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High expression of the RNA-binding protein RBPMS2 in gastrointestinal stromal tumors

Ilona Hapkova a, b, Josef Skarda c, Caroline Rouleau a, d, An Thys e, Cécile Notarnicola a, Maria Janikova c, Florence Bernex a, Miroslav Rypka b, Jean-Marie Vanderwinden e, Sandrine Faure a, Jaroslav Vesely b, Pascal de Santa Barbara a, e

⁎ INSERM U1046, Université Montpellier 1, Université Montpellier 2, Montpellier, France
† Department of Pathophysiology, Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic
‡ Department of Clinical and Molecular Pathology, Faculty of Medicine and Dentistry, Palacky University and University Hospital, Olomouc, Czech Republic
§ CHU Montpellier, Service d’Anatomie Pathologique, Montpellier, France

Laboratory of Neurophysiology, Faculty of Medicine, Université Libre de Bruxelles, Brussels, Belgium

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ABSTRACT

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal neoplasms of the gastrointestinal tract and are often associated with KIT or PDGFRα gene mutations. GIST cells might arise from the interstitial cells of Cajal (ICCs) or from a mesenchymal precursor that is common to ICCs and smooth muscle cells (SMCs). Here, we analyzed the mRNA and protein expression of RNA-Binding Protein with Multiple Splicing-2 (RBPMS2), an early marker of gastrointestinal SMC precursors, in human GISTs (n = 23) by in situ hybridization, quantitative RT-PCR analysis and immunohistochemistry. The mean RBPMS2 mRNA level in GISTs was 42-fold higher than in control gastrointestinal samples (p < 0.001). RBPMS2 expression was not correlated with KIT and PDGFRα expression levels, but was higher in GISTs harboring KIT mutations than in tumors with wild type KIT and PDGFRα or in GISTs with PDGFRα mutations that were characterized by the lowest RBPMS2 levels. Moreover, RBPMS2 levels were 64-fold higher in GIST samples with high risk behavior than in adult control gastrointestinal samples and 6.2-fold higher in high risk than in low risk GIST specimens. RBPMS2 protein level was high in 87% of the studied GISTs independently of their histological classification. Finally, by inhibiting the KIT signaling pathway in GIST882 cells, we show that RBPMS2 expression is independent of KIT activation. In conclusion, RBPMS2 is up-regulated in GISTs compared to normal adult gastrointestinal tissues, indicating that RBPMS2 might represent a new diagnostic marker for GISTs and a potential target for cancer therapy.

Introduction

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal neoplasms of the gastrointestinal tract (Corless et al., 2011) and are highly resistant to conventional chemotherapy and radiotherapy. These tumors are characterized by the presence of activating mutations in KIT (75–80% frequency) or Platelet-Derived Growth Factor Receptor Alpha (PDGFRα) (5–10% of tumors), two genes encoding receptors for growth factors that are normally activated only in specific situations (Hirota et al., 1998). Imatinib mesylate, a small-molecular tyrosine kinase inhibitor that targets phosphorylation/activation of KIT and PDGFRα and also constitutively activated KIT and PDGFRα proteins, has proven efficient in GIST treatment (Joensuu et al., 2001); however, resistance to such therapy is increasing. Therefore, the development of new-targeted therapies is strongly encouraged (Renouf et al., 2009).

It is now largely documented that post-regulatory RNA events play crucial roles in modulating differentiation and remodeling of smooth muscle tissues (Notarnicola et al., 2012; Xin et al., 2009). RNA-protein complexes control multiple steps of this process, including mRNA cellular localization, splicing, translational regulation and degradation (St Johnston, 2005). For instance, the expression of an alternative splicing isoform of the natural killer (NK) cell receptor Nkp30 correlates with the prognosis of GISTs, independently from the KIT mutations (Delahaye et al., 2011). Moreover, RNA-binding proteins (RBPs), which play important roles in regulating RNA metabolism, may also be deregulated in diseases, particularly in cancers during the initiation and progression phases (van Kouwenhove et al., 2011). The RNA Recognition Motif (RRM) proteins form a large RBP family which includes also RNA-Binding Protein for Multiple Splicing-2 (RBPMS2), an early marker
of gastrointestinal smooth muscle precursor cells that we identified recently (Notarnicola et al., 2012). We showed that ectopic expression of RBPMS2 in differentiated SMCs hinders their ability to contract, favors their proliferation and leads to their dedifferentiation, demonstrating that RBPMS2 expression must be tightly regulated to avoid SMC dedifferentiation (Notarnicola et al., 2012).

These results and the fact that GISTs are thought to arise from Interstitial Cells of Cajal (ICCs) or from a mesenchymal precursor that is common to ICCs and smooth muscle cells (SMCs) (Faure and de Santa Barbara, 2011; Le Guen et al., 2009; Sanders et al., 2006) prompted us to examine the mRNA and protein expression of RBPMS2 in different categories of human adult GISTs and in the GIST882 cell line. We found that RBPMS2 is up-regulated in GISTs compared to normal adult gastrointestinal tissues.

**Materials and methods**

**Tumor samples and KIT and PDGFRA mutational analysis**

Paraffin-embedded tumor samples from primary GISTs of 23 adult patients before Imatinib treatment were collected in the Department of Clinical and Molecular Pathology of the Olomouc University Hospital (Olomouc, Czech Republic). The risk of clinically aggressive behavior was evaluated according to the consensus approach published by Fletcher and coworkers (Fletcher et al., 2002). Control samples were normal gastrointestinal tissue specimens isolated from adult epithelial-derived tumors. For KIT and PDGFRA mutational analysis, genomic DNA was extracted from the paraffin-embedded GIST samples using the QIAamp DNA FFPE Tissue Kit (Qiagen). Specific PCR primers were used.

### Table 1

<table>
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<th>Target transcript</th>
<th>Forward primer (5′–3′)</th>
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<td>GCT TCC GCA AGG AGT A</td>
<td>TCG TGG GCA TCT GCA TT</td>
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<td>PDGFRα</td>
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<td>CCC TCA CTC TCT TCT AAG GTC</td>
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<td>HPRT</td>
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<td>TGA CCA AGG AAA GCA AAG TCT G</td>
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**Fig. 1.** Expression of RBPMS2 transcripts in adult normal rectum and GIST samples. Hematoxylin–eosin (H&E) staining (upper panels) and in situ hybridization of smooth muscle layers sections from normal rectum and GIST samples (middle and lower panels show different magnifications of the same sections) with an anti-human RBPMS2 riboprobe. RBPMS2 transcripts are not detected in adult normal rectal musculature (arrows), whereas they are strongly expressed in tumor cells (red arrows). Bars, 50 μm (upper panels), 500 μm (middle panels) and 200 μm (lower panels).
to analyze KIT exons 9, 11, 13 and 17 and PDGFRA exons 12, 13, 17 and 18 as previously described (Willmore-Payne et al., 2005). The GIST tissue microarray (DA1, SuperBioChips Laboratories) contained 48 GIST and 9 normal adult gastrointestinal tissue samples. The microarray GIST samples were KIT-positive by immunodetection, but their KIT mutation status was unknown.

RNA isolation and quantitative RT-PCR

Total RNA was extracted from paraffin-embedded GIST samples using the High Pure RNA Paraffin kit (Roche Diagnostic). For quantitative RT-PCR analysis, gene expression levels were measured using the LightCycler technology (Roche Diagnostics). PCR primers (Table 1) were designed using the LightCycler Probe Design software (version 3.5) relative to standard curves. Data were represented as the mean level of gene expression relative to the expression of the reference gene GAPDH (Roche Diagnostic). For cell culture experiments, RNA isolation was done with the RNeasy Mini kit (Qiagen) and quantitative RT-PCR was performed using Power Sybergreen (Applied Biosystems). Data were represented as the mean level of gene expression relative to the expression of the reference genes ACTIN and GAPDH as a loading control. The human GIST cell line GIST882 was maintained in DMEM ( Gibco ) supplemented with 10% Fetal Bovine Serum (FBS), 2% Penicillin-Sterptomycin (Lonza) and incubated with 1 μM of Imatinib (inhibitor of KIT activity) (LC Laboratory) or 5 μM of U0126 (MEK inhibitor) (Sigma-Aldrich), as previously described (Gromova et al., 2011). The human Embryonic Kidney 293 (HEK293) cell line was grown in DMEM supplemented with 10% FBS and transfected, using JetPEI-TEX (Polyplus, France), with 5 μg of a construct in which the full length human RBPM2 cDNA was subcloned in the pc32 vector with an in frame N-terminal HA tag and the CMV promoter. Cells were analyzed after 24 h. For western blot analyses, cells were lysed and protein extracts (20 μg) were separated on 10% polyacrylamide gels (Bio-Rad Laboratories), transferred onto nitrocellulose membranes (Amersham Hybond-ECL) and incubated with anti-RBPM2 (home-made), anti-Tubulin (Abcam) and anti-α-HA (Santa Cruz Biotechnologies) primary antibodies overnight. After several washes, membranes were incubated with the relevant horseradish peroxidase-conjugated secondary antibodies (Perkin Elmer). Detection was performed by chemiluminescence (Santa Cruz Biotechnologies) on Kodak films. Tubulin expression served as a loading control.

Immunohistochemistry and in situ hybridization

Immunofluorescence and immunohistochemistry analysis of paraffin-embedded GIST sections were performed as described (Rouleau et al., 2009; Rouleau et al., 2012). Briefly, sections were deparaffinized with Histoclear (VWR, France) and rehydrated through a series of graded alcohols to PBS. For immunodetection, heat-induced antigen retrieval was carried out in 10 mM sodium citrate solution (pH 9.0). Endogenous peroxidases were inactivated by incubation in 3% O2 (Sigma-Aldrich) for 30 minutes. Anti-RBPM2 (home-made), -KIT (NeoMarker), -αSMA (Sigma-Aldrich), -CD34 (Clinsciences), -S100 (Clinsciences), -KIT (Zymed), -Desmin (Euromedx) and

Table 2

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<th>Site</th>
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<th>Size (mm)</th>
<th>Risk</th>
<th>KIT or PDGFRA mutations</th>
<th>KIT RT-PCR</th>
<th>RBPM2 RT-PCR</th>
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<td>++</td>
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<td>WT</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
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<td>Low Risk</td>
<td>WT</td>
<td>+</td>
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<td>WT</td>
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<td>+</td>
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<td>WT</td>
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<td>++</td>
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<td>Exon 11 del/ins KIT</td>
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**Fig. 2**: Clinicopathological features and RBPM2 mRNA expression in the adult GIST cohort. (A) Results of immunohistochemical staining for KIT (CD117), TMEM16A, CD34, αSMA (smooth muscle actin), DESMIN and S100 (n = 23 GIST samples). (B and C) KIT (B) and PDGFRA (C) expression level by quantitative RT-PCR in GIST samples (n = 20) and in normal samples from different regions of the gastrointestinal tract as controls (n = 5). KIT and PDGFRA mut., GIST samples with a mutation in KIT or PDGFRA; GIST WT, GIST samples with wild type KIT or PDGFRA; NA, not available. (D) RBPM2 transcript level was determined by quantitative RT-PCR in GIST (n = 20) and in non-tumor samples from different regions of the gastrointestinal tract (n = 5, control). GIST WT, GIST samples with wild type KIT or PDGFRA; RBPM2 RT-PCR, RBPM2 RT-PCR in GIST samples with a mutation in KIT or PDGFRA. (E) Comparison of the RBPM2 transcript levels in the GIST and non-tumor samples described in A with the Mann–Whitney test (p < 0.001). (F) Mean expression level of RBPM2, KIT and PDGFRA in GISTs that were classified based on their KIT and PDGFRA mutational status. (G) Mean expression level of RBPM2, KIT and PDGFRA in GISTs that were classified according to the risk of aggressive behavior.
### Table A

<table>
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<tr>
<th>Immunohistochemistry</th>
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<th>Negative, n (%)</th>
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<td>S100</td>
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### Graphs

- **Graph B**: Fold change in KIT relative expression across different categories.
- **Graph C**: Fold change in PDGFRA relative expression across different categories.
- **Graph D**: Fold change in RBM4252 relative expression across different categories.
- **Graph E**: Levels of RBM4252 relative expression.
- **Graph F**: Fold change in RBM4252 relative expression for GISTs with KIT mutation, GISTs with PDGFRA mutation, and GISTs WT.
- **Graph G**: Fold change in RBM4252 relative expression for GISTs Low Risk, GISTs High Risk, and Control.
TMEM16A (also called DOG1) (Abcam) primary antibodies were used. Specific mouse or rabbit anti-IgG biotinylated secondary antibodies were used with the avidin–peroxidase reagent (Vector) and antibody reactions were detected with 3,3′-Diaminobenzidine (Sigma-Aldrich). As control, each GIST sections were tested without primary antibodies. Hematoxylin and Eosin (H&E) staining was performed.

**A**

Human RBPMS2 protein

MSNLKPDGEHGGSTGTGSGAGSSEGGALEEEVRTLFVSGLPVDKPRELYLFRPF
KYGEGSLIKLTAQRPVGFVIPDSCRAAGAEAAKNALNIGIRFDPENPQTLLEFAKA
NTKMAKSKLMATPNSVHPALGAHFIARDPYPYDLMGALIPASPEAWAPYPLOY
TTELTPAHAAFTYPTATAAAAALHAQVRWYPSSDTQQGWKRYRQFC

**B**

![Immunohistochemistry Images](image_url)

**C**

![Immunohistochemistry Images](image_url)

**D**

![Immunohistochemistry Images](image_url)

**E**

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<td>20 (87)</td>
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**F**

![Immunohistochemistry Images](image_url)
according to standard procedures. In situ hybridization experiments using paraffin sections were carried out as described (Come et al., 2006; Notarnicola et al., 2012). Anti-sense riboprobes were generated by PCR amplification of human RBPMS2 cDNA using specific primer sets and subcloned in pGEM T Easy Vector (Promega, France) as previously published (Notarnicola et al., 2012). Images were acquired using a Nikon-AZ100 stereomicroscope and a Carl-Zeiss AxioImager microscope.

**Statistical analysis**

Statistical analysis was carried out using the Mann–Whitney test as previously described (Notarnicola et al., 2012).

**Results**

**RBPMS2 transcripts are detected in adult GISTs**

To determine whether the RNA-binding protein RBPMS2 was expressed in GISTS, we first analyzed by in situ hybridization a commercial tissue microarray that included 48 KIT-positive GISTs (from low to high risk) and 9 normal adult gastrointestinal tissues (Fig. 1). The level of RBPMS2 transcripts was very low in smooth muscles of normal adult gastrointestinal tissues (black arrows, Fig. 1). Conversely, RBPMS2 was strongly expressed in 36 of the 48 GIST samples (75%), independently of their localization and the risk of aggressive behavior (red arrows, Fig. 1).

**Clinicopathological features of the GIST cohort**

Due to the limited clinical data and the absence of information on the KIT and PDGFRA mutational status for the GISTs included in the microarray and in order to better characterize RBPMS2 expression in GISTS, we analyzed GIST samples from a cohort of patients from Olomouc University Hospital. The group included 16 males and 7 females (male/female ratio: 1.44) with a mean age of 63.4 years (range: 36 to 81 years) who had exclusively primary GISTs located in the esophagus (n = 4), stomach (n = 5), small intestine (n = 8), rectum (n = 3), colon (n = 2), and soft tissues (n = 1) (Table 2). All GISTs included in this study were characterized by using classical histopathological and immunohistochemical approaches with anti-KIT, -SMA, -CD34, -Desmin, -S100 and -TMEM16A (DOG-1) antibodies (Fig. 2A; and data not shown). GISTS were divided in spindle (n = 11), epithelioid (n = 4) and mixed cell (epithelioid and spindle) (n = 8) tumors based on the cell morphology (Table 2). Mitotic cells were detected with the anti-Ki67 antibody (summary in Fig. 2A) and tumors were classified as low risk (n = 10) or high risk (n = 13) as previously described (Miettinen and Lasota, 2006). KIT and PDGFRA mutational analysis was available for 21 GISTS and showed that 10 tumors (47.6%) had KIT mutations (three GISTS with KIT mutations in exon 9, six tumors with mutations in exon 11 and one tumor with a mutation in exon 13), four (19%) had a PDGFRA mutation (one missense and two silent mutations in exon 11, one missense mutation in exon 12) and seven (33.4%) had wild type (WT) KIT and PDGFRA (Table 2). Quantitative RT-PCR analysis of KIT and PDGFRA expression levels indicated, as previously published, that in all the analyzed GIST samples (n = 20) KIT and PDGFRA were up-regulated in comparison to normal gastrointestinal samples of adult colon, stomach and small intestine (Fig. 2B and C).

**RBPMS2 is strongly expressed in malignant GISTS**

We then analyzed the levels of RBPMS2 transcripts in this GIST cohort and in gastrointestinal control samples by quantitative RT-PCR. In most of the analyzed GIST samples (19 of 20) RBPMS2 expression level was higher than in control samples (Fig. 2D; Table 2) and the mean RBPMS2 level in GISTs was 42-fold higher than in control samples (p < 0.001) (Fig. 2E). The level of KIT and PDGFRA did not significantly correlated with the amount of RBPMS2 expression in the tumors (compare Fig. 2B and C and D; data not shown). Conversely, RBPMS2 expression was higher in GISTS harboring KIT mutations than in tumors with wild type KIT and with wild type KIT or PDGFRA mutations (Fig. 2F). GISTS with PDGFRA mutations were characterized by the lowest RBPMS2 expression levels (Fig. 2F). Finally, RBPMS2 levels were 64-fold higher in GIST samples with high risk of aggressive behavior than in adult control digestive samples (Fig. 2G) and 6.2-fold higher in high risk than in low risk GISTS (Fig. 2G).

**RBPMS2 protein is highly expressed in adult GISTS**

In order to examine RBPMS2 protein expression, we generated antibodies directed against the C-terminus of human RBPMS2 (Fig. 3A). The efficiency and the specificity of these anti-RBPMS2 antibodies were confirmed by western blot and immunofluorescence analyses in HEK293 cells that express HA tagged RBPMS2 (Fig. 3B, and C). In western blots, the anti-RBPMS2 antibody identified a single band of 27 kDa, corresponding to the predicted size of human RBPMS2 fused to the HA tag (Fig. 3B). In addition, this band was also detected with the anti-HA antibody (Fig. 3B). Moreover, the anti-RBPMS2- and — HA antibodies both detected an epitope localized in the cytoplasmic compartment of such cells (Fig. 3C). Finally, the RBPMS2 signal was specifically lost when the anti-RBPMS2 antibody was pre-incubated with the RBPMS2 C-terminal peptide used to produce the antibody (data not shown).

We then examined the expression of RBPMS2 by immunohistochemistry in control gastrointestinal and GIST samples (Fig. 3D–F). RBPMS2 was faintly but reproducibly detected in the gastrointestinal musculature with the exception of the myenteric plexus (arrow, Fig. 3D). In contrast, it was strongly detected in 87% of the analyzed GIST samples (Fig. 3E) and the expression level was comparable in spindle, epithelioid and mixed cell tumor variants (Fig. 3F).

**RBPMS2 expression and regulation in GIST882 cells**

To position RBPMS2 in the KIT signaling pathway in GISTS, we first compared RBPMS2 expression in GIST882 (a GIST cell line homozygous for the oncogenic KIT mutation K641E with strong KIT expression and high level of KIT activity) (Tuveson et al., 2001), HELa and 1321N1 (human astrocytoma) cells as well as in two prostate cancer cell lines (PC3 and LNCaP). RBPMS2 mRNA level was relatively high in

![Fig. 3. RBPMS2 protein expression in adult GISTS. Characterization of the anti-human RBPMS2 antibody by immunofluorescence and western blotting (A–C). (A) Sequence of the human RBPMS2 protein (amino acids 1–209). Amino acids 195–207 of the C-terminus (in red) correspond to the sequence of the synthesized peptide used for producing the anti-RBPMS2 antibody. (B) Western blot analysis of HEK293 cells following transfection of empty vector (lane 1), the HA-TC10 (lane 2) or the HA-RBPMS2 construct (lane 3). 20 μg of whole protein extracts were processed for each condition. Tubulin (50 kDa) was used as loading protein control. HA-RBPMS2 was detected as a 27 kDa band with both anti-HA and anti-RBPMS2 antibodies. In addition, lower band corresponding to a C-terminal product of degradation was observed with only anti-HA antibodies. (C) HEK293 cells were transfected with the HA-RBPMS2 construct and then processed for immunofluorescence analysis using mouse anti-HA and anti-RBPMS2 primary antibodies and anti-mouse Alexa488 and anti-rabbit Alexa555 IgG, respectively. Nuclei were stained with Hoechst. Cytoplasmatic localization of the signal is observed with both antibodies. (D) Representative example of RBPMS2 protein expression in a colonc, high risk GIST sample. Histological staining (H&E) and labeling with anti-KIT (CD117) and anti-RBPMS2 antibodies. In normal colon (control), RBPMS2 is expressed in vessels and the enteric myenteric plexus (arrow), but not in visceral SMCs. (E) Summary of KIT (CD117) and RBPMS2 protein expression in the GIST cohort (n = 23). NA, not available. (F) Analysis of the expression of RBPMS2 in the GIST samples that were classified based on their morphological features (spindle, mixed and epithelioid cells). Arrowhead shows vessels.
of mesenchymal cells that will give rise to GIST tumors. A thorough analysis of KIT/ PDGFRA mutational status, risk of aggressive behavior and histological pattern (spindle, epithelioid and mixed cell phenotype).

We found that RBPM2 mRNA and protein expression was significantly higher in GIST samples than in control gastrointestinal tissues, particularly in high-risk tumors. The levels of KIT and PDGFRA did not significantly correlate with the amount of RBPM2 expression. However, RBPM2 mRNA levels were higher in GISTS harboring KIT mutations than in tumors with PDGFRA mutations or with wild type KIT and PDGFRA. This difference could be due to the different origins of KIT and PDGFRA expressing cells and would suggest that RBPM2 and KIT expression are linked. Moreover, in the GIST882 cell line that carries the KIT mutation K641E and shows high KIT activity, inhibition of KIT activity using Imatinib treatment did not alter the expression of RBPM2. In contrast, using primary embryonic digestive smooth muscle cultures we found that RBPM2 over-expression induces a 2-fold increase of KIT mRNA level (Notarnicola et al., 2012; Notarnicola and de Santa Barbara, unpublished data). All these data suggest that KIT and RBPM2 could be expressed in the same mesenchymal cells during the early development and remodeling of mesenchymal-derived gastrointestinal cells, or that they are part of the same signaling pathway, or share a regulatory circuitry. More experiments are needed to confirm these hypotheses and to position RBPM2 relative to the KIT signaling pathway.

Chromosomal alterations during GIST progression have been described and could be involved in GIST prognosis (Corless et al., 2011). Specifically, losses and gains on chromosome 15 between 15q22.1 and 15q25.3 have been clearly correlated with poor outcome (Ylipää et al., 2010). As the RBPM2 gene is localized on chromosome 15q22.31, we can hypothesize based on our results that elimination of a negative regulatory sequence necessary for correct RBPM2 expression could alter the endogenous expression of RBPM2, thus favoring dedifferentiation of mesenchymal cells that will give rise to GIST tumors. A thorough characterization of the correlation between alterations of this genome region and RBPM2 expression is now required.

In conclusion, our study shows that most of the analyzed GISTS are characterized by abnormally elevated expression of RBPM2, suggesting that RBPM2 could be an indicator for tumor progression and a potential target for cancer therapy in GIST.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Contribution to authorship

Immunohistochemistry and in situ hybridization were carried out by IH with the help of CR, FB and SF under the supervision of PdSB. GIST collection and analyses were carried out by JS, MJ, and MR under the supervision of PdSB and JV. GIST cell line analyses were carried out by AT under the supervision of JMV. PdSB wrote the paper.

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