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Original Article

Somatostatin receptors in gastrointestinal stromal tumors: new prognostic biomarker and potential therapeutic strategy

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Abstract: Somatostatin receptors (SSTRs) already act as important roles in gastroenteropancreatic neuroendocrine tumors (GEP-NETs) with high expression levels for prognosis predicting and octreotide LAR treatment purposes but less noticed in gastrointestinal stromal tumors (GISTs). Our study aims to fully evaluate the expression levels and prognostic values of SSTRs in GIST patients. For SSTRs expression detection, qPCR were used in 25 fresh GIST specimens, and then, 453 GIST samples (405 GISTs with operation only and 48 with imatinib adjuvant therapy after surgery) were collected for tissue microarrays (TMAs) construction and confirmed by immunohistochemistry (IHC). Clinicopathological data were confirmed by pathological diagnosis and clinical recorders, recurrence-free survivals (RFS) were evaluated in 453 GIST patients. With IHC performed, SSTR1 and SSTR2 present high positive proportion (81.9% and 87.6%) in 453 GISTs in our study, and positive expression rates of SSTR3, SSTR4 and SSTR5 are 56.1%, 8.8% and 47.2%, respectively. SSTR2 and SSTR5 negative expression are associated with decreased RFS when compared to positive cases by Kaplan-Meier survival analyses with log-rank test and univariate analysis in GISTs, furthermore, SSTR2 was an independent prognostic indicator for GISTs by multivariate analysis. In our study, detection of SSRT2 and SSTR5 expression helps to predict different prognosis in GIST patients. SSTR2 is a novel independent prognostic biomarker for GISTs. With high expression performance of SSTRs in GISTs, new therapeutic strategies such as octreotide or pasireotide LAR could be taken into consideration in selected advanced GIST patients.

Keywords: Gastrointestinal stromal tumor, somatostatin receptor, octreotide, pasireotide, prognosis

Introduction

Gastrointestinal stromal tumors (GISTs) is the most common types of gastrointestinal mesenchymal tumors with increased incidence in recent years [1-3]. Abnormal activation of tyrosine kinase proteins KIT or PDGFRa transcribed by oncogenic mutations were shown in most of GISTs [4]. NIH consensus criteria, Modified NIH criteria or AFIP criteria are common criteria wildly accepted as risk-stratification schemes for predicting prognosis in GISTs, with similar accuracy [5]. The mitosis count, tumor size and tumor site were important prognostic indicators in these schemes [5]. Following surgical resection, GIST patients often suffered disease recurrence and with no response to chemotherapy or radiation therapy. With wide application of imatinib mesylate (IM) in clinical practice for GISTs, the mortality rate of GIST patients has decreased significantly. Nevertheless, the recurrence or metastasis rates still remain high [6-8], and almost all advanced GIST patients eventually develop resistance to imatinib treatment [9]. Although sunitinib act as second-line treatment for advanced GISTs progressing on imatinib [10], and some other small molecule targeted therapies are in clinical trails, the reality is there are still few options we can choose in current GIST therapeutic strategies.

Somatostatin (SST) is a naturally growth hormone inhibitory neuropeptide with potent and broad antisecretory actions, and also performed negative regulation of cell proliferation in both normal and tumor cells [11-13]. Anti-

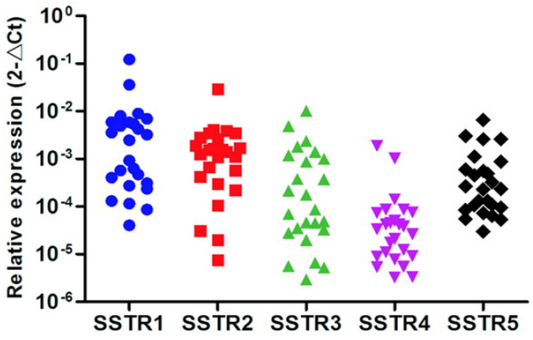

Figure 1. Relative expression levels of SSTRs in GISTs by quantitative real-time PCR.

Table 1. Clinicopathological characters of GISTs for real-time PCR

	Number	(%)
Age (years)		
≤ 50	3	12.0
> 50	22	88.0
Gender		
Male	14	56.0
Female	11	44.0
Tumor site		
Stomach	19	76.0
Small bowel	6	24.0
Tumor size (cm)		
2.1-5.0	5	20.0
5.1-10.0	13	52.0
> 10.0	7	28.0
Mitoses per 50 HPFs		
≤ 5	16	64.0
6-10	4	16.0
> 10	5	20.0
Modified NIH criteria		
Low risk	6	24.0
Intermediate risk	6	24.0
High risk	13	52.0
Total	25	100.0

proliferation effect of SST is cytostatic or cytotoxic by binding to seven trans-membrane G-protein coupled receptors (GPCRs), which were named as somatostatin receptors (SSTRs) with five subtypes SSTR 1-5 [14, 15]. SST analogs such as octreotide or octreotide long-acting repeatable (octreotide LAR), similarly to somatostatin structure, was already developed and successfully used for control neuroendocrine symptoms and tumor progression in advanced or metastasis gastroenteropancreatic neuroendocrine tumors (GEP-NETs), which present high positive expression rates of SSTRs [16-18]. Detecting the expression of SSTRs helps to predict not only the efficacy of octreotide LAR treatment in GEP-NETs but also the prognosis in tumors [11, 19]. However, the research about the roles of SSTRs in GISTs was very limited. Our study aims to fully evaluate the expression levels and prognostic values of SSTRs in 453 GIST patients.

Materials and methods

Ethics statement

This project was approved by ethics committee of Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine for the use of samples. Informed consents were obtained from all patients before study inclusion.

Patients and specimens

25 fresh GIST tissues obtained from patients during surgical resection between January 2013 to May 2014 were collected for detecting mRNA expression level of SSTRs by quantitative real-time PCR.

GIST patients inclusion criteria for immunohistochemistry and prognostic evaluation of SSTRs were as follows: 1) a confirmative pathologic diagnosis of GISTs; 2) underwent R0 resection, R0 resection in our study defined as margin-free resection and no metastasis detected before and during the surgery; 3) no radiotherapy, chemotherapy, nor other anticancer therapies prior to the surgery; and 4) complete clinicopathologic and follow-up data were available. The risk of potential malignancy in recurrence was calculated according to the modified NIH criteria [20], which classified GISTs into very low, low, intermediate, and high-risk categories. High-risk GIST patients with imatinib adjuvant therapy required at least 12 months uninterrupted drugs taking with 400 mg/day in our study. The parameters, including patient age, gender, tumor site, tumor size and number of mitoses/50 high-power fields (HPF) were recorded in the official pathology database.

453 paraffin-embedded tumor tissue samples from GIST patients (405 GISTs with operation only and 48 high-risk GISTs received imatinib adjuvant therapy after radical surgery), which met the inclusion criteria, were collected at Ren Ji Hospital, Shanghai Jiao Tong University,

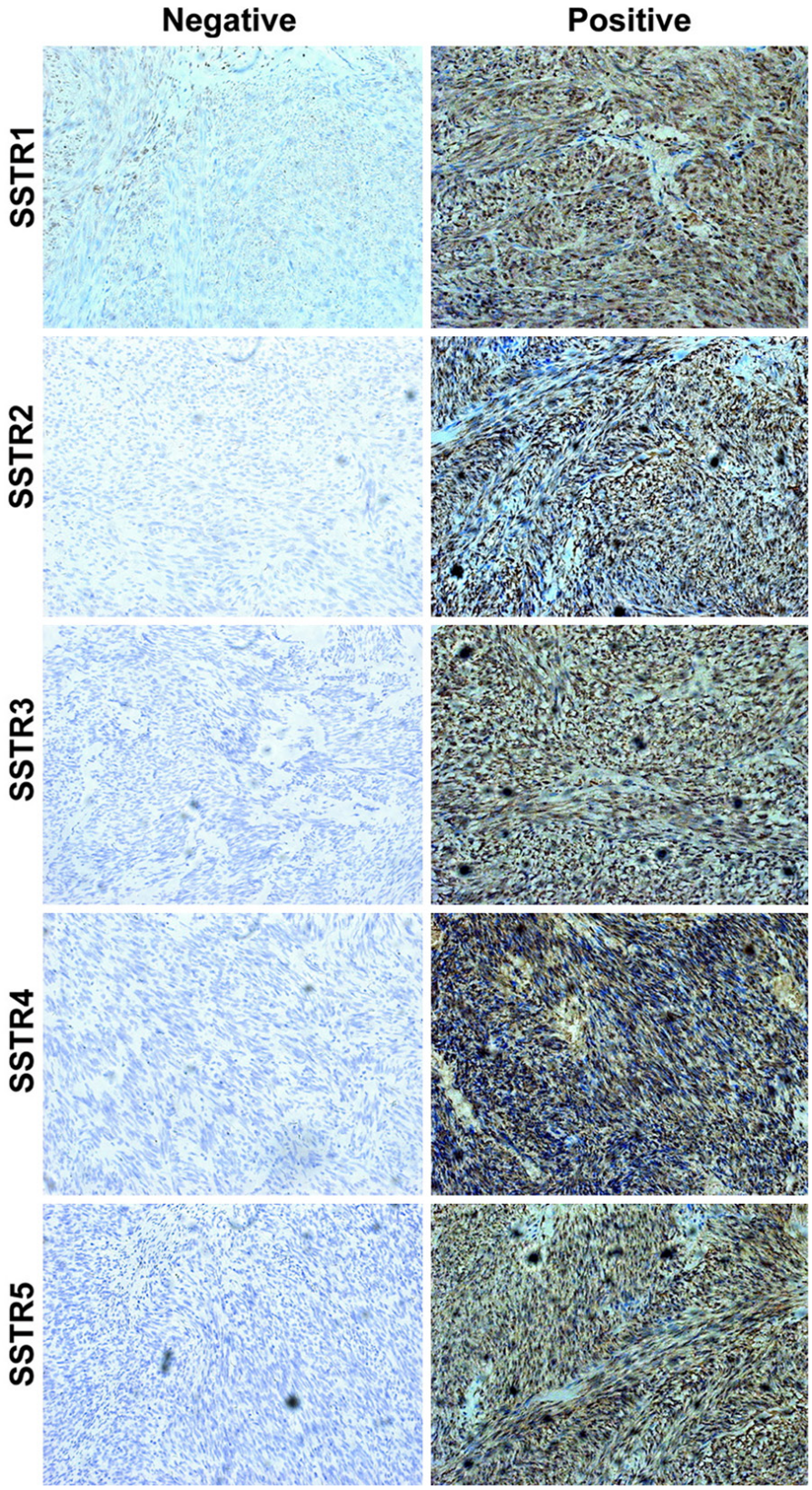

Figure 2. Representative immunohistochemical stains for SSTRs in GISTs.

School of Medicine from June 2004 to May 2013 for tissue microarrays (TMAs) construction and immunohistochemistry staining. Complete follow-up data until May 2014 for GIST patients were available. Recurrence free survival (RFS) was calculated from the date of tumor resection until the detection of tumor recurrence or last observation. The median follow-up of 405 GISTs with operation only was 53 months (range, 8-113 months). In high-risk GISTs with imatinib adjuvant therapy, The median follow-up was 45 months (range 2274 months). Computed tomography (CT) and/or magnetic resonance imaging (MRI) were used to verify tumor recurrence in suspected cases.

Total RNA extraction and quantitative real-time PCR

Total RNA was extracted from 25 fresh GIST tissues using Trizol reagent (Takara) followed the manufacturer instructions. The reverse-transcription reactions were carried out with random primers and M-MLV Reverse Transcriptase (Taka-

Table 2. Clinicopathological characters of 405 GISTs with operation only and 48 high-risks with imatinib adjuvant therapy for IHC

	Operation only		Imatinib adjuvant therapy	
	Number	(%)	Number	(%)
Age (years)				
≤ 50	81	20.0	7	14.6
> 50	324	80.0	41	85.4
Gender				
Male	215	53.1	28	58.3
Female	190	46.9	20	41.7
Tumor site				
Stomach	238	58.8	18	37.5
Small bowel	129	31.9	23	47.9
Colorectum	19	4.7	3	6.3
Others	19	4.7	4	8.3
Tumor size (cm)				
≤ 2.0	36	8.9	1	2.1
2.1-5.0	194	47.9	3	6.3
5.1-10.0	115	28.4	24	50.0
> 10.0	60	14.8	20	41.7
Mitoses per 50 HPFs				
≤ 5	327	80.7	17	35.4
6-10	43	10.6	16	33.3
> 10	35	8.6	15	31.3
Modified NIH criteria				
Very low risk	32	7.9	0	0
Low risk	187	46.2	0	0
Intermediate risk	62	15.3	0	0
High risk	124	30.6	48	100.0
Total	405	100.0	48	100.0

ra). The 25 cases of cDNA were used for quantitative real-time PCR reaction in SYBR-Green method. The specific primer sequences of SSTR1-5 and 18 s were as follow: SSTR1 [forward: 5'-TATCTGCCTGTGCTACGTGC-3'; reverse: 5'-GATGACCGACAGCTGACTCA-3'], SSTR2 [forward: 5'-ATGCCAAGATGAAGACCATCAC-3'; reverse: 5'-TGAACTGATTGATGCCATCCA-3'], SSTR3 [forward: 5'-CTGGGTAACTCGCTTGGTCATCTA-3'; reverse: 5'-AGCGCCAGGTTGAGGATGTA-3'], SSTR4 [forward: 5'-GTGATCCTTCGCTACGCCAA-3'; reverse: 5'-CACGGTGAGACAGAAGACGC-3'], SSTR5 [forward: 5'-GTGACAACAGGACGCTGGT-3'; reverse: 5'-TGGTGACGGTCTTCATCTTG-3'] and 18s [forward: 5'-TGCGAGTACTCAACACCAACA-3'; reverse: 5'-GCATATCTTCGGCCCACA-3']. 18 s was used as an internal control. Relative SSTRs expression levels were quantified by the $2^{-\Delta Ct}$ method.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using a two-step protocol. After citrate buffer (pH 6.0) antigen retrieval, tissues were incubated with SSTR1 antibody (rabbit polyclonal antibody, Abcam), SSTR2 antibody (rabbit monoclonal antibody, Epitomics), SSTR3 antibody (rabbit polyclonal antibody, Abcam), SSTR4 antibody (rabbit polyclonal antibody, Pierce) or SSTR5 antibody (rabbit monoclonal antibody, Epitomics) overnight at 4°C. Next day, following incubated with goat anti-rabbit IgG-HRP (HUABIO) secondary antibody for one hour at room temperature, sections were developed in DAB solution under microscopic observation and counterstained with hematoxylin.

Judgment for immunoreactivity of SSTRs in GISTs was referred from Edris et al's research [21]. TMAs were scored as follows: 0: absence of any staining; 1: weak staining whether diffusely or focally present in the tumor; 2: strong staining whether diffusely or focally present in the tumor. Score 0-1 was considered as negative (-) and a score of 2 was positive (+) for subsequent statistical analyses [21].

Statistical analysis

Statistical analyses were conducted by using SPSS (version 21.0) and MedCalc (version 11.4.2.0). RFS was calculated according to Kaplan-Meier method and log-rank test was used for comparing the survival distributions. Univariate and multivariate analyses were based on the cox proportional hazards regression model. All statistical tests were two-sided. *P* value less than 0.05 was considered statistically significant.

Results

SSTRs mRNA and protein expression in GISTs

For determining the differences in mRNA expressions of SSTRs in GISTs, mRNA transcript levels were analyzed by quantitative real-time PCR from 25 GIST samples. Scatter dot plot for mRNA relative expression levels ($2^{-\Delta Ct}$)

Table 3. IHC expression for SSTRs in 453 GIST patients (405 with operation only and 48 high-risks with imatinib adjuvant therapy)

	Total	SSTR1				SSTR2				SSTR3				SSTR4				SSTR5			
		—	(%)	+	(%)	—	(%)	+	(%)	—	(%)	+	(%)	—	(%)	+	(%)	—	(%)	+	(%)
Age (years)																					
≤ 50	88	13	2.9	75	16.6	7	1.5	81	17.9	37	8.2	51	11.3	80	17.7	8	1.8	39	8.6	49	10.8
> 50	365	69	15.2	296	65.3	49	10.8	316	69.8	162	35.8	203	44.8	333	73.5	32	7.1	200	44.2	165	36.4
Gender																					
Male	243	42	9.3	201	44.4	27	6.0	216	47.7	112	24.7	131	28.9	221	48.8	22	4.9	125	27.6	118	26.0
Female	210	40	8.8	170	37.5	29	6.4	181	40.0	87	19.2	123	27.2	192	42.4	18	4.0	114	25.2	96	21.2
Tumor site																					
Stomach	256	44	9.7	212	46.8	33	7.3	223	49.2	131	28.9	125	27.6	234	51.7	22	4.9	138	30.5	118	26.0
Small bowel	152	29	6.4	123	27.2	18	4.0	134	29.6	47	10.4	105	23.2	136	30.0	16	3.5	72	15.9	80	17.7
Colorectum	22	7	1.5	15	3.3	2	0.4	20	4.4	12	2.6	10	2.2	21	4.6	1	0.2	16	3.5	6	1.3
Others	23	2	0.4	21	4.6	3	0.7	20	4.4	9	2.0	14	3.1	22	4.9	1	0.2	13	2.9	10	2.2
Tumor size (cm)																					
≤ 2.0	37	10	2.2	27	6.0	3	0.7	34	7.5	23	5.1	14	3.1	37	8.2	0	0.0	18	4.0	19	4.2
2.1-5.0	197	27	6.0	170	37.5	16	3.5	181	40.0	89	19.6	108	23.8	178	39.3	19	4.2	90	19.9	107	23.6
5.1-10.0	139	25	5.5	114	25.2	16	3.5	123	27.2	54	11.9	85	18.8	130	28.7	9	2.0	73	16.1	66	14.6
> 10.0	80	20	4.4	60	13.2	21	4.6	59	13.0	33	7.3	47	10.4	68	15.0	12	2.6	58	12.8	22	4.9
Mitoses per 50 HPFs																					
≤ 5	344	55	12.1	289	63.8	26	5.7	318	70.2	157	34.7	187	41.3	315	69.5	29	6.4	158	34.9	186	41.1
6-10	59	15	3.3	44	9.7	13	2.9	46	10.2	18	4.0	41	9.1	50	11.0	9	2.0	41	9.1	18	4.0
> 10	50	12	2.6	38	8.4	17	3.8	33	7.3	24	5.3	26	5.7	48	10.6	2	0.4	40	8.8	10	2.2
Modified NIH criteria																					
Very low risk	32	7	1.5	25	5.5	2	0.4	30	6.6	20	4.4	12	2.6	32	7.1	0	0.0	16	3.5	16	3.5
Low risk	187	28	6.2	159	35.1	15	3.3	172	38.0	88	19.4	99	21.9	168	37.1	19	4.2	80	17.7	107	23.6
Intermediate risk	62	8	1.8	54	11.9	1	0.2	61	13.5	28	6.2	34	7.5	58	12.8	4	0.9	28	6.2	34	7.5
High risk	172	39	8.6	133	29.4	38	8.4	134	29.6	63	13.9	109	24.1	155	34.2	17	3.8	115	25.4	57	12.6
Total	453	82	18.1	371	81.9	56	12.4	397	87.6	199	43.9	254	56.1	413	91.2	40	8.8	239	52.8	214	47.2

Table 4. Univariate & multivariate cox proportional hazards model to predict factors associated with RFS in GISTs with operation only

Variable	Univariate		Multivariate	
	Hazard Ratio (95% CI)	*P* value	Hazard Ratio (95% CI)	*P* value
Total				
Age	1.414 (0.692-2.887)	0.342	0.883 (0.406-1.918)	0.753
Gender	0.413 (0.231-0.738)	0.003**	0.485 (0.267-0.881)	0.017*
Tumor site	1.728 (1.309-2.281)	< 0.001**	1.353 (1.004-1.823)	0.047*
Tumor size	4.280 (2.990-6.127)	< 0.001**	2.887 (1.939-4.299)	< 0.001**
Mitosis count	4.251 (3.180-5.681)	< 0.001**	2.351 (1.594-3.468)	< 0.001**
SSTR1	0.707 (0.373-1.339)	0.287	1.282 (0.567-2.896)	0.551
SSTR2	0.176 (0.100-0.310)	< 0.001**	0.333 (0.166-0.669)	0.002**
SSTR3	1.297 (0.754-2.230)	0.347	1.833 (0.955-3.519)	0.069
SSTR4	0.776 (0.281-2.147)	0.819	0.713 (0.250-2.036)	0.528
SSTR5	0.446 (0.255-0.782)	0.005**	0.892 (0.449-1.770)	0.743
High-risk sub-group				
Age	1.120 (0.524-2.393)	0.770	0.836 (0.367-1.906)	0.671
Gender	0.584 (0.315-1.081)	0.087	0.537 (0.287-1.007)	0.053
Tumor site	1.062 (0.770-1.466)	0.714	1.179 (0.850-1.636)	0.324
Tumor size	1.582 (1.001-2.500)	0.049*	1.752 (1.070-2.869)	0.026*
Mitosis count	2.060 (1.480-2.868)	< 0.001**	1.819 (1.201-2.756)	0.005**
SSTR1	0.878 (0.450-1.712)	0.702	1.253 (0.546-2.874)	0.595
SSTR2	0.236 (0.130-0.430)	< 0.001**	0.361 (0.181-0.721)	0.004**
SSTR3	1.108 (0.573-1.810)	0.951	1.759 (0.885-3.496)	0.107
SSTR4	0.635 (0.229-1.762)	0.383	0.782 (0.272-2.246)	0.648
SSTR5	0.508 (0.274-0.941)	0.031*	0.743 (0.360-1.530)	0.420

*, $P < 0.05$; **, $P < 0.01$.

of SSTRs in GISTs were shown in **Figure 1** and the clinicopathological characters for real-time PCR were shown in **Table 1**.

To confirm SSTRs expressions, we performed IHC study using TMAs that contained 453 GISTs (405 GISTs with operation only and 48 high-risk GISTs with imatinib adjuvant therapy). Representative stains of SSTR1-5 were shown in **Figure 2**. Positive expression proportion of SSTR1 and SSTR2 were 81.9% (371/453) and 87.6 % (397/405) in our study, which indicate high expression performance of SSTR1 and SSTR2 in GIST patients. Positive expression rates of SSTR3, SSTR4 and SSTR5 are 56.1%, 8.8% and 47.2% respectively in our study. These IHC results are similar to our real-time PCR findings. Clinicopathological characters of 453 GISTs could be referred from **Tables 2** and **3**. In 405 GISTs with operation only, sub-group study was designed based on modified NIH criteria. High-risk sub-group of GIST patients which suffered worst prognosis than very low, low and intermediate-risk was the most important population deserved attention in GISTs. Given the IHC reactivity of SSTRs observed, we focused remainder of our study on examining the prognostic value of SSTRs in GISTs.

SSTR2 acts as an independent prognostic indicator for GISTs

In GISTs with operation only, we found SSTR2 negative expression significantly associated with disease recurrence by univariate and multivariate cox proportional hazards model analyses ($P < 0.001$ and $P = 0.002$, **Table 4**). Further sub-group univariate and multivariate studies also showed SSTR2 was an independent prognostic indicator for high-risk GISTs ($P < 0.001$ and $P = 0.004$, **Table 4**). Kaplan-Meier survival analyses with log-rank test for RFS showed sharply decreased curves in SSTR2 negative expression tumors compared with positive cases in GISTs (**Figure 3A**) and high-risk sub-group (**Figure 3B**).

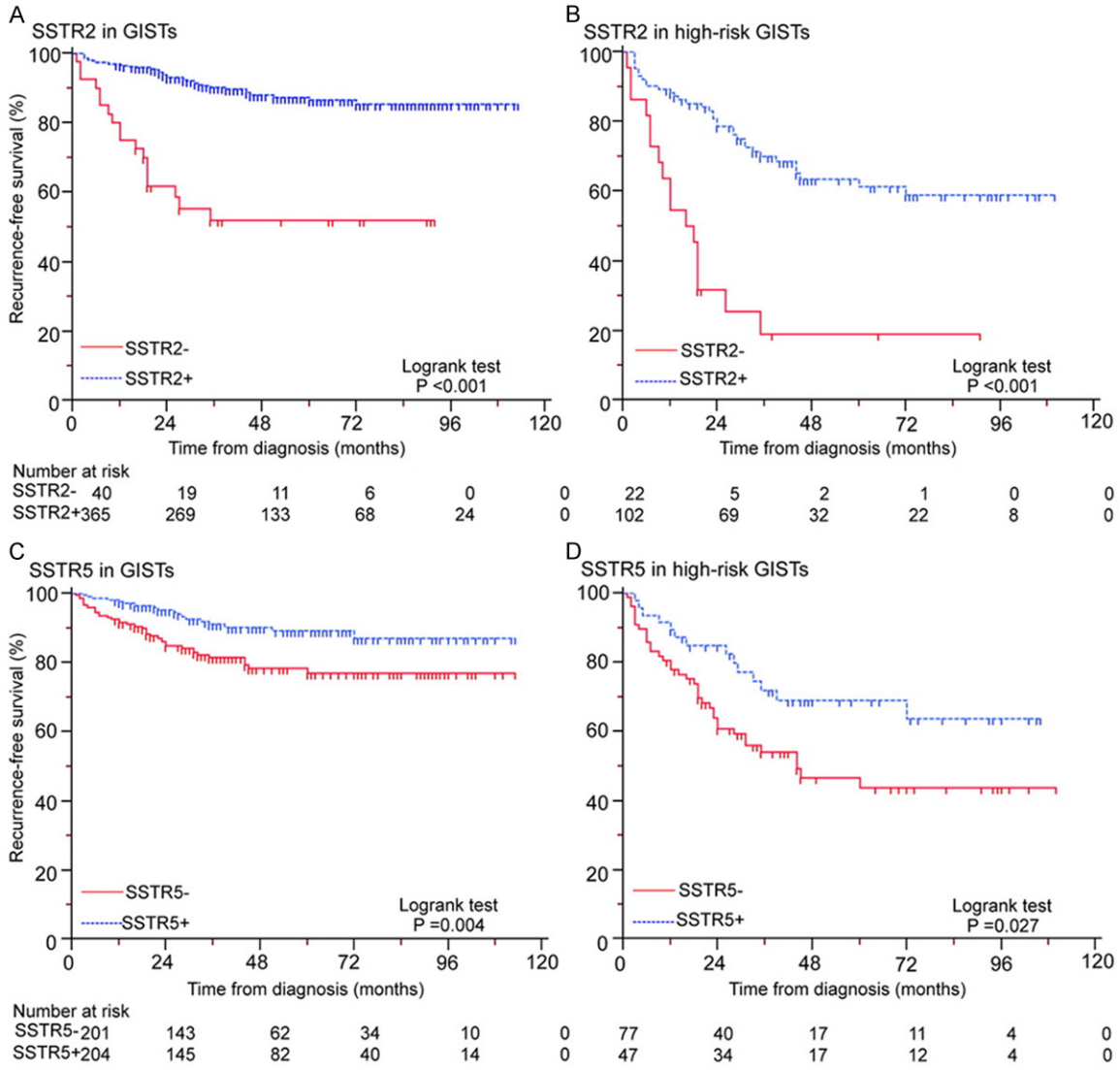

Figure 3. SSTR2 negative expression predicts poor prognosis in GISTs (A) and high-risk sub-group (B), and SSTR5 negative expression also predicts poor prognosis in GISTs (C) and high-risk sub-group (D).

SSTR5 negative expression can predict poor prognosis of GISTs

Kaplan-Meier survival analysis with log-rank test and univariate analysis show GIST tumors with SSTR5 negative expression were associated with decreased RFS when compared to cases expressed SSTR5 positively in GISTs and high-risk sub-group (**Figure 3C** & **3D**, **Table 4**), but there is no statistic significance between SSTR5 and RFS by multivariate analysis (**Table 4**).

Expressions of SSTRs show no relationship with efficacy of imatinib adjuvant therapy

Although negative expression of SSTR2 and SSTR5 can predict adverse prognosis in GISTs with operation only, no relationship was found between SSTRs and RFS in high-risk GIST patients with imatinib adjuvant therapy by univariate analysis (**Table 5**).

Discussion

SSTRs act as important roles in GEP-NETs with high expression levels for prognosis predicting and octreotide LAR treatment purposes [19, 22]. However, the studies about relationship between SSTRs and GISTs were very limited. Arne et al's research detected SSTR1-5 expression levels in 34 GISTs which presented 100% positive expression rates of SSTR1, 2 and relative lower expression rates of SSTR3, 4, 5 in GISTs [23]. Here, we present the first large-

Table 5. Univariate cox proportional hazard model to predict factors associated with RFS in high-risk GISTs with imatinib adjuvant therapy

Variable	Hazard Ratio (95% CI)	*P* value
Age	0.684 (0.188-2.498)	0.566
Gender	0.993 (0.332-2.974)	0.990
Tumor site	1.340 (0.701-2.561)	0.375
Tumor size	1.811 (0.704-4.659)	0.218
Mitosis count	1.514 (0.749-3.059)	0.248
SSTR1	1.435 (0.440-4.682)	0.550
SSTR2	0.466 (0.153-1.417)	0.179
SSTR3	0.616 (0.205-1.846)	0.387
SSTR4	1.688 (0.210-13.566)	0.622
SSTR5	1.170 (0.321-4.269)	0.812

scale characterization of SSTRs expression in GIST patients. Initial detection of GISTs mRNA expression were conducted by quantitative real-time PCR, and then 453 GIST cases (405 cases with operation only and 48 high-risk cases received imatinib adjuvant therapy after radical surgery) were confirmed by IHC on TMAs. SSTR1 and SSTR2 also present high positive proportion (81.9% and 87.6%) in 453 GISTs in our study, and positive expression rates of SSTR3, SSTR4 and SSTR5 are 56.1%, 8.8% and 47.2%, respectively.

The next we focused on examining the prognostic value of SSTRs in GISTs. Negative expression of SSTR2 and SSTR5 showed adverse prognosis of GIST patients by univariate cox model analysis and Log-rank test, furthermore, SSTR2 acted as an independent prognostic indicator in GISTs in our study by multivariate analysis. This result was similar in GEP-NETs, which indicated SSTR2 negative expression also as an independent adverse prognostic indicator although high positive expression rate presented [19, 22]. There were no significant associations for prognosis were found between SSTR1, SSTR3 or SSTR4 and GISTs, and no relationship between SSTRs and imatinib adjuvant therapy.

With high positive expression performance of SSTR1 (81.9%) and SSTR2 (87.6%), and approximately half of the GISTs expression SSTR3 (56.1%) and SSTR5 (47.2%) in our study, relative therapeutic strategies are reasonably considerable. Agitating SSTR1, 2, and 3 can transduce their antiproliferative functions by stimulating one or more PTPs, which have inhibitory effects on mitogenic MAPK and survival PI3K pathways [24]. Octreotide LAR, mainly agitate SSTR2 (IC50, 0.75 nM) and SSTR5 (IC50, 7 nM) [25], was demonstrated its antiproliferative effect in the randomized, double-blind, placebo-controlled PROMID study in patients with advanced intestinal NETs [18]. By application of pasireotide (SOM230), which is new generation of SST analog with high-efficiency of agitating abilities in SSTR1 (IC50, 9.3 nM), SSTR2 (IC50, 1 nM), SSTR3 (IC50, 0.5 nM) and SSTR5 (IC50, 0.16 nM) [25], 56.5% (13/23) patients with advanced NETs refractory or resistant to octreotide LAR showed stable disease status in a phase II study [26]. Like GEP-NETs, GISTs is also a relatively rare neoplastic disorder with limited therapeutic options. Such high positive expression performance of SSTR1 and SSTR2, and approximate half of the GISTs with SSTR3 and SSTR5 expression, may provide new possible choices by applications of octreotide or pasireotide LAR in selected advanced GISTs alone or accompany with current imatinib/sunitinib treatment procedure.

In summary, detection of SSTR2 and SSTR5 help to predict outcomes of GIST patients. Negative expression of SSTR2 is an independent adverse prognostic indicator in GISTs. High expression performance of SSTRs in GISTs may provide new therapeutic strategies in selected advanced GISTs.

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Disclosure of conflict of interest

None.

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