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LncRNA HIF1A-AS2 mediates imatinib resistance by regulating autophagy in gastrointestinal stromal tumor cells

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The aim of this study was to explore the role and mechanism of long non-coding RNA (lncRNA) HIF1A antisense RNA 2 (HIF1A-AS2) in regulating imatinib (IM) resistance in gastrointestinal stromal tumor (GIST) cells under hypoxia. The expression of HIF1A-AS2 was silenced by siRNA in GIST cells. Cytotoxicity, apoptosis, and autophagy were evaluated under normoxic and hypoxic conditions. The expression levels of HIF1A-AS2, HIF1A, apoptosis-associated genes, and autophagy-associated genes were determined by qRT-PCR analysis and western blot. We found that lncRNA HIF1A-AS2 was highly expressed in GIST tissues and cells. Knockdown of HIF1A-AS2 increased the sensitivity of GIST cells to IM and increased apoptosis. Moreover, a hypoxic environment decreased the sensitivity of GIST cells to IM, and the knockdown of HIF1A-AS2 reversed this effect. Mechanistically, the knockdown of HIF1A-AS2 inhibited IM-mediated autophagy. Finally, HIF1A was found to positively regulate HIF1A-AS2 under hypoxic conditions. Collectively, these data demonstrate that hypoxia-induced HIF1A-AS2 promotes IM resistance in GIST cells by regulating autophagy.

Key words: HIF1A-AS2; gastrointestinal stromal tumor; imatinib; autophagy

Gastrointestinal stromal tumors (GIST) are the most common type of gastrointestinal mesenchymal tumors, accounting for 1–2% of gastrointestinal malignancies [1]. GIST can arise in any level of the gastrointestinal tract but is most commonly found in the stomach (55.6%) and small intestine (31.8%) [2]. Most GIST harbors gain of function KIT or PDGFR mutations [3]. Surgical resection is the main treatment for GIST [4]. For recurrent, metastatic, and primary unresectable disease, targeted therapy is the first choice. However, GIST is often resistant to conventional chemotherapy. Imatinib (IM) is used as a first-line drug for the treatment of advanced or metastatic GIST and has been shown to greatly prolong the survival of patients with advanced GIST [5]. However, GIST frequently develops resistance after prolonged IM treatment. Therefore, a better understanding of the mechanisms underlying IM resistance in GIST will help identify new approaches to improve treatment efficacy and prolong survival for patients with GIST.

In many solid tumors, the rapid proliferation of tumor cells causes intratumoral hypoxia, which is thought to be a factor that contributes to the development of resistance to chemotherapy [6]. Hypoxia-inducible factor-1α (HIF1A) is a master mediator of hypoxia response and is activated under hypoxic conditions. HIF1A promotes chemotherapy resistance in cancers by activating the transcription of downstream genes [7–10]. Upregulation of HIF1A has been shown to promote IM resistance of GIST cells by binding to the promoter region of the PGD gene [11].

Long noncoding RNAs (lncRNAs), which are RNA molecules >200 bp in length that do not code for a protein, have recently been identified as potentially targetable mediators of drug resistance [12, 13], and are reported to be involved in tumor drug resistance [14–16]. The main regulatory mechanisms through which lncRNAs mediate drug resistance include regulating microRNAs, cell cycle, apoptosis, altering drug efflux, DNA damage repair, induction of signaling pathways, and autophagy [17–19]. Therefore, it may be possible that lncRNAs are involved in the molecular mechanisms underlying IM resistance in GIST. Indeed, lncRNA RP11-616M22.7 and HOTAIR have been

reported to reduce IM resistance in GIST cells [20, 21]. However, studies on the role of lncRNAs in IM resistance in GIST remain limited.

Here, we analyzed the differential expression of lncRNAs in IM mesylate-resistant GIST samples and primary GIST samples. We identified lncRNA HIF1A antisense RNA 2 (HIF1A-AS2) as being highly expressed in GIST tissues and cells. Moreover, we investigated the molecular mechanisms of HIF1A-AS2 in IM resistance under hypoxic and normoxic conditions.

Materials and methods

Cell culture. Human GIST-T1 and GIST882 cells were purchased from Cosmo Bio, Co., Ltd., (Tokyo, Japan). Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (Gibco). Cells were cultured at 37°C and 5% CO_2 with 20% oxygen in a humidified incubator, or 1% oxygen in a hypoxia incubator, for different time durations (0, 6, 24, 48 h).

CCK-8 assay. GIST-T1 and GIST 882 cells were seeded at 5×10^4 cells per well in 96-well plates and cultured with a complete culture medium containing different concentrations of IM (0, 4, 8, 16, 32, 64, 128, 256 nM). 48 hours following treatment, cell viability was monitored using the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, CCK-8 reagent (1:10, 10 μl) was added into each well and cells were incubated for 2–3 hours in conventional cell culture conditions. Finally, absorbance at 450 nm was measured using a microplate reader.

Microarray analysis. We isolated total RNA from 6 tissue samples by using the MirVanaTM RNA Isolation kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The quantification of RNA was assessed using a NanoDrop ND-2000 (Thermo Fisher Scientific, Inc.), and RNA integrity was determined using an Agilent 2100 Bioanalyzer system (Agilent Technologies, Inc., Santa Clara, CA, USA). The expression profile of lncRNAs was detected by the Human OE lncRNA Microarray Technology (Affymetrix; Thermo Fisher Scientific, Inc.) (including 63,542 lncRNAs and 27,134 mRNAs). The sample labeling, microarray hybridization, and washing were performed according to the previous report [22].

Quantitative real-time PCR (qRT-PCR). After the cells were incubated with or without IM (30 nM) for 48 hours, total RNA was extracted from cells using TRIzol reagent (Takara, Japan). Afterward, cDNA was obtained by reverse transcription using a PrimeScript™ RT Master Mix (Perfect Real Time) kit (Takara). Real-time quantitative PCR was performed on an ABI 7900 instrument (Applied Biosystems, Foster City, CA, USA). All primers for qRT-PCR were synthesized by Shanghai Sangon Biological Engineering Technology and Services; the primer sequences are listed below: HIF1A-AS2-F 5'-CTTCAGAGAAGCTCTAGCC-3'; HIF1A-AS2-R 5'-ATGGGATGAGTGAAGCAG-3'; Beclin-1-F 5'-CTCCCGAGGTGAAGAGCATC-3'; Beclin-1-R 5'-AATGGAGCTGTGAGTTCCTGG-3'; SQSTM1-F 5'-GAAGCTGCCTTGTACCCACATC-3'; SQSTM1-R 5'-GAAGCTGCCTTGTACCCACATC-3'; ATCB-F 5'-TGGCACCCAGCACAATGAA-3'; ATCB-R 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'.

Cell transfection. GIST-T1 and GIST 882 were transfected with small interfering RNA (siRNA) targeting HIF1A-AS2 (si-HIF1A-AS2), si-HIF1A, or negative control siRNA (NC) (GenePharma) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The transfected cells were subsequently incubated with or without IM (30 nM) for 48 h under normoxic or hypoxic conditions. Cells were collected and used for subsequent experiments.

Apoptosis assay. After different treatments, cells were digested, harvested, and re-suspended in PBS. Apoptosis was measured using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) cell apoptosis detection kit (Abcam). Cells were stained with FITC and PI. Proportions of apoptotic cells were quantified using a BD FACS Aria flow cytometry instrument (BD Bioscience, San Jose, CA, USA). The results are expressed as the rate of apoptosis (the percentage of early + late apoptotic cells).

Western blots assay. After different treatments, protein samples were prepared by lysing cells in RIPA lysis buffer (Thermo Fisher Scientific Inc., Rockford, IL, USA). Protein concentrations were quantified using a BCA Protein assay kit (Thermo Fisher). Protein samples were resolved by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Transfer membranes were blocked with 5% nonfat milk in PBS with 0.1% Tween-20 (PBST). Then, PVDF membranes were incubated with primary antibodies at 4 °C overnight followed by the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h. Finally, protein bands were visualized by chemiluminescence (Millipore). The primary antibodies used in the study were as followed: Bax (ab32503, Abcam, Cambridge, MA, USA); Bcl-2 (15071S, Cell Signaling Technology (CST), Danvers, MA, USA); β-Actin (4970s, CST); Phospho-ULK1 (Ser555) (5869s, CST); Beclin-1 (3495s, CST); SQSTM1/p62 (88588s, CST); LC3B (83506S, CST); HIF1A (36169s, CST). The HRP-conjugated secondary antibodies were as follows: goat anti-mouse IgG (7076s, CST) and goat anti-rabbit IgG (7074s, CST).

Statistical analysis. All statistical analyses were performed using GraphPad Prism 7. Two or multiple data sets were analyzed using Student's t-test or one-way ANOVA. A p-value of $p<0.05$ was considered statistically significant.

Results

HIF1A-AS2 was associated with hypoxia-induced IM resistance. The sensitivity of GIST-T1 cells to IM was evalu-

ated by CCK-8 assay under normoxic or hypoxic conditions. Hypoxia induced resistance to IM (Figure 1A). The inhibitory concentration of IM (IC_{50}) under hypoxic conditions was higher (IC50: 87.59 nM) than under normoxic conditions (IC50: 33.34 nM). In order to determine whether lncRNAs may be involved in hypoxia-induced IM resistance, we performed microarray analysis to identify lncRNAs that were significantly differentially expressed between IM mesylate-resistant GIST samples and primary GIST samples. We found a total of 112 differentially expressed genes between IM mesylate-resistant GIST samples and primary GIST samples, including 63 upregulated genes and 49 downregulated genes (Figure 1B, criteria: adjusted p<0.05 and |log2 fold change|>0). The upregulated lncRNAs included, of note, HIF1A-AS2 (Log2FC=3.96). In addition, 260 lncRNAs were shown by the cluster heatmap (Figure 1C). We next evaluated the expression changes of HIF1A-AS2 in cell lines cultured in hypoxic conditions for different times. HIF1A-AS2 expression increased after exposure to hypoxia (Figure 1D), and reached maximal expression at 6 h exposure. We also examined expression changes of HIF1A-AS2 in GSIT-T1 cells with or without IM treatment. After IM treatment, the expression of HIF1A-AS2 increased four-fold compared with untreated cells (Figure 1E). These results suggest that HIF1A-AS2 is associated with hypoxia-induced IM resistance in GIST cells.

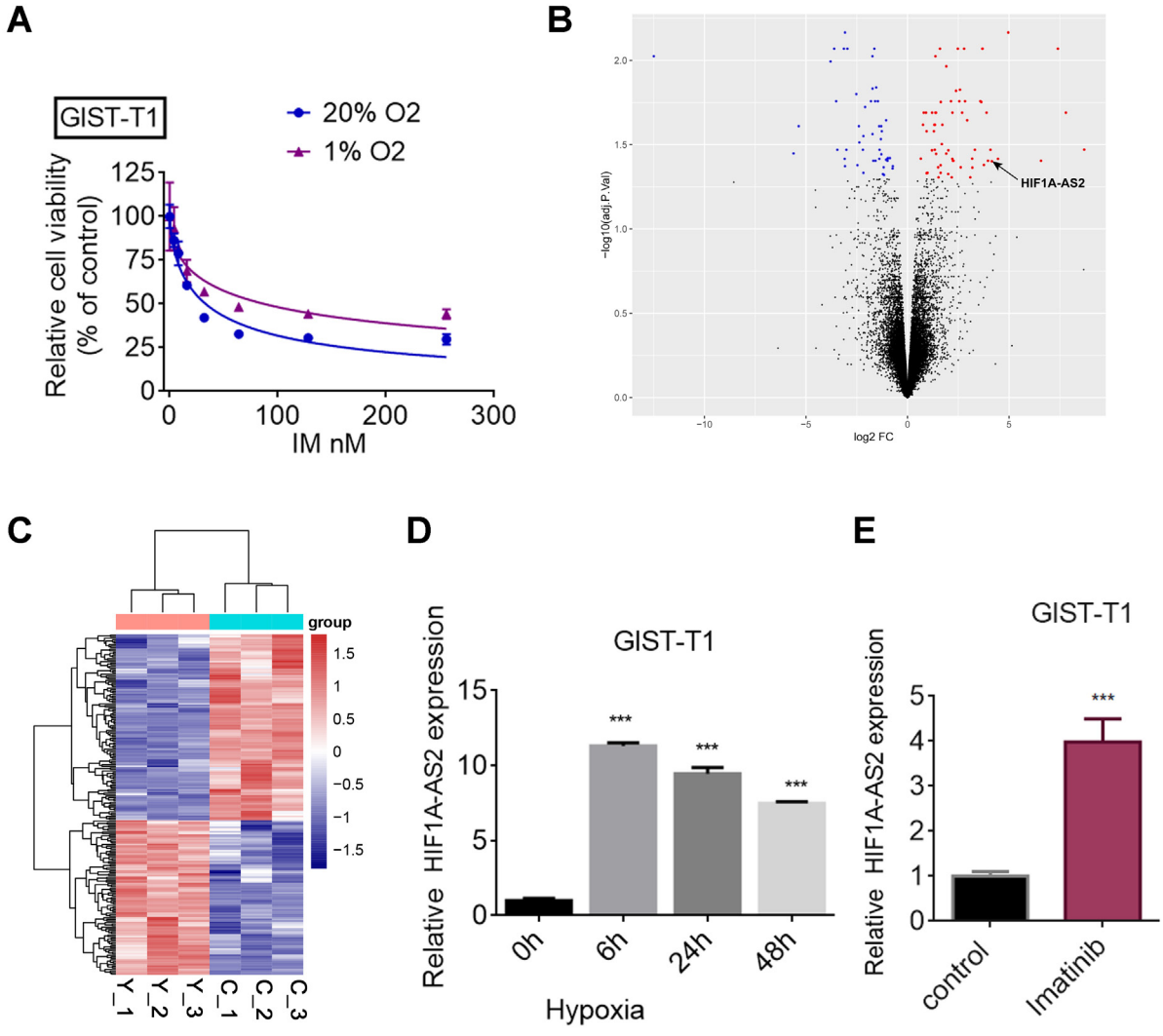

Figure 1. HIF1A-AS2 is associated with hypoxia-induced IM resistance in GIST cells. A) Cell viability of GIST-T1 was measured after treatment with different concentrations of IM under normoxia or hypoxia. B) Volcano plot analysis of the differentially expressed lncRNAs between IM mesylate-resistant gastrointestinal stromal tumor samples vs. primary gastrointestinal stromal tumor samples. C) Cluster analysis of the differentially expressed lncRNAs between IM mesylate-resistant gastrointestinal stromal tumor samples vs. primary gastrointestinal stromal tumor samples. Y, primary gastrointestinal stromal tumor samples; C, IM mesylate-resistant gastrointestinal stromal tumor samples. D) The relative expression of HIF1A-AS2 in GIST-T1 cells at 0 h, 6 h, 24 h, 48 h of hypoxia. E) The relative expression of HIF1A-AS2 in GIST-T1 cells treated with 30 nM IM for 48 h. ***p<0.001

HIF1A-AS2 knockdown inhibits IM resistance in GIST cells, promotes apoptosis, and inhibits autophagy. To explore the function of HIF1A-AS2 in IM resistance of GIST cells, GIST-T1 and GIST 882 cells were transfected with si-HIF1A-AS2-1, si-HIF1A-AS2-2, or NC siRNA. Transfection efficiency was confirmed using qRT-PCR 48 h after transfection (Figure 2A). HIF1A-AS2 knockdown increased the response of GIST cells to IM, as evidenced by the decrease of IM IC_{50} (Figure 2B). HIF1A-AS2 siRNA enhanced apoptosis, as demonstrated by an approximately 40% increased apoptosis rate in GIST-T1 cells and GIST 882 cells (Figures 2C, 2D). Bax and Bcl-2 are a pair of apoptosis-related genes in the BCL-2 family. Bax is a pro-apoptosis gene while Bcl-2 is an anti-apoptosis gene. Differences in protein levels of Bax and Bcl-2, as indicated by western blot, further confirmed the effect that HIF1A-AS2 knockdown has on increasing apoptosis (Figure 2E). These data demonstrate that HIF1A-AS2 knockdown inhibits IM resistance in GIST cells and promotes apoptosis.

HIF1A-AS2 knockdown reversed hypoxia-induced IM resistance. We then further explored the effects of HIF1A-AS2 on hypoxia-mediated IM resistance. We transfected GIST cells with HIF1A-AS2 siRNA and exposed the transfected cells to IM and hypoxic conditions. The silencing efficiency of HIF1A-AS2 in both cell lines under hypoxic conditions was confirmed by qRT-PCR. HIF1A-AS2 expression was upregulated by hypoxia in both cell lines and was significantly reduced following HIF1A-AS2 knockdown

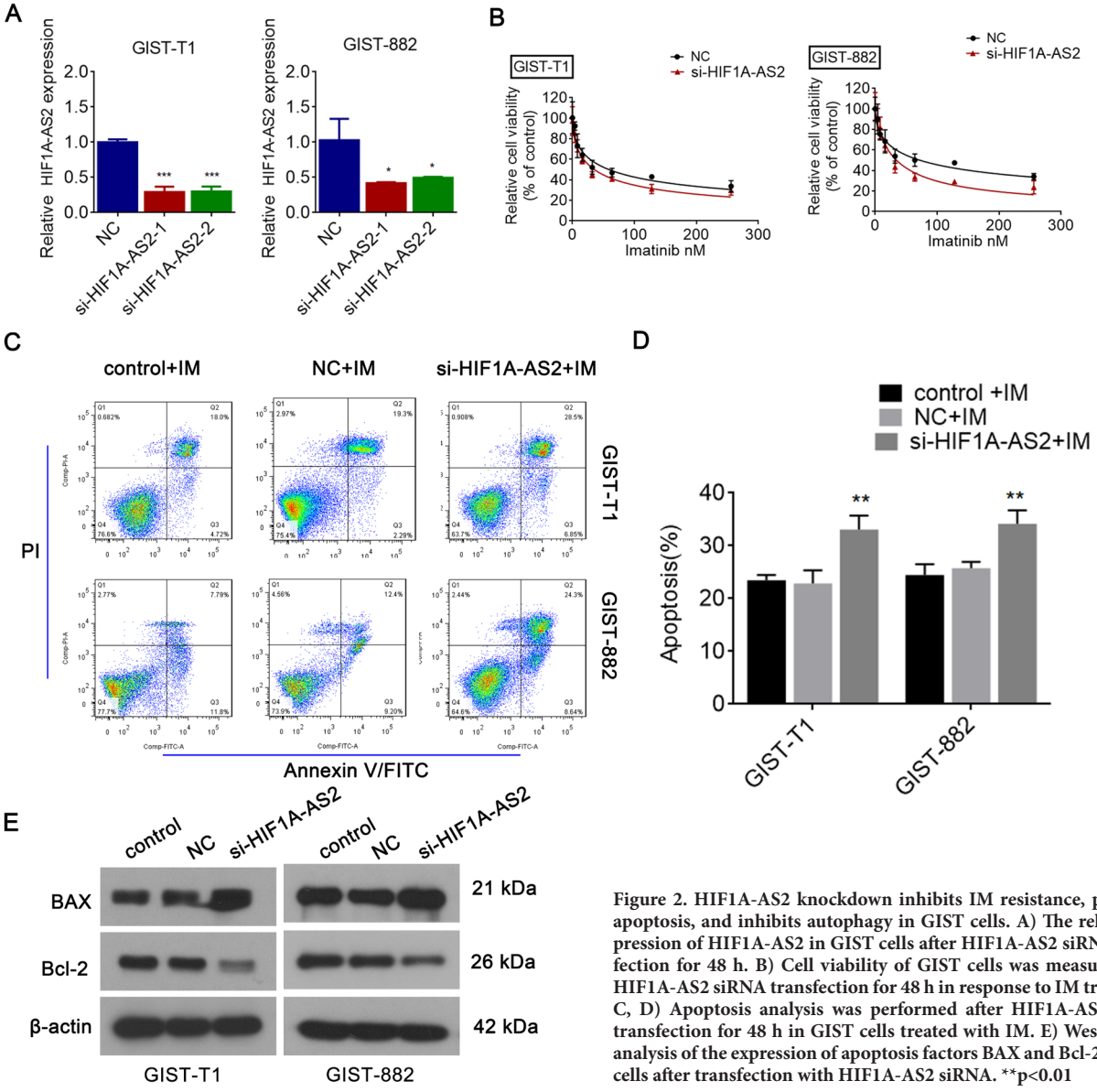

Figure 2. HIF1A-AS2 knockdown inhibits IM resistance, promotes apoptosis, and inhibits autophagy in GIST cells. A) The relative expression of HIF1A-AS2 in GIST cells after HIF1A-AS2 siRNA transfection for 48 h. B) Cell viability of GIST cells was measured after HIF1A-AS2 siRNA transfection for 48 h in response to IM treatment. C, D) Apoptosis analysis was performed after HIF1A-AS2 siRNA transfection for 48 h in GIST cells treated with IM. E) Western blot analysis of the expression of apoptosis factors BAX and Bcl-2 in GIST cells after transfection with HIF1A-AS2 siRNA. **p<0.01

(Figure 3A). Moreover, hypoxia increased cell proliferation and inhibited cell apoptosis compared with normoxia, but this effect was effectively negated when HIF1A-AS2 expression was knocked down (Figures 3B, 3C). Together, these data indicate that hypoxia-induced HIF1A-AS2 promotes GIST cell resistance to IM.

The effects of HIF1A-AS2 on IM-induced autophagy. It has been reported that autophagy plays an important role in mediating therapeutic resistance in GIST. Here, we first verified that IM can induce autophagy in GIST cells. As shown in Supplementary Figure S1A, autophagy-related proteins (p-ULK, Beclin-1, and LC3I/LC3IIratio) were increased with prolonged exposure to IM, and P62 levels gradually decreased. Further, immunofluorescence demonstrated downregulation of P62 after IM treatment (Supplementary Figures S1B, S1C). Furthermore, we evaluated whether HIF1A-AS2 also plays a role in IM-induced autophagy. We transfected GIST cells with HIF1A-AS2 siRNA and then exposed the transfected cells to IM. Protein expression analysis indicating the downregulation of p-ULK and Beclin-1 and the upregulation of P62 demonstrates that autophagy induced by IM could be reversed after siRNA-knockdown of HIF1A-AS2 (Figure 4A). Similarly, PCR results showed that Beclin-1 expression was downregulated and P62 expression was increased after HIF1A-AS2 knockdown, compared with the IM treatment group (Figure 4B). Next, we used the autophagy inhibitor, chloroquine (CQ), to block autophagy. The cells were then treated with HIF1A-AS2 siRNA. As shown in Supplementary Figures S2A and S2B, there was no difference in autophagy markers between the CQ group and the CQ+HIF1A-AS2 siRNA group. Moreover, the CCK-8 assay showed that there was no difference in sensitivity to IM between the CQ group and the CQ+HIF1A-AS2 siRNA group (Figure 4C).

HIF1A suppresses HIF1A-AS2 expression. We next explored the mechanism of HIF1A-AS2 upregulation under hypoxic conditions. HIF1A is the main transcriptional regulator of cellular and developmental response to hypoxia [23]. It has previously been reported that there are putative hypoxia response elements in the HIF1A-AS2 promoter regions [24]. Therefore, we speculate that the upregulation of HIF1A-AS2 is related to HIF1A expression and activity. To test our hypothesis, we evaluated the change of HIF1A-AS2 expression in HIF1A siRNA-treated GIST cells under hypoxia. Knockdown efficiencies were determined by

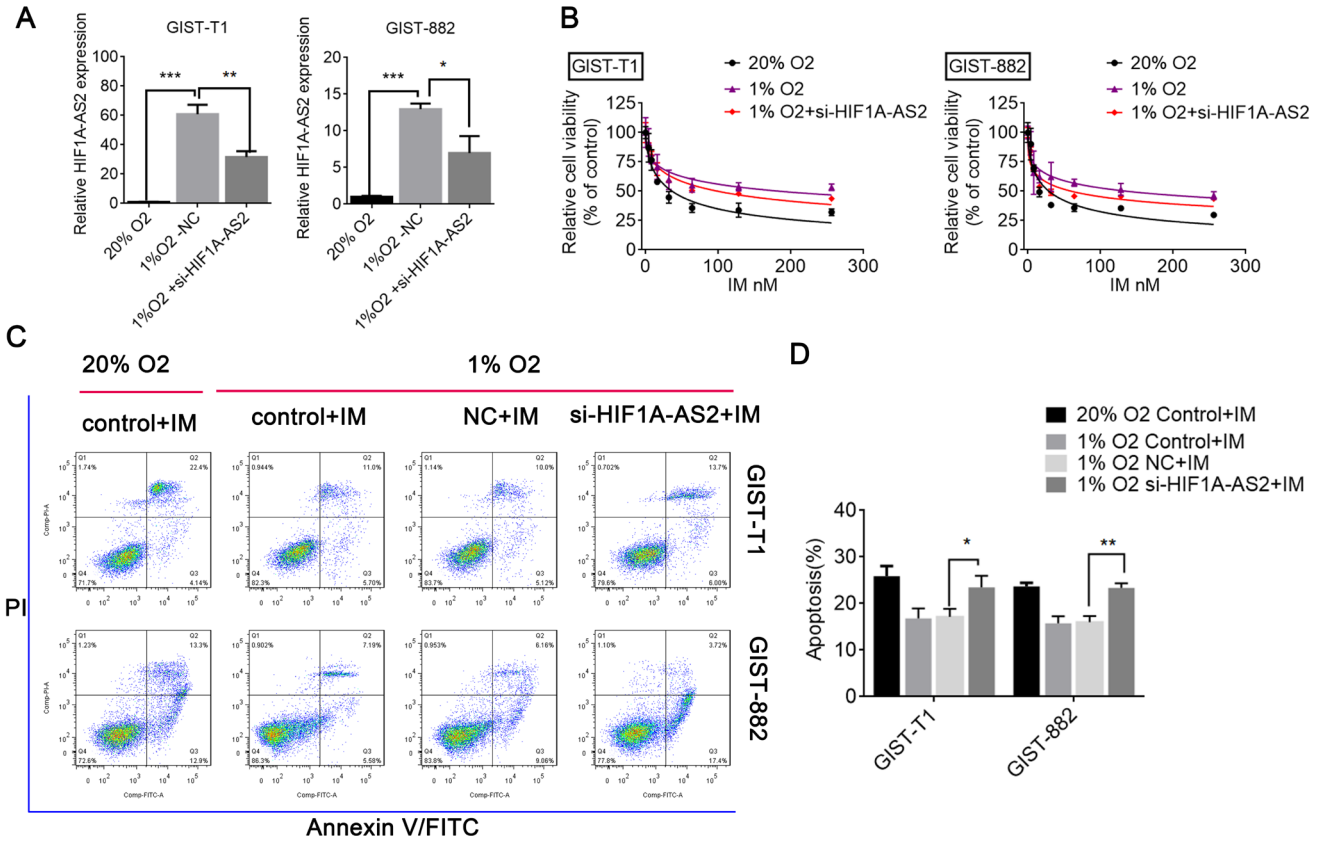

Figure 3. HIF1A-AS2 knockdown reverses hypoxia-induced IM resistance. A) The relative expression of HIF1A-AS2 in GIST cells after HIF1A-AS2 siRNA transfection under normoxic or hypoxic conditions. B) Cell viability of GIST cells was measured after HIF1A-AS2 siRNA transfection under normoxia or hypoxia and treatment with IM. C, D) Apoptosis was analyzed after HIF1A-AS2 siRNA transfection in GIST cells under normoxia or hypoxia and treatment with IM. *p<0.05, **p<0.01, ***p<0.001

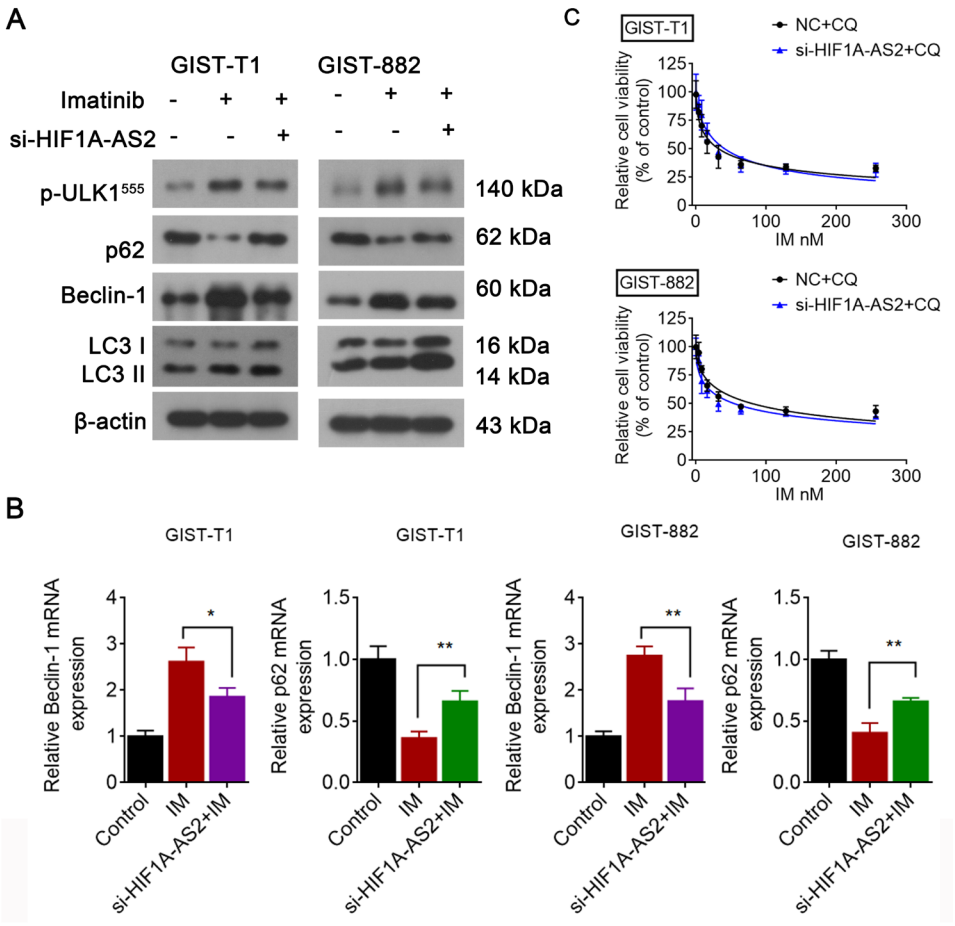

Figure 4. The effects of HIF1A-AS2 on IM-induced autophagy. A) Western blot analysis of expression of autophagy-related proteins (p-ULK1, LC-3, P62, and Beclin-1) in si-HIF1A-AS2-transfected GIST cells in response to IM. B) qRT-PCR analysis of expression of P62 and Beclin-1 in si-HIF1A-AS2-transfected GIST cells in response to IM. C) Cell viability of GIST cells was measured after HIF1A-AS2 siRNA and CQ treatment. *p<0.05, **p<0.01. Abbreviation: CQ-chloroquine

western blot analysis for the HIF1A protein. We found that hypoxia increased HIF-1α protein levels which were inhibited by HIF1A siRNA under hypoxic conditions (Figure 5A). The change in HIF1A-AS2 expression was consistent with the change in HIF1A (Figure 5B). These results indicate that HIF1A positively regulates HIF1A-AS2 under hypoxic conditions.

Discussion

Imatinib resistance is a serious obstacle in the treatment of GIST, and finding therapeutic strategies to overcome drug resistance is an urgent need. In the current study, we found that expression of the lncRNA HIF1A-AS2 is elevated in IM mesylate-resistant GIST samples compared with primary GIST samples. We also observed high expression of HIF1A-AS2 in GIST cell lines under hypoxic conditions. Furthermore, HIF1A-AS2 knockdown reinforced the IM sensitivity of GIST cells via inhibiting autophagy, indicating that HIF1A-AS2 is an important regulator in the hypoxia signaling pathway and is a potential therapeutic target to enhance the efficacy of IM in the treatment of GIST.

Hypoxia contributes to resistance to chemotherapy in tumors. For example, the toxicity of IM to GIST T1 cells under hypoxic conditions was lower than in normoxic conditions. Previous studies have revealed that lncRNAs are implicated in chemoresistance, such as the role of lncRNA CASC9 in gefitinib resistance in lung cancer [18, 25]. Therefore, we identified lncRNAs, which may be involved in hypoxia-mediated IM resistance in GIST. Through microarray analysis, we found that HIF1A-AS2 is a differentially upregulated lncRNA gene in IM mesylate-resistant GIST samples. HIF1A-AS2 is elevated in many human cancers, including gastric cancer, lung adenocarcinoma, renal carcinoma, and breast cancer [26–29]. Therefore, HIF1A-AS2 may serve as a potential actionable biomarker. Additionally, we also found that HIF1A-AS2 is increased in GIST cells after hypoxia or IM treatment, suggesting that HIF1A-AS2 is correlated with hypoxia-induced IM resistance.

Knockdown of HIF1A-AS2 increases the drug sensitivity of lung cancer cells [30]. Similar to this observation, we found that silencing HIF1A-AS2 enhances the sensitivity of GIST cells to IM. Furthermore, hypoxia caused an increase of HIF1A-AS2 expression and a decrease in the sensitivity of GIST cells to IM. However, when GIST cells were transfected with HIF1A-AS2 siRNA, the effect of hypoxia on IM resistance was reversed. Collectively, our data demonstrate that HIF1A-AS2 is involved in hypoxia-mediated IM resistance in GIST.

HIF1A-AS2 is a natural antisense transcript of HIF1A. In the present study, we found that hypoxia induces high expression of HIF1A and HIF1A-AS2. We show that the knockdown of HIF1A is able to suppress the HIF1A-AS2 expression under hypoxic conditions, indicating that HIF1A-AS2 acts as a HIF1A-regulated gene. This is similar to a previous study which demonstrated that both HIF1A and HIF1A-AS2 are elevated under hypoxia and inhibition of HIF1A also reduced levels of HIF1A-AS2 [24]. However, this study also revealed that silencing HIF1A-AS2 induced the expression of HIF1A mRNA and protein under hypoxia, indicating that HIF1A-AS2 negatively regulates HIF1A. Tokujiro et al. also showed that HIF-1α protein levels increase HIF1A-AS2 transcript levels, which in turn increases HIF-1A mRNA instability and eventually decreases HIF-1α protein expression [31]. These indicate that the regulatory mechanism between HIF1A-AS2 and HIF1A is complicated and merits further elucidation.

In summary, our study reveals that lncRNA HIF1A-AS2 is involved in hypoxia-induced IM resistance in GIST by regulating autophagy. Targeting lncRNA HIF1A-AS2 may serve as a promising treatment for GIST.

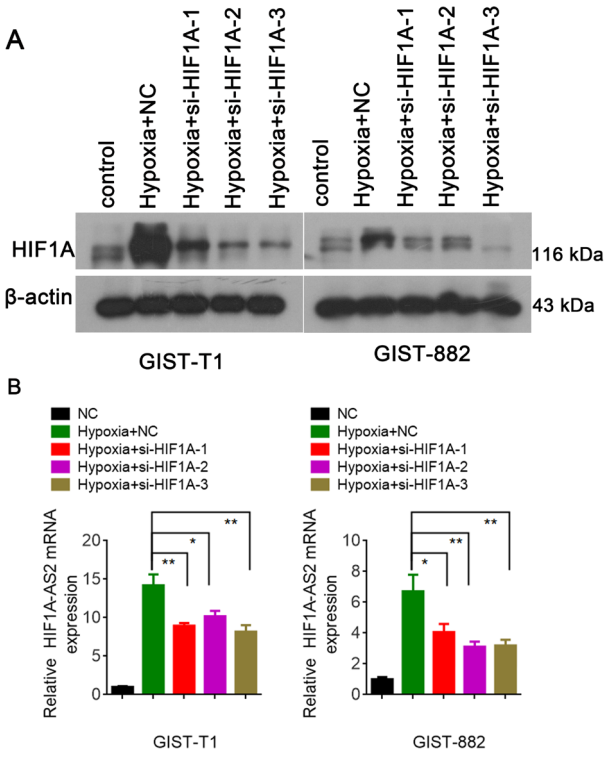

Figure 5. The effects of HIF1A knockdown on HIF1A-AS2 expression under hypoxic conditions. A) Western blot analysis of the HIF1A protein expression in si-HIF1A transfected GIST cells under hypoxic conditions and treatment with IM. B) HIF1A-AS2 expression was measured by RT-qPCR following the silencing of HIF1A under hypoxia. *p<0.05, **p<0.01

Supplementary information is available in the online version of the paper.

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LncRNA HIF1A-AS2 mediates imatinib resistance by regulating autophagy in gastrointestinal stromal tumor cells

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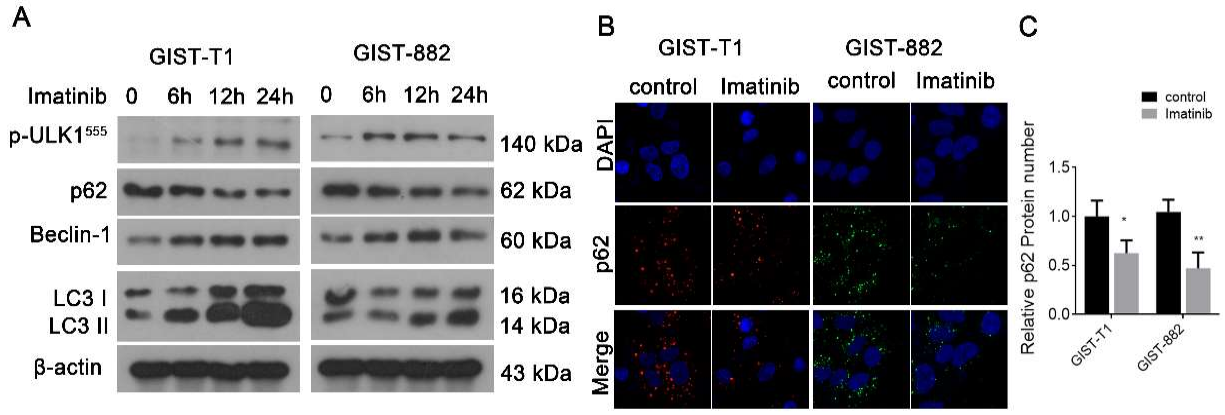
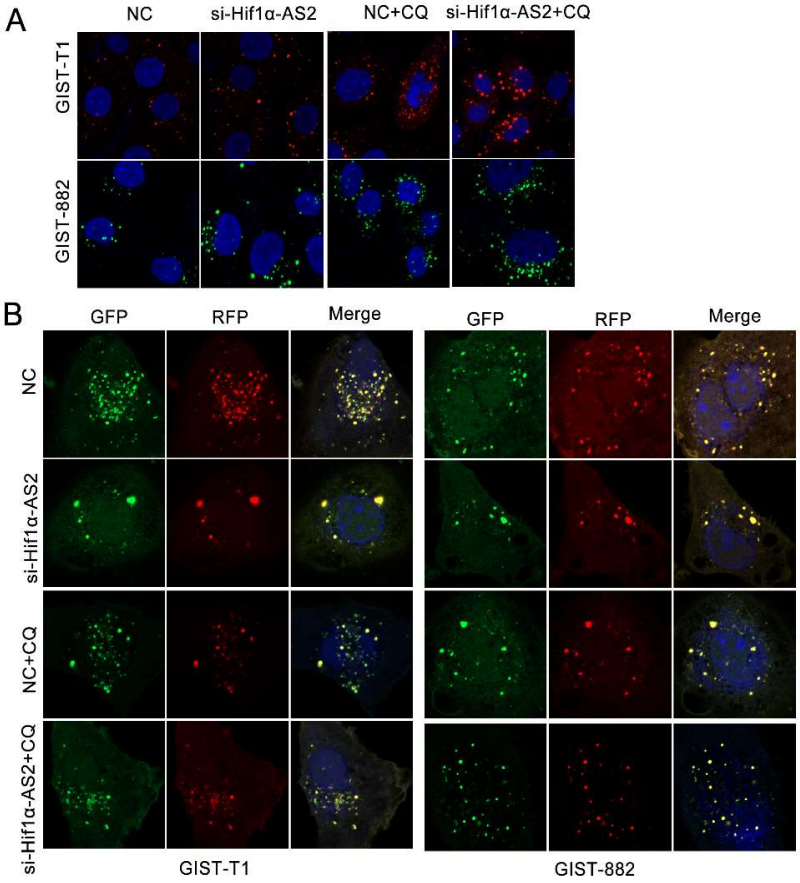
Supplementary Information



Supplementary Figure S1. Imatinib induced autophagy in GIST cells. A) WB analysis of the autophagy protein expression (p-ULK1, LC-3, P62 and Beclin-1) in GIST cells response to imatinib at 0 h, 6 h, 12 h, 24 h. B, C) Immunofluorescence detection revealed the change of P62 after imatinib treatment.



Supplementary Figure S2. The effects of HIF1A-AS2 on autophagy. A) Immunofluorescence detection revealed the change of P62 after GIST cells treated with si-HIF1A-AS2 or CQ. B) RFP-GFP dual fluorescence systems revealed the change of LC3 after GIST cells treated with si-HIF1A-AS2 or CQ.