatment, a subset of patients do not experience a clinical benefit from treatment, highlighting the need for a more personalized approach through refined patient stratification. Based on our study, stratification should be actively considered in order to identify patients who will benefit from pazopanib or
immunotherapies. Our findings lay the basis for patient stratifications with respect to therapeutic strategies for STS, which may be useful in future clinical trials investigating the effects of novel agents.

Ethical Statement
The study was approved by the committee of Samsung Medical Center (IRB file # 2014-03-173), and Severance Hospital (IRB file #4-2018-0743), Seoul, Republic of Korea on the use of human samples for experimental studies. Written informed consent was obtained from all study participants prior to enrollment. The research conformed to the principles of the Helsinki Declaration.

Author Contributions
Conceived and designed the analysis: Hong JY, Kim HS, Lee J.
Collected the data: Hong JY, Yun KH, Lee YH, Kim SH, Baek W, Kim HS, Lee J.
Contributed data or analysis tools: Cho HJ, Kim SK, Lee Y, Choi YL, Kwon M, Kim HS, Lee J.
Performed the analysis: Cho HJ, Kim SK, Lee Y, Choi YL, Kwon M, Kim HS, Lee J.
Wrote the paper: Hong JY, Kim HS.

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Conflicts of Interest

Conflict of interest relevant to this article was not reported.

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A subset of biospecimens analyzed for this study were provided by Samsung Medical Center Biobank.
References

Table 1. Patient characteristics

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<td>I</td>
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<td>II</td>
<td>8</td>
<td>3 (37.5%)</td>
<td>5 (62.5%)</td>
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<td>III</td>
<td>23</td>
<td>11 (47.8%)</td>
<td>12 (52.2%)</td>
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<td>Number of previous chemotherapy regimens</td>
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<td>6</td>
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<td>1 (16.7%)</td>
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<tr>
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<td>15</td>
<td>11 (73.3%)</td>
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ECOG PS, Eastern Cooperative Oncology Group Performance Status; UPS, undifferentiated pleomomorphic sarcoma; MPNST, malignant peripheral nerve sheath tumor; LMS, leiomyosarcoma; FNCLCC, Fédération Nationale des Centres de Lutte Contre le Cancer; NA, not available. aOthers: desmoplastic small round cell tumor, myxoid fibrosarcoma, high grade endometrial stromal cell sarcoma, clear cell sarcoma, low grade myofibroblastic sarcoma, and solitary fibrous tumor. b8th edition of the American Joint Committee on Cancer guideline of tumor, node, and metastasis (TNM) classification. cThe associations were analyzed by the chi-square test or Fisher’s exact test (\(^*\)).
**Fig. 1.** Molecular landscape and response to pazopanib-based treatment. Integrated plot of clinical and molecular features. From top to bottom, panels indicate: Waterfall plot represents percentage of maximum tumor reduction as assessed according to RECIST 1.1 criteria; the number of mutations; clinical characteristics including primary site, histological subtype, best response, treatment (pazopanib vs pazopanib and durvalumab combination), and percentage of alterations (fusions, mutations, and somatic copy number alterations). Patient identity number is provided in S1 Table. TMB, tumor mutation burden; BR, best response; DDLPS, dedifferentiated liposarcoma; UPS, Undifferentiated pleomorphic sarcoma; G-LMS, gynecological leiomyosarcomas; ST-LMS, soft tissue leiomyosarcomas; MPNST, malignant peripheral nerve sheath tumors; DSRCT, desmoplastic small round cell tumor; SS, synovial sarcoma; CN, copy number.
Fig. 2. Response to pazopanib or pazopanib and durvalumab combination. (A) Waterfall plot represents percentage of maximum tumor reduction after treatment, as assessed according to RECIST 1.1 criteria. (B) Swimmer plot. Each lane represents a single patient’s data. X axis represents the duration of treatment for each patient. (C) Spider plots of the percentage change in the sum of target lesions by subject.
Fig. 3. CDK4, FRS2, and HDLBP copy number alterations are genomic determinants of pazopanib resistance. (A) Prevalence of tumors harboring CDK4 gains (pink) and amplifications (red) in responders and non-responders. PR, partial response; SD, stable disease; PD, progressive disease. (B) Univariate cox regression analysis with genetic variables. Error bars represent the 95% confidence interval. X-axis indicates log10-transformed hazard ratio. AMP, amplification/gain; DEL, deletion/loss. An asterisk (*) indicates p-value < 0.05. (C) Kaplan-Meier curve of progression-free survival (months) based on CDK4 amplification. (D) Univariate cox regression analysis among histologic variants. Error bars represent the 95% confidence intervals. X-axis indicates hazard ratio (log10-scaled). DDLPS, dedifferentiated liposarcoma; LMS, leiomyosarcomas; MPNST, malignant peripheral nerve sheath tumors; SS, synovial sarcoma; UPS, Undifferentiated pleomorphic sarcoma; Others, other sarcoma subtypes. An asterisk (*) indicates p-value < 0.05.
Fig. 4. Transcriptomic correlates of clinical response to pazopanib. (A) Gene Set Enrichment Analysis (GSEA) between pazopanib-sensitive and resistant patients. Significantly enriched gene sets (false discovery rate < 0.05) were clustered based on their similarity. (B) Volcano plot representation of genes differentially expressed between pazopanib responders and non-responders. Genes with > 1 log2 fold change and an adjusted p-value < 0.05 are colored in red (Responder DEGs), and those with < -1 log2 fold change and an adjusted p-value < 0.05 are colored in blue (Non-Responder DEGs). (C) The association between mRNA expression levels (log2(FPKM + 1)) and copy number variations of CDK4. P-values were calculated using the two-sided Student t-test.
Fig. 5. Transcriptomic correlates of clinical response to the pazopanib-durvalumab combination. (A) Heat map and unsupervised hierarchical clustering describing tumor microenvironment cell infiltration. Color scale indicates Z-normalized MCPscores of each type of microenvironment cell across samples. Color bar above the heatmap indicates responders (pink) and non-responders (blue) to the pazopanib-durvalumab combination. (B) Immune cell fraction analysis of responding and non-responding patients receiving the pazopanib-durvalumab combination. Immune cell fractions were estimated using MCPcounter scores. An asterisk (*) indicates p-value < 0.05. P-value is from Student’s t-test and not adjusted for multiple tests. (C) Gene Set Enrichment Analysis (GSEA) between sensitive and resistant patients to the pazopanib-durvalumab combination. Significantly enriched gene sets (false
discovery rate < 0.05) were clustered based on their similarity. (D-E) GSEA plot showing BIOCARTA NK cell pathway and KEGG NK cell-mediated cytotoxicity pathway enrichment in the responder group. FDR, false discovery rate; NES, normalized enriched score. (F) Volcano plot representation of differentially expressed gene analysis between responders and non-responders to the pazopanib-durvalumab combination. Genes with >1 log2 fold change and an adjusted p-value < 0.05 are colored in red, and those with < -1 log2 fold change and an adjusted p-value < 0.05 are colored in blue.
Supplementary Methods

1. Whole-exome sequencing for tumor tissue

For the generation of standard exome capture libraries, we used the Agilent SureSelect Target Enrichment protocol for Illumina paired-end sequencing library (Version C2, December 2018) together with 1ug input gDNA. In all cases, the SureSelect Human All Exon V6 probe set was used. The quantification of DNA and the DNA quality is measured by PicoGreen and agarose gel electrophoresis. We used 200ng of DNA diluted in EB Buffer and sheared to a target peak size of 150–200 bp using the Covaris LE220 focused-ultrasonicator (Covaris, Woburn, MA) according to the manufacturer's recommendations. Load the 8 microTUBE Strip into the tube holder of the ultrasonicator and shear the DNA using the following settings: mode, frequency sweeping; duty cycle, 10%; intensity, 5; cycles per burst, 200; duration, 60 sec × 6 cycles; temperature, 4°C–7°C. The fragmented DNA is repaired, an ‘A’ is ligated to the 3′ end, agilent adapters are then ligated to the fragments. Once ligation had been assessed, the adapter ligated product is PCR amplified. For exome capture, 250 ng of DNA library was mixed with hybridization buffers, blocking mixes, RNase block and 5 µl of SureSelect all exon capture library, according to the standard Agilent SureSelect Target Enrichment protocol. Hybridization to the capture baits was conducted at 65°C using heated thermal cycler lid option at 105°C for 24 hours on PCR machine. The captured DNA was then washing and amplified. The final purified product is then quantified using qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantification kits for Illumina Sequencing platforms) and qualified using the TapeStation DNA screentape D1000 (Agilent). And then we sequenced using the HiSeq™ 2500 platform (Illumina,San Diego)

2. WES data analysis

Fastq files generated by WES were mapped on human genome (hg19) through Burrows-Wheeler Aligner (BWA) (version 0.7.12-r1039) with BWA-MEM algorithm [1]. The aligned bam files were sorted by coordinates using SAMtools (v0.1.19) [2]. Then, Genome Analysis Toolkit (GATK, v3.6 and v.4.13) performed duplicate marking, indel realignment, and base recalibration for coordinate-sorted hg19-aligned reads [3]. For somatic mutation detection, MuTect2 from GATKv4.13 was used by comparing BAM files of tumor and those of matched normal samples. Possible germline events were filtered by gnomAD [4] (population allele
fraction $< 2.5 \times 10^{-6}$, and variant effect predictors (VEP) [5] annotated the remained events. For SMC03 sample, which lacks matched normal control, MuTect2 was performed with tumor-only mode, followed by possible germline event elimination. For further analysis, called mutations with mutant reads $\leq 4$ were filtered out. For the hypermutated tumor sample, mutational signature (COSMIC v2) analysis was performed by R package, deconstructSigs (v1.8.0) [6].

3. WES-based copy number variation analysis

Python package, ngCGH was used to detect WES-based copy number ratios of tumor samples to their corresponding normal samples with window size = 1000 sequencing reads. We defined copy number gain and amplification if the copy ratio is greater that 1.5 and 2, respectively. On the contrary, if the genes showed the copy ratio less than 0.75 and 0.5, the gene were classified as copy number loss and deletion, respectively. To confirm whether this classification of gene copy number variations (CNVs) was reliable or not, we investigated the association between gene CNVs and mRNA expression. As a result, CDK4/FRS2 gene expression levels of CDK4/FRS2-amplified tumors were significantly higher than those of tumors with no CDK4/FRS2 gain or amplification (Wilcoxon rank sum $p = 1.5 \times 10^{-6}$ and 0.0018, respectively) (Fig. 4C and S5 Fig). HDLBP loss tumors showed significant down-regulation of HDLBP gene expression compared to tumors with no HDLBP loss/deletion (Wilcoxon rank sum $p = 1.1 \times 10^{-6}$) although the HDLBP-deleted tumor displayed comparable gene expression levels with no HDLBP loss/deletion tumors.

Also, accurate CDK4 and MDM2 co-amplification status is critical since their co-amplification is used for the molecular diagnosis of STS, and therefore, we re-investigated their amplification status with consideration of tumor purity and ploidy. we adopted ABSOLUTE [7] algorithm to calculate tumor purity, ploidy, and absolute copy number values from ngCGH results (S5 Table) except for four samples (SMC7, SMC12, YCC8 and YCC17) as ABSOLUTE failed to model their data. Based on tumor ploidy and total copy number values from ABSOLUTE, we classified CNV as well. If tumor ploidy $\geq 4$, CNVs were defined as amplification and gain when copy number $> \text{ploidy} + 4$ and $> \text{ploidy} + 2$, respectively. If copy number $< \text{ploidy} - 4$ or ploidy $\times 0.5$, CNV was defined as deletion, and if copy number $< \text{ploidy} - 2$ or ploidy $\times 0.75$, CNV was defined as loss. For tumors with ploidy less than 4, CNV was defined as amplification, gain, loss, and deletion if copy number $> \text{ploidy} + 2$, copy number $> \text{ploidy} + 1$, copy number $< 1.5$, and copy number $< 1$, respectively (S7 Fig). Although there are
some inconsistencies between log2R-based classification and total copy number-based classification, log2R-based CDK4/MDM2 amplification and total copy number-based CDK4/MDM2 amplification were observed in exactly same tumors, and all of them showed moderate-level amplifications (11~14 copies). Since the HDLB5-deleted tumor SMC16 (log2R classification) showed comparable gene expression level with no HDLB5 loss/deletion, we investigated the total copy number of HDLB5 in this sample and confirmed that SMC16 was classified into deletion according to total copy number-based classification as well (HDLB5 total copy number of SMC16 was 0).

4. RNA-sequencing

Total RNA concentration was estimated by Quant-IT RiboGreen (Invitrogen). To determine the DV200 (% of RNA fragments > 200 bp) value, samples were run on the TapeStation RNA ScreenTape (Agilent). Overall, 100 ng of total RNA was subjected to sequencing library construction using a TruSeq RNA Access library prep kit (Illumina, San Diego, CA) according to the manufacturer's protocol. Briefly, the total RNA was first fragmented into small pieces using divalent cations under elevated temperature. The cleaved RNA fragments were copied into first strand cDNA using SuperScript II reverse transcriptase (Invitrogen, #18064014) and random primers. This was followed by second strand cDNA synthesis using DNA polymerase I, RNase H, and dUTP. These cDNA fragments were subjected to an end-repair process, addition of a single ‘A’ base, and subsequently, ligation of the adapters. The products are then purified and enriched with PCR to create the cDNA library. All libraries were normalized and six were pooled into a single hybridization/capture reaction. Pooled libraries were incubated with a cocktail of biotinylated oligos, corresponding to coding regions of the genome. Targeted library molecules were captured via hybridized biotinylated oligo probes using streptavidin-conjugated beads. After two rounds of hybridization/capture reactions, the enriched library molecules were subjected to a second round of PCR amplification. The captured libraries were quantified using KAPA Library Quantification kits for Illumina Sequencing platforms according to the qPCR Quantification Protocol Guide (KAPA BIOSYSTEMS, #KK4854), and assessed using the TapeStation D1000 ScreenTape (Agilent Technologies, # 5067-5582). Indexed libraries were then submitted to an Illumina HiSeq2500 (Illumina, Inc., San Diego, CA), and paired-end (2 × 100 bp) sequencing was performed by Macrogen Incorporated.
5. RNA-Sequencing data analysis

Sequence reads from RNA-sequencing were aligned on hg19 by STAR (v2.6.1d) [8]. For gene expression profiling, Cufflinks (v2.2.1) quantified the aligned reads in Fragments Per Kilobase Million (FPKM) [9]. For further analysis, log2-transformed FPKM values were used except for differentially expressed gene (DEG) analysis. To extract DEGs between responders and non-responders, normalized reads counts per gene were obtained with DEGseq [10] (R package), and then the resulting reads counts were applied to DEseq2 [11] (R package) to obtain DEGs. To calculate the Microenvironment Cell Populations-counter scores in tumor samples, MCPcounter (R package) was used [12]. Geneset enrichment analysis (GSEA) was performed by GSEA-P [13]. GSEA results were visualized on Cytoscape (v3.7.1) Enrichment Map [14]. Single sample GSEA (ssGSEA) algorithm in GSVA [15] (R package) was applied to estimate geneset activation score in tumor samples for HU_ANGIOGENESIS_UP MSigDB geneset [16] and Responder and Non-Responder DEGs. Gene fusion detection was performed by STAR-Fusion (v1.5.0) with STAR-aligned bam files [17].

References
S1 Fig. CONSORT diagram
S2 Fig. Tumor mutational burden of STS in this study (n=34, somatic mutation only) and other cancer types in the TCGA dataset. X-axis represents cancer types, and Y-axis represents the number of mutations (log10 scale). The numbers above the plot indicate the sample size of the dataset. Maftools was used for this analysis in R.
S3 Fig. Mutational signature for YCC#18. (A) The fraction of single base substitutions in each trinucleotide context (top). The reconstructed fraction of mutations obtained by multiplying the weight of signatures (middle). The error between top and middle panels (bottom). (B) Mutational signature fraction.
S4 Fig. Progression free survival analysis with genomic determinants. (A-B) Kaplan-Meier curve of progression-free survival (months) based on FRS2 and HDLBP copy number alterations. (C) Multivariate cox regression analysis with genetic variables and histologic variants. Error bars represent the 95% confidence intervals. X-axis indicates log10-transformed hazard ratio. AMP, amplification/gain; DEL, deletion/loss. An asterisk (*) indicates p-value < 0.05.
**S5 Fig.** Progression free survival analysis according to TP53 mutational status in all patients and pazopanib monotherapy-treated patients. P-values were calculated using the log-rank test.
S6 Fig. VEGFA expression level according to TP53 mutational status in all patients and pazopanib monotherapy-treated patients. P-values were calculated using the Wilcoxon rank sum test.
S7 Fig. The association between mRNA expression levels (log2(FPKM + 1)) and copy number variations of FRS2 and HDLBP. P-values were calculated using the two-sided Student t-test.
S8 Fig. PD-L1/PD-1 expression level in pazopanib and durvalumab-treated patients (n=17). Horizontal lines within the violin plots represent 0.25, 0.50 (median), and 0.75 quantiles. P-values were calculated using the two-sided Wilcoxon’s rank-sum test.
S9 Fig. The landscape of ploidy-based copy number variations in this study cohort. Sample order of this landscape is the same with that of Fig. 1. Classification of copy number variations is described in Supplementary Methods.