Immunohistochemical Expression of STAM2 in Gastrointestinal Stromal Tumors

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Abstract. *Background: Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the digestive tract, believed to originate from the interstitial cells of Cajal or their stem cell-like precursors. Recent studies incidentally found the expression in interstitial cells of Cajal of the signal-transducing adaptor molecule-2 (STAM2), which is an endosomal protein acting as a regulator of receptor signaling and trafficking. Here, we investigated the immunohistochemical expression of STAM2 in GIST. Materials and Methods: To evaluate the level of STAM2 expression, the percentage of cells staining positively for STAM2 and their staining intensity were graded on a scale of 0-3 and then multiplied to give the staining index as: 0=none; 1-3=low; 4-6=moderate and 9=high. Results: In 51 analyzed GIST samples, expression of STAM2 was observed in 45 cases (88.2%). Based on antibody screening, we observed a positive correlation between the expression of GIST marker stem cell growth factor receptor, also known as tyrosine-protein kinase KIT or CD117, and STAM2 expression ($r=0.387$, $p<0.003$). To identify possible STAM2 function in GIST, we performed correlation analysis between STAM2 expression and tumor size, primary tumor site, tumor type, mitotic count, Ki-67 proliferative index, risk stratification and development of recurrent/metastatic disease. Among these parameters, only correlation between the percentage of STAM2-positive cells and mitotic count was statistically significant ($r=-0.362$, $p<0.01$). Conclusion: Further studies are required to unravel the role of STAM2 in the oncogenic cell phenotype of GIST.*

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Key Words: Gastrointestinal stromal tumor, STAM2, KIT (CD117), immunohistochemistry.

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the digestive tract, recognized as a distinct entity after the demonstration of stem cell growth factor receptor (KIT or CD117)-activating mutations which they harbor. Until then, these tumors were classified as leiomyoma, leiomyosarcoma or schwannoma. They are most commonly found in the stomach and small intestine, but can arise anywhere along the gastrointestinal tract, and rarely in the omentum and mesentery (1). GISTs are believed to originate either from interstitial cells of Cajal, which normally generate the pacemaker activity for gastrointestinal motility, or from their stem cell-like precursors (2).

GISTs are intra-mural tumors which are histologically subclassified into spindle cell, epithelioid or mixed-type. Immunohistochemically, most GISTs (95%) exhibit a positive cytoplasmic/membranous reaction to KIT, which is the major diagnostic tool. The other markers, aside from discovered on gastrointestinal stromal tumors protein-1 (DOG1), which is expressed in almost all GISTs irrespective of the type of activating mutation, have limited relevance in routine practice owing to their low specificity (CD34, smooth muscle actin, S-100, desmin, heavy caldesmon, calponin, protein kinase C theta) (3-7).

On the molecular level, GISTs can harbour activating mutations of *KIT* (75-80%) and *PDGFRA* (5-7%), which encode the stem cell growth factor receptor (KIT) and platelet-derived growth factor receptor-α (PDGFRα) tyrosine kinases, respectively. These mutations lead to constitutive activation of the respective tyrosine kinase, with subsequent activation of downstream signaling pathways (8, 9).

The wide spectrum of biological behavior of GISTs, ranging from purely benign to characteristic sarcomas, and the introduction of imatinib – a tyrosine kinase inhibitor that blocks the activity of both KIT and PDGFRα – have emphasized the importance of patient risk stratification. Currently, the most accurate classification used to assess

patient prognosis after surgical resection and to select patients for adjuvant systemic therapy is described by Joensuu and is based on the tumor size, mitotic count, primary tumor site and tumor rupture (10).

Signal-transducing adaptor molecule-2 (STAM2) is an endosomal protein and a regulator of receptor signaling and trafficking. Together with STAM1 and the hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), STAM2 forms the endosome-associated complex ESCRT0 implicated in sorting of mono-ubiquitinated endosomal cargo for degradation in the lysosome. Since STAM2 is tyrosine-phosphorylated by a wide range of receptor tyrosine kinases (RTKs) and cytokine receptors (11, 12), it might also play a regulatory role in cell signaling, being at the intercross of signaling pathways and membrane transport in the cell. STAM2 operates downstream of janus tyrosine kinases (JAKs), and upon interleukin-2 and granulocyte-macrophage colony-stimulating factor stimulation, plays a role in signaling leading to DNA synthesis and Myc proto-oncogene protein induction (13).

Involvement of ESCRT complexes in cytokinesis very likely explains some of the diseases associated with impaired ESCRT functions (14-16). A growing number of experiments indicate that enhanced RTK signaling is associated with tumorigenesis. HRS, an ESCRT0 member and a STAM2-binding molecule involved in the vesicular transport of storing RTKs, was found to be highly expressed in several types of human cancer, such as those of stomach and colon (17).

A variety of signaling pathways and molecules have been shown to be required for normal development and function of the gastrointestinal system. We designed this study to investigate the expression of STAM2 in GIST due to the recent discovery of *STAM2* gene expression in the interstitial cells of Cajal (18) and the importance of the regulation of growth factor and cytokine signaling for the maintenance of a healthy gut.

Materials and Methods

Materials. The study included 51 consecutive cases of GIST diagnosed between 1999 and 2012 at the Department of Pathology, Sestre Milosrdnice Clinical Hospital Centre, Zagreb, Croatia. All cases were revised and the risk stratification was uniformed according to the classification set forth by Joensuu (10). There were 28 male and 23 female patients, with an age range from 20 to 85 years (mean=64.5 years). Most tumors were localized in the stomach (60.8%), followed by the small intestine (29.4%), large intestine (7.8%) and peritoneum (2.0%). Tumor size ranged between 0.4 and 25.0 cm (mean 6.8 cm). The number of mitoses per 50 high-power fields (HPF) ranged from 0 to 50 (mean 6/50 HPF). The proliferation rate was assessed immunhistochemically using Ki67 and ranged from 1.0 to 25.0% (mean 7.9%). Stratified by risk (10), most cases were in the low-risk group (41.2%), followed by high- (39.2%), intermediate- (13.7%) and very low-risk (5.9%). The majority of cases were histologically-subclassified as spindle cell type (68.6%), mixed type (29.4%) and only one epithelioid (2.0%). Only one case was immunohistochemically-negative for KIT (2.0%), two were focally positive (4.0%), and the rest were diffusely positive (94.0%).

Follow-up data were available for 23 patients, for a range of 1 to 133 months (mean 43 months). Among the follow-up group, 18 patients were well and without signs of recurrence, while three patients developed recurrent disease and two metastatic disease to the liver and pelvis (in the period of 6 to 40 months after surgery). Two patients died due to progression of GIST, while one patient died of causes unrelated to GIST. Five patients received chemotherapy following surgical excision lasting from 2 to 40 months.

Immunohistochemistry. Specimens were fixed in 10% buffered formalin, embedded in paraffin, cut at 5-μm thickness and stained routinely with hematoxylin and eosin.

Immunohistochemical staining was performed using standard procedures on a DAKO TechMate Horizon automated immunostainer (DAKO, Copenhagen, Denmark). The pre-treatment of sections was performed using Dako PT link (deparaffinization, rehydration and epitope retrieval). After blocking the endogenous peroxidase activity by 5 min incubation with 3% hydrogen peroxide, the sections were incubated with primary rabbit polyclonal antibodies (ab63372; Abcam, Cambridge, UK) against STAM2 (dilution 1:100) for 30 min at room temperature. The sections were then incubated with biotinylated immunoglobulin and peroxidase-labelled streptavidin (LSAB+ Kit, HRP; Dako). Color was developed by incubation with 3,3'-diaminobenzidine tetrahydrochloride and slides were counterstained by hematoxylin. Normal intestine was used as a positive control.

Immunohistochemical staining for KIT and Ki67 was performed routinely during pathohistological processing using standard procedures on a DAKO TechMate Horizon automated immunostainer. The results of expression were taken retrospectively from pathology reports.

The percentage of positive cells was scored in the entire tumor section at low magnification (×40), while the staining intensity was determined on chosen slides at medium (×100) and high (×400) magnifications. To evaluate the level of STAM2 expression, the percentage of positive staining cells (PPC) and the staining intensity (SI) were graded on a scale of 0-3: PPC was scored as 0 for no positive cells, 1 for up to 10% positive cells, 2 for 10-50% positive cells and 3 for more than 50% positive cells; SI was given as 0 for no staining,1 for weak staining, 2 for moderate staining and 3 for strong staining. For each sample, PPC and SI scores were multiplied to give the immunohistochemical staining index (ISI), which was labeled as following: 0=zero; 1-3=low; 4-6=moderate and 9=high. All samples were examined independently by three observers and any difference was resolved by joint review.

Statistical analysis. The results for continuous variables are presented as mean values and standard deviation, while categorical variables were presented as frequencies. Association between variables were analyzed by the Pearson's correlation coefficient. One-tailed test was used to evaluate the strength of the relationship between STAM2 and CD117 expression. *p*-Values of 0.003 or less were considered statistically significant. All other significance tests were two-tailed.

Table I. *Summary of immunohistochemical expression of signal-transducing adaptor molecule-2 (STAM2) in 51 samples of gastrointestinal stromal tumors.*

Percentage of positively-stained cells(PPC)				Staining index (SI) (%)				Immunohistochemical staining index#			
0	1	2	3	0	1	2	3	0	L	M	H
11.8	9.8	19.6	58.8	11.8	33.3	41.18	13.73	11.8	35.3	39.2	13.7

#(ISI=PPC×SI) (%). L:Low (ISI=0-3); M: moderate (ISI = 4-6); H: high (ISI=9).

Results

The expression of STAM2 was observed in 45 cases (88.2%), as a granular staining pattern in the cytoplasm (Figure 1). The marker was also expressed in interstitial cells of Cajal and gastric and intestinal glands. The results of immunohistochemical analysis are summarized in Table I.
Based on antibody screening we observed a positive correlation between the expression of GIST marker KIT and STAM2 expression (r=0.387, $p<0.003$). The correlation between PPC and SI of STAM2 was highly significant (r=0.723, $p<0.00000002$) as well.

To gain insight into possible STAM2 function in GIST, we performed correlation analysis between STAM2 expression (PPC, SI, ISI) and the following parameters: tumor size, primary tumor site, tumor type, mitotic count, Ki-67 proliferative index, risk stratification and recurrent/metastatic disease. Pearson's coefficients for those correlations ranged from −0.0127 to −0.328. The relatively low correlation coefficients indicate that results were not statistically significant and that no correlation could be established between STAM2 expression and clinical outcome. Only the correlation between the PPC for STAM2 and mitotic count was statistically significant (r=−0.362, $p<0.01$).

There was also no statistically significant correlation found between STAM2 expression in patients who developed recurrent or metastatic disease patients with disease-free follow-up (r=−0.08427).

Discussion

GISTs are generally KIT- and KIT ligand (stem cell factor)-positive mesenchymal tumors, which differentiates them from other smooth muscle tumors, nerve sheath tumors, inflammatory myofibroblastic tumors, undifferentiated sarcomas and others arising in the same location. Although KIT is highly specific for GIST, it is also expressed in other tumor types, particularly melanoma, small cell lung cancer, testicular teratocarcinoma and angiosarcoma (19, 20). Most GISTs exhibit diffuse cytoplasmic staining for KIT, but less commonly a membranous and dot-like (Golgi) staining pattern is seen. The latter is associated with *KIT* mutation and is more frequent in GISTs with homozygous mutations (21).

About 5% of GISTs are immunohistochemically-negative for KIT and these seem to have predilection for the stomach or omentum (22). In KIT-negative cases, other markers such as DOG1 should be considered, while molecular analysis of *KIT* and *PDGFRA* is diagnostic.

The presence of STAM2 in the interstitial cells of Cajal was recently shown in the gastrointestinal tract of the mouse (18). Our results in the present study reconfirmed its expression in human small and large intestine. Since GISTs are thought to derive from interstitial cells of Cajal lineage (2), we designed this study to investigate whether STAM2 is also expressed in GIST cells. This hypothesis was tested in a series of immunohistochemical studies on human GIST sections. Analysis of those samples indicated that *STAM2* was highly expressed in GIST cells, suggesting its possible involvement in tumorigenesis. Parallels in *STAM2* gene expression further support the relationship between GISTs and interstitial cells of Cajal, and the hypothesis that these cells could be the cell-of-origin of GISTs.

Interstitial cells of Cajal are specialized mesenchymal cells in the gastrointestinal tract, where they play a pacemaker role in coordinating intestinal peristalsis (23). These cells express KIT, which is a cytokine receptor belonging to the RTK family (23, 24), on the plasma membrane. KIT receptor, also known as a proto-oncogene, is important for normal Cajal cell development since activating mutations of this gene are associated with GISTs (25).

Human and mouse STAM2 were reported to be ubiquitously expressed in a variety of tissues and cell lines (26, 27) and to be involved in the down-regulation of mono-ubiquitinated RTKs (13). Degradation of ubiquitinated RTKs requires sorting from the early endosome to the multivesicular body and finally the lysosomes. Several ubiquitin-interacting molecules, including STAM2 and the large multiprotein complex ESCRT, carry out this process. Furthermore, an inappropriate activation of RTKs has been linked to a large number of pathological conditions and tumor types (28).

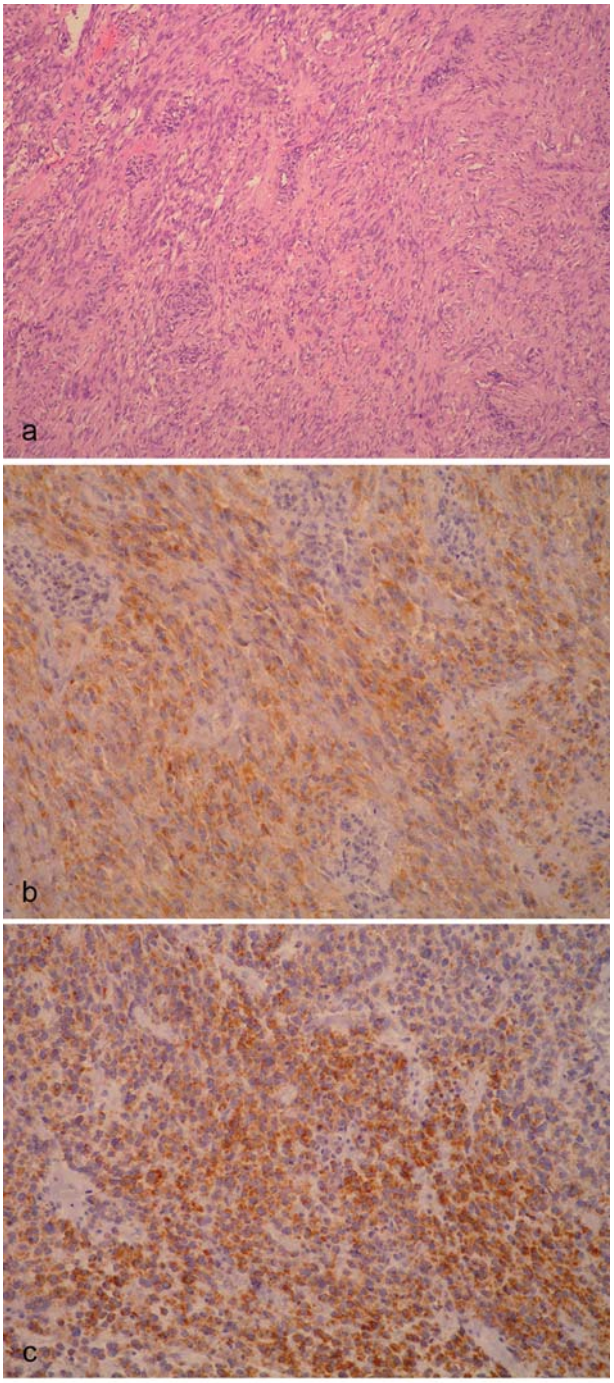

Figure 1. *Hematoxylin-eosin stained section of gastrointestinal stromal tumor (GIST) (a, magnification×100) and the sections of spindle cell type (b, ×200) and mixed type (c, ×200) of GIST with a high immunohistochemical staining index for signal-transducing adaptor molecule-2 (STAM2).*

STAM2-interacting protein HRS contributes to promoting malignant characteristics of cancer cells *in vivo* and *in vitro*. It is highly expressed in cancerous lesions of the human stomach, colon, skin, liver, and cervix (29). HRS plays a critical role in malignancy by regulating degradation of a tumor-suppressor protein E-cadherin (29). The loss of E-cadherin leads to the translocation of β-catenin from the cytoplasm to the nucleus, which then affects the T-cell factor/lymphoid enhancing factor transcription factors, subsequent activation of growth-promoting genes such as c-MYC (30). HRS is most likely involved in regulation of E-cadherin degradation through the lysosomal pathway. The loss of HRS leads to aberrant accumulation of E-cadherin, which suggests the mechanism that scavenges such aggregates may be impaired in affected cells (29).

STAM2 is also involved in the regulation of intra-cellular signal transduction for DNA synthesis and induction of proto-oncogene *c-MYC*. It has been shown that overexpression of STAM2 up-regulates *c-MYC*, which is mediated by some interleukins and growth factors, such as interleukin-2 and granulocyte-macrophage colony-stimulating factor (13). However, there are no data about *STAM2* expression in tumors *in vivo*.

Several further lines of evidence relate the ESCRT proteins to tumorigenesis. Tumor susceptibility gene 101 (*TSG101*), which is the ESCRTI component, was also found to be overexpressed in human tumors. It was originally identified as a tumor-suppressor gene. Reduced *TSG101* expression leads to metastatic tumors in mice, and mutations of *TSG101* were observed in many cases of acute myeloid leukemia and human prostate cancer, although later studies did not confirm these findings (31, 32). In this regard, the mechanisms underlying the positive contributions of *TSG101* to tumorigenesis have remained unclear. *TSG101* and some other ESCRT proteins share some features of the so-called 'two-faced' proteins, which can have dual roles and can regulate signaling pathways in seemingly opposite ways under different physiological conditions (32).

Altogether, these data strongly suggest that the ESCRT machinery, as well as STAM2, may be involved in tumor pathogenesis arising from defects in cytokinesis. However, the relative contribution of each ESCRT subunit in this process is likely to be dependent on cell type.

In our study, the expression pattern of STAM2 was granular and cytoplasmic, which is consistent with several previous findings that STAM2 is a protein localized in the cytosol and on the early endosome membrane (26, 33). There was only one KIT-negative GIST among our cases, which was also STAM2-negative.

STAM2 was found to be expressed in GIST cells of various histotypes, but whether high STAM2 expression in GIST cells is related to its specific function could not be confirmed. No correlation was found between immunohistochemical staining index and clinicopathological data. Correlation coefficients between *STAM2* expression and most of the changes in clinical outcome were low and non-significantly different.

Among all parameters, the only exception was mitotic count. A statistically significant negative correlation was found between percentage of *STAM2*-positive staining cells and mitotic count. The latter might suggest a possible oncosuppressive effect of STAM2 in GIST cells.

Further studies analyzing the signal transduction associated with STAM2 in GIST cells, with clinical follow-up on a larger number of patients, and the usage of more sophisticated methods are needed to uncover mechanisms by which STAM2 contributes to oncogenic cell phenotypes and to elucidate its possible prognostic and therapeutic value. It will be intriguing to determine whether STAM2 may be a novel marker of GIST.

Conflicts of Interest

The Authors declare no conflict of interest.

Acknowledgements

This work was supported in part by the Ministry of Science, Education and Sports, Republic of Croatia, grant number: 108-1081870-1902.

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Received January 29, 2014
Revised March 8, 2014
Accepted March 10, 2014