Inhibition of stromal-interacting molecule 1-mediated store-operated Ca^{2+} entry as a novel strategy for the treatment of acquired imatinib-resistant gastrointestinal stromal tumors

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Gastrointestinal stromal tumors (GIST) are the most common mesenchymal tumor of the digestive tract. The incidence of GIST and so-called subclinical GIST is nearly 10\%-30\% in adults.\textsuperscript{1-3} KIT and PDGFRA mutations are recognized as the driving factors of GIST,\textsuperscript{4,5} with the decisive therapy being selective receptor tyrosine kinase inhibitors. Imatinib has been approved by the Food and Drug Administration for the treatment of GIST since 2002 and is regarded as the first-line drug worldwide, including in the National Comprehensive Cancer Network, European Society for Medical Oncology, and Asian consensus.\textsuperscript{6-8} Although more than 80\% of patients benefit from imatinib monotherapy, half still develop acquired resistance within 2 years of treatment, leading to recurrence or metastasis, and increasing mortality and morbidity.\textsuperscript{9} Interstitial cells of Cajal (ICC) are the peacemaker cells in the gastrointestinal tract and are where GIST originates from.\textsuperscript{10} Intracellular Ca^{2+} plays a key role in the function of ICC;\textsuperscript{11} therefore, it is important to study the influence of intracellular Ca^{2+} in GIST tumorigenesis, proliferation and drug response. Store-operated Ca^{2+} entry (SOCE) is mediated via store-operated channels and is the principal approach for Ca^{2+} entry.\textsuperscript{12} Stromal-interacting molecule 1 (STIM1) is...
a critical component of SOCE. STIM1 is an endoplasmic reticulum (ER) Ca\(^{2+}\) sensor. Once the ER Ca\(^{2+}\) concentration is depleted, STIM1 is activated and aggregates to the pore subunit of the Ca\(^{2+}\) channel (such as Orai1) in the plasma membrane, forming a pore channel for Ca\(^{2+}\) influx. From the opening of the Ca\(^{2+}\) channel, ER stores can be refilled again.\(^{13,14}\) Recently, a growing body of literature has reported that SOCE is involved in the proliferation and migration of various cancers.\(^{15-17}\) A study of ovary carcinoma showed that SOCE participates in resistance to cisplatin.\(^{18}\) However, the mechanism by which SOCE promotes the malignancy of tumor cells remains inconclusive and the role of STIM1-mediated SOCE in GIST is unclear.

In this study, we investigated the correlation between the STIM1 expression level and imatinib resistance in GIST patients. We found that STIM1 was upregulated in imatinib-resistant GIST cells compared to sensitive parental cells, and knockdown or overexpression of STIM1 has significant effects on SOCE, proliferation and drug response in imatinib-resistant GIST cells. We further revealed that STIM1-mediated SOCE could induce apoptosis via the MEK/ERK pathway.

2 | MATERIALS AND METHODS

2.1 | Patients and specimens

A total of 35 specimens were collected from pathologically-confirmed GIST patients between 2012 and 2017 from the Department of General Surgery, Xinhua Hospital, School of Medicine, Shanghai Jiao Tong University, China. Fresh GIST tissues and paired nontumor tissues used for quantitative RT-PCR (qRT-PCR) and western blotting were frozen and stored in liquid nitrogen within 15 minutes after removal. No patients in this study received neoadjuvant therapy before their operations. Imatinib treatment after radical surgery was performed as described in the guidelines, with a proportion of the patients developing imatinib resistance after 6 months, defined as acquired resistance. All patients received follow-up. This study was approved by the Ethics Committee of the Xinhua Hospital and all patients provided informed consent.

2.2 | Cell culture

Human GIST cell lines GIST-T1 and GIST-882 were obtained from the Shanghai Cancer Institute. Both cell lines were grown in DMEM (Gibco, Gaithersburg, MD, USA) that contained 10% FBS (Gibco). Cells were cultured at 37°C in 5% CO\(_2\) and at 95% relative humidity. Imatinib-resistant sublines emerged from cell cultures with gradually increasing doses of imatinib. Imatinib-sensitive cell lines GIST-882 and GIST-T1 were cultured in medium with gradually increasing doses of imatinib (.2, .4 and 1 μmol/L) and were obtained after 1, 2 and 4 months, respectively. Relative resistance assessment was applied to detect the stability of the resistant phenotype originating from each culture continuously in medium with increasing concentrations of imatinib for up to 6 months. Imatinib (STI571) was purchased from Selleck (Shanghai, China).

2.3 | Quantitative RT-PCR

Total RNA was isolated from tissue samples or cultured cells using TRIzol Reagent (Takara, Shiga, Japan). cDNA was synthesized with PrimeScript Reverse Transcriptase (Takara, Osaka, Japan) following the manufacturer’s instructions. Real-time PCR was performed with the StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA) with SYBR Green (Takara, Dalian, China). GAPDH was used as the internal standard. Primer sequences used for amplification were listed in supporting information (Supplementary Table S1).

2.4 | Western blotting

Protein was extracted using radioimmunoprecipitation assay buffer (Cell Signaling, Danvers, MA, USA). A total of 25 μg of protein was loaded onto a 12% sodium dodecyl sulfate polyacrylamide...


2.5 siRNA, plasmid and Lv-shRNA transfection

Stromal-interacting molecule 1 were designed and synthesized by Biotend (Shanghai, China). The siRNA targeting human STIM1 are listed below: siRNA1: 5′-UCAAUCGGCAAAAACUCUGdTdT, siRNA2: 5′-AAGGCUCUCUCUAACUGAdTdT-3′, siRNA3: 5′-AAUCGGAAUUGGUCAAAUcTdT-3′; siRNA was transfected into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. STIM1 plasmids (Longqian Biotech, Shanghai, China) or control plasmids were transfected into cells using the ViaFect Transfection Reagent (Promega, Madison, WI, USA). Purified lentiviruses encoding small hairpin RNA targeting STIM1 (5′-GGAGGATAATGGCTCTATT-3′) were constructed by Genechem (Shanghai, China). GIST-882-R cells were infected at a multiplicity of infection of 40 for 24 hours. STIM1 expression levels were detected by qRT-PCR and western blotting.

2.6 Cell viability assays

The Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) assay was used for the assessment of cell viability. Cells were plated at 2 × 10³ cells per well in 96-well plates. For proliferation assays, absorbance at 450 nm was measured for 5 days. To determine the half maximal inhibitory concentration (IC50), cells were rinsed the next day and cultured with various doses of imatinib for 48 hours. Colony formation assays were performed to assess anchorage-independent growth. A total of 500 cells were seeded in 6-well plates and cultured for 10 days. Then, the cells were fixed, stained and photographed.

2.7 Calcium imaging

Cells were stained with 4 µM Fluo-4-AM (Invitrogen) before imaging and then resuspended with Hanks’ balanced salt solution (with Ca²⁺, Mg²⁺, without phenol red, pH 7.4; Yeasen, Shanghai, China) to remove extracellular Fluo-4-AM. A Zeiss LSM 710 (Zeiss, Jena, Germany) measured fluo-4-Ca²⁺ fluorescence at 488-nm emission. SOCE in Fluo-4-AM-loaded cells was detected using a calcium imaging system 200 s after the intracellular Ca²⁺ responses were stimulated with 2-µM thapsigargin (TG).

2.8 Flow cytometry analysis of cell apoptosis

Apoptosis was inspected with an Annexin V-FITC Apoptosis Detection kit (BD Biosciences) according to the manufacturer’s instructions. Cells were collected and twice washed with PBS, gently resuspended in 100 µL Annexin V binding buffer (1x) containing 2.5 µL FITC (BD Pharmingen) and 5 µL of 50 µg/mL PI, and then incubated at room temperature in the dark for 15 minutes. The stained cells were analyzed by flow cytometry (BD Biosciences) and data were analyzed with FlowJo (Flowjo Studio, Carrboro, NC, USA).

2.9 Xenograft nude mouse model

Animal studies were approved by the Ethics Committee of Xinhua Hospital. Nude nu/nu mice, 4-6 weeks old, were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). GIST-882-R cells were stably infected with Lv-shNC (negative control)/Lv-shSTIM1. A total of 5 × 10⁶ viable cells were injected into the right axilla of nude mice. Tumor sizes were measured weekly using a vernier caliper. After 4 weeks, the mice were killed and the tumors were dissected out and weighed.

| TABLE 1 Association of STIM1 expression with the clinicopathological characteristics of GIST |
| Variable | Category | Number of cases | Number of high-level cases | χ² | P |
|----------|----------|-----------------|---------------------------|----|---|
| Age      | <60      | 19              | 10                        | .274 | .600 |
|          | ≥60      | 16              | 9                         | .077 | .782 |
| Sex      | Male     | 20              | 11                        | .775 | .384 |
|          | Female   | 15              | 8                         | .901 | .347 |
| Tumor location | Stomach | 18              | 10                        | .274 | .600 |
|          | Nonstomach | 17          | 9                         | .878 | .384 |
| Tumor size | ≤2 cm | 7               | 4                         | .674 | .879 |
|          | >2 cm ≤ 5 cm | 11          | 5                         | .054 | .817 |
|          | >5 cm ≤ 10 cm | 13          | 8                         | .746 | .389 |
|          | >10 cm | 4               | 2                         | .013 | .910 |
| Mitotic rate | ≤5 | 23              | 14                        | 1.172 | .311 |
|          | >5      | 12              | 5                         | 1.016 | .313 |
| Rupture  | Yes      | 6               | 1                         | 4.130 | .034 |
|          | No       | 29              | 18                        | 1.016 | .313 |
| Risk classification (NIH) | Very low | 5               | 4                         | 1.154 | .707 |
|          | Low     | 4               | 2                         | .013 | .910 |
|          | Moderate | 12             | 6                         | 1.016 | .313 |
|          | High    | 14              | 7                         | .013 | .910 |
| Imatinib resistance | Yes | 9               | 8                         | 5.846 | .022 |
|          | No      | 26              | 11                        | 1.016 | .313 |
| Total    |          | 35              | 19                        |        |   |

Bold values indicate statistical significance; *P < .05. *Fisher’s exact test. GIST, gastrointestinal stromal tumors; STIM1, stromal-interacting molecule 1.
2.10 | Statistical analysis

The SPSS 22.0 software program for Windows was used for statistical analysis. The difference between the STIM1 expression level and clinicopathologic parameters was found using Pearson’s $\chi^2$ test or Fisher’s exact test. The independent Student’s t test was used when the data were normally distributed. Each experimental value was expressed as the mean ± standard deviation. A P-value of less than .05 was considered statistically significant. All data points represent the mean of triplicate experiments.

3 | RESULTS

3.1 | Stromal-interacting molecule 1 overexpression was related to acquired imatinib resistance in gastrointestinal stromal tumor patients

To investigate the role of STIM1 in GIST, we first compared the expression levels of STIM1 mRNA in 35 pairs of GIST tissue by qRT-PCR. The relative STIM1 expression levels were significantly higher in tumor tissue samples than those in corresponding nontumor tissue samples ($P < .01$) (Figure 1A,B). Based on the fold change, we divided patients into a high-level group (fold change $\geq 2$) and a low-level group (fold change $< 2$). Further clinicopathological association examination of the 35 GIST patients showed that STIM1 was significantly associated with acquired imatinib resistance ($P = .022$) (Table 1). STIM1 expression levels in GIST patients who developed imatinib resistance were significantly higher than in those who did not develop imatinib resistance ($P < .01$) (Figure 1C). Furthermore, western blotting confirmed that STIM1 protein expression levels in GIST tissues were higher than those in the corresponding non-GIST tissues (Figure 1D).

3.2 | Overexpressing of stromal-interacting molecule 1 and enhanced store-operated Ca$^{2+}$ entry were detected in imatinib-resistant gastrointestinal stromal tumor cells

To reveal the function of STIM1, we established 2 cell line models of acquired resistance following continuous in vitro exposure to imatinib using GIST-T1 and GIST-882 cells. We first investigated the
peak of the Ca²⁺ elevation and found that SOCE was higher in imatinib-resistant cells than that in imatinib-sensitive cells (Figure 2A, B). STIM1, Orai1 and TRPC channel expression in imatinib-resistant cells and their parental counterparts were compared using qRT-PCR (Supplementary Figure S1); only the STIM1 expression level had significant change. Among the 4 cell lines, STIM1 expression decreased in imatinib-sensitive GIST-882 and GIST-T1 cells, whereas it was overexpressed in the homologous imatinib-resistant cells (Figure 2C). Consistent protein levels were observed in western blotting (Figure 2D).

3.3 | Knockdown of stromal-interacting molecule 1 (STIM1) suppressed proliferation of imatinib-resistant gastrointestinal stromal tumor cells in vitro

We transfected GIST-882-R and GIST-T1-R cell lines with 3 different siRNA against STIM1. The efficiency of each siRNA was assessed by qRT-PCR and, from this, the third siRNA was employed (Figure 3A). Western blot analysis confirmed the knockdown efficiency (Figure 3B). We used CCK-8 and colony formation assays to explore the influence of STIM1 knockdown on GIST cell proliferation. Figure 3C shows that the viability of GIST-882-R and GIST-T1-R cells were markedly inhibited by STIM1 depletion (P < .05). In addition, compared with the si-NC (negative control) groups, the downregulation of STIM1 reduced the capacity of colony formation in GIST-882-R and GIST-T1-R cells (Figure 3D).

3.4 | Overexpression of stromal-interacting molecule 1 enhanced store-operated Ca²⁺ and imatinib sensitivity in gastrointestinal stromal tumor cells

We transfected STIM1 overexpression vectors into GIST-882 and GIST-T1 cells to determine the functions of STIM1. qRT-PCR and western blotting verified that the expression level of STIM1 was
upregulated after transfection (Figure 4A,B). We then detected that the IC₅₀ value of imatinib was higher in the STIM1-transfected cells than that in the empty vector-transfected cells. The IC₅₀ value of GIST-882 increased from .2001 ± .0012 μmol/L to .9868 ± .0107 μmol/L, and the IC₅₀ value of GIST-T1 increased from .4257 ± .0053 μmol/L to 1.7610 ± .1054 μmol/L after transfection (Figure 4C). STIM1 is one of the essential components of SOCE; therefore, we also investigated the effect of STIM1 overexpression on SOCE and found that STIM1 overexpression significantly increased the peak of the Ca²⁺ elevation resulting from Ca²⁺ influx (Figure 4D).

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3.5 | Blockage of store-operated Ca²⁺ inhibits growth of imatinib-resistant gastrointestinal stromal tumors in vivo

To evaluate the effect of STIM1 on imatinib-resistant GIST growth in vivo, we chose GIST-882-R cell lines for stable transfection with shRNA lentivirus vectors to STIM1. qRT-PCR and western blotting verified the knockdown efficiency of the system (Figure 5A,B, respectively). In addition, we confirmed that STIM1 downregulation decreased the functional SOCE in GIST-882-R cells (Figure 5C). With the above verification, GIST-882-R cell lines stably expressing shRNA-STIM1 or the negative control were injected into the axilla of nude mice, and the tumor volume was regularly monitored for the following 4 weeks (Figure 5D). Our results showed that the growth of STIM1-knockdown xenografts was significantly inhibited compared to the tumors formed by control cells (Figure 5E,F). These results showed that blockage of SOCE is essential for growth inhibition of imatinib-resistant GIST.

3.6 | Stromal-interacting molecule 1-mediated store-operated Ca²⁺ exerted an antiapoptosis effect through the MEK/ERK pathway

To explore the molecular mechanism of the proliferate inhibition induced by STIM1, flow cytometry was used to investigate the effect of STIM1 on apoptosis by measuring the apoptotic index, which is defined as the percentage of apoptotic cells. The results showed that apoptotic and dead cells increased significantly in the cells with STIM1 siRNA transfection. The apoptosis indexes of the si-NC group and the si-STIM1 group in GIST-882-R cells were 1.44% and 9.42%, respectively; and the apoptosis indexes of the si-NC group and the si-STIM1 group in GIST-T1-R cells were 2.48% and
13.42%, respectively (P < .05; Figure 6A). Considering that the deregulation of MEK/ERK phosphorylation represents an important antiapoptotic mechanism in various tumors, we studied the phosphorylation status of proteins involved in the above signaling pathway using western blotting. We found that p-MEK and p-ERK expression levels decreased significantly when STIM1 was silenced (Figure 6B). These results indicated that the MEK/ERK pathway might be regulated by the STIM1-mediated SOCE in imatinib-resistant GIST cells.

4 | DISCUSSION

Imatinib has revolutionized the treatment of GIST; however, primary and secondary resistance to imatinib is still a major cause of treatment failure. Several mechanisms have been found to be responsible for imatinib resistance, including secondary point mutations, gene amplification, autophagy and apoptosis, and other mechanisms. These mechanisms are complex, often heterogeneous, and not fully understood. Among all possible reasons, second point mutations in the kinase or loop domain of KIT or PDGFRA are considered the main cause. However, more than a few imatinib-resistant GIST do not detect such mutations, indicating the existence of additional mechanisms and the existence of additional mechanisms. Here, we report on a novel mechanism of acquired resistance to imatinib, which was induced by enhanced Ca2+ entry via STIM1-mediated SOCE. This hypothesis was verified both in vivo and in vitro by regulating the STIM1 expression level. The results showed that STIM1 was related to acquired imatinib resistance in GIST. Inhibition of STIM1-mediated SOCE suppressed the
proliferation of imatinib-resistant GIST cell lines and xenografts. Further study indicated that STIM1 plays a critical role in apoptosis of imatinib-resistant GIST cell lines, which was confirmed by detecting the expression of the MEK/ERK pathway. In fact, Ca²⁺ entry participates in a variety of fundamental cellular mechanisms and ensures that cells acclimatize to external environmental change.24 Recent research has suggested that deregulation of SOCE contributes to tumor proliferation, invasion and migration. SOCE channels have been proven to be potential targets for several malignant tumors.16,25,26 STIM1, which is the essential component of SOCE and the focus of the present study, is located on human chromosome region 11p15.5, and the loss of 11p13-11p15.5 has been associated with several malignancies. Therefore, further research on why overexpressed STIM1 exerts an antidrug effect in GIST is required. According to previous research in prostate cancer,27,28 although different studies have contrary results for the expression level of STIM1, STIM1 plays an oncogene role. Difference in sample size and detecting methods may explain these discrepancies. The present study examined STIM1 expression in both translational and post-translational levels and matched it to the clinicopathological characteristics of GIST patients. We failed to find correlation between STIM1 and risk stratification (Table 1); however, we found that STIM1 was related to acquired imatinib resistance in the studied cohort. Therefore, we investigated the function of STIM1-mediated SOCE in imatinib-resistant GIST.

Based on our data, we suggest that the antiapoptotic effect induced by SOCE is one of the mechanisms leading to imatinib resistance. Apoptosis has been widely investigated in GIST and is closely related to tumor progression. Wang et al29 found a significant negative correlation between apoptosis and the degree of GIST differentiation. Moreover, apoptosis is also involved in imatinib response. Liu et al30 report that GIST cells survived imatinib treatment by escaping from apoptosis. Another study reports that apoptosis induced by miR-518a-5p affected the cellular response to the drug, causing imatinib resistance in GIST.31 We have also found previously that STIM1-mediated SOCE contributes to apoptosis via phosphorylation of the MEK/ERK pathway, which is a classical signaling pathway that participates in cell growth, EMT and apoptosis.32 STIM1-knockdown cells in the present study had lower levels of phosphorylated MEK and ERK than that of control cells, whereas total protein levels were unaffected. Thus, the results suggest that the MEK/ERK pathway might be involved in the apoptosis of acquired imatinib-resistant GIST.

In summary, to determine a strategy to fight imatinib-resistant GIST, many aspects still need to be explicated and understood despite the achievements researchers have already made. Our research improves the understanding of how STIM1-mediated SOCE is involved in acquired imatinib-resistant GIST and expounds the biological functions of STIM1. Future studies on the effects of SOCE inhibitors or STIM1-targeted drugs will be of further assistance for the development of clinical therapies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest for this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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