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Overexpression of High Mobility Group Box 1 in Gastrointestinal Stromal Tumors with KIT Mutation

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ABSTRACT

Gain-of-function mutations of KIT are common genetic events in gastrointestinal stromal tumors (GISTs). To investigate the molecular characteristies of KIT mutations in GISTs, 20 GISTs (14 GISTs with KIT mutation and 6 GISTs without KIT mutation) were analyzed by two-dimensional electrophoresis and matrix-associated laser desorption ionization mass spectrophotometry-time of flight. Comparative analysis of the respective spot patterns on two-dimensional electrophoresis showed that HMGB1, an intranuclear protein that interacts with several transcription factors and plays a role in tumour metastasis after its secretion, was overexpressed in GISTs with KIT mutation. All of the 14 GISTs with KIT mutation, and only 2 of 6 GISTs without KIT mutation, revealed HMGB1 expression. Of the GISTs with KIT mutation, 12 (86%) showed strong expression of HMGB1, more than three times higher in intensity than the maximum observed in the 6 GISTs without KIT mutation by two-dimensional electrophoresis analysis. The overexpression of HMGB1 was further supported by Western blot analysis, and directly related to matrix metalloproteinase 2 overexpression. Our results indicate that the overexpression of HMGB1 is common in GISTs and is related to the KIT mutation, and that this may play a role in the tumorigenesis of GISTs because overexpressed HMGB1 could accelerate genes related to tumor growth and invasion.

INTRODUCTION

GISTs are the most common mesenchymal tumors composed of uncommitted mesenchymal cells (1). Recent studies have described the morphological and immunohistochemical similarities between the ICCs and GISTs (2, 3). Immunohistochemically, tumor cells of GISTs characteristically express CD34 and KIT, ICCs are located in and near the circular muscle layer of the stomach and intestine, thus suggesting that GISTs originate from stem cells that differentiate toward ICCs. Among the molecular genetic changes reported to be related to GISTs, gain-of-function mutations of the KIT proto-oncogene are the most frequent and important (3–5). KIT encodes a type III receptor kinase (6), the ligand of which is stem cell factor (7). Mutations in KIT result in ligand-independent kinase activity and autophosphorylation of KIT (8, 9). The KIT mutation is known to be present in 30–92% of GISTs (5, 10–12). Mutations are reported to be most frequent in exon 11 and rare in exons 9 and 13. Deletions, insertions, and point mutations have been reported, and mutations of exon 11 and exon 13 were reported to be gain-of-function mutations (3, 5, 11). Recent studies have also demonstrated that STI571, an inhibitor of tyrosine kinase, was effective at treating GISTs (13).

Although there is much experimental and clinical evidence that gain-of-function mutations of KIT evoke uncontrolled cell proliferation and stimulate downstream signaling pathways, the specific downstream pathways related to tumor development and/or progression of GISTs and the target molecules have not been elucidated. We, therefore, carried out a proteomic study on 20 GISTs and compared the results with respect to the KIT mutation status.

MATERIALS AND METHODS

Tissue Samples. Twenty GISTs of the stomach were included in this study. All of the cases were identified prospectively and consecutively in the Department of Pathology at Yonsei University Medical Center between September 1995 and November 2000 for molecular marker studies. Among these 20 cases, 14 had previously been analyzed for chromosomal changes (14). Information from hospital charts and clinicians was obtained on demographic features and tumor sites. The patients included 9 females and 11 males ranging in age from 35 to 79 years (Table 1). Thirteen colorectal carcinomas were also selected for comparison.

For DNA and protein extraction, fresh tumors and adjacent nontumorous smooth muscle or mucosal tissues were obtained immediately after surgical excision and were stored at −70°C before use. To enrich the tumor cell population, areas with more than 90% of tumor cells were selected from H&E-stained slides using the cryostat microdissection technique. Genomic DNA was prepared using the SDS-proteinase K and phenol-chloroform extraction method.

Conventional pathological parameters (tumor size, number, differentiation) were examined prospectively without prior knowledge of the molecular data. The GISTs were divided into three groups according to the criteria of Lewin et al. (1). The guidelines for the diagnosis of gastric stromal tumor malignancy are composed of two unequivocal factors (histologically confirmed metastasis and invasion of adjacent organs) and seven high-risk factors (larger than 5.5 cm in diameter; more than five mitoses per 50 high power fields; presence of tumor necrosis; nuclear pleomorphism; dense cellularity; microscopic invasion into the lamina propria or blood vessels; and the presence of alveolar or cell balls). Tumors having more than one unequivocal or two high-risk features were categorized as malignant GISTs, tumors having only one high-risk feature were categorized as borderline GISTs, and tumors having neither unequivocal nor high-risk features were categorized as benign GISTs. According to these criteria, 4 cases were categorized as benign, 10 cases as borderline, and 6 cases as malignant GIST (Table 1).

KIT Mutation Analysis. Somatic mutations in exons 9, 11, 13, and 17 of KIT were detected using PCR-based assay with previously described primers (5, 10, 11). PCR was carried out in a mixture of 20 µl containing 1.5 mM MgCl2; 20 pmol of primer; 0.2 mM each dATP, dGTP, and dTTP; 5 mM dCTP; 1 µCi of [α-32P]dCTP (3000 Ci/mmol; DuPont New England Nuclear, Boston, MA); 50 ng of sample DNA; 1× PCR buffer; and 1.25 units of Taq DNA polymerase (Life Technologies, Inc., Grand Island, NY). After denaturation at 95°C for 5 min, DNA amplification was performed for 25–30 cycles. Each cycle consisted of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, and elongation at 72°C for 15 s. PCR products were separated in 6%
polycrylamide gels, followed by autoradiography for single-strand conforma-
tional polymorphism analysis. The PCR products were also sequenced using an
ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Two-Dimensional Electrophoresis. GIST tissues were suspended in sample
buffer containing 40 mM Tris, 7 M urea, 2 M thiourea, 4% 3-[3-Cholami-
dopropyldimethylammonio]-1-propane sulfonate (CHAPS), 100 mM 1,4-
dithioerythritol, and protease inhibitor cocktail (Roche, Mannheim, Germany).
Suspending solutions were sonicated for 30 s and centrifuged at 100,000 × g for 45
min. One mg of total GIST protein was used for each electrophoresis. Aliquots of proteins in sample buffer were applied onto immobilized pH 3–10 nonlinear
gradient strips (Amersham Pharmacia Biotech, Uppsala, Sweden). Isoelectric
focusing was conducted at 80,000 Vh. The second-dimension electrophoresis was carried out in 9–16% linear gradient polyacrylamide gels (18 cm × 20
cm × 1.5 mm) as described previously (15). After protein fixation in 40%
methanol and 5% phosphoric acid for 12 h, gels were stained with Coomassie
Blue G250 for 24 h. Gels were destained with H2 O and scanned in a Bio-Rad
G710 densitometer, and data were converted into electronic files, which were
analyzed with Melanie III computer software (GenBio, Geneva, Switzer-
land).

Identification of Protein Spots. For mass spectrometry fingerprinting,
protein spots were directly cut out of the gels, destained, and treated with
trypsin. Aliquots of the peptide mixtures obtained from trypsin treatment were
applied onto a target disk and allowed to air-dry. Spectra were obtained using
an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Protein database searching was performed with MS-Fit5 using
the homogenate was centrifuged for 30 s. The supernatant was collected and
used for cytoplasmic protein assay. The pellet was resuspended in 100
μl of 10% NP40 were added. After vigorous vortexing for 10 s,
and centrifuged at 30,000 × g for 30 min. The supernatant was collected and
used for nuclear protein assay. Twenty μg of total protein lysates and cytoplasmic extracts and 5 μg of nuclear protein extracts were loaded into each lane, size-fractionated by SDS-PAGE, and
transferred to a polyvinyldene difluoride membranes that were blocked with
Tris-buffered saline-Tween 20 containing 5% skim milk. Primary antibodies,
KIT (C-19, polyclonal; Santa Cruz Biotech, Santa Cruz, CA), HMGB1 (poly-
clonal; BD Biosciences, Franklin Lakes, NJ), MMP2 (42–5D11, monoclonal;
Calbiochem, Temecula, CA), glyceraldehyde-3-phosphate dehydrogenase
(G3PDH; polyclonal; Trevigen, Gaithersburg, MD) were diluted 1:5000 in
blocking buffer and incubated for 1 h at room temperature. After washing,
membranes were incubated for 1 h with horseradish peroxidase-conjugated
secondary antibody (Amersham Pharmacia Biotech), washed, and then developed
with ECL-Plus (Amersham Pharmacia Biotech).

Immunohistochemical Analysis. Formalin-fixed and paraffin-embedded
tissues were used for the immunostaining of KIT and HMGB1. Deparaftiniza-
tion and rehydration were performed using xylene and alcohol. The sections
were treated with 0.3% hydrogen peroxide for 3 min and with blocking
antibody for 30 min. The same primary antibodies used for Western blotting
were applied in immunohistochemistry. The antibody against KIT protein was
used at 1:1000 (v/v), whereas the antibody against HMGB1 was at 1:100
(v/v). Avidin-biotin complex methodology was used. The chromogen was
diaminobenzidine, and counterstaining was done with methyl green. The
evaluation of KIT was categorized as expressed and absent. The expression of
HMGB1 was categorized as overexpressed and normal. Cases with definite
nuclear staining in more than 30% of the tumor cells and/or cytoplasmic
expression were categorized as overexpressed, and cases with definite nuclear
staining in less than 30% of the tumor cells were categorized as normal.

RESULTS

Mutation Status of KIT. KIT mutations were found in 14 of 20
tumors. All of the mutations were present in exon 11. No mutations
were found in exons 9, 13, or 17. Deletion mutations were most
frequent and were found in eight cases, whereas point mutations were
found in four cases and insertions in two cases. Large deletions were
present between codons 550 and 558, and single amino acid deletions
were present at codons 560 and 579. The point mutations found were
V557R, V559D, and V559A, which have been previously reported in
GISTs.

Table 1 Clinico pathological features, KIT mutation status, and HMGB1 expression levels of GISTs

<table>
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<th>Sample no.</th>
<th>Tumor size (cm)</th>
<th>Tumor type</th>
<th>Anatomic site</th>
<th>Sex</th>
<th>Age</th>
<th>Western blot</th>
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<td>2.3</td>
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* 2DE, two-dimensional electrophoresis; del, deletion; ins, insertion.
Two-Dimensional Electrophoresis Analysis of GISTs. Proteome analysis was performed on GISTs by high-resolution two-dimensional electrophoresis. More than 1000 protein spots were detected on the two-dimensional electrophoresis gels and localized in the ranges of \( pI 3-10 \) and a relative molecular mass of \( 10-200 \) kDa. We were able to identify more than 250 protein species in two-dimensional electrophoresis gels using MALDI-TOF spectrometry. Because matched normal tissues with the same histogenetic origin cannot be obtained in GISTs, we performed computer-assisted comparative analysis of the respective Coomassie Blue spot patterns of GISTs with and without KIT mutation or of benign and malignant GISTs. Accordingly, we found that HMGB1 [formerly named HMG1 (Ref. 18; Fig. 1A] was overexpressed in GISTs with KIT mutation.

Identification of HMGB1 as a Tumor Marker of GISTs with KIT Mutation. We focused on the expression of HMGB1 because some of the reported functions of HMGB1 are related to tumor growth and metastasis. Both the \( pI \) and the relative molecular mass of HMGB1 in each two-dimensional electrophoresis gel were determined by comparison with standard two-dimensional electrophoresis gels from the Swiss-Two-Dimensional-PAGE database. As shown in Fig. 1B, HMGB1 was overexpressed in GISTs with KIT mutation compared with the GISTs without KIT mutation. The relative expression level of HMGB1 in all 20 of the samples analyzed by two-dimensional electrophoresis was quantified using Melanie III computer software (GenBio). In the 14 GISTs with KIT mutation, all of the cases showed HMGB1 expression and 12 (86%) showed strong expression level, more than three times higher in intensity than the maximum observed in the six GISTs without KIT mutation (case 16). In the six GISTs without KIT mutation, four cases showed no detectable HMGB1 expression, and the remaining two cases showed weak expression. The expression of HMGB1 in colorectal carcinomas and matched normal mucosae was ubiquitous; however, marked variations were found. Of the 13 colorectal carcinomas, all of the tumors and matched normal mucosae showed detectable HMGB1 expression, and seven tumors showed more than three times higher intensity than the matched normal mucosae. Comparison of HMGB1 expression by volume percentage showed higher level expression in colorectal carcinomas and GISTs than in the nontumorous colon mucosae; the average volume percentage was 0.23 ± 0.19 for GISTs, 0.31 ± 0.14 for colorectal carcinomas, and 0.08 ± 0.06 for nontumorous colon mucosae. To further examine the molecular characteristics of HMGB1, the HMBG1 spot in two-dimensional electrophoresis was

![Image](https://example.com/image.jpg)

Fig. 1. Two-dimensional electrophoresis of HMGB1. A, two-dimensional electrophoresis of GIST tissue (case 13). The proteins from GIST were extracted and separated on pH 3–10 nonlinear immobilized pH-gradient strips and were run through a 9–16% SDS/polyacrylamide gel. The gel was stained with Coomassie Blue G250, and protein spots were examined by MALDI-TOF. B, two-dimensional electrophoresis gel images of HMGB1. Eight representative tissues of 20 cases are shown. Arrows, the protein spot corresponding to HMGB1. C, MALDI-TOF peptide mass spectrum of the tryptic digest of HMGB1; T, trypsin autolytic fragments.
isolated and subjected to trypsin digestion followed by MALDI-TOF analysis. HMGB1 was validated by mass fingerprinting of the selected peaks of peptides by applying low tolerance (<20 ppm) with recalibration (Fig. 1C). For HMGB1, a total of six peaks were detected with 37% coverage (Table 2). This information led us to believe that the proteins detected in 20 samples by two-dimensional electrophoresis were authentic HMGB1. These HMGB1 spots on two-dimensional electrophoresis also showed the expected mass profiles that were well matched with the theoretical values of HMGB1.

**Western Blot and Immunohistochemical Analysis for KIT and HMGB1.** To determine the relative protein expression levels of both KIT and HMGB1, in which the former was not detectable in two-dimensional electrophoresis under the same conditions, Western blot analysis was carried out using antibodies against KIT and HMGB1 (Fig. 2). KIT expression was strong in GISTs with KIT mutation, and this overexpression was directly related to mutation, regardless of the mutation type. The relative expression ratio of KIT was 3.6 in cases with KIT mutation and 0.5 in cases without KIT mutation. As expected, HMGB1 was found to be consistently overexpressed in GISTs with KIT mutation. In accordance with the two-dimensional electrophoresis results, 11 of 14 GISTs with KIT mutation showed more than a 2-fold overexpression of HMGB1 compared with the maximally expressed HMGB1 level in those without KIT mutation (case 16). To determine whether the overexpression of HMGB1 is related to MMP expression, we performed Western analyses for MMP2 and found that all 12 of the cases with HMGB1 overexpression also strongly expressed MMP2 (Fig. 2). This result is consistent with the previous report by Taguchi et al. (19), in which secreted HMGB1 was found to induce MMP expression. The results of Western blot analysis for HMGB1 expression in colorectal carcinomas were also correlated with the results of the two-dimensional electrophoresis. HMGB1 expression was found in most of the normal mucosa and tumors, and overexpression was noted in 10 of 13 tumors. The nuclear and cytoplasmic expression of HMGB1 was separately measured in five GISTs (three GISTs with KIT mutation and two without KIT mutation) by using fractionated nuclear and cytoplasmic protein lysates.

All three of the GISTs with KIT mutation showed variable amounts of HMGB1 expression in the nuclear and cytoplasmic proteins, whereas weak or undetectable cytoplasmic expression was found in the two GISTs without KIT mutation, and one GIST without KIT mutation also showed no detectable nuclear HMGB1 expression (Fig. 3). Interestingly, KIT expression was correlated to the cytoplasmic expression level of HMGB1 and MMP2 (Fig. 3). Immunohistochemically, HMGB1 was detected mainly in the nuclei of the tumor cells, cytoplasm of some tumor cells, and some nuclei of the normal cells (Fig. 4). In 14 GISTs with KIT mutation, 12 showed nuclear HMGB1 expression, and 4 showed cytoplasmic HMGB1 expression. In six GISTs without KIT mutation, two showed nuclear HMGB1 expression and one showed cytoplasmic HMGB1 expression. In the colorectal carcinomas, stronger HMGB1 expression compared with that of the normal mucosal cells was found in most of the tumor cells; however, the level of expression between tumors was variable.

**DISCUSSION**

In this study, 20 GISTs were investigated for the mutation status of KIT and the characteristics of their protein expression. We found frequent mutations in exon 11 of KIT in our GISTs, and strong expression of KIT and HMGB1 in cases with KIT mutation.

Frequent mutations of KIT in GISTs had been reported by many laboratories (3, 5, 10, 11). In agreement with the previous reports, we found frequent mutations (14 of 20, 70%) in our GISTs. Most of the mutations were deletions (8 of 14, 57%) in exon 11, as has been previously reported. Exon 11 constitutes the juxtamembrane region of KIT, which serves as an antidermination domain. Deletions, insertions, or point mutations in this domain result in the activation of tyrosine kinase activity by allowing ligand-independent receptor dimerization (8, 9). Recently germ-line and somatic point mutations of exon 13 (K642E) were reported and were found to be activating mutations in the kinase domain of KIT gene (11). In this study, we provide direct evidence of KIT protein overexpression in all of our GISTs with KIT mutation. KIT overexpression in GISTs is a well-
HMGB1 OVEREXPRESSION IN GISTs

Fig. 3. Analysis of HMGB1 expression in nuclear and cytoplasmic proteins. A, HMGB1 expression in total protein lysates of five GISTs. Cases 13T, 7T, and 19T were positive for KIT mutation and cases 10T and 12T had no KIT mutation. B, analysis of HMGB1, KIT, and MMP2 expression in the nuclear and cytoplasmic protein fraction of five GISTs. Preparation of total protein lysates, nuclear protein fraction, and cytoplasmic protein fraction was performed as described in “Materials and Methods.” Tn, nuclear protein lysates from GIST tissue; Tc, cytoplasmic protein lysates from GIST tissue. kDa, Mr in thousands.

known characteristic, and is currently used as a diagnostic marker. Most of the previous studies regarding KIT expression in GISTs in human tissues were performed by using immunohistochemistry, and quantitative evaluations of KIT expression and comparisons between this and KIT mutations has not been performed. We applied Western blot by using fresh tumor samples, thus enabling relative quantification by Western blot, and we compared the results with KIT mutation data. We found that KIT overexpression was directly related to KIT mutation regardless of the mutation type. The ubiquitous KIT activation in GISTs by activating KIT mutation (12) and its roles have previously been suggested epidemiologically (10, 12, 20). In addition, there have been a number of reports on the aberrant expression of KIT in other tumors (21–23) and on the relationship between KIT activation and the protection of apoptosis and the enhancement of tumor invasion (24). On the basis of these reports, we suggest that mutations of exon 11 of KIT induce the overexpression and the constitutive activation of KIT, which subsequently plays a role in tumor transformation and progression.

Initially, this work was designed as part of our attempts to identify a set of protein biomarkers involved in the progression of malignant tumors by proteome analysis. We used GISTs for the following reasons: (a) there are well-defined diagnostic criteria for differentiating benign, borderline, and malignant tumors; (b) most GISTs show similar chromosomal, morphological, and immunohistochemical characteristics; and (c) there are high percentages of tumor cells in GIST. During the course of this work, we found that several proteins are overexpressed in GISTs with KIT mutation, and, thus, we focused on the cause and consequence of HMGB1 overexpression in GISTs with KIT mutation. The implications of our proteome analysis are at least 3-fold. The direct link between KIT mutation and HMGB1 expression should be regarded as a novel finding. We found HMGB1 expression in all 14 of the GISTs with KIT mutation, and 12 of these showed very strong expression by two-dimensional electrophoresis analysis. In GISTs without KIT mutation, HMGB1 was not expressed in four of six GISTs. Interestingly, two GISTs with insertion mutations in the distal portion of exon 11 of KIT showed low levels of HMGB1 expression. From these results, we suspect that the constitutive activity of KIT caused by these two insertion mutations in the distal portion of exon 11 would be milder than that caused by mutations in the proximal portion of the juxtamembrane domain. Although the number of inserted sequences is not identical to ours, similar insertion mutations at the same sites have been reported previously (12), and further functional study of these two insertion mutations is needed.

We demonstrated that the tumor cells are the origin of overexpressed HMGB1 in our GISTs by immunohistochemical analysis. HMGB1 was abundantly expressed in the nuclei of the tumor cells and/or some of the tumor cell cytoplasms, and the expression was correlated with the result of two-dimensional electrophoresis and Western blot analysis. We also demonstrated marked variation of HMGB1 expression in colorectal carcinomas. Variations in HMGB1 expression at the RNA level had been reported in breast carcinomas (25), gastric carcinomas (26), and hepatocellular carcinomas (27). Overexpression of HMGB1 and cisplatin resistance had been reported (28), and the role of HMGB1 in the repair of cisplatin-DNA adduct had been proposed (28, 29). The marked intertumoral variation of HMGB1 expression level and the reported roles of HMGB1 in tissue differentiation and tumor progression raise the possibility that the roles of HMGB1 in tumor differentiation and progression may be different according to the HMGB1 expression level.

One of the important roles of the KIT mutation of GISTs in tumorigenesis may be the activation of signal transduction pathways of the cell cycle. In this study, we found that KIT, HMGB1, and MMP2 are concomitantly overexpressed in GISTs with KIT mutation, and, thus, HMGB1 and MMP2 may be downstream target molecules in GISTs with KIT mutation. HMGB1 was originally identified as a chromosomal DNA-binding protein (30). Apart from this intranuclear function, HMGB1 was also shown to be localized in the extracellular medium of different cell types as matrix-bound and in soluble molecules (31) and to have an extracellular function in inflammation and tumor metastasis (19, 32, 33). On the basis of the reported function of HMGB1, the role of HMGB1 overexpression in GIST tumorigenesis

Fig. 4. Immunohistochemical analysis of HMGB1 in GISTs. Expression of HMGB1 is noted in the nuclei of epithelial cells and inflammatory cells in the gastric mucosa (A) and in the nuclei of smooth muscle cells (B). Microscopic feature of a GIST with KIT mutation (C), which shows strong nuclear and cytoplasmic HMGB1 expression (D). Histology of a GIST without KIT mutation (E) showing no HMGB1 expression in tumor cells. HMGB1 expression is evident only in the nuclei of the vascular smooth muscle cells and capillary endothelial cells (F).
can be explained in two ways. First, overexpressed HMGB1 can influence the expression and function of several related genes. HMGB1 is mainly localized in the nucleus and interacts with several transcription factors, by binding to the minor groove of DNA (33), increasing the binding affinity of several transcription factors, (34) and down-regulating the binding affinity of p53 and p73 in the human BAX promoter (35). Thus, we suggest that the overexpression of HMGB1 can contribute to tumorigenesis by altering tumor suppressor gene function. Second, HMGB1 directly activates signal transduction pathways related to cellular proliferation and/or metastasis. In addition to its intracellular role, HMGB1 is also secreted and/or released by certain cells and plays important roles in tumor growth, invasion, and metastasis (19, 33). Secreted and/or released HMGB1 binds to receptor for advanced glycation end products (RAGE), as a receptor-ligand pair, and this complex activates several molecules, including mitogen-activated protein kinase (MAPK) signaling molecules and MMPs. The suppression of tumor growth and metastasis by blocking RAGE-ampotherin (synonym of HMGB1) complex in mice had been reported (19). Therefore, it is likely that, after necrosis some of the overexpressed HMGB1 may exist in secreted forms or released forms that are capable of facilitating tumor growth and metastasis. Although we could not confirm that a portion of the HMGB1 in our GISTs was in a secreted form, we demonstrated the presence of HMGB1 by showing that MMP2 is selectively overexpressed according to HMGB1 expression in our GISTs. Taken together, our results suggest that KIT mutation in GISTs is strongly related to HMGB1 overexpression which may contribute to tumor growth and invasion.

REFERENCES