Activation of ERK1/2 Causes Pazopanib Resistance via Downregulation of DUSP6 in Synovial Sarcoma Cells

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Synovial sarcoma (SS) is a rare high-grade malignant mesenchymal tumour with a relatively poor prognosis despite intensive multimodal therapy. Although pazopanib, a multi-kinase inhibitor, is often used for advanced SS, most cases eventually become resistant to pazopanib. In the present study, we investigated the mechanisms of acquired pazopanib resistance in SS. To examine acquired pazopanib resistance, two SS cell lines, SYO-1 and HS-SY-II, were isolated after multiple selection steps with increasing concentrations of pazopanib. SYO-1 was also used in vivo. Then, pazopanib-resistant clones were investigated to assess potential mechanisms of acquired pazopanib resistance. Stable pazopanib-resistant clones were established and exhibited enhanced cell cycle progression, cell growth with increased ERK1/2 phosphorylation, and higher sensitivity than parental cells to a MEK-inhibitor, trametinib, both in vitro and in vivo. Furthermore, addition of low-dose trametinib partially reversed the pazopanib resistance. In the pazopanib-resistant clones, dual specificity phosphatase 6 (DUSP6) was downregulated. Inhibition of DUSP6 expression in parental HS-SY-II cells partially recapitulated acquired pazopanib resistance. Acquired pazopanib resistance in SS was associated with activation of ERK1/2 through downregulation of DUSP6 expression. Simultaneous treatment with pazopanib and a MEK inhibitor could be a promising strategy to overcome pazopanib resistance in SS.

Synovial sarcoma (SS) accounts for 7–10% of soft-tissue sarcomas and has a characteristic chromosomal translocation, t(X;18)(p11.2;q11.2), coding the chimeric protein SYT-SSX1. The treatment of SS consists of adjuvant chemotherapy, radical surgery, and/or radiotherapy. Several active agents against SS have been reported, and in general, doxorubicin- or ifosfamide-based regimens are applied as first-line adjuvant chemotherapies in SS2,3. Despite intensive treatment, patients with this tumour have relatively poor prognoses, with 5-year overall survival rates of 50–80%4–6.

Pazopanib is a multi-kinase inhibitor that inhibits several receptor tyrosine kinases (RTKs) such as platelet-derived growth factor receptor α (PDGFRα), PDGFRβ, vascular endothelial growth factor (VEGF) receptor 1 (VEGFR1), VEGFR2, VEGFR3, and c-KIT. In the PALETTE study, a randomised, double-blinded, placebo-controlled phase III trial of pazopanib in pretreated metastatic sarcoma, pazopanib resulted in a statistically significant improvement in progression-free survival (PFS) of approximately 3 months. Based on this result, pazopanib became a promising agent for second-line chemotherapy in patients with advanced soft-tissue sarcomas, including SS. However, disease progression eventually occurs in the majority of patients due to the development of pazopanib resistance. The mechanisms of acquired pazopanib resistance in SS have been minimally investigated and remain unclear, and it is important to elucidate the underlying mechanisms so as to define new therapeutic strategies for SS.

The present study was undertaken in order to establish pazopanib-resistant SS cells and examine the mechanisms of acquired pazopanib resistance.
Establishment of pazopanib-resistant SS clones. Continuous stepwise selection of the two SS cell lines was undertaken with up to 20 μM pazopanib, and development of pazopanib resistance was periodically tested using a chemosensitivity assay. After approximately 6 months, stable, highly pazopanib-resistant clones were established in both cell lines as depicted in Fig. 1a. It has been reported that pazopanib mainly targets PDGFRα and induces G1/S arrest by inhibition of the PDGFRα–PI3K–Akt pathway in SS cells. We therefore performed cell cycle analysis using flow cytometry in order to determine the cell cycle status of pazopanib-resistant and parental cells in the presence or absence of pazopanib. In the presence of pazopanib, parental clones exhibited a decrease in the proportion of cells in S phase in a dose-dependent manner. On the other hand, pazopanib-resistant clones exhibited an increase in the proportion of cells in S phase and a decrease of those in G1.

Figure 1. Establishment of pazopanib-resistant SS clones. (a) SS clones were incubated with various doses of pazopanib for 48 hours. Viable cells were counted using a Z1 Coulter particle counter. Values represent mean ± S.D. *P < 0.05; **P < 0.01, vs parental clones. (b,c) Cells were incubated with pazopanib (0, 1, and 10 μM) for 24 hours and fixed in 70% ethanol. After staining with PI, the DNA content of each phase (e.g., G1, S, or G2/M) was analysed by flow cytometry. Values represent mean ± S.D. *P < 0.05; **P < 0.01, vs parental clones. (d) Pazopanib resistance of SYO-1 parental and pazopanib-resistant clones in vivo. Values represent mean ± S.D. *P < 0.05; n.s. not significant, vs pazopanib-free condition. Pazo-R represents pazopanib-resistant clone.
Pazopanib-resistant clones showed enhanced cell growth and activated phosphorylation of ERK1/2. We next performed a cell proliferation assay, since a previous study reported that accelerated cell cycle transitions were associated with deregulated cell proliferation\(^1\), and our cell cycle analysis showed that pazopanib-resistant clones were more frequently in S phase in the absence of pazopanib (Fig. 1b and c). Surprisingly, promotion of cell growth was observed in addition to cell cycle progression in pazopanib-resistant clones, compared with the parental clones in the absence of pazopanib (Fig. 2a). Consistent with \textit{in vitro} experiments, mice inoculated with pazopanib-resistant SYO-1 cells showed a significant increase in tumour burden compared with the mice inoculated with parental cells (Fig. 2b). In order to address the mechanisms of cell cycle promotion and cell growth in pazopanib-resistant SS clones, as an initial approach we used a human phospho-antibody array (Human Phospho–Kinase Array, Proteome Profiler Array Kit) and semi-quantitate the levels of phosphorylation to study a subset of phosphorylation events in two SS cell lines to investigate signalling pathway profiles. In pazopanib-resistant clones, phosphorylation of ERK1/2 was increased in comparison with parental clones, while phosphorylation of Akt in the resistant clones was comparable or decreased (Fig. 2c and Table 1). Consistent with the kinase array results, Western blot analysis revealed increased ERK1/2 phosphorylation in pazopanib-resistant clones (Fig. 2d and Table 1).

A MEK1/2 inhibitor strongly inhibited cell growth and partially reversed acquired pazopanib resistance in pazopanib-resistant clones. We examined whether activated ERK1/2 might be a good therapeutic target for pazopanib-resistant SS cells. To determine the role of ERK1/2 in SS cells, MAPK signalling pathways were inhibited with a chemical inhibitor, and cell cycle and cell proliferation were examined. We used trametinib, an oral, reversible, selective allosteric inhibitor of MEK1/2 activation and kinase activity, to inhibit the ERK signalling pathway\(^1\). First, Western blotting showed that phosphorylation of ERK1/2 in pazopanib-resistant clones was inhibited to the same extent by trametinib as parental clones (Fig. 3a), indicating that trametinib inhibited the ERK1/2 signalling pathway in both parental and pazopanib-resistant SS clones. Second, we examined the effect of trametinib on the cell cycle. Pazopanib-resistant clones showed a higher proportion of cells in the G1 phase than parental clones after treatment with 10 nM trametinib (Fig. 3b). Third, we performed a chemosensitivity assay with trametinib. Interestingly, trametinib more effectively inhibited the cell growth of pazopanib-resistant clones than parental clones (Fig. 3c). Fourth, we examined the \textit{in vivo} efficacy of trametinib on pazopanib-resistant SYO-1 clones in the mouse model. As we expected, pazopanib-resistant xenograft mice treated with 0.1 mg/kg trametinib showed a significant decrease in tumour burden while parental xenograft mice treated with the same dose of trametinib showed no significant decrease (Fig. 3d). Finally, to examine whether inhibition of activated ERK1/2 would overcome pazopanib resistance, the pazopanib-resistant SS cells were simultaneously treated with pazopanib and a low dose of trametinib. In the pazopanib-resistant SS cells, even low-dose trametinib partially reversed the resistance to pazopanib chemosensitivity (Fig. 3e). In the SYO-1 mouse model, pazopanib-resistant clones showed higher sensitivity to 10 mg/kg pazopanib along with low-dose trametinib (Fig. 3f). These results suggest that trametinib can be an effective drug for pazopanib-resistant SS, and increased phosphorylation of ERK1/2 is a key to acquired pazopanib resistance.

Mutational status of the RAS–RAF–MEK–ERK pathway and PDGFR\(\alpha\) in pazopanib-resistant clones. To address the mechanism of activation of ERK1/2 in pazopanib-resistant SS cells, we examined aspects of the signalling pathway upstream of ERK1/2. First, we investigated the phosphorylation status of ERK1/2 and PDGFRs, the latter of which is a main target of pazopanib in SS since the PDGF signalling pathway can initiate the MAPK pathway and activate ERK1/2\(^1\). Interestingly, Western blot analysis showed that phosphorylation of PDGFR\(\alpha\) was inhibited in all parental and pazopanib-resistant SS clones in the presence of pazopanib (Fig. 4a). However, pazopanib alone could not sufficiently inhibit phosphorylation of ERK1/2 in only pazopanib-resistant clones (Fig. 4a). Next, we examined kinases upstream of ERK1/2. The activation profile of the RAS–RAF–MEK–ERK pathway was similar between pazopanib-resistant and parental clones in Western blot analysis, with the exception of phospho-ERK1/2 (Fig. 4b). Furthermore, we conducted whole-exome sequencing in the pazopanib-resistant and parental clones to search for any activating mutations of the RAS–RAF–MEK–ERK pathway or any gatekeeper mutations of PDGFR\(\alpha\). There were no such mutations in either the pazopanib-resistant clones (Supplementary Figure 1) or parental clones (data not shown), and no common pazopanib-resistant-specific mutations (data not shown). These findings suggest that upstream signalling elements of ERK1/2, including gatekeeper mutations of PDGFR\(\alpha\), are not involved in the activation of ERK1/2 in pazopanib-resistant SS cells, and pazopanib-resistant clones are resistant to pazopanib inhibition of ERK1/2 phosphorylation. Next, we were interested in specific regulators of ERK1/2. It is known that sustained activation of ERK1/2 does not always correlate with upstream kinases, and dual specificity phosphatases (DUSPs) regulate MAPK activity\(^4\). We performed gene expression microarray analysis of SYO-1 parental and pazopanib-resistant clones to comprehensively evaluate molecules downstream from ERK1/2 in each clone. We identified down-regulation of the DUSP6 gene in the SYO-1 pazopanib-resistant sample using the same criteria described in the Methods section regarding all the DUSPs (Table 2). We next performed quantitative PCR and Western blot analysis to examine mRNA and protein expression of DUSP6 in all SS clones. The resistant clones showed lower expression levels of DUSP6 compared with the parental clones (Fig. 4c and d). Taken together, these findings
suggest that activation of ERK1/2 is sustained, at least in part, via downregulation of DUSP6 expression in pazopanib-resistant SS cells.

**Downregulation of DUSP6 in parental SS cells recapitulates pazopanib resistance.** To examine the relevance of DUSP6 to ERK1/2 phosphorylation, cell growth promotion, and pazopanib resistance, we knocked down the expression of DUSP6 in parental clones using the CRISPR/Cas9 system. As shown in Fig. 4e, phosphorylation of ERK1/2 was increased as a result of decreased DUSP6 expression in HS-SY-II cells transfected with small guide RNA (sgRNA) targeting DUSP6.

We next examined the phenotypes of CRISPR-treated HS-SY-II cells. These cells showed enhanced cell growth (Fig. 4f), and in the chemosensitivity assay they exhibited a little greater resistance to pazopanib (Fig. 4g) than...
control cells. Taken together, these results suggest that downregulation of \textit{DUSP6} caused enhanced cell growth and pazopanib resistance via activation of ERK1/2 in HS-SY-II.

**Discussion**

Pazopanib is an oral multi-targeted tyrosine kinase inhibitor, and the first molecular target drug approved for soft-tissue sarcoma in Japan. Although a subgroup analysis showed that patients with synovial sarcoma, leiomyosarcoma, vascular tumours, alveolar soft part sarcoma, solitary fibrous tumour, and desmoplastic small round cell tumour had better progression-free survival with pazopanib\(^\text{15}\), most sarcomas eventually acquire drug resistance to pazopanib. Pazopanib targets VEGFRs, PDGFRs, and c-Kit \(^\text{16}\). Since previous studies found that

| Target            | SYO-1  | SYO-1 pazo | HS-SY-II | HS-SY-II pazo |
|-------------------|--------|-----------|----------|--------------|
| p38               | 27185  | 50538     | 22286    | 20333        |
| ERK1/2            | 100019 | 288321    | 89270    | 97567        |
| JNK1/2/3          | 77395  | 77065     | 24433    | 22714        |
| GSK 3a/b          | 143858 | 108616    | 24208    | 23893        |
| p53 (S46)         | 167783 | 171765    | 44826    | 48486        |
| EGFR              | 39309  | 48547     | 20694    | 18995        |
| MSK1/2            | 50319  | 95069     | 62443    | 43471        |
| AMPKα1            | 72491  | 50196     | 17451    | 19173        |
| Akt1/2/3 (S473)   | 274974 | 89608     | 17563    | 16950        |
| Akt1/2/3 (T308)   | 48944  | 37706     | 16570    | 21254        |
| p53 (S15)         | 118583 | 124327    | 54242    | 68071        |
| TOR               | 48586  | 56227     | 38804    | 24692        |
| CREB              | 102784 | 75882     | 180734   | 99465        |
| HSP27             | 31474  | 36865     | 17000    | 15359        |
| AMPKα2            | 81370  | 86270     | 63793    | 66588        |
| b-catenin         | 293000 | 127186    | 17943    | 16732        |
| p70 S6 kinase (T389) | 14568   | 9719     | 12116    | 14177        |
| p53               | 89306  | 75529     | 17425    | 16322        |
| c-Jun             | 107244 | 40943     | 17061    | 21136        |
| Src               | 46052  | 53229     | 26714    | 18600        |
| Lyn               | 50851  | 59028     | 49061    | 34769        |
| Lck               | 27997  | 34987     | 16145    | 15046        |
| STAT2             | 112232 | 134533    | 95794    | 85316        |
| STAT 5a           | 58079  | 67275     | 39774    | 40098        |
| p70 S6 kinase (T421/S424) | 47915  | 34216     | 16237    | 16797        |
| RSK1/2/3          | 60293  | 40917     | 18258    | 20572        |
| eNOS              | 25031  | 17306     | 14288    | 16073        |
| Fyn               | 43334  | 50826     | 39685    | 27175        |
| Yes               | 53604  | 53433     | 34761    | 23466        |
| Fgr               | 28021  | 26194     | 15085    | 14106        |
| STAT6             | 76754  | 96172     | 46382    | 47204        |
| STAT 3b           | 51351  | 81959     | 22535    | 21215        |
| STAT3             | 33042  | 20225     | 18115    | 19723        |
| p27               | 31729  | 12446     | 11176    | 13434        |
| PLCg1             | 28054  | 19782     | 13090    | 13218        |
| Hck               | 94072  | 64611     | 39452    | 23454        |
| Chk-2             | 61067  | 59698     | 35305    | 23094        |
| FAK               | 90811  | 63448     | 26943    | 17407        |
| PDGFRβ            | 28910  | 33964     | 13902    | 15861        |
| STAT5a/b          | 62719  | 81523     | 22467    | 21518        |
| STAT3             | 53225  | 25804     | 11957    | 13286        |
| WNK1              | 698519 | 160631    | 29746    | 24156        |
| PYK2              | 17108  | 10415     | 13935    | 15843        |
| PRAS40            | 371550 | 136339    | 24842    | 15820        |
| HSP60             | 298441 | 180503    | 88998    | 64145        |

Table 1. Result of semi-quantitation of all tested phospho protein in phospho-kinase array. Pazo represents pazopanib-resistant clone.
Figure 3. Trametinib, a MEK1/2 inhibitor, strongly inhibited growth and partially reversed pazopanib resistance of pazopanib-resistant clones. (a) Trametinib-induced inhibition of phosphorylation of ERK1/2 in SS clones was assessed by Western blot analysis with anti-ERK1/2 and anti-phospho-ERK1/2 antibodies. SS clones were pre-treated using 10 nM trametinib for 2 hours. (b) Cells were incubated with trametinib (10 nM and 100 nM) for 24 hours and fixed in 70% ethanol. After staining with PI, the DNA content of each fraction (e.g., G1, S, or G2/M) was analysed by flow cytometry. Values represent mean ± S.D. *P < 0.05; **P < 0.01, vs parental clones. (c) SS clones were incubated with various doses of trametinib for 48 hours. Viable cells were counted using a Z1 Coulter particle counter. Values represent mean ± S.D. *P < 0.05; **P < 0.01, vs parental clones. (d) Trametinib sensitivity of SYO-1 parental and pazopanib-resistant clones in vivo. **P < 0.01; n.s. not significant, vs trametinib-free condition. (e) Pazopanib-resistant SS clones were incubated with various doses of pazopanib and/or 1 nM trametinib for 48 hours. Viable cells were counted using a Z1 Coulter particle counter. Values represent mean ± S.D. *P < 0.05, vs trametinib-free condition. (f) Anti-tumour effect of 10 mg/kg pazopanib and/or 0.01 mg/kg trametinib in SYO-1 pazopanib-resistant clones in vivo. Values represent mean ± S.D. *P < 0.05, vs drug-free condition. Pazo-R represents pazopanib-resistant clone.
immunohistochemistry of SS shows high protein expression of PDGFRs and VEGFRs. Pazopanib potentially offers advantages in the treatment of SS. Therefore, establishing cells with acquired pazopanib resistance and using SS cell lines to elucidate the mechanism underlying this drug resistance are critical to developing novel treatment strategies.

In the present study, the dose-sensitivity to pazopanib of parental SS clones was comparable to that in a previous study that showed an IC50 value between 2 and 10 μM (Fig. 1a). We succeeded in developing SS cell lines with acquired pazopanib resistance with an IC50 of more than 20 μM (Fig. 1a). It is reported that the recommended dose of pazopanib is 800 mg orally once daily, and a mean target trough concentration was 34 μM in patients with solid tumours. Additionally, pazopanib of the almost same dose is administered in patients with...
renal cell carcinoma which is another adaption disease of pazopanib. Therefore, we consider that our experiments at the concentration of 20 μM is clinically relevant.

Among the two SS cell lines utilised in this study, only SYO-1 is tumourigenic, and we therefore used it to perform preclinical animal experiments. The established pazopanib-resistant clones proved to have several characteristics. In addition to pazopanib resistance, promotion of cell growth was observed in pazopanib-resistant SS cell lines (Fig. 2a). Further, the resistant clones exhibited increases in phosphorylated ERK1/2 (Fig. 2c, d, and Table 1). In addition, we found that a couple of blots were also decreased commonly both SYO-1 and HS-SY-II resistant cells compared to their parental cells (Table 1). The semi-quantitation revealed that CREB, PRAS40, HSP60 and WNK1 were decreased prominently in SYO-1 (Table 1). Because CREB, PRAS40, HSP60 and WNK1 were considered to contribute to aggressive phenotypes such as tumour progression and metastases, we speculated that their downregulation would be less likely to cause the aggressive phenotype of resistant clones. Further investigations are warranted to determine their roles in pazopanib resistance.

It is known that the MAPK pathway regulates diverse cellular processes such as cell growth, apoptosis, malignant transformation, and drug resistance. MAPK signalling is tightly regulated and is activated primarily by extracellular growth factor stimulation. Activation of ERK1/2 can be induced by activation of its upstream kinases, RAS, RAF, and MEK1/2. While many reports have shown that the MAPK pathway plays important roles in cancer drug resistance, only a few have reported the involvement of the MAPK pathway in acquired tyrosine kinase inhibitor resistance. The inactivation of the MAPK pathway has been proposed as one of the most promising approaches to overcoming acquired resistance to various cytotoxic drugs. Indeed, we found that trametinib exerted greater antitumour effects against pazopanib-resistant SS cells than the parental cells, not only in vitro but also in vivo (Fig. 3b, c and d), and that low dose of trametinib along with pazopanib effectively inhibited the growth of pazopanib-resistant SS clones in vitro (Fig. 3e) and in vivo (Fig. 3f). Thus our data as well as those of others suggest that the MAPK pathway is a good molecular target in cases of acquired pazopanib resistance in SS. A phase II clinical trial has just been initiated to evaluate trametinib in combination with pazopanib in patients with advanced gastrointestinal stromal tumour (GIST) (ClinicalTrials.gov Identifier: NCT02342600).

### Table 2. Result of gene expression microarray of SYO-1 parental and pazopanib-resistant clones about DUSPs.

| Probe ID | Gene Symbol | SYO-1/Parent signal | SYO-1/Pazo-R signal | Z score | ratio |
|----------|-------------|---------------------|---------------------|---------|-------|
| A_23_P137904 | DUSP6 | 4520.012333 | 1190.453333 | -3.48108 | 0.26374 |
| A_24_P182394 | DUSP10 | 9.69494667 | 3.61817945 | -1.4046 | 0.18118 |
| A_23_P150018 | DUSP5 | 207.195 | 93.695067 | -1.37885 | 0.45184 |
| A_24_P367602 | DUSP5P1 | 790.066333 | 446.243367 | -1.32193 | 0.56481 |
| A_23_P120254 | DUSP22 | 906.2421 | 697.353133 | -0.60877 | 0.7694 |
| A_23_P7896 | DUSP22 | 26.7696667 | 18.45077333 | -0.4864 | 0.68923 |
| A_23_P376759 | DUSP11 | 263.968667 | 232.368667 | -0.24048 | 0.87366 |
| A_24_P154771 | DUSP15 | 923.3841 | 877.506133 | -0.22705 | 0.90798 |
| A_33_P3331345 | DUSP11 | 75.93203333 | 69.89328 | -0.11843 | 0.919949 |
| A_33_P3383656 | DUSP11 | 678.1746667 | 653.914667 | -0.08842 | 0.964233 |
| A_23_P190333 | DUSP19 | 23.91798333 | 23.26543333 | -0.04734 | 0.972722 |
| A_23_P134935 | DUSP4 | 250.8295 | 245.6786 | -0.04314 | 0.979465 |

Probe ID | Gene Symbol | SYO-1/Parent signal | SYO-1/Pazo-R signal | Z score | ratio |
|----------|-------------|---------------------|---------------------|---------|-------|
| A_24_P417189 | DUSP9 | 66.95873 | 137.8416667 | 1.239243 | 2.058606 |
| A_23_P143650 | DUSP18 | 62.21632333 | 123.5135 | 1.176759 | 1.985227 |
| A_33_P3272698 | DUSP23 | 10.63196867 | 28.16985667 | 1.030808 | 2.649543 |
| A_23_P110712 | DUSP11 | 185.036869 | 303.3611493 | 0.846196 | 1.639463 |
| A_24_P189739 | DUSP16 | 93.9441667 | 120.9678 | 0.429173 | 1.287653 |
| A_33_P3559012 | DUSP8 | 556.4892 | 669.6873667 | 0.422461 | 1.203415 |
| A_24_P37409 | DUSP2 | 111.2015 | 132.5071 | 0.295323 | 1.191595 |
| A_23_P51508 | DUSP12 | 2528.703333 | 2771.286333 | 0.233366 | 1.095391 |
| A_32_P98238 | DUSP8 | 21.00351333 | 21.97329333 | 0.187192 | 1.046172 |
| A_23_P129956 | DUSP3 | 1343.944667 | 1413.815333 | 0.127322 | 1.051989 |
| A_23_P207537 | DUSP14 | 1199.725 | 1237.316333 | 0.075948 | 1.031333 |

Probe ID | Gene Symbol | SYO-1/Parent signal | SYO-1/Pazo-R signal | Z score | ratio |
|----------|-------------|---------------------|---------------------|---------|-------|
| A_23_P10447I | DUSP13 | 2.957820333 | 2.977214667 | NULL | NULL |
| A_23_P369890 | DUSP15 | 2.980815333 | 6.45668667 | NULL | NULL |
| A_23_P256077 | DUSP21 | 3.38911667 | 3.486214667 | NULL | NULL |
| A_23_P146134 | DUSP26 | 9.328484 | 3.483556 | NULL | NULL |
| A_33_P3294645 | DUSP27 | 4.514399333 | 3.32608667 | NULL | NULL |
| A_24_P51855 | DUSP72 | 3.698769333 | 3.48907667 | NULL | NULL |
| A_33_P3263625 | DUSP8 | 14.8356 | 5.108212667 | NULL | NULL |

Table 2. Result of gene expression microarray of SYO-1 parental and pazopanib-resistant clones about DUSPs. Pazo-R represents pazopanib-resistant clone.
Similarly, simultaneous administration of trametinib and pazopanib was found to exhibit anti–tumour activity in a preclinical thyroid cancer model. Further research regarding the clinical applications of trametinib in patients with advanced SS is required.

The causative molecular mechanisms of increased phosphorylated ERK1/2 have been proposed to be the activation of kinases upstream of ERK1/2 and/or the inhibition of downstream phosphatases. With regard to upstream molecules in the MAPK pathway, gatekeeper mutations of PDGFRα cause phosphorylation of ERK1/2 through the RAS–RAF–MEK pathway. It has been reported that in cases of GIST, gatekeeper mutations of PDGFRα are frequently observed in resistance to other tyrosine kinase inhibitors such as imatinib. However, a number of our findings suggest that it is unlikely that activation of ERK1/2 results from upstream signalling elements and activating mutations in acquired pazopanib-resistant SS cells: PDGFRα was not activated more in pazopanib-resistant SS cells than in parental SS cells (Fig. 4a), pazopanib was still able to inhibit the phosphorylation of PDGFRα in pazopanib-resistant SS cells (Fig. 4a), phosphorylation of ERK1/2 was partially inhibited by pazopanib (Fig. 4a), and there were no identified gatekeeper mutations of PDGFRα in both of the resistant clones (Supplementary Figure 1). Furthermore, Western blot analysis showed no differences between pazopanib-resistant and parental SS cells in expression levels and phosphorylation of KRAS, BRAF, and MEK1/2 (Fig. 4b).

With regard to downstream specific regulators in the MAPK pathway, DUSPs have been reported to modulate the duration, magnitude, and subcellular compartmentalization of MAPK activity. It is known that DUSPα dephosphorylates and inactivates MAP kinases. Using a microarray system, we found that DUSP6 was down-regulated in pazopanib-resistant SYO-1 cells (Table 2). Consistent with this microarray result, mRNA expression and protein expression of DUSP6 were down-regulated in all pazopanib-resistant clones (Fig. 4c and d). Forced knockdown of DUSP6 in parental HS-SY-II cells reproduced the increases in ERK1/2 phosphorylation (Fig. 4e). A previous study showed that DUSP6 negatively and specifically modulated ERK1/2 kinase activity. It has also been reported that down-regulation of DUSP6 expression is involved in drug resistance in ovarian cancer, and up-regulation of DUSP6 mediated by p53 caused a cellular senescent phenotype. Taken together with our results, these findings suggest that increased ERK1/2 phosphorylation in pazopanib-resistant SS cells is at least in part due to down-regulation of DUSP6 expression.

As mentioned above, it has been reported that the activated MAPK pathway is associated with acquired cancer drug resistance. Harada et al. reported that acquired resistance to sorafenib, a multi-kinase inhibitor, resulted from the continuous activation of MAPK pathway. In this study, we revealed that trametinib inhibited cell growth of pazopanib-resistant SS cells more than the parental cells (Fig. 3c). In addition, inhibition of ERK1/2 by low-dose trametinib partially reversed pazopanib resistance in vitro (Fig. 3e) and in vivo (Fig. 3f). Conversely, DUSP6 knockdown in parental HS-SY-II cells apparently induced pazopanib resistance, accompanied by an increase in phosphorylated ERK1/2 (Fig. 4e and g), which recapitulated the acquired resistance to pazopanib in SS cells. Therefore, we conclude that prolonged exposure to pazopanib induces downregulation of DUSP6 by an unknown mechanism, and sustained phosphorylation of ERK1/2 promotes cell cycle progression and proliferation in SS cells.

Bridgeman et al. reported that the addition of trametinib could overcome acquired sunitinib resistance in renal cell carcinoma xenograft models, which supports our conclusion that inhibition of ERK1/2 phosphorylation may be a key to overcome the acquired resistance to antiangiogenic tyrosine kinase inhibitors.

In this study, we found that pazopanib-resistant cells exhibited a marked increase in phosphorylated ERK1/2 (Fig. 2d), and the efficacy of trametinib on inhibiting the cell growth of pazopanib-resistant clones increased compared with the parental clones (Fig. 3c). Furthermore, down-regulation in DUSP6 expression levels were shown in pazopanib-resistant cell lines (Fig. 4c and d). These results suggest that the increase in ERK1/2 phosphorylation and down-regulation in DUSP6 expression are certainly keys to acquired pazopanib resistance, but the mechanisms of DUSP6 down-regulation are unknown. Further investigations are needed to determine what factors are responsible for the emergence of DUSP6 down-regulation.

In conclusion, this is the first report to address acquired pazopanib resistance in soft-tissue sarcoma. This study reveals a critical role of canonical MAPK signalling in the acquisition of pazopanib resistance through the down-regulation of DUSP6 in SS cells. MEK1/2 inhibition with trametinib is a promising strategy to overcome pazopanib resistance in SS.

Methods

Cell lines. The human SS cell lines HS-SY-II and SYO-1 were used in this study. HS-SY-II was kindly provided by Dr. Sonobe (Department of Pathology, Kochi University, Nangoku) and SYO-1 by Dr. Kawai (Department of Musculoskeletal Oncology and Rehabilitation, National Cancer Center Hospital, Tokyo). Each cell line was maintained in RPMI 1640 (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT, USA), 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO2.

Reagents. The primary antibodies are summarised in Supplementary Table S1.

Pazopanib was purchased from SYNkinase (San Diego, CA, USA) and trametinib was purchased from MedChem Express (Monmouth Junction, NJ, USA).

Establishment of pazopanib-resistant SS clones. Pazopanib-resistant SS clones were isolated after multiple selection steps in the presence of increasing concentrations of pazopanib for approximately 6 months. Pazopanib concentrations were increased from 1 to 20 μM, because 20 μM was the highest concentration in which...
pazopanib could be solubilised in tissue culture media, and 20 μM is clinically achievable and lower than the pazopanib concentration of 40 μM found clinically in patients.

**Chemosensitivity assay.** For the chemosensitivity assay, 2 × 10^5 cells were seeded in 6-well plates. After 24 hours incubation, various concentrations of pazopanib or trametinib were added to the media. After 48 hours incubation, the number of cells was counted with a Z1 Coulter particle counter (Beckman Coulter, Brea, CA, USA).

**Cell cycle analysis by flow cytometry.** Cells were harvested with pazopanib or trametinib, incubated at 37°C overnight, washed with PBS twice, and fixed with 70% ethanol. Then cells were centrifuged, washed with PBS twice, resuspended in PBS with 10 μg/mL RNase A and 50 μg/mL propidium iodide, and incubated for 30 minutes on ice. Alterations in cell distribution were analyzed using a BD Accuri™ C6 Flow Cytometer (BD Biosciences, San Jose, CA, USA). For each sample, 20,000 events were scored.

**Mouse tumour xenograft model.** Female 5-week-old BALB/C nude mice were obtained from Charles River Japan (Fukuoka, Japan) and maintained in a “specific pathogen”-free environment throughout the experiment. Each SYO-1 clone (1 × 10^7) was suspended in 200 μl BD Matrigel™ Basement Membrane Matrix obtained from BD Biosciences (San Jose, CA, USA) and injected subcutaneously. When the tumour volume reached 100–200 mm³, mice were randomly divided into three groups, each consisting of at least four mice. Pazopanib or trametinib suspended in dimethyl sulfoxide (DMSO) and PBS were administered orally three times weekly at 0, 10, 30, or 50 mg/kg, and 0, 0.1, 0.3, or 3 mg/kg, respectively. Tumour volume was measured twice a week by calipers using the following equation: tumour volume (mm³) = (length × width²) × 0.5. At the end of the experiments, all the animals were euthanised. Experiments involving animals were performed in compliance with the guidelines established by the Animal Care and Use Committee of Kyushu University. The Institutional Review Board at Kyushu University approved the use of xenograft model for this study.

**Cell proliferation assay.** Cells were seeded in 96-well plates at a density of 2 × 10^3 cells per well, and the number of viable cells in each well was measured using the CellTiter-Glo™ Luminescent Cell Viability Assay (Promega, Madison, WI, USA).

**Phospho-kinase analysis.** Proteome Profiler Human Phospho-Kinase Array Kits were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Each clone grown in a 10-cm culture dish was incubated with drug-free media for 24 hours and the cell lysate was prepared and analysed as previously described. Semi-quantitation was performed with ATTO CS Analyzer 3.0 (ATTO, Motoasakusa, Taito, Tokyo, Japan).

**Western blot analysis.** Cells were incubated with 5 μM pazopanib or 10 nM trametinib for 2 hours, washed twice with ice-cold PBS, scraped, and centrifuged in microcentrifuge tubes. The cells were lysed using Celllytic M (Sigma-Aldrich, St Louis, MO, USA) with a protease and phosphatase inhibitor cocktail (Complete Mini, PhosSTOP; Roche Diagnostics, Mannheim, Germany). Western blot analysis was performed as described previously.

**Target selection and sequencing.** Exome sequencing was conducted for six DNA samples from each clone. Genomic DNA was extracted using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), sheared into approximately 150–200 bp fragments, and used to make a library for multiplexed paired-end sequencing (Illumina, Tokyo, Japan). The constructed library was hybridised to biotinylated cRNA oligonucleotide baits from the SureSelect Human All Exon V6 Kit (Agilent Technologies, Santa Clara, CA, USA) for exome capture. Targeted sequences were purified by magnetic beads, amplified, and sequenced on an Illumina HiSeq2500 platform in a paired-end 100-bp configuration.

**Mapping and single-nucleotide variant/Indel calling.** Adapter sequences were removed from reads by cutadapt (v1.2.1). After quality control, reads were mapped to the reference human genome (hg19) using BWA (ver.0.7.10). The mapping result was corrected using Picard (ver.1.73) for removing duplicates, and using GATK (ver.1.6–13) for local alignment and quality score recalibration. Single-nucleotide variant (SNV) and Indel calls were performed with multi-sample calling using GATK, and filtered to coordinates with Variant Quality Score Recalibration (VQSR) passed and variant call quality score ≥ 30. Somatic SNV calls were performed by comparing tumour and normal pairs using SomaticSniper (v1.0.2.3). Annotations of SNVs and Indels were based on dbSNP142, CCDS (NCBI, Nov 2014), RefSeq (UCSC Genome Browser, Nov 2014), Gencode (UCSC Genome Browser, ver. 19), and 1000Genomes (Nov 2014). Variants were further filtered according to the following predicted functions: frameshift, nonsense, read-through, missense, deletion, insertion, or insertion-deletion.

**Gene expression microarrays.** The total RNA was isolated from each cell using TRIzol Reagent (Invitrogen) and purified using SV Total RNA Isolation System (Promega). RNA samples were quantified by an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the quality was confirmed with an Experion System (Bio-Rad Laboratories, Hercules, CA, USA). The cRNA was amplified, labelled, and hybridised to a 60-mer oligomicroarray. All hybridised microarray slides were scanned using an Agilent scanner. Relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software (9.5.1.1).

**Data analysis and filter criteria.** Raw signal intensities and flags for each probe were calculated from hybridization intensities (gProcessedSignal) and spot information (gIsSaturated, etc.), according to the procedures recommended by Agilent. (Flag criteria on GeneSpring Software. Absent (A): 1Feature is not...
positive and significant and ‘Feature is not above background’. Marginal (M): ‘Feature is not Uniform,’ ‘Feature is Saturated,’ and ‘Feature is a population outlier.’ Present (P): others.) The raw signal intensities of two samples were log-transformed and normalised by quantile algorithm with the ‘preprocessCore’ library package\(^\text{44}\) of Bioconductor software\(^\text{45}\). We selected probes with a ‘P’ flag call in both samples. To identify up- or down-regulated genes, we calculated Z-scores\(^{46}\) and ratios (non-log scaled fold-change) from the normalised signal intensities of each probe for comparison between SYO-1 parental samples and pazopanib-resistant samples. Then we established criteria for regulated genes: up-regulated genes, Z-score ≥ 2.0 and ratio ≥ 1.5-fold; down-regulated genes, Z-score ≤ −2.0 and ratio ≤ 0.66. A total of 1107 probes were obtained.

RNA preparation and quantitative PCR. Total RNA from each clone was extracted using the RNasey Lipid Tissue Mini kit (QIAGEN). Quantitative PCR was carried out using a LightCycler 1.5 as previously described (Perfect Real Time, Takara Bio, Otsu, Japan)\(^\text{46}\). The primers are summarised in Supplementary Table S2. Data were standardised using GAPDH as a housekeeping gene. A negative control was also prepared using distilled water instead of a DNA template. The assay was performed in triplicate and was repeated in at least three separate experiments. The expression of mRNA was calculated using LightCycler version 3.5 software (Roche Diagnostics).

CRISPR/Cas9 system. The following were purchased from Thermo Fisher Scientific (Waltham, MA, USA): template DNA designed for gRNA, MEGAscript\textsuperscript{TM} Kit, MEGAclean\textsuperscript{TM} Kit, Lipofectamine\textsuperscript{TM} CRISPRMAX\textsuperscript{TM} Transfection Reagent, and GeneArt\textsuperscript{TM} Platinum Cas9 Nuclease. Transfection to the parental clones was carried out using Cas9 protein and lipid-mediated transfection according to the manufacturer’s instructions\(^{48}\).

Statistical analysis. The Wilcoxon signed-rank test was used for two-group comparisons. \(P < 0.05\) was considered to be statistically significant. Data in graphs are given as means ± standard deviation (S.D.). All statistical analyses were performed with the Statistical Analysis System (SAS) software package (JMP9, SAS Institute Inc., Cary, NC, USA).

References
1. Fisher, C. Synovial sarcoma. Ann Diagn Pathol 2, 401–421 (1998).
2. Stelzer, J., Seynaeve, C. & Verweij, J. Using single-agent therapy in adult patients with advanced soft tissue sarcoma can still be considered standard care. The oncologist 10, 833–841 (2005).
3. Taschler, M., Loos, W. J., Seynaeve, C., Verweij, J. & Stelzer, S. The pharmacologic basis of ifosfamide use in adult patients with advanced soft tissue sarcomas. The oncologist 12, 1351–1360 (2007).
4. Sultan, I. et al. Comparing children and adults with synovial sarcoma in the Surveillance, Epidemiology, and End Results program, 1983 to 2005: an analysis of 1268 patients. Cancer 115, 3537–3547 (2009).
5. Bergh, P. et al. Synovial sarcoma: identification of low and high risk groups. Cancer 85, 2596–2607 (1999).
6. Spillane, A. J., A’Hearn, R., Judson, I. R., Fisher, C. & Thomas, J. M. Synovial sarcoma: a clinicopathologic, staging, and prognostic assessment. Journal of clinical oncology 18, 3794–3803 (2000).
7. Kumar, R. et al. Pharmacokinetic pharmacodynamic correlation from mouse to human with pazopanib, a multikinase angiogenesis inhibitor with potent antitumor and antiangiogenic activity. Molecular cancer therapeutics 6, 2012–2021 (2007).
8. Stelzer, J. et al. Pazopanib, a multikinase angiogenesis inhibitor, in patients with relapsed or refractory advanced soft tissue sarcoma: a phase II study from the European organisation for research and treatment of cancer-sof tissue and bone sarcoma group (EORTC study 62043). Journal of clinical oncology 27, 3126–3132 (2009).
9. van der Graaf, W. T. et al. Pazopanib for metastatic soft-tissue sarcoma of paclitaxel-based chemotherapy-refractory disease: a phase II, double-blind, placebo-controlled trial. Lancet 379, 1879–1886 (2012).
10. Hosaka, S. et al. A novel multi-kinase inhibitor pazopanib suppresses growth of synovial sarcoma cells through inhibition of the PI3K-AKT pathway. Journal of orthopaedic research 30, 1493–1498 (2012).
11. Evan, G. I. & Vousden, K. H. Proliferation, cell cycle and apoptosis in cancer. Nature 411, 342–348 (2001).
12. Teizer, R. & Trautetmib. Recent results in cancer research. Fortschr Schriften der Krebsforschung. Progres dans les recherches sur le cancer 201, 241–248 (2014).
13. Bartlett, N. J., Liu, J., Wang, J. & Post, M. PDGE-PP-mediated activation of p42/44MAPK is independent of PDGF receptor tyrosine phosphorylation. American journal of physiology. Lung cellular and molecular physiology 281, L786–798 (2001).
14. Campo, S. & Arkin, J. Dual specificity phosphatases: a gene family for control of MAP kinase function. FASEB J 14, 6–16 (2000).
15. Kasper, B. et al. Long-term responders and survivors on pazopanib for advanced soft tissue sarcomas: subanalysis of two European Organisation for Research and Treatment of Cancer (EORTC) clinical trials 62043 and 62072. Annals of oncology 25, 719–724 (2014).
16. Schulte, F. A., Choueiri, T. K. & Sternberg, C. N. Pazopanib: Clinical development of a potent anti-angiogenic drug. Critical reviews in oncology/hematology 77, 163–171 (2011).
17. Kallman, E. et al. VEGFR2 predicts decreased patients survival in soft tissue sarcomas. Pathology, research and practice 211, 726–730 (2015).
18. Harvitz, H. I. et al. Phase I trial of pazopanib in patients with advanced cancer. Clinical cancer research 15, 4220–4227 (2009).
19. Sternberg, C. N. et al. Pazopanib in locally advanced or metastatic renal cell carcinoma: results of a randomized phase III trial. Journal of clinical oncology 28, 1061–1068 (2010).
20. Xiao, X., Li, B. X., Mitton, B., Ikeda, A. & Sakamoto, K. M. Targeting CREB for cancer therapy: friend or foe. Curr Cancer Drug Targets 10, 384–391 (2010).
21. Moniz, S. & Jordan, P. Emerging roles for WNK kinases in cancer. Cell Mol Life Sci 67, 1265–1276 (2010).
22. Lu, Y. Z., Deng, A. M., Li, L. H., Liu, G. Y. & Wu, G. Y. Prognostic role of phosphorylated PRAS40 (Thr246) expression in gastric cancer. Archives of medical science 10, 149–153 (2014).
23. Li, X. S., Xu, Q., Fu, X. Y. & Luo, W. S. Heat shock protein 60 overexpression is associated with the progression and prognosis in gastric cancer. PloS one 9, e107507 (2014).
24. Boguslawski, G., McGlynn, P. W., Harvey, K. A. & Kovala, A. T. SU1498, an inhibitor of vascular endothelial growth factor receptor 2, causes accumulation of phosphorylated ERK kinases and inhibits their activity in vivo and in vitro. J Biol Chem 279, 5716–5724 (2004).
25. McCubrey, J. A. et al. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. Biochim Biophys Acta 1773, 1263–1284 (2007).
26. Wertz, I. & Seger, R. The ERK Cascade: Distinct Functions within Various Subcellular Organelles. Genes Cancer 2, 195–209 (2011).
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Seiji Okada supervised this study, and Yukihide Iwamoto supervised this study. All authors reviewed this manuscript. Tomoya Matsunobu is a corresponding author of this manuscript, Yoshihiro Matsumoto supervised this study, and Hideki Kaneko helped with this study.

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