

lncRNA *HAR1B* has potential to be a predictive marker for pazopanib therapy in patients with sarcoma

HIDEHARU YAMADA^{1,2}, MASANOBU TAKAHASHI^{1,2}, MUNENORI WATANUKI³, MIKA WATANABE⁴,
SAKURA HIRAIDE^{1,2}, KEN SAIJO^{1,2}, KEIGO KOMINE^{1,2} and CHIKASHI ISHIOKA^{1,2,5}

¹Department of Clinical Oncology, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Miyagi 980-8575; ²Department of Medical Oncology, Tohoku University Hospital, Sendai, Miyagi 980-8574; ³Department of Orthopaedic Surgery, Tohoku University Graduate School of Medicine, Sendai, Miyagi 980-8575; ⁴Department of Pathology, Tohoku University Hospital, Sendai, Miyagi 980-8574; ⁵Department of Clinical Oncology, Tohoku University Graduate School of Medicine, Sendai, Miyagi 980-8575, Japan

Received September 4, 2020; Accepted February 10, 2021

DOI: 10.3892/ol.2021.12716

Abstract. Bone and soft-tissue sarcomas are rare and are highly heterogeneous mesenchymal malignancies. It is therefore challenging to acquire the clinical data of patients with specific histological subtypes of sarcoma using large clinical trials, and there is a need to further establish the diagnosis and treatment of sarcomas. The results of the current study revealed that long non-coding RNA (lncRNA) highly accelerated region 1B (*HAR1B*) may serve as a predictive biomarker for pazopanib treatment in bone and soft-tissue sarcomas. Using multiplex reverse transcription-quantitative PCR and microarray analyses, the results demonstrated that *HAR1B* and HOX transcript antisense RNA (*HOTAIR*) were differentially expressed in pazopanib-sensitive cells and responders. It was further revealed that small interfering RNA-knockdown of *HAR1B* led to an increased resistance to pazopanib in sarcoma cell lines. Gene expression profiles associated with pazopanib sensitivity included cellular molecular pathways, such as genes involved in von-Willebrand factor-related signaling. The current study demonstrated that lncRNA *HAR1B* expression in sarcoma cell lines affected cellular sensitivity to pazopanib in patients with sarcoma.

Introduction

Bone and soft-tissue sarcomas are rare and highly heterogeneous mesenchymal malignancies, encompassing more than 70 distinct histological subtypes with various clinical features. They include liposarcoma, leiomyosarcoma, synovial sarcoma, angiosarcoma, malignant peripheral nerve sheath tumors, osteosarcoma, Ewing sarcoma, chondrosarcoma, and many others (1,2). Soft-tissue sarcomas account for only 1% of all malignancies (3), and bone sarcomas are even 8-10 times less common than soft-tissue sarcomas (4).

Chemotherapy is the main treatment modality used in unresectable sarcomas. This modality includes conventional cytotoxic drugs, such as doxorubicin, ifosfamide, dacarbazine, epirubicin, gemcitabine, temolozomide, docetaxel, trabectedin, and eribulin (5-7). Moreover, the multi-target tyrosine kinase inhibitor pazopanib, which inhibits vascular endothelial growth factor receptors (VEGFRs), platelet-derived growth factor receptors, KIT, and other receptor tyrosine kinases, has recently been adopted in the treatment of soft-tissue sarcomas (8). However, for many sarcomas, no standard pathological or molecular biomarker exists that can predict the clinical outcomes of patients on different drug therapies, and these drugs have limited effects. Because sarcomas are rare, it is challenging to collect clinical data from prospective clinical trials with patients with specific histological subtypes of sarcomas. Therefore, the diagnoses and treatment of sarcoma needs to be further developed.

In recent years, long non-coding RNA (lncRNA) has attracted great attention as a potential diagnostic, prognostic, and predictive biomarkers in the treatment of various cancers. lncRNAs are transcripts composed of more than 200 nucleotides that do not encode proteins (9). The number of known human lncRNAs is gradually on the rise, with about 96,000 currently described, according to the NONCODEV5 database (10). Accumulating evidence has demonstrated an association between the altered expression of some lncRNAs with various cancer types. These include *GAS5*, *LINC-PINT*, *MEG3*, *HOTAIR* and *MALAT1*, which are involved in various

Correspondence to: Professor Chikashi Ishioka, Department of Clinical Oncology, Tohoku University Graduate School of Medicine, Seiryomachi 2-1, Aoba-ku, Sendai, Miyagi 980-8575, Japan
E-mail: chikashi@tohoku.ac.jp

Dr Masanobu Takahashi, Department of Clinical Oncology, Institute of Development, Aging and Cancer, Tohoku University, Seiryomachi 4-1, Aoba-ku, Sendai, Miyagi 980-8575, Japan
E-mail: masanobu.takahashi.a7@tohoku.ac.jp

Key words: sarcoma, long non-coding RNA, pazopanib, highly accelerated region 1B, HOX transcript antisense RNA

cellular functions such as proliferation, survival, metastasis, and genomic stability (11). Moreover, some of these lncRNAs may be used as prognostic biomarkers, such as *HOTAIR* in breast cancer (12) and gastrointestinal cancers (13), and *MALAT1* in lung cancer (14). Likewise, in osteosarcoma, some lncRNAs have been reported as prognostic biomarkers (15-17). In addition to detecting lncRNA expression levels in tumor tissues, detecting lncRNA in the plasma might be useful in the diagnosis and to develop treatment strategies for patients with cancer (18). Thus, the clinical application of lncRNAs as biomarkers in the treatment of patients with cancer is currently researched. However, few reports have identified the biological roles of lncRNAs or their clinical utility in sarcomas, particularly soft-tissue sarcomas.

In this study, we screened for lncRNAs that are specifically dysregulated in bone and soft-tissue sarcoma cell lines and patients, by using a multiplex polymerase chain reaction (qPCR) assay and genome-wide RNA expression analysis. Furthermore, using knockdown systems, we sought to clarify whether lncRNAs play a role in drug sensitivity to pazopanib in sarcoma cell lines. We identified a lncRNA *highly accelerated region 1B (HARIB)*, which is highly expressed in cell lines sensitive to pazopanib and in patients with sarcoma who benefited from pazopanib therapy, and found that its suppression led to an increased resistance to pazopanib in sarcoma cell lines.

Materials and methods

Cell lines. A total of 16 bone or soft-tissue sarcoma cell lines were used in this study. These include SW872 (liposarcoma), HT1080 (fibrosarcoma), SK-LMS-1 (leiomyosarcoma), A204 (rhabdomyosarcoma), RD (rhabdomyosarcoma), ISO-HAS-B (angiosarcoma), HS-sch2 (malignant peripheral nerve sheath tumor, MPNST), FMS-1 (MPNST), SFT8611 (MPNST), SFT9817 (MPNST), YST-1 (MPNST), S462 (NF-1 associated MPNST), HS-SY-II (synovial sarcoma), Yamato-SS (synovial sarcoma), SaOs2 (osteosarcoma), and MG63 (osteosarcoma). SW872, HT1080, SK-LMS-1, A204, and RD were purchased from the American Type Culture Collection. HS-sch2, HS-SY-II and Yamato-SS were purchased from the Riken Bioresource Center (Ibaraki, Japan). YST-1, SaOs2, and MG63 were purchased from Tohoku University Cell Resource Center for Biomedical Research Cell Bank (Miyagi, Japan). FMS-1 (19) was provided by Dr Michiyuki Hakozaki from the First Department of Pathology, Fukushima Medical University School of Medicine (Fukushima, Japan). SFT8611 and SFT9817 (20) were kindly provided by Dr Mikiko Aoki and Dr Kazuki Nabeshima at the Department of Pathology, Fukuoka University School of Medicine (Fukuoka, Japan). S462 (21) was provided by Dr Lan Kluwe from the Department of Oral and Maxillofacial Surgery, the University Medical Center Hamburg-Eppendorf (Hamburg, Germany). ISO-HAS-B (22) was provided by Dr Mikio Masuzawa from the School of Allied Health Science, Kitasato University (Kanagawa, Japan). The cell lines were regularly authenticated by short tandem repeat analysis. SW872, SK-LMS-1, RD, ISO-HAS-B, HS-sch2, S462, HS-SY-II, Yamato-SS, and MG63 were maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose. HT1080,

A204, FMS-1, YST-1 and SaOs2 were maintained in an RPMI-1640 medium. SFT8611 and SFT9817 were maintained in a DMEM/F12 medium. These media included 10% fetal bovine serum. The cells were grown at 37°C under 5% CO₂.

MTT assay. Pazopanib was purchased from Adooq Bioscience (GW-786034). Pazopanib was diluted in dimethyl sulfoxide to 10 mM and the required concentrations were added to the respective media.

Using a 96-well plate, the following cell numbers of each sarcoma cell line were seeded in individual wells: 2,500 of SW872, 500 of HT1080, 3,000 of SK-LMS-1, 2,000 of A204, 2,500 of RD, 2,000 of ISO-HAS-B, 1,000 of S462, 2,000 of FMS-1, 1,000 of SFT8611, 2,500 of SFT9817, 2,500 of HS-sch2, 3,000 of YST-1, 2,000 of HS-SY-II, 3,000 of Yamato-SS, 2,000 of SaOs2, and 1,000 of MG-63. To calculate the pazopanib inhibitory potency (IC₅₀) for the 16 sarcoma cell lines, at least seven different doses of pazopanib were used. Cells were treated with pazopanib and were seeded 24 h after onto the plates. The cell viability was measured after 72 h using the Cell Counting Kit-8 (Dojindo Laboratories), according to the manufacturer's protocol. Cell viability (%) was calculated by dividing the median value for each pazopanib dose (conducted at least in triplicates) by the value of untreated cells. IC₅₀ values were calculated using the dosages from the two-cell viability values surrounding 50%. From the results obtained from three or more independent experiments, we calculated the IC₅₀ data.

lncRNA expression profiles in sarcoma cell lines analyzed by multiplex real-time RT-PCR. The total RNA obtained from the 16 sarcoma cell lines was extracted using RNeasy Mini kit (Qiagen) and quantified by NanoDrop-1000 v3.8.1 (Thermo Fisher Scientific, Inc.). The LncProfiler qPCR Array Kit (System Bioscience) recognizes 90 cancer-related or stem cell-related lncRNAs, and was used to analyze lncRNA expression profiles in the 16 sarcoma cell lines, according to the manufacturer's protocol. In brief, cDNA was synthesized using the GeneAmp PCR system 9700 (Thermo Fisher Scientific, Inc.). The 90 cancer-related or stem cell-related lncRNAs and 5 control RNAs for normalization (composed of *18S rRNA*, *RNU43*, *GAPDH*, *LAMIN A/C*, and *U6*) were quantified by real-time PCR using CFX96 (Bio-Rad). The thermal cycling was programmed for 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. The geometric mean values for the expression of the four control RNAs, except *18S rRNA* whose expressions was inconsistent among the cell lines, were used to normalize the relative expression values of each lncRNA. Expression data are expressed as mean values from the results obtained from at least two independent experiments in each cell line. When the fold change (FC) in mean lncRNA expression levels of the sensitive cells versus those of the resistant cells was >1.5 or <0.67, with P<0.10, the lncRNAs were considered to be candidates for factors that affect cellular sensitivity to pazopanib.

Patients. We recruited 39 patients with bone or soft-tissue sarcoma who were on pazopanib therapy between December 2012 and June 2018 in the Tohoku University Hospital. Patients were regarded eligible if they were aged 20 years or older; had histologically confirmed unresectable, recurrent, or metastatic

bone or soft-tissue sarcoma of extremity, trunk, retroperitoneal, or any organs; formalin-fixed paraffin-embedded (FFPE) tissues were available. Pathological diagnosis was performed by a pathologist (M.M.). Tumor tissues were generally resected with at least 5-mm margin from normal tissues. DNA and RNA were extracted from the macro-dissected tumor cells. Written informed consent was obtained from all patients. Their records were retrospectively reviewed for information on the clinical characteristics of the patients and tumors, changes in tumor size, overall response rate, progression-free survival (PFS) after the initiation of drug therapies, and overall survival (OS). The change in tumor size of one or two measurable lesions, overall response rate, and PFS were calculated based on Response Evaluation Criteria in Solid Tumor ver1.1 (23).

Global gene and lncRNA expression analyzed by microarrays. Total RNA from FFPE sarcoma tissues were extracted using the RNeasy FFPE kit (Qiagen). The levels of RNA degradation were analyzed with a Bioanalyzer-2100 (Agilent Technologies), and the RNA quality was confirmed based on the manufacturer's protocol (Agilent Technologies, <https://www.agilent.com/>). The genome-wide gene and lncRNA expression levels of the 23 bone or soft-tissue sarcoma tissues were analyzed using SurePrint G3 Hyman Gene Expression 8x60K ver. 3.0 microarray (Agilent Technologies), which covers 58,341 probes including 26,083 coding genes and 30,606 lncRNAs. To analyze the gene and lncRNA expressions of the 16 sarcoma cell lines, we performed microarray analyses using SurePrint G3 Hyman Gene Expression 8x60K ver. 3.0, according to the manufacturer's protocol (Agilent Technologies). The microarray data were extracted and analyzed using the Feature Extraction ver. 10.7 (Agilent Technologies; https://www.agilent.com/cs/library/usermanuals/public/G4460-90026_FE_Reference.pdf) and the GeneSpring ver. 14.5 (Agilent Technologies). We classified the differentially expressed probes between responders and non-responders, either with FC >1.5 or <0.67 (P<0.1), as the lncRNAs that might be related to pazopanib sensitivity.

Knockdown of HAR1B by siRNA and its influence on cellular sensitivity to pazopanib. HS-SY-II and Yamato-SS cells were used in the HAR1B knockdown assay to determine whether alterations in HAR1B expression affected cellular sensitivity to pazopanib treatment. The cells were transfected with si-HAR1B (Mission siRNA HAR1B, #SASI_Hs02_00378868_AS; Sigma-Aldrich; Merck KGaA) or si-negative control (MISSION siRNA Universal Negative Control#1, #SIC001) at a final concentration of 5.7 nM using Lipofectamine 2000 (#11668019; Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

Total RNA was extracted from the cells using the RNeasy Mini kit (Qiagen). cDNA was prepared using the 5x PrimeScript RT Master Mix (Takara), 48 and 72 h after transfection in HS-SY-II and Yamato-SS cells, respectively. Real-time PCR primers for HAR1B (VC00026) were purchased from Sigma-Aldrich; Merck KGaA. HAR1B expression levels were normalized by GAPDH expression detected by primers for GAPDH (forward: ACCCAGAAGACTGTGGATGG, reverse: CAGTGAGCTTCCCGTTCAG). The PowerUp SYBR-Green Master Mix (Applied Biosystems) was used for real-time PCR.

The thermal cycling was programmed for 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Each sample was amplified in triplicate and the mean expression values were obtained from at least three independent experiments.

Twenty-four hours after transfection, 0, 1, 2, and 4 μ M of pazopanib for HS-SY-II cells, and 0, 1, 2, and 5 μ M for Yamato-SS cells, were respectively added to the medium, and cell viability was measured by the MTT assay, as described above, after 48 h for HS-SY-II cells, or 72 h for Yamato-SS cells. Each sample was amplified in triplicate during each run, and the mean viability values were obtained from at least four independent experiments in each cell line.

Statistical analyses. Significance analyses of gene expression and cell viability assays were conducted using unpaired or paired t-tests. Hierarchical clustering and heat-map generation were performed using R (version 3.6.1, R Development Core Team, <http://www.R-project.org/>) in multiplex lncRNA real-time RT-PCR analysis, and GeneSpringGX 14.5 (Agilent Technologies, https://www.agilent.com/cs/library/usermanuals/public/GeneSpring_manual.pdf) in microarray analyses. Survival analyses was performed using JMP Pro ver.14.2.0 (SAS Institute Inc.). Kaplan-Meier analyses were used to estimate the distributions of PFS or OS, and a log-rank test was used to analyze the statistical differences in survival.

Gene enrichment analysis and functional annotation clustering were performed using the David analysis (24), according to the instruction (ver. 6.8, <https://david.ncicrf.gov>). In brief, we uploaded a list of each Entrez Gene ID of our 306 genes into the DAVID webpage. The threshold for the number of genes was 2. The gene enrichment was quantitatively measured by modified Fisher's exact test. There were 65 annotation terms with P-value <0.01. For functional annotation clustering, an enrichment score of 1.30 or higher is considered statistically significant.

Results

Sensitivity to pazopanib in 16 sarcoma cell lines. To elucidate cellular sensitivity to pazopanib, we first performed MTT assays and calculated the IC₅₀ values of the 16 bone or soft-tissue sarcoma cell lines. The most sensitive cell line was A204 (IC₅₀ value of 0.08 μ M), and the most resistant cell line was SFT8611 (IC₅₀ value of 141 μ M) (Fig. 1A). Preclinical models showed that pazopanib activity depended on reaching a steady-state concentration of >40 μ M (25), and a phase I clinical trial showed that in most patients, 800 mg pazopanib once daily as a current standard clinical dose helped achieve a plasma concentrations of >34 μ M pazopanib after 24 h (26). Based on these previous data, we categorized the cell lines as 'sensitive' cell lines with regard to pazopanib (IC₅₀ values <20 μ M: A204, HS-SY-II, Yamato-SS, and YST-1) and 'resistant' (the other 12 cell lines) (Fig. 1A).

Screening for candidate lncRNAs differentially expressed between pazopanib-sensitive and pazopanib-resistant cell lines. We then analyzed the expression profiles of 90 lncRNAs among the 16 cell lines using multiplex real-time RT-PCR. As shown in Fig. 1B, each of the 16 cell lines had distinct lncRNA expression profiles. The expression profiles

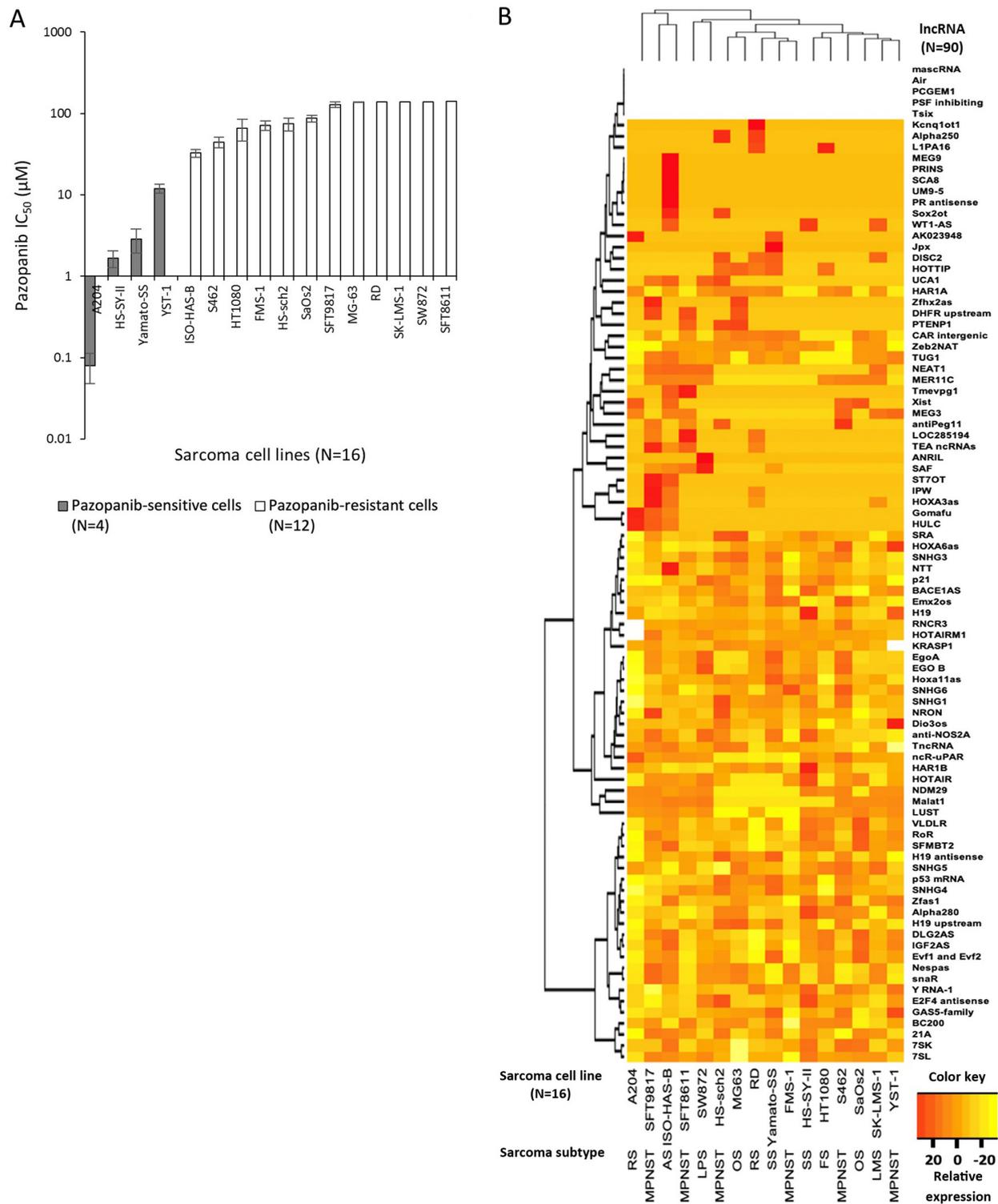


Figure 1. Sensitivity to pazopanib and lncRNA expression in 16 bone or soft-tissue sarcoma cell lines. (A) IC_{50} values of 16 sarcoma cell lines treated with pazopanib were analyzed by an MTT assay. Four sarcoma cell lines with IC_{50} values $<20 \mu M$ were classified as pazopanib-sensitive cell lines, while the other 12 cell lines were categorized as pazopanib-resistant cell lines. Data are presented as the logarithmic value of the mean \pm SEM. (B) Expression of 90 lncRNAs in 16 sarcoma cell lines analyzed by multiplex qPCR analysis. Clustering analysis using the complete method revealed that clustering did not depend on the histological subtype. Each lncRNA expression value was normalized to a geometric mean value of four normalization genes, *RNU43*, *GAPDH*, *LAMIN A/C* and *U6*. Five lncRNAs were not detected in any of the 16 sarcoma cell lines. Data are shown as relative expression mean values. lncRNA, long non-coding RNA; RS, rhabdomyosarcoma; MPNST, malignant peripheral nerve sheath tumor; AS, angiosarcoma; LPS, liposarcoma; OS, osteosarcoma; SS, synovial sarcoma; FS, fibrosarcoma; LMS, leiomyosarcoma.

did not seem to depend solely on histological subtype, although the number of cell lines was too small to draw a firm conclusion.

We next tried to identify lncRNAs differentially expressed between the sensitive and resistant cell lines. The expression of each lncRNA expression was compared between the sensitive

and resistant cells using unpaired t-tests (Table SI). When the FC in mean lncRNA expression levels of the sensitive cells versus those of the resistant cells was >1.5 or <0.67 , with $P<0.10$, we hypothesized that the lncRNAs were candidates for factors that affect cellular sensitivity to pazopanib. A total of 12 lncRNAs including *BACE1AS*, *MER11C*, *H19*, *GAS5-family*, *Dio3os*, *Y RNA-1*, *AK023948*, *HAR1B*, *Jpx*, *Gomafu*, *HULC*, and *HOTAIR*, fulfilled this criteria (Table SI). The expression of all lncRNAs except *MER11C* was upregulated in the sensitive cell lines.

Patients with bone or soft-tissue sarcomas were divided into responders and non-responders. In a phase III study, PALETTE, the median PFS of patients with soft-tissue sarcomas was 4.6 months (8). Considering the PALETTE data, we defined the 39 patients with at least 6 months of PFS and/or with at most 0% of the maximum change in tumor size as 'responders'. The other patients were defined as 'non-responders'. The 39 patients with bone or soft-tissue sarcoma who received pazopanib therapy were categorized into 16 responders and 23 non-responders.

Microarray analyses to validate candidate lncRNAs that might be related to pazopanib sensitivity. We tried to determine whether there were also differentially expressed lncRNAs between responders and non-responders who received pazopanib. Among the 39 patients, samples from 23 patients with a sufficient quantity and quality of RNA were analyzed using microarray analyses (Table I). Seven patients showed tumor shrinkage, and PR was observed in three patients among the 18 with measurable lesions (Fig. 2A). The median PFS of responders and non-responders was 10.2 months [95% confidence interval (95% CI), 5.6-21.0 months] and 1.9 months (95% CI 1.1-2.8 months), as shown in Fig. 2B. The median OS of responders and non-responders was 22.0 months (95% CI, 6.6-55.8 months) and 5.6 months (95% CI 1.3-11.0 months), as shown in Fig. 2C.

In microarray analyses using SurePrint G3 Hyman Gene Expression 8x60K microarrays, we classified the differentially expressed probes between responders and non-responders, either with FC >1.5 or <0.67 ($P<0.1$), as the lncRNAs that might be related to pazopanib sensitivity. Among 14,661 probes of the lncRNAs analyzed, 2,417 probes (2,417/14,661, 16%) fulfilled these criteria (Fig. 2D). The results showed that some proportion of lncRNAs was differentially expressed between responders and non-responders, and that at least some lncRNAs might be involved in sensitivity to pazopanib in patients with bone or soft-tissue sarcomas.

We next attempted to validate whether the lncRNAs differentially expressed between the pazopanib-sensitive and pazopanib-resistant cell lines were also differentially expressed between responders and non-responders with bone or soft-tissue sarcomas who received pazopanib. Among 90 lncRNAs analyzed by multiplex real-time RT-PCR, 32 lncRNAs were detected in the microarray analyses. Among them, *HAR1B* and *HOTAIR* fulfilled the criteria of FC >1.5 or <0.67 , with $P<0.1$ (Fig. 3, Table II). *HAR1B* expression levels in sensitive cells were higher than in resistant cells, with 279 of FC and $P=0.08$ (Fig. 3A), and higher in responders than those in non-responders with 2.25 of FC and $P=0.09$ (Fig. 3B). *HOTAIR* expression levels in sensitive cells were higher than in resistant cells with 360 of FC and $P=0.08$ (Fig. 3C), and

higher in responders than those in non-responders with 1.74 of FC and $P=0.06$ (Fig. 3D).

Knockdown of HAR1B results in increased pazopanib resistance in sarcoma cell lines. Based on the above results, we hypothesized that the two lncRNAs, *HAR1B* and *HOTAIR*, might be related to pazopanib sensitivity in sarcomas. We thus attempted to elucidate whether forced alteration of the lncRNA expression levels would affect pazopanib sensitivity in sarcoma cells. For this purpose, we decided to focus on *HAR1B* rather than *HOTAIR*, because the FC of *HAR1B* was slightly higher than that of *HOTAIR* in responders (FC 2.25 vs. 1.74), and the functional significance of *HAR1B* in tumorigenesis remains unclear.

Transfection of siRNA against *HAR1B* led to a 42% decrease in the expression level in pazopanib-sensitive HS-SY-II cells (Fig. 4A). This level of *HAR1B* knockdown led to a modest but statistically significant increase in the viability of cells treated with 2- μ M pazopanib (69 vs. 59%, $P=0.003$, Fig. 4B). In another sensitive cell line Yamato-SS, knockdown of *HAR1B* also led to a modest but statistically significant increase in the viability of cells treated with 2- μ M pazopanib (68 vs. 62%, $P=0.005$, Fig. 4C and D). In contrast, in the pazopanib-resistant cell line SW872, *HAR1B* knockdown did not affect pazopanib sensitivity (data not shown).

Gene enrichment and functional annotation clustering analyses reveal that some functional pathways, including a von-Willebrand factor-related pathway, correlate with pazopanib-sensitivity-related expression profiles. Our findings suggest that *HAR1B* might be related, even partially, to pazopanib sensitivity in sarcoma cells, and that a certain threshold of *HAR1B* expression might be required for pazopanib efficacy. However, the precise molecular mechanisms of how *HAR1B* is involved in pazopanib sensitivity remain to be elucidated. To address this issue, we next attempted to elucidate how whole genome-wide gene and lncRNA expression profiles including *HAR1B* expression differ between sensitive and resistant cells and between responders and non-responders.

In microarray analyses using SurePrint G3 Hyman Gene Expression 8x60K microarrays of 16 bone or soft-tissue sarcoma cell lines, 4,962 probes (4,538 genes/lncRNAs) were upregulated in sensitive cells (FC >1.5 with $P<0.1$). In microarray analyses including 23 bone or soft-tissue sarcoma tissues, 3,733 probes (3,652 genes/lncRNAs) were upregulated in responders (FC >1.5 with $P<0.1$); 351 genes and 109 lncRNAs, including *HAR1B*, upregulated in both sensitive cell lines and responders. Using the 306 genes/lncRNAs with NCBI Entrez Gene IDs, we performed gene enrichment analysis, which revealed 65 significant annotation terms ($P<0.01$) (Table SII). Many of them included terms related to the 'von Willebrand factor' (Table SII). The most statistically significant term was 'VWC out,' which includes five genes-*MUC2*, *MUC5B*, *MUC6*, *NELL1*, and *VWCE*. We next performed functional annotation analysis using the result of the gene enrichment analysis and detected seven statistically significant clusters with enrichment scores >1.3 (Table III). The functional clusters included von-Willebrand factor-related, cell membrane-related, EGF-related, receptor-related, and neurogenesis-related clusters.

Table I. Summary of patient/tumor characteristics and treatment outcomes for 23 patients/tumors analyzed via microarray analyses.

Factors	Total, n (%) (n=23)	Responder, n (%) (n=13)	Non-Responder, n (%) (n=10)
Sex			
Male	14 (61)	7 (54)	7 (70)
Female	9 (39)	6 (46)	3 (30)
Age (years)			
Median	65	65	62
Range	20-76	20-76	31-76
ECOG Performance Status			
0	9 (39)	7 (54)	2 (20)
1	13 (57)	5 (38)	8 (80)
2	1 (4)	1 (8)	0
>2	0 (0)	0 (0)	0 (0)
Treatment line			
1st line	1 (4)	1 (8)	0 (0)
2nd line	12 (52)	7 (54)	5 (50)
3rd line	7 (30)	3 (23)	4 (40)
4th line	3 (13)	2 (15)	1 (10)
Pathology	5 (22)	2 (15)	3 (30)
Myxoid LPS			
LMS	3 (13)	2 (15)	1 (10)
UPS	3 (13)	2 (15)	1 (10)
SFT	2 (9)	1 (8)	1 (10)
OS	2 (9)	1 (8)	1 (10)
ASPS	2 (9)	2 (15)	0 (0)
US	1 (4)	1 (8)	0 (0)
AS	1 (4)	1 (8)	0 (0)
ES	1 (4)	1 (8)	0 (0)
ESFT	2 (9)	0 (0)	2 (20)
CCS	1 (4)	0 (0)	1 (10)
Primary site	12 (52)	8 (62)	4 (40)
Extremity			
Trunk	2 (9)	1 (8)	1 (10)
Retroperitneum	1 (4)	1 (8)	0 (0)
Thoracic cavity	1 (4)	1 (8)	0 (0)
Liver	1 (4)	1 (8)	0 (0)
Pancreas	1 (4)	1 (8)	0 (0)
Abdominal cavity	1 (4)	0 (0)	1 (10)
Oral	1 (4)	0 (0)	1 (10)
Pelvis	1 (4)	0 (0)	1 (10)
Sternum	1 (4)	0 (0)	1 (10)
Eye	1 (4)	0 (0)	1 (10)

ECOG, Eastern Cooperative Oncology Group; LPS, liposarcoma; LMS, leiomyosarcoma; UPS, undifferentiated pleomorphic sarcoma; SFT, solitary fibrous tumor; OS, osteosarcoma; ASPS, alveolar soft part sarcoma; US, undifferentiated sarcoma; AS, Angiosarcoma; ES, epithelioid sarcoma; ESFT, Ewing sarcoma family tumor; CCS, clear cell sarcoma.

Discussion

This study is the first to demonstrate that lncRNAs may serve as molecular biomarkers to predict the clinical outcomes of patients with sarcomas who receive molecularly

targeted therapy. We made the following observations: (1) Multiplex qPCR analysis identified 12 lncRNAs that were differentially expressed between pazopanib-sensitive and pazopanib-resistant cells; (2) comprehensive gene and lncRNA expression analyses revealed that *HARIB*

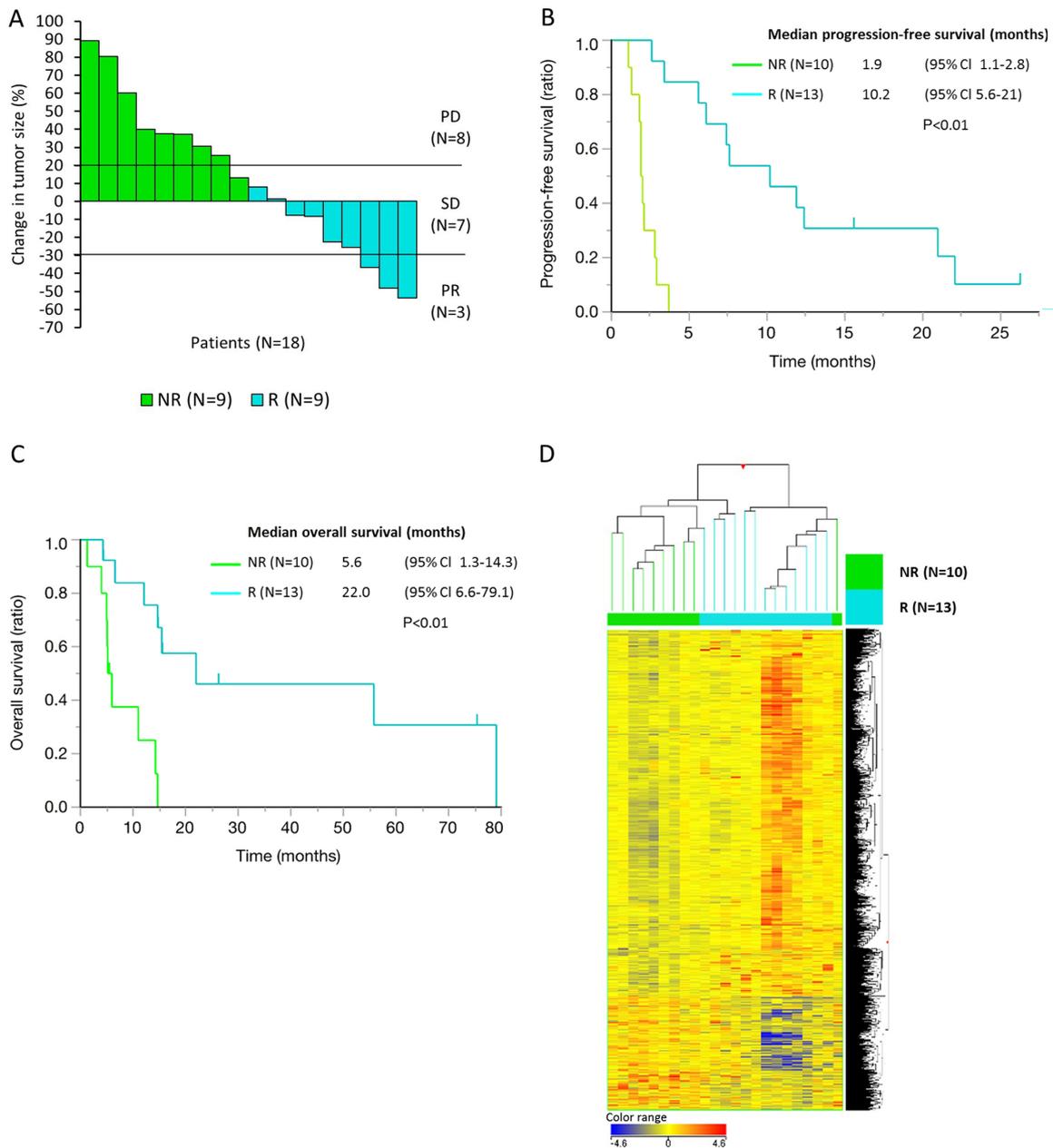


Figure 2. Clinical outcomes in 23 responders and non-responders among patients with bone or soft-tissue sarcoma who received pazopanib treatment. The gene and lncRNA expression profiles differed between responders and non-responders. (A) Change in tumor size (%) and tumor response by pazopanib treatment in 18 patients with measurable tumor lesions. A total of 9 patients were categorized as responders, with $\leq 0\%$ change in tumor size and/or ≥ 6 months of PFS, while the others were categorized as non-responders. (B) Progression-free survival and (C) overall survival of 23 patients for whom whole genome gene/lncRNA expression profiles were analyzed by microarrays. (D) A total of 2,417 lncRNAs (probes) differentially expressed genes (fold change >1.5 or <0.67 with $P<0.1$) between responders ($n=13$) and non-responders ($n=10$) were detected in the microarray analysis. Data are presented as normalized intensity values. Hierarchical clustering analysis divided the patients into two groups, responders and non-responders, except for one non-responder. lncRNA, long non-coding RNA; PD, progressive disease; SD, stable disease; PR, partial response; NR, non-responder; R, responder; CI, confidence interval.

and *HOTAIR* were also differentially expressed between responders and non-responders who received pazopanib therapy; (3) we clarified the functional role *HARIB* through knockdown by siRNA, which led to an increased pazopanib resistance in sarcoma cell lines; and (4) gene expression profiles related to pazopanib sensitivity include various cellular molecular pathways, including von-Willebrand factor-related signaling. These results suggest that lncRNA *HARIB* is involved, even partially, in sensitivity to pazopanib through some mechanisms, which might be related to the regulation of angiogenesis, and that *HARIB* may be effective

as a predictive biomarker for patients with bone or soft-tissue sarcomas who received pazopanib therapy.

Growing evidence suggests that lncRNAs exert oncogenic or tumor-suppressive effects in various cancers (11,27-30). Some lncRNAs are also involved in drug sensitivity, exemplified by *H19* for paclitaxel, *NEAT1* for 5-FU, and *ARA* for anthracycline in breast cancer (31). However, the biological roles of lncRNAs and their clinical significance in sarcomas, particularly in soft-tissue sarcomas, remains to be elucidated.

We have shown in our study that *HARIB* is upregulated in pazopanib-sensitive cells and in responders, and the *HARIB*

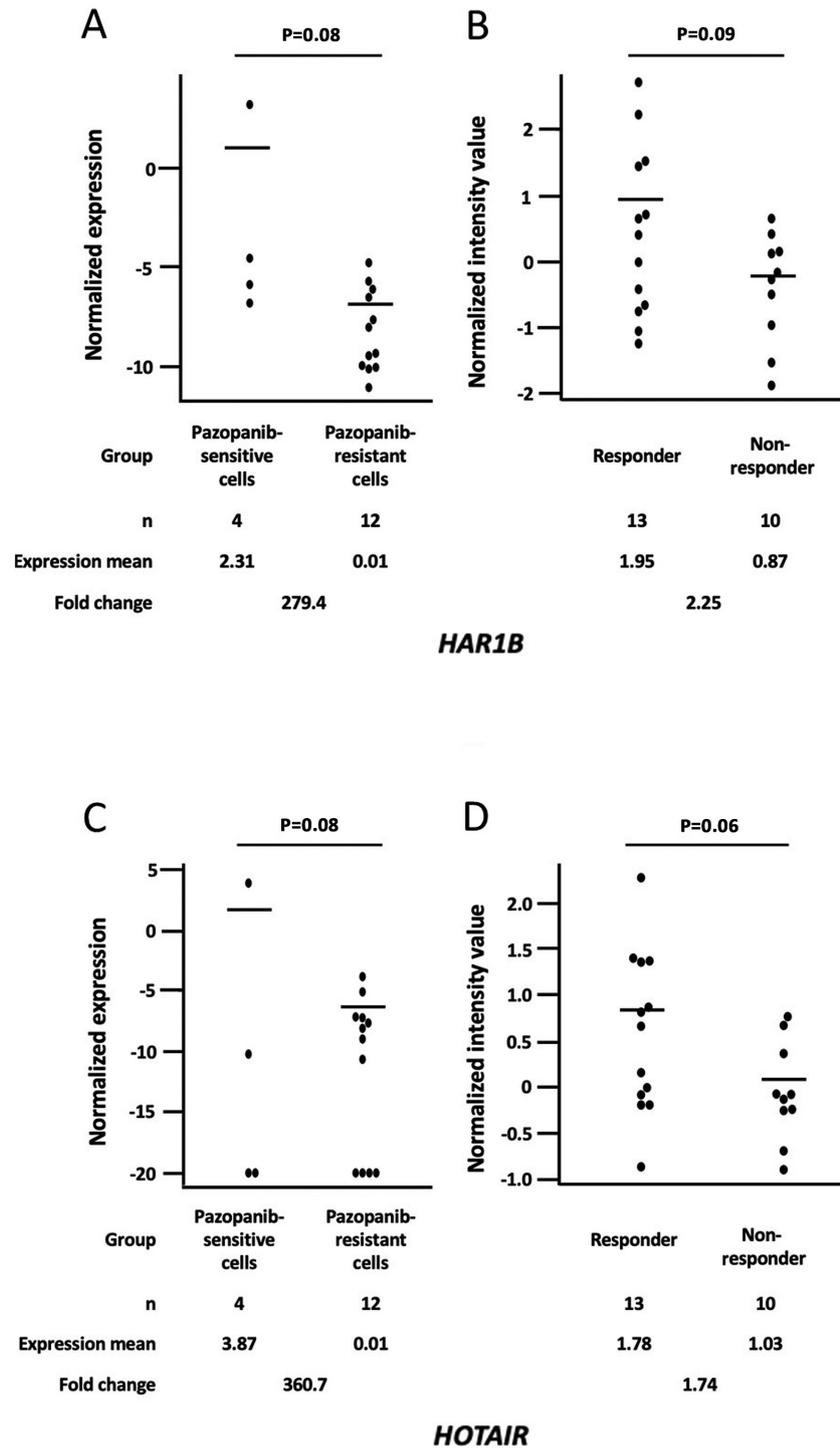


Figure 3. *HAR1B* and *HOTAIR* were upregulated in pazopanib-sensitive cells and in responders. *HAR1B* and *HOTAIR* were differently expressed and upregulated long non-coding RNAs (fold change >1.5 or <0.67 with $P<0.1$) in pazopanib-sensitive cells compared with pazopanib-resistant cells and in the responders compared with non-responders. *HAR1B* expression in (A) sarcoma cell lines and (B) sarcoma tissues is presented. *HOTAIR* expression in (C) sarcoma cell lines and (D) as a scatter plot of sarcoma tissues. Data are presented as relative expression levels normalized to four normalization genes in sarcoma cell lines, or normalized intensity values for sarcoma tissues. P-values were calculated by unpaired t-tests. Black dots represent each sample data point. The horizontal line in the scatter plot represents relative expression or normalized intensity values for each expression mean. *HAR1B*, highly accelerated region 1B; *HOTAIR*, HOX transcript antisense RNA.

knockdown confers resistance to pazopanib, but the exact mechanisms underlying this altered expression are unknown. *HAR1B* is a 6,827-bp lncRNA, located in 20q13.33. *HAR1B* is a pair of *HAR1A*, overlapping oppositely transcribed genes, and has three exons (32). A ‘highly accelerated region’ was found as a specifically evolved region in humans, and *HAR1A*

was found to be expressed specifically in the Cajal-Retzius neurons in the developing human neocortex, suggesting that *HAR1A*, and possibly *HAR1B*, plays a role in neurogenesis (32). However, details of the functions of *HAR1A* and *HAR1B*, particularly their molecular functions and clinical significance in tumorigenesis, have not been well studied.

Table II. Differentially expressed lncRNAs between responders and non-responders.

lncRNA	Expression in sarcoma cell ^a		Expression in sarcoma tissue ^b	
	Mean fold change (sensitive/resistant)	P-value ^c	Mean fold change (responders/non-responders)	P-value ^c
<i>HAR1B</i>	279.41	0.08	2.25	0.09
<i>NEAT1</i>	0.00	0.16	2.01	0.01
<i>NEAT1</i>			1.48	0.14
<i>NEAT1</i>			1.41	0.06
<i>UCA1</i>	0.00	0.44	1.98	0.07
<i>UCA1</i>			1.70	0.13
<i>TSIX</i>	1.00	^d	1.80	0.12
<i>TSIX</i>			1.12	0.39
<i>TSIX</i>			0.97	0.95
<i>TSIX</i>			0.79	0.18
<i>HOTAIRM1</i>	0.48	0.37	1.74	0.02
<i>HOTAIRM1</i>			1.15	0.70
<i>HOTAIRM1</i>			1.06	0.15
<i>SNHG4</i>	1.00	0.99	1.74	0.02
<i>SNHG4</i>			1.51	0.48
<i>HOTAIR</i>	360.69	0.08	1.74	0.06
<i>HOTAIR</i>			1.35	0.28
<i>HOTAIR</i>			1.30	0.12
<i>HOTAIR</i>			1.26	0.73
<i>HOTAIR</i>			1.08	0.94
<i>JPX</i>	301.29	0.08	1.60	0.83
<i>JPX</i>			0.75	0.22
<i>JPX</i>			0.38	<0.01
<i>HAR1A</i>	1.15	0.82	1.49	0.17
<i>HAR1A</i>			1.02	0.58
<i>EMX2OS</i>	0.82	0.57	1.44	0.52
<i>EMX2OS</i>			1.18	0.10
<i>SOX2-OT</i>	0.01	0.50	1.44	0.46
<i>SOX2-OT</i>			0.96	0.86
<i>SOX2-OT</i>			0.81	0.64
<i>LINC-ROR</i>	1.15	0.77	1.40	0.04
<i>IGF2-AS</i>	0.99	0.99	1.36	0.02
<i>TUG1</i>	0.39	0.55	1.31	0.60
<i>TUG1</i>			1.04	0.33
<i>TUG1</i>			0.85	0.24
<i>TUG1</i>			0.55	0.06
<i>MEG3</i>	1.23	0.87	1.26	0.27
<i>MEG3</i>			1.12	0.33
<i>MEG3</i>			0.88	0.55
<i>MEG3</i>			0.85	0.97
<i>MEG3</i>			0.82	0.69
<i>MEG3</i>			0.60	0.20
<i>HOXA11-AS</i>	1.28	0.42	1.25	0.01
<i>PCGEMI</i>	1.00	^d	1.13	0.31
<i>BACE1-AS</i>	1.62	0.02	1.08	0.92
<i>MALAT1</i>	0.40	0.40	1.06	0.98
<i>MALAT1</i>			0.93	0.43
<i>MALAT1</i>			0.92	0.56
<i>MALAT1</i>			0.79	0.05
<i>HOTTIP</i>	1.29	0.80	1.05	0.65
<i>WT1-AS</i>	6.07	0.16	0.92	0.80
<i>GAS5</i>	1.84	0.04	0.90	0.63

Table II. Continued.

lncRNA	Expression in sarcoma cell ^a		Expression in sarcoma tissue ^b	
	Mean fold change (sensitive/resistant)	P-value ^c	Mean fold change (responders/non-responders)	P-value ^c
<i>GAS5</i>			0.48	0.17
<i>SNHG5</i>	0.68	0.52	0.87	0.64
<i>SNHG5</i>			0.77	0.39
<i>SNHG5</i>			0.53	0.02
<i>SNHG5</i>			0.38	0.03
<i>SNHG6</i>	0.86	0.76	0.83	0.54
<i>SNHG6</i>			0.63	0.14
<i>SNHG3</i>	0.83	0.54	0.77	0.16
<i>H19</i>	32.79	0.04	0.75	0.43
<i>H19</i>			0.61	0.25
<i>IPW</i>	0.00	0.58	0.73	0.30
<i>DISC2</i>	1.00	1.00	0.71	0.17
<i>KCNQ1OT1</i>	0.03	0.58	0.67	0.13
<i>KCNQ1OT1</i>			0.64	0.02
<i>XIST</i>	1.33	0.82	0.64	0.79
<i>ZFAS1</i>	1.25	0.55	0.54	0.06
<i>DIO3OS</i>	4.40	0.07	0.30	0.09

A total of 69 lncRNA probes (32 lncRNAs) were common in both the cell and tissue assays, and are listed above. Mean fold change was calculated by dividing responder (or sensitive cell) expression data by that of non-responder (or resistant cell). ^aExpression data in multiplex qPCR in cells. ^bExpression data in microarray in tissues. ^cCalculated by an unpaired t-test, ^dNot calculable.

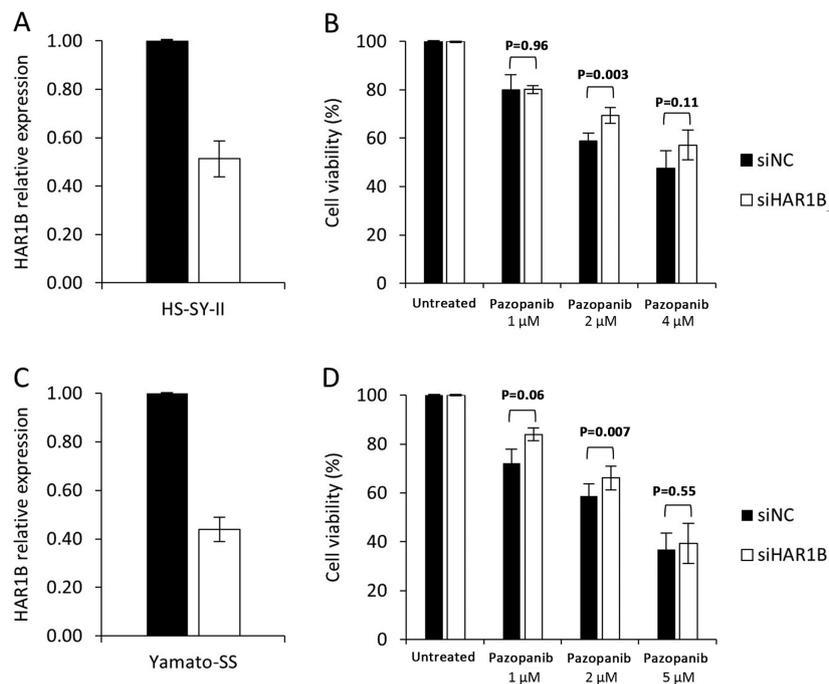


Figure 4. *HAR1B* knockdown by siRNA confers resistance to pazopanib treatment in sarcoma cell lines. Pazopanib-sensitive HS-SY-II and Yamato-SS cells with high *HAR1B* expression were selected for the *HAR1B* knockdown assay. The two cell lines were transiently transfected with siRNA for *HAR1B* or NC. (A) RT-qPCR analysis confirmed that siHAR1B transfection led to a decrease in *HAR1B* expression by ~58% when compared with siNC transfection in HS-SY-II cells. (B) siHAR1B transfection led to significantly higher cell viabilities compared with siNC transfection in HS-SY-II cells treated with 2 μ M pazopanib. (C) RT-qPCR analysis confirmed that siHAR1B transfection led to a decrease in *HAR1B* expression by ~49% compared to siNC transfection in Yamato-SS cells. (D) siHAR1B transfection led to significantly higher cellular viability than siNC transfection in Yamato-SS cells treated with 2 μ M pazopanib. Data are presented as the mean \pm SEM of relative expression normalized by *GAPDH* expression in A and C, or cell viability (%) in B and D. The P-value was calculated by paired t-tests. *HAR1B*, highly accelerated region 1B; siRNA or si, small interfering RNA; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR.

Table III. Functional clusters identified by functional annotation clustering.

A, Cluster number 1, 6 annotation terms included, enrichment score of 1			
Top 5 categorized annotation term of each cluster ^a	P-value ^b	Source database ^c	Accession no.
VWC_out	<0.01	SMART	SM00215
VWFC domain	<0.01	InterPro	IPR001007
domain:VWFC 1	<0.01	UniProt	None
domain:VWFC 2	<0.01	UniProt	None
VWC	<0.01	SMART	SM00214
B, Cluster number 1, 18 annotation terms included, enrichment score of 2.11			
Top 5 categorized annotation term of each cluster ^a	P-value ^b	Source database ^c	Accession no.
Cell membrane	<0.01	UniProt	KW-1003
Signal peptide	<0.01	UniProt	None
Signal	<0.01	UniProt	KW-0732
Glycoprotein	<0.01	UniProt	KW-0325
Disulfide bond	<0.01	UniProt	None
C, Cluster number 3, 19 annotation terms included, enrichment score of 1.89			
Top 5 categorized annotation term of each cluster ^a	P-value ^b	Source database ^c	Accession no.
EGF-like, conserved site	<0.01	InterPro	IPR013032
EGF-like calcium-binding domain	<0.01	InterPro	IPR001881
EGF-like domain	<0.01	InterPro	IPR000742
EGF_CA	<0.01	SMART	SM00179
domain:EGF-like 4	<0.01	UniProt	None
D, Cluster number 4, 27 annotation terms included, enrichment score of 1.56			
Top 5 categorized annotation term of each cluster ^a	P-value ^b	Source database ^c	Accession no.
VWC_out	<0.01	SMART	SM00215
VWFC domain	<0.01	INTERPRO	IPR001007
domain:TIL	<0.01	UniProt	None
domain:VWFD 3	<0.01	UniProt	None
domain:VWFD 2	<0.01	UniProt	None
E, Cluster number 5, 17 annotation terms included, enrichment score of 1.50			
Top 5 categorized annotation term of each cluster ^a	P-value ^b	Source database ^c	Accession no.
Signaling receptor activity	<0.01	Gene Ontology	GO:0038023
Molecular transducer activity	<0.01	Gene Ontology	GO:0060089
Glycoprotein	<0.01	UniProt	KW-0325
Topological domain: Extracellular	<0.01	Gene Ontology	None
Intrinsic component of plasma membrane	<0.01	Gene Ontology	GO:0031226
F, Cluster number 6, 23 annotation terms included, enrichment score of 1.40			
Top 5 categorized annotation term of each cluster ^a	P-value ^b	Source database ^c	Accession no.
Neurogenesis	<0.01	Gene Ontology	GO:0022008
Neuron differentiation	<0.01	Gene Ontology	GO:0030182

Table III. Continued.

F, Cluster number 6, 23 annotation terms included, enrichment score of 1.40			
Top 5 categorized annotation term of each cluster ^a	P-value ^b	Source database ^c	Accession no.
Generation of neurons	<0.01	Gene Ontology	GO:0048699
Cell projection organization	<0.01	Gene Ontology	GO:0030030
Axon development	0.02	Gene Ontology	GO:0061564
G, Cluster number 7, 12 annotation terms included, enrichment score of 1.833			
Top 5 categorized annotation term of each cluster ^a	P-value ^b	Source database ^c	Accession no.
Inositol phosphate metabolic process	<0.01	Gene Ontology	GO:0043647
Polyol metabolic process	<0.01	Gene Ontology	GO:0019751
Alcohol metabolic process	<0.01	Gene Ontology	GO:0006066
Organic hydroxy compound metabolic process	0.01	Gene Ontology	GO:1901615
Carbohydrate metabolic process	0.04	Gene Ontology	GO:0005975

Seven functional annotation clusters with an enrichment score >1.3 are listed. ^aAmong each cluster, 5 annotation terms are listed from lower enrichment P-values with its accession no. and source database referred from DAVID (<https://david.ncicrf.gov>). ^bA modified Fisher Exact P-value was generated from gene enrichment analysis. ^cEach database is available at the following URLs: SMART, <http://smart.embl-heidelberg.de>; InterPro, <https://www.ebi.ac.uk/interpro>; UniProt, <https://www.uniprot.org>; Gene Ontology, <http://geneontology.org>. vWF, von Willebrand factor; VWC, von Willebrand factor type C domain; VWFC, von Willebrand factor type C; EGF, epidermal growth factor; EGF_CA, Calcium-binding EGF-like domain; VWFD, von Willebrand factor type D.

Liu *et al* reported that *HARIA* is upregulated in about 3% of breast cancers, and that the upregulation of nine lncRNAs, including *HARIA*, correlated with an increased risk of recurrence (33). In contrast, Ma *et al* reported that in thyroid cancer, *HARIA* downregulation correlated with an increase in risk of recurrence (34). Compared to *HARIA*, *HARIB* is a less studied lncRNA, but Shi *et al* have recently shown that the downregulation of *HARIA* and *HARIB* correlated with worse OS in hepatocellular carcinoma (35). These findings suggest that altered expressions of *HARIA* and *HARIB* are somehow involved in the tumorigenesis of various cancers, and that their clinical significance depends differentially on cancer type.

In our gene enrichment and functional clustering analyses, genes whose expression levels were related to pazopanib sensitivity included cellular molecular pathways, such as von-Willebrand factor-related, cell membrane-related, EGF-related, receptor-related, and neurogenesis-related pathways. Von-Willebrand factor, which is expressed in endothelial cells, plays an essential role in hemostasis, and has also been shown to regulate angiogenesis through the control of VEGFR-2 signaling (36). Gel-forming mucin protein MUC2 shares N- and C-terminal domains with the von Willebrand factor (37). MUC5B and MUC6 are the other gel-forming mucin proteins (37). NELL1 and, VWCE, or WWC1, also contain von Willebrand factor type C domains (38,39). Our functional clustering analysis revealed that their transcriptional upregulation correlated well with pazopanib sensitivity, suggesting that von Willebrand factor domain-containing proteins are involved in pazopanib sensitivity in sarcomas. Our functional clustering analysis also revealed that neurogenesis-related

pathways are linked to pazopanib sensitivity, which seems consistent with the finding that *HARIB* is possibly involved in human neurogenesis (32).

This study has several limitations. First, the number of patients with bone and soft-tissue sarcomas was limited. A larger cohort of patients will be required to validate our results. Second, although our findings suggest that *HARIB* affects sensitivity to pazopanib in sarcoma cell lines and patients with sarcoma, the precise molecular mechanisms by which this occurs remain to be elucidated.

In conclusion, our study demonstrates that lncRNA *HARIB* expression affects cellular sensitivity to pazopanib in sarcoma cell lines and in patients with sarcoma. Further studies are warranted to validate the clinical utility of *HARIB* as a predictive biomarker for the treatment of patients with sarcomas, and to clarify the molecular mechanisms by which *HARIB* is involved in pazopanib sensitivity. Such studies could lead to the development of more efficient molecular diagnostics and molecularly targeted therapies in bone and soft-tissue sarcomas.

Acknowledgements

The authors would like to thank Ms Hiromi Nakano, Ms Noriko Takenaga and Ms Nobuko Saeki (Institute of Development, Aging and Cancer, Tohoku University) for their technical assistance. The authors would also like to thank Dr Mikio Masuzawa (School of Allied Health Science, Kitasato University) and Dr Lan Kluwe (Laboratory for Tumor Genetics, University Medical Center Hamburg-Eppendorf) for providing the cell lines.

Funding

The present study was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan (grant nos. 18K07993 and 19H03508).

Availability of data and materials

The microarray data that support the findings of this study are openly available in the GEO database at <https://www.ncbi.nlm.nih.gov/geo/>, reference number (GSE156344). The other datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HY, MT, MuW, MiW, KK and CI conceived and designed the experiments. HY and MT confirmed authenticity of all the raw data. MT and CI wrote the manuscript. HY, MT, KS and SH performed experiments. HY and MT analyzed the data. MuW and MiW obtained tissues. HY and MuW obtained informed consent from patients. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The current study was approved by the Ethics Committee of Tohoku University (approval no. 2019-1-993). Written informed consent was obtained from all patients prior to enrolment.

Patient consent for publication

Patient consent for publication was obtained.

Competing interests

CI received research funding from the Tokyo Cooperative Oncology Group. CI also received contributions from Chugai Pharmaceutical, Asahi Kasei Pharma Corporation, Ono Pharmaceutical, MSD, Pfizer, AstraZeneca, Bristol-Myers Squibb, Janssen Pharmaceutical, Taiho Pharmaceutical, Eisai Pharmaceutical, Daiichi Sankyo Company, Limited and Takeda Pharmaceutical. MT received research funding from Ono Pharmaceutical Company. CI is a representative of the Tohoku Clinical Oncology Research and Education Society, a specified nonprofit corporation. The remaining authors declare that they have no competing interests.

References

- Jo VY and Fletcher CD: WHO classification of soft tissue tumours: An update based on the 2013 (4th) edition. *Pathology* 46: 95-104, 2014.
- Taylor BS, Barretina J, Maki RG, Antonescu CR, Singer S and Ladanyi M: Advances in sarcoma genomics and new therapeutic targets. *Nat Rev Cancer* 11: 541-557, 2011.
- Linch M, Miah AB, Thway K, Judson IR and Benson C: Systemic treatment of soft-tissue sarcoma-gold standard and novel therapies. *Nat Rev Clin Oncol* 11: 187-202, 2014.
- Whelan JS and Davis LE: Osteosarcoma, chondrosarcoma, and chordoma. *J Clin Oncol* 36: 188-193, 2018.
- Takahashi M, Komine K, Imai H, Okada Y, Saijo K, Takahashi M, Shirota H, Ohori H, Takahashi S, Chiba N, *et al*: Efficacy and safety of gemcitabine plus docetaxel in Japanese patients with unresectable or recurrent bone and soft tissue sarcoma: Results from a single-institutional analysis. *PLoS One* 12: e0176972, 2017.
- Kawai A, Araki N, Sugiura H, Ueda T, Yonemoto T, Takahashi M, Morioka H, Hiraga H, Hiruma T, Kunisada T, *et al*: Trabectedin monotherapy after standard chemotherapy versus best supportive care in patients with advanced, translocation-related sarcoma: A randomised, open-label, phase 2 study. *Lancet Oncol* 16: 406-416, 2015.
- Schoffski P, Chawla S, Maki RG, Italiano A, Gelderblom H, Choy E, Grignani G, Camargo V, Bauer S, Rha SY, *et al*: Eribulin versus dacarbazine in previously treated patients with advanced liposarcoma or leiomyosarcoma: A randomised, open-label, multicentre, phase 3 trial. *Lancet* 387: 1629-1637, 2016.
- van der Graaf WT, Blay JY, Chawla SP, Kim DW, Bui-Nguyen B, Casali PG, Schöffski P, Aglietta M, Staddon AP, Beppu Y, *et al*: Pazopanib for metastatic soft-tissue sarcoma (PALETTE): A randomised, double-blind, placebo-controlled phase 3 trial. *Lancet* 379: 1879-1886, 2012.
- Uszczynska-Ratajczak B, Lagarde J, Frankish A, Guigó R and Johnson R: Towards a complete map of the human long non-coding RNA transcriptome. *Nat Rev Genet* 19: 535-548, 2018.
- Fang S, Zhang L, Guo J, Niu Y, Wu Y, Li H, Zhao L, Li X, Teng X, Sun X, *et al*: NONCODEV5: A comprehensive annotation database for long non-coding RNAs. *Nucleic Acids Res* 46 (D1): D308-D314, 2018.
- Huarte M: The emerging role of lncRNAs in cancer. *Nat Med* 21: 1253-1261, 2015.
- Sørensen KP, Thomassen M, Tan Q, Bak M, Cold S, Burton M, Larsen MJ and Kruse TA: Long non-coding RNA HOTAIR is an independent prognostic marker of metastasis in estrogen receptor-positive primary breast cancer. *Breast Cancer Res Treat* 142: 529-536, 2013.
- Abdeahad H, Avan A, Pashirzad M, Khazaei M, Soleimanpour S, Ferns GA, Fuji H, Ryzhikov M, Bahrami A and Hassanian SM: The prognostic potential of long noncoding RNA HOTAIR expression in human digestive system carcinomas: A meta-analysis. *J Cell Physiol* 234: 10926-10933, 2019.
- Ji P, Diederichs S, Wang W, Böing S, Metzger R, Schneider PM, Tidow N, Brandt B, Buerger H, Bulk E, *et al*: MALAT-1, a novel noncoding RNA, and thymosin beta4 predict metastasis and survival in early-stage non-small cell lung cancer. *Oncogene* 22: 8031-8041, 2003.
- Sun XH, Yang LB, Geng XL, Wang R and Zhang ZC: Increased expression of lncRNA HULC indicates a poor prognosis and promotes cell metastasis in osteosarcoma. *Int J Clin Exp Pathol* 8: 2994-3000, 2015.
- Tian ZZ, Guo XJ, Zhao YM and Fang Y: Decreased expression of long non-coding RNA MEG3 acts as a potential predictor biomarker in progression and poor prognosis of osteosarcoma. *Int J Clin Exp Pathol* 8: 15138-15142, 2015.
- Li Z, Dou P, Liu T and He S: Application of long noncoding RNAs in osteosarcoma: Biomarkers and therapeutic targets. *Cell Physiol Biochem* 42: 1407-1419, 2017.
- Matsuzaki J and Ochiya T: Circulating microRNAs and extracellular vesicles as potential cancer biomarkers: A systematic review. *Int J Clin Oncol* 22: 413-420, 2017.
- Hakozaki M, Hojo H, Sato M, Tajino T, Yamada H, Kikuchi S and Abe M: Establishment and characterization of a novel human malignant peripheral nerve sheath tumor cell line, FMS-1, that overexpresses epidermal growth factor receptor and cyclooxygenase-2. *Virchows Arch* 455: 517-526, 2009.
- Aoki M, Nabeshima K, Nishio J, Ishiguro M, Fujita C, Koga K, Hamasaki M, Kaneko Y and Iwasaki H: Establishment of three malignant peripheral nerve sheath tumor cell lines, FU-SFT8611, 8710 and 9817: Conventional and molecular cytogenetic characterization. *Int J Oncol* 29: 1421-1428, 2006.
- Frahm S, Mautner VF, Brems H, Legius E, Debiec-Rychter M, Friedrich RE, Knöfel WT, Peiper M and Kluwe L: Genetic and phenotypic characterization of tumor cells derived from malignant peripheral nerve sheath tumors of neurofibromatosis type 1 patients. *Neurobiol Dis* 16: 85-91, 2004.
- Masuzawa M, Fujimura T, Hamada Y, Fujita Y, Hara H, Nishiyama S, Katsuoka K, Tamauchi H and Sakurai Y: Establishment of a human hemangiosarcoma cell line (ISO-HAS). *Int J Cancer* 81: 305-308, 1999.

23. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, *et al*: New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). *Eur J Cancer* 45: 228-247, 2009.
24. Huang da W, Sherman BT and Lempicki RA: Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4: 44-57, 2009.
25. Kumar R, Knick VB, Rudolph SK, Johnson JH, Crosby RM, Crouthamel MC, Hopper TM, Miller CG, Harrington LE, Onori JA, *et al*: Pharmacokinetic-pharmacodynamic correlation from mouse to human with pazopanib, a multikinase angiogenesis inhibitor with potent antitumor and antiangiogenic activity. *Mol Cancer Ther* 6: 2012-2021, 2007.
26. Hurwitz HI, Dowlati A, Saini S, Savage S, Suttle AB, Gibson DM, Hodge JP, Merkle EM and Pandite L: Phase I trial of pazopanib in patients with advanced cancer. *Clin Cancer Res* 15: 4220-4227, 2009.
27. Li ZX, Zhu QN, Zhang HB, Hu Y, Wang G and Zhu YS: MALAT1: A potential biomarker in cancer. *Cancer Manag Res* 10: 6757-6768, 2018.
28. Qu X, Alsager S, Zhuo Y and Shan B: HOX transcript antisense RNA (HOTAIR) in cancer. *Cancer Lett* 454: 90-97, 2019.
29. Marín-Béjar O, Marchese FP, Athie A, Sánchez Y, González J, Segura V, Huang L, Moreno I, Navarro A, Monzó M, *et al*: Pint lincRNA connects the p53 pathway with epigenetic silencing by the Polycomb repressive complex 2. *Genome Biol* 14: R104, 2013.
30. Sánchez Y, Segura V, Marín-Béjar O, Athie A, Marchese FP, González J, Bujanda L, Guo S, Matheu A and Huarte M: Genome-wide analysis of the human p53 transcriptional network unveils a lncRNA tumour suppressor signature. *Nat Commun* 5: 5812, 2014.
31. Campos-Parra AD, López-Urrutia E, Orozco Moreno LT, López-Camarillo C, Meza-Menchaca T, Figueroa González G, Bustamante Montes LP and Pérez-Plasencia C: Long non-coding RNAs as new master regulators of resistance to systemic treatments in breast cancer. *Int J Mol Sci* 19: 2711, 2018.
32. Pollard KS, Salama SR, Lambert N, Lambot MA, Coppens S, Pedersen JS, Katzman S, King B, Onodera C, Siepel A, *et al*: An RNA gene expressed during cortical development evolved rapidly in humans. *Nature* 443: 167-172, 2006.
33. Liu H, Li J, Koirala P, Ding X, Chen B, Wang Y, Wang Z, Wang C, Zhang X and Mo YY: Long non-coding RNAs as prognostic markers in human breast cancer. *Oncotarget* 7: 20584-20596, 2016.
34. Ma B, Liao T, Wen D, Dong C, Zhou L, Yang S, Wang Y and Ji Q: Long intergenic non-coding RNA 271 is predictive of a poorer prognosis of papillary thyroid cancer. *Sci Rep* 6: 36973, 2016.
35. Shi Z, Luo Y, Zhu M, Zhou Y, Zheng B, Wu D, Wang S, Xie X, Lin H and Yu X: Expression analysis of long non-coding RNA HAR1A and HAR1B in HBV-induced hepatocellular carcinoma in Chinese patients. *Lab Med* 50: 150-157, 2019.
36. Starke RD, Ferraro F, Paschalaki KE, Dryden NH, McKinnon TA, Sutton RE, Payne EM, Haskard DO, Hughes AD, Cutler DF, *et al*: Endothelial von Willebrand factor regulates angiogenesis. *Blood* 117: 1071-1080, 2011.
37. Nilsson HE, Ambort D, Backstrom M, Thomsson E, Koeck PJ, Hansson GC and Hebert H: Intestinal MUC2 mucin supramolecular topology by packing and release resting on D3 domain assembly. *J Mol Biol* 426: 2567-2579, 2014.
38. Nakamura Y, Hasebe A, Takahashi K, Iijima M, Yoshimoto N, Maturana AD, Ting K, Kuroda S and Niimi T: Oligomerization-induced conformational change in the C-terminal region of Nel-like molecule 1 (NEL1) protein is necessary for the efficient mediation of murine MC3T3-E1 cell adhesion and spreading. *J Biol Chem* 289: 9781-9794, 2014.
39. Xu ER, Blythe EE, Fischer G and Hyvönen M: Structural analyses of von Willebrand factor C domains of collagen 2A and CCN3 reveal an alternative mode of binding to bone morphogenetic protein-2. *J Biol Chem* 292: 12516-12527, 2017.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.