Secretomics identifies follistatin as a predictive biomarker for response to treatment with tyrosine kinase inhibitors in synovial sarcoma

Zhiwei Qiao¹, Fusako Kito², Yoko Takai², Rieko Oyama² and Tadashi Kondo¹, ²*

¹ Division of Rare Cancer Research, National Cancer Center Research Institute
² Department of Innovative Seeds Evaluation, National Cancer Center Research Institute

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SUMMARY

Sarcoma is a rare malignancy with an aggressive clinical course. Tyrosine kinase inhibitors (TKIs) have emerged as effective drugs in targeted therapy for malignancies; in particular, pazopanib was recently approved for the treatment of sarcoma. However, as only a limited proportion of patients exhibit favorable response to treatment with TKIs, predictive biomarkers of response to these drugs are urgently needed. In this study, we attempted to identify predictive biomarkers for response to TKIs in synovial sarcoma. We performed a magnetic bead–based assay (Bio-Plex) using synovial sarcoma cell culture supernatant and validated the results by ELISA and western blotting. Cellular protein and mRNA expression levels of candidate biomarkers were evaluated by western blotting and RT-PCR. Gene expression profiling of candidate biomarkers was conducted by meta-analysis of publicly available gene expression data from 149 patients with synovial sarcoma. We found that follistatin (FST) was significantly highly expressed in TKI-resistant cells. Moreover, cell proliferation was decreased following gene silencing of FST. Meta-analysis revealed that the mRNA expression of FST varied among the 149 patients with synovial sarcoma, and that 23 genes were co-expressed with FST; these included genes encoding receptor tyrosine kinase-like orphan receptor 1, Sal-like protein 4, and signal transducer CD24. This study suggested that FST represents a candidate predictive biomarker for response to treatment with TKIs in synovial sarcoma. Secretomics is a promising approach for predictive biomarker exploration. The utility of FST as a predictive biomarker for response to treatment with TKIs in synovial sarcoma should be further validated using clinical samples.

Key words: follistatin, synovial sarcoma, tyrosine kinase inhibitor, predictive biomarker, secretomics

INTRODUCTION

Sarcomas constitute a heterogeneous group of bone and soft tissue malignancies that account for approximately 15% of all pediatric and 1% of all adult cancers.¹ Current treatments for sarcomas are based on radical surgery and standardized chemo- and radiotherapy.²,³ Owing to its high specificity towards tumor cells and relatively few adverse effects, targeted therapy represents a promising treatment for sarcoma. Tyrosine kinases (TKs) play a vital role as regulators of cancer cell proliferation, apoptosis, and angiogenesis, and are therefore recognized as potential targets for anti-cancer therapies.⁴ Tyrosine kinase inhibitors (TKIs) are a class of targeted drugs used for the treatment of cancer.⁵ TKIs have been reported to elicit remarkable therapeutic effects in patients with sarcoma. Pazopanib was recently approved for the treatment of sarcoma; however, not all patients have been found to respond to treatment with pazopanib⁶. Therefore, the development of biomarkers of response to sarcoma treatment with TKIs is necessary.

Previous studies have reported various biomarkers and genetic aberrations related to response to TKIs. Rosell et al. reported that mutations in the TK domain of the epidermal growth factor receptor (EGFR) predict response to treatment with gefitinib and erlotinib in lung cancer.⁷ Amplification or overexpression of EGFR2 serves as a predictive biomarker of response to lapatinib in breast cancers.⁸ Vascular endothelial growth factor D expression is a potential predictive biomarker of bevacizumab treatment in colorectal cancer.⁹ The BCL2L11 deletion polymorphism serves as a predictive biomarker of response to TKIs in chronic myeloid leukemia and non-small-cell lung cancer, in addition.
to mediating intrinsic resistance to these drugs. Moreover, Koehler et al. demonstrated that mutations in TP53 serve as a predictive biomarker of response to pazopanib in patients with advanced sarcomas such as leiomyosarcoma, mesenchymal chondrosarcoma, and liposarcoma. Although few of these previously reported predictive biomarkers have proved to be of clinical value, these reports suggest the possible utility of predictive biomarkers in cancer treatment. However, to date, no predictive biomarkers are available for the assessment of response to treatment of sarcoma with TKIs.

Secreted proteins, whose detection and quantification may be performed by non-invasive and low-cost methods such as enzyme-linked immunosorbent assay (ELISA), represent a fundamental source of biomarkers. Numerous secreted proteins have been reported to be of utility as biomarkers for the prediction of response to drug treatment. Yao et al. reported that IL-18 serves as a novel biomarker for doxorubicin resistance. Teng et al. identified biomarkers from secretomes for response to cisplatin treatment in epithelial ovarian carcinoma. These observations suggest the utility of secretomics in biomarker development. However, to date, secretomics-based approaches have not been applied to the discovery of biomarkers for response to TKI treatment in sarcoma.

The aim of this study was to identify predictive biomarkers for response to TKI treatment in sarcoma, with a particular focus on synovial sarcoma, which is one of the most common soft tissue sarcomas in adolescents and young patients. A magnetic bead-based assay was performed to identify predictive biomarkers in synovial sarcoma, and a meta-analysis was conducted to identify the gene expression pattern of the candidate biomarkers.

Methods

Cells and culture

This study utilized four synovial sarcoma cell lines: the SYO-1 cell line was a gift from Akira Kawai (National Cancer Center, Tokyo, Japan), HS-SY-II from Hiroshi Sonobe (Kochi Medical School, Kochi, Japan), 1273/99 from Olle Larsson (Karolinska Institute, Stockholm, Sweden), and YaFuSS from Tatsuya Ishibe (University of Tokyo, Tokyo, Japan). Cells were cultured in DMEM low-glucose medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), at 37°C in a humidified atmosphere with 5% CO₂. Whole cell lysates were subjected to protein quantification and analyzed by western blotting.

Analysis of cell viability

The tumor cells plated on 96-well plates (10,000 cells/well) were incubated in the presence of TKIs (cabozantinib, cediranib, dasatinib, imatinib, pazopanib, sunitinib, bosutinib, nilotinib, ponatinib, and sorafenib) (Selleck Chemicals, Houston, TX, USA), at various concentrations (0, 0.02, 0.2, 2, or 20 μM), for 72 h. Then, cell proliferation was measured using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s recommendations.

Bio-Plex assay

The Bio-Plex Pro™ Human Cancer Biomarker Panel 1 (Number 171AC500M), Bio-Plex Pro™ Human Cancer Biomarker Panel 2 (Number 171AC600M), and Human Inflammation Panel 1 (Number 171AL001M) (Bio-Rad laboratories, Inc., Hercules, CA, USA) were used to measure the concentrations of 71 proteins (Supplementary Table 1) in culture medium from synovial sarcoma cell lines, according to the manufacturer’s instructions. Data were acquired using the Bio-Plex suspension system (Bio-Rad Laboratories).

Enzyme-linked immunosorbent assay (ELISA)

The concentration of follistatin (FST) in conditioned medium of the four synovial sarcoma cell lines was measured by ELISA, according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA). The absorbance was measured at 450 nm to quantify FST levels, and concentrations of FST were calculated from the standard curve. The Student’s t-test for two independent groups was used to address any differences between groups at baseline. A p value of <0.05 was considered to represent statistical significance.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using QIAzol lysis reagent (Qiagen, Venlo, the Netherlands). cDNA was synthesized using 1 μg of total RNA with SuperScript III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA, USA). cDNA amplification was performed using the following primers for FST: 5'-GAA CTG AGC AAG GAG GAG TG-3' (forward) and 5'-CAC TTT CCC TCA TAG GCT AAT CC-3' (reverse); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-ACC ACA GTC CAT GCC ATC AC-3' (reverse); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-ACC ACA GTC CAT GCC ATC AC-3' (forward) and 5'-TCC ACC ACC CTG TTG CTG TA-3' (reverse). PCR was performed as follows: 38 cycles for FST and 29 cycles for GAPDH, of 50 s at 94°C, 30 s at 59°C, and 90 s at 72°C. The PCR products were separated on 1.5% agarose gels and stained with ethidium bromide. The bands were analyzed using ImageJ (US National Institutes of Health, Bethesda, MD, USA).

Western blotting

Cells were lysed in lysis buffer containing 7 M urea, 2 M thiourea, 3% CHAPS, 1% Triton X-100, and 1 mM dithiothreitol. Whole cell lysates were subjected to protein quantification and analyzed by western blotting. Ten micrograms of each protein sample was separated by SDS-PAGE on a
RESULTS

The 1273/99 cell line was resistant to TKIs compared with the other three cell lines.

Four synovial sarcoma cell lines (SYO-1, HS-SYII, 1273/99, and YaFuSS) were treated with various concentrations of 11 TKs for 72 h, and cell viability was determined. The 72-h half maximal inhibitory concentrations (IC50) of 11 TKs in the four cell lines were calculated (Supplementary Table 2) and depicted in heatmap format (Fig. 1). The 1273/99 cells showed higher IC50 values for all 11 TKIs examined than the other three cell lines. The data therefore indicate that 1273/99 cells are resistant to the 11 TKIs tested.

Follistatin (FST) expression was upregulated in the 1273/99 cell line.

In order to explore the candidate biomarkers for TKI resistance, we measured 71 proteins in conditioned medium from the four synovial sarcoma cell lines using the Bio-Plex system. We detected 28 proteins in all four synovial sarcoma cell lines. We then performed hierarchical clustering analysis to reveal the expression patterns of the 28 proteins in four synovial sarcoma cell lines (Fig. 2A). The 28 proteins were grouped into four clusters; among these, 9 proteins in cluster 1 were expressed at higher levels in the 1273/99 cell line than in the other three cell lines, 6 proteins in cluster 2 were expressed at higher levels in the HS-SYII cell line than in the other three cell lines, 8 proteins in cluster 3 were expressed at higher levels in the SYO-1 cell line than in the other three cell lines, and 5 proteins in cluster 4 exhibited higher expression levels in the YaFuSS cell line than in the other three cell lines. We hypothesized that proteins in cluster 1 represented candidate biomarkers for prediction of response to treatment with TKIs.

In the present study, we focused on FST, which was included in cluster 1. FST has been previously reported to be a potential biomarker for detection of tumors, or a candidate therapeutic target in lung adenocarcinoma, hepatocellular carcinoma.

Gene silencing assay

siRNAs were purchased from Sigma, and non-specific control siRNA duplexes (AllStar Negative Control siRNA) from Qiagen. The target sequences were designed as follows: FST siRNA1: sense, GAUCUAUGGAUAGCC UATT, and anti-sense, UAGGCUAUCCAUAAGAUCTG; FST siRNA2: sense, GGUCCUGUACAAGACCGAATT, and anti-sense, UUCGGUCUUGUACAGGACCTG; A total of 1×10⁴ synovial sarcoma cells were seeded into each well of a 96-well plate. Cells were transfected with the appropriate siRNA using RNAi max transfection reagents (Thermo Fisher, Waltham, MA, USA) in accordance with the manufacturer’s protocol. Twenty-four hours later, the culture medium was replaced with DMEM low-glucose medium (Sigma). The cells were harvested for western blotting or treated with 5 μM pazopanib for 72 h after transfection.

Meta-analysis

Gene expression data for patients with synovial sarcoma were obtained from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). We used two microarray data sets as follows: 57 samples from GSE 54187 and 92 samples from GSE40025. The microarray data were normalized and standardized using the Linear Models for Microarray Data (Limma) package (http://bioconductor.org/packages/limma/).

Statistical analysis

All data are presented as the mean ± SE and were analyzed using the GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). The Student’s t test or Mann–Whitney U test was used for between-group comparisons. Spearman’s correlation test was used to estimate the correlation between the expression of each gene in synovial sarcoma tissue and the FST expression value. P<0.05 was considered to indicate statistical significance.
patients with synovial sarcoma (Fig. 4) revealed variations in expression profiles between the patients. Then, we performed Spearman correlation analysis, and found 23 genes whose expression levels were highly correlated with FST expression in patients with synovial sarcoma (r>0.6, P<0.001).

**DISCUSSION**

The proteome is a functional translation of the genome that directly regulates cell phenotypes, and is therefore a rich source of biomarkers27). Accordingly, proteomics-based analyses have distinct advantages over other so-called -omics approaches such as genomics, epigenetics, transcriptomics, and metabolomics. In this study, we performed a magnetic bead–based assay (Bio-Plex) to identify predictive biomarkers for response to treatment with TKIs in synovial sarcoma. Among the 71 proteins analyzed using this assay, we found that FST was related to response to TKI treatment. To our knowledge, this is the first study to report FST as a candidate predictive biomarker for response to treatment with TKIs in sarcoma.

Reduced expression of FST inhibited cell growth in synovial sarcoma.

We further investigated the functional roles of FST in synovial sarcoma. We confirmed that FST levels were reduced after transfection with small interfering RNAs against FST in the four synovial sarcoma cell lines (Fig. 3A). We found that cell viability was significantly inhibited by downregulation of FST mRNA expression in all four synovial sarcoma cell lines (Fig. 3B). We additionally examined the synergistic effect of FST silencing and pazopanib treatment, which has been approved by the FDA for sarcoma26). As shown in Supplementary Fig. 2, no synergistic effects were identified in any cell lines examined.

**Meta-analysis of the expression profiles of FST in synovial sarcoma**

In order to examine the expression profiles of FST in patients with synovial sarcoma, we performed a meta-analysis using the microarray data deposited in the GEO database. Examination of the mRNA expression of FST in 149 patients with synovial sarcoma (Fig. 4) revealed variations in expression profiles between the patients. Then, we performed Spearman correlation analysis, and found 23 genes whose expression levels were highly correlated with FST expression in patients with synovial sarcoma (r>0.6, P<0.001).

**DISCUSSION**

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The Bio-Plex system, which comprises 100 distinctly colored bead sets created using two fluorescent dyes in varying ratios, is a multiplex analysis system that permits the multiplexing of distinct assays in a single sample. Beads are conjugated with a reagent specific to a particular bioassay. The Bio-Plex system has been used to simultaneously analyze numerous kinds of biomolecules for biomarker discovery. Using this assay system, Martinetti et al. investigated circulating prognostic biomarkers, such as SDF-1.
and CEA, in patients with colorectal cancer\textsuperscript{28}. Harle et al. used the Bio-Plex system to identify predictive biomarkers for anti-EGFR monoclonal antibodies in metastatic colorectal cancers\textsuperscript{29}. In this study, we identified secreted proteins associated with TKI treatment using the Bio-Plex system, and found 9 proteins that were secreted from TKI-resistant synovial sarcoma cells at high levels.

FST, an autocrine glycoprotein that was originally isolated from ovarian follicular fluid, inhibits the release of follicle-stimulating hormone from the pituitary\textsuperscript{30,31}. In addition, this protein, which specifically binds activin, is involved in the regulation of multiple physiological and pathological functions. Two FST molecules encircle one activin molecule, thereby neutralizing activin activity by burying its receptor-binding sites\textsuperscript{32}. Activin is a member of the transforming growth factor beta (TGF-b) superfamily and is involved in numerous cellular processes such as differentiation, proliferation, apoptosis, and regulation of immune responses in the tissue\textsuperscript{33}. FST binds activin to inhibit its activity\textsuperscript{34}, and dysregulation of the FST and activin axis is one of the most significant factors underlying tumor genesis. FST has been reported as a biomarker or therapeutic target in cancers such as breast cancer\textsuperscript{35}, ovarian cancer\textsuperscript{36,37}, hepatocellular carcinoma\textsuperscript{23}, and glioblastoma\textsuperscript{38}. Recently, Chen et al. reported that FST is a novel biomarker for the diagnosis of lung adenocarcinoma\textsuperscript{22}. Furthermore, Sepporta et al. demonstrated that FST is a potential therapeutic target in prostate cancer\textsuperscript{39}. Here, we report that increased FST expression is significantly associated with resistance to TKI treatment in synovial sarcoma. Further studies are needed to address the role of FST in synovial sarcoma.

In the present study, we performed a meta-analysis of gene expression data derived from public repositories. Mining of public data has proved a useful approach in cancer research\textsuperscript{40–42}. We found that the gene expression pattern of FST varied among the 149 clinical synovial samples and CEA, in patients with colorectal cancer\textsuperscript{28}. Harle et al. used the Bio-Plex system to identify predictive biomarkers for anti-EGFR monoclonal antibodies in metastatic colorectal cancers\textsuperscript{29}. In this study, we identified secreted proteins associated with TKI treatment using the Bio-Plex system, and found 9 proteins that were secreted from TKI-resistant synovial sarcoma cells at high levels.

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examined, and identified 23 genes whose expression levels were strongly associated with FST levels. Among these, three genes have been previously reported to be associated with TKI resistance: the receptor tyrosine kinase-like orphan receptor 1 (ROR1), Sal-like protein 4 (SALL4), and signal transducer CD24. ROR1 was previously reported to sustain signaling for multiple receptor tyrosine kinases and overcome TKI resistance in non-small cell lung cancers. Hüpfeld et al. reported that TK inhibition facilitates cooperation of transcription factor SALL4 and ATP-binding cassette transporters A3 in eliciting drug resistance in intrinsic chronic myelogenous leukemia cells. CD24 contributes to resistance to lapatinib in HER2-positive breast cancer cells. Further studies are necessary to investigate the coordinate effect of these genes with FST in patients with TKI-resistant cancers.

In conclusion, secretomics-based studies represent a promising approach for predictive biomarker exploration. In the present work, secretomics studies identified FST as a candidate predictive biomarker for TKI resistance. The clinical utility of FST requires further validation using clinical samples.

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ABBREVIATIONS

TKI; tyrosine kinase inhibitors, siRNA; small interfering RNA, FST; follistatin, EGFR; epidermal growth factor receptor, ELISA; enzyme-linked immunosorbent assay, GEO; Gene Expression Omnibus, TGF-b; transforming growth factor beta, ROR1; receptor tyrosine kinase-like orphan receptor 1, SALL4; Sal-like protein 4

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