Comprehensive Analysis of Genes Involved in the Malignancy of Gastrointestinal Stromal Tumors

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Abstract. *Background: During tumorigenesis of gastrointestinal stromal tumors (GISTs), the most frequent changes are reported to be gain-of-function mutations in the C-KIT proto-oncogene. However, we speculated that additional genetic alterations are required for the progression of GISTs. Patients and Methods: Using 15 cases diagnosed with GISTs, we searched for novel indicator genes by microarray analyses using an Oligo GEArray® PI3K-AKT Signaling Pathway Microarray Kit. In addition, we analyzed the mutational status of C-KIT and the proliferation status indicated by the Ki-67 index. Results: The tumor localizations of the 15 GISTs were as follows: 8 in the stomach; 2 in the small intestine; 2 in the mesentery; 1 in the duodenum; 1 in the rectum; and 1 in liver. Regarding the C-KIT gene analysis, mutations in exon 11 were detected in 11 out of 13 patients. In 1 out of the 13 patients, mutations were detected in both exons 11 and 13. No genetic abnormalities were identified in 1 patient. The Ki-67 labeling indices were significantly lower for the low-risk and intermediate-risk groups than for the high-risk group (p=0.0440). No specific genes were overexpressed in the >1% Ki-67 group. Regarding the primary lesion sites, the following 6 genes were overexpressed in tumors in the stomach: RBL2, RHOA, SHC1, HSP90AB1, ACTB and BAS2C. Conclusion: Gene analysis is currently only useful for diagnostic assessment and predicting therapeutic effects. However, it may be possible for new malignancy-related factors to be identified by comparing and investigating gene expression levels and other factors using such analyses.*

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Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors that arise in the gastrointestinal tract, and account for about 1% of gastrointestinal tumors (1). During tumorigenesis of GISTs, the most frequent changes are reported to be gain-of-function mutations in the *C-KIT* proto-oncogene. These mutations can result in autophosphorylation, namely *KIT* ligand-independent kinase activity (2). Approximately 95% of GISTs express the receptor tyrosine kinase *KIT*, which is useful for distinguishing these tumors from other sarcomas that develop in the abdomen (3). In recent years, it has been established that 75-80% of GISTs harbor mutations in the *KIT* gene, and that the resulting *KIT* mutants play important roles in the development of these tumors (3). Furthermore, therapeutic targeting of *KIT* mutants with the tyrosine kinase inhibitor imatinib (Gleevec, Glivec; Novartis Pharma AG, Basel, Switzerland) has proven to be an effective treatment for patients with advanced unresectable GISTs. However, the efficacy varies depending on the locations of the mutations (4). Recently, approximately 5% of GISTs were found to be characterized by mutations in the related receptor tyrosine kinase platelet-derived growth factor receptor alpha *(PDGFRA)* exons 12 and 18 (5, 6). Very recently, Lasota *et al*. (7) reported *PDGFRA* exon 14 mutations in 11 of 200 GISTs that were negative for mutations in *C-KIT* exons 9, 11, 13 and 17 and *PDGFRA* exons 12 and 18. However, approximately 5% of all GISTs do not have detectable mutations in either the *C-KIT* or *PDGFRA* genes.

Although *KIT* gene mutations specific to GISTs have been used as important diagnostic indicators, histological factors such as tumor diameter and tumor cell mitosis/density have mostly been used to assess GIST malignancy (8). Therefore, we speculated that additional genetic alterations are required for the progression of GISTs. However, malignancy has not been assessed based on genetic abnormalities. In the present study, we aimed to identify novel indicator genes by microarray analyses using an Oligo GEArray® PI3K-AKT Signaling Pathway Microarray Kit. In addition, we analyzed the mutational status of *C-KIT* and the proliferation status indicated by the Ki-67 index.

Table I. *Fletcher's GIST malignancy classification system.*

Risk	Tumor size (cm)	Mitotic count (per 50 HPF)
Very low risk	<2	<5
Low risk	2-5	<5
Intermediate risk	<5	6-10
	5-10	<5
High risk	>5	>5
	>10	Any
	Any	>10

HPF: High-power field.

Patients and Methods

Patients and tumor tissues. The study comprised 15 patients (7 men and 8 women) diagnosed with GISTs between July 2005 and July 2008, and included 2 patients with recurrent GISTs. The specimens excised by surgical resection were histopathologically assessed and classified into four groups using Fletcher's GIST malignancy classification system (Table I) (8).

The risk grades of the GISTs were evaluated according to Fletcher *et al*. (8). Tumors of <2 cm in diameter with 0-4 mitoses/50 high power fields (HPF) were considered to be very low risk, while tumors of 2-5 cm in diameter with 0-4 mitoses/50 HPF were considered to be low risk. Tumors of <5 cm in diameter with 6-10 mitoses/50 HPF or tumors of 5-10 cm in diameter with 0-4 mitoses/50 HPF were considered to be intermediate risk. Tumors of >5 cm in diameter with >5 mitoses/50 HPF, tumors of >10 cm in diameter with any number of mitoses or tumors with >10 mitoses/50 HPF were considered to be high risk. Tumors with necrosis were also classified as high risk.

C-KIT gene analysis. Genomic DNA was extracted from formalin-fixed paraffin-embedded tumor tissues using standard proteinase K digestion and phenol/chloroform extraction methods, and used for subsequent molecular analyses. To avoid contamination from normal tissues, only tumor tissues were scraped and obtained from each paraffin section, by reference to serial sections stained with hematoxylin and eosin.

Exons 9, 11, 13 and 17 of *C-KIT* were amplified by PCR using previously published primer sets (Table II). Each of the amplified fragments was purified from a polyacrylamide gel after electrophoresis, and subjected to direct sequencing. All the sequencing reactions were carried out in both the forward and reverse directions.

Immunohistochemistry of Ki-67. Resected specimens were fixed with 10% (v/v) formaldehyde and embedded in paraffin blocks. Immunohistochemical staining was performed on sections using a standard avidin-biotin-peroxidase complex (ABC) method. Briefly, 4-μm sections were cut from the paraffin blocks, deparaffinized and treated with a 0.3% (v/v) H_2O_2/methanol solution for 30 min at room temperature to block endogenous peroxidase activity. After rehydration through a graded ethanol series, the sections were microwaved in 10 mM phosphate citrate buffer (pH 8.0) at 90˚C for 15 min, and cooled to 30˚C. After rinsing in 0.1 M phosphate-buffered saline (PBS; pH 7.4), nonspecific binding sites were blocked by incubation with 10% (v/v) normal horse serum for 30 min. Next, the sections were incubated with an anti-human Ki-67 murine monoclonal antibody (Dako, Carpinteria, CA, USA) as the primary antibody overnight at 4˚C. Subsequent immunohisto-chemical staining steps were performed using an ABC system (Vectastain; Vector Laboratories Inc, Burlingame, CA, USA). Briefly, incubation with biotinylated anti-rabbit IgG as the secondary antibody was carried out for 30 min. The chromogen utilized was 3,3'-diaminobenzidine tetrahydrochloride, which was applied as a 0.02% (w/v) solution containing 0.005% (v/v) H2O2 in 50 mM ammonium acetate/citric acid buffer (pH 6.0). The sections were lightly counterstained with hematoxylin.

TUNEL staining. TUNEL staining was carried out using Apoptag® Plus Peroxidase *In Situ* Kit (Chemicon, Temecula, CA). Briefly, deparaffinized hydrated esophageal biopsy sections were treated with proteinase K (20 μg/ml) in 0.05 M PBS (pH 7.4) at room temperature for 15 min. After washing with distilled water, the sections were incubated with 3% H_2O_2 for 5 min. Each slide was then covered with equilibration buffer at room temperature for 1 h, incubated with working-strength terminal transferase (TdT) enzyme at 37˚C for 1 h in a humidified chamber, and washed with 0.05 M PBS. The sections were then incubated with a peroxidase-conjugated anti-digoxigenin antibody at room temperature for 30 min in a humidified chamber, washed with 0.05 M PBS and incubated with diaminobenzidine (DAB) solution for 6 min. After washing with distilled water, the sections were counterstained with Mayer's hematoxylin (Sigma Chemical Co, St. Louis, MO, USA) and coverslipped.

Oligo GEArray. RNA was extracted from each frozen specimen using an acid guanidinium-phenol-chloroform method. Microarray analyses were conducted using an Oligo GEArray® PI3K-AKT Signaling Pathway Microarray Kit (SuperArray, Frederick, MD, USA). For each total RNA sample (2 μg), a TrueLabeling-AMPTRM 2.0 Linear RNA Kit (SuperArray) was used to synthesize cDNA, and an IVT enzyme mix was used to prepare biotin-labeled cRNA. The samples were hybridized with membranes containing spots for biotin-labeled cRNA and 122 DNAs (Table III) overnight at 60˚C. The hybridized membranes were placed in a CDP-Star substrate solution for 5 min at room temperature, and luminescent signals were detected using a charge-coupled device camera system. The GEArray® Expression Analysis Suite (SuperArray) was used to analyze the results. Since the tumor diameter was smallest in Case 1, the expression levels were compared with those in Case 1. The cases were divided into two groups with respect to their median gene expressions to compare the risk classifications, primary tumor sites and Ki-67 labeling indices.

Statistical analysis. The Ki-67 labeling indices were calculated as the percentages of cells with nuclear staining of Ki-67 in 10 HPFs (HPF; ×400). The TUNEL indices were calculated as the numbers of TUNEL-positive cells among more than 1.000 cells counted. The relationships between the Ki-67 labeling indices and other parameters were determined by the analysis of variance method.

Results

Clinicopathological characteristics. The clinicopathologic characteristics of the patients are shown in Table IV. The mean age of the 15 patients was 59.3 years (range, 25-80 years). The GIST localizations were as follows: 8 in the

Table II. *Sequences of the primers used for mutational analyses of the C-KIT gene.*

Exon	Forward	Reverse
Exon 9	5'-ATG CTC TGC TTC TGT ACT GCC-3'	5'-AGA GCC TAA ACA TCC CCT TA-3'
Exon 11	5'-CCA GAG TGC TCT AAT GAC TG-3'	5'-CTG TTA TGT GTA CCC AAA AAG G-3'
Exon 13	5'-CAT CAG TTT GCC AGT TGT GC-3'	5'-ACA CGG CTT TAC CTC CAA TG-3'
Exon 17	5'-TGT ATT CAC AGA GAC TTG GC-3'	5'-GGA TTT ACA TTA TGA AAG TCA CAG G-3'

stomach; 2 in the small intestine; 2 in the mesentery; 1 in the duodenum; 1 in the rectum; and 1 in liver. The mean tumor size (maximum diameter) was 6.3 cm (range, 0.7-16 cm). According to the risk categories, 6 cases were classified as high risk, 4 as intermediate risk, 4 as low risk and 1 as very low risk.

C-KIT gene analysis. Among the 15 cases, mutations in *C-KIT* exons 9, 11, 13 and 17 were analyzed in 13 patients, excluding 2 patients who underwent emergency surgery (Table IV). In 11 out of the 13 patients, mutations in exon 11 were detected. In 1 out of the 13 patients, mutations were detected in both exons 11 and 13. One patient had no genetic abnormalities. No correlations existed between the *C-KIT* genetic mutations and the risk classifications.

Immunohistochemistry of Ki-67. The Ki-67 labeling indices were 0.89 in the low-risk group included very-low-risk group, 3.54 in the intermediate-risk group and 5.31 in the high-risk group. The Ki-67 labeling index was significantly lower for the low-risk and intermediate-risk groups than for the high-risk group (p=0.0440).

TUNEL staining. In all patients for whom immunostaining was conducted, the apoptosis index evaluated by TUNEL staining was <1% and no correlations with the risk classifications were identified.

Oligo GEArray. Among the 15 patients, the gene expressions were analyzed in 11 patients, excluding Cases 1, 2, 14 and 15. A total of 122 genes in the 11 specimens were expressed at low or high level. The cases were divided into two groups with respect to their Ki-67 labeling indices (cutoff value, 1%). The following 30 genes were also expressed as higher in the >1% Ki-67 group: *RPS27A, ADAR, APC, AXIN1, CCND1, CDC42, CDKN1B, CUTL1, EIF4B, FOS, FZD1, HSPB, HSP90AA2, ILK, INPPL1, ITGB1, MAP2K1, MYD88, NFKB1, NFKBIA, PIK3R1, RBL2, RHOA, RPS6KA1, TSC2, YWHAH, ZFYVE21, B2M, HSP90AB1,* and *ACTB*. Among these 30 genes, *PIK3RI* and *B2M* were underexpressed in the ≤1% Ki-67 group (Figure 1). These gene expressions were analyzed in relation to the risk classifications, and the following 5 genes were overexpressed in the three high-risk patients: *EIF4A1, HSPB, ITGB, RHOA*, and *WASL*. Among these 5 genes, only *WASL* was underexpressed in the low-risk patients (Figure 2). Regarding the primary lesion sites, the following 6 genes were overexpressed in tumors in the stomach: *RBL2, RHOA, SHC1, HSP90AB1, ACTB,* and *BAS2C* (Figure 3).

Discussion

GIST is a generic term for mesenchymal tumors arising in the gastrointestinal tract. Rosai *et al*. (9) proposed a classification system and diagnostic and therapeutic criteria are currently being established, but there remains room for discussion.

In the present study, mutations in exon 11 were highly detected and the Ki-67 labeling index was significantly higher for the high-risk group. Regarding the results using Oligo GEArray, positive association between gene expression and the Ki-67 labeling index were found in *PIK3RI* and *B2M*. The association between gene expressions and the risk classifications was found only in *WASL*. Regarding the primary lesion sites, the six genes were overexpressed in tumors of the stomach.

In GIST patients with exon 11 *KIT* mutations, previous studies have reported high levels of imatinib sensitivity (10, 11). Most patients in the present study showed exon 11 *KIT* mutations, and imatinib was expected to be effective (12). However, although *KIT* gene analyses may be part of the standard diagnostic procedure for GISTs, they may not indicate imatinib sensitivity. In fact, *C-KIT* gene analyses were not found to be a better indicator of malignancy than the risk classifications based on tumor diameter and mitosis (8). In terms of immunostaining, TUNEL staining was not useful for assessing malignancy, while the Ki-67 labeling index was higher for the high-risk group, similar to the findings of previous studies (13, 14). Our findings show that the Ki-67 labeling index is an indicator of malignancy in GIST.

The present analyses identified two candidate genes, *PIK3R1* and *B2M*, that were overexpressed in the >1% Ki-67 group and underexpressed in the ≤1% Ki-67 group. *PIK3R1* is a gene involved in cellular proliferation,

Table III. *Target RNAs analyzed in this study.*

Gene symbol	Gene name
RPS27A	Ribosomal protein S27a
ADAR	Adenosine deaminase, RNA-specific
AICDA	Activation-induced cytidine deaminase
AKT1	V-akt murine thymoma viral oncogene homolog 1
AKT2	V-akt murine thymoma viral oncogene homolog 2
AKT3	V-akt murine thymoma viral oncogene homolog 3
APC	Adenomatosis polyposis coli
APPL1	Adaptor protein, phosphotyrosine interaction, pH domain and leucine zipper containing 1
ASAH1	*N*-Acylsphingosine amidohydrolase (acid ceramidase) 1
AXIN1	Axin 1
BAD	BCL2-antagonist of cell death
BTK	Bruton agammaglobulinemia tyrosine kinase
CASP9	Caspase 9, apoptosis-related cysteine protease
CCND1	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)
CD14	CD14 antigen
CDC42	Cell division cycle 42 (GTP binding protein, 25 kDa)
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)
CHUK	Conserved helix-loop-helix ubiquitous kinase
CSNK2A1	Casein kinase 2, alpha 1 polypeptide
THEM4	Thioesterase superfamily member 4
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88 kDa
CUTL1	Cut-like 1, CCAAT displacement protein
EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2
EIF3S10	Eukaryotic translation initiation factor 3, subunit 10 theta, 150/170 kDa
EIF4A1	Eukaryotic translation initiation factor 4A, isoform 1
EIF4B	Eukaryotic translation initiation factor 4B
EIF4E	Eukaryotic translation initiation factor 4E
EIF4EBP1	Eukaryotic translation initiation factor 4E binding protein 1
EIF4G1	Eukaryotic translation initiation factor 4 gamma, 1
ELK1	ELK1, member of ETS oncogene family
FASLG	Fas ligand (TNF superfamily, member 6)
FKBP1A	FK506 binding protein 1A, 12 kDa
FOS	V-Fos FBJ murine osteosarcoma viral oncogene homolog
FOXO1A	Forkhead box O1A (rhabdomyosarcoma)
FOXO3A	Forkhead box O3A
FRAP1	FK506 binding protein 12-rapamycin associated protein 1
FZD1	Frizzled homolog 1 (Drosophila)
GHR	Growth hormone receptor
GJA1	Gap junction protein, alpha 1, 43 kDa (connexin 43)
GNB1	Guanine nucleotide binding protein (G protein), beta polypeptide 1
GRB10	Growth factor receptor-bound protein 10
GRB2	Growth factor receptor-bound protein 2
GSK3B	Glycogen synthase kinase 3 beta
HRAS	V-Ha-ras Harvey rat sarcoma viral oncogene homolog
HSPB1	Heat-shock 27 kDa protein 1
HSP90AA2	Heat-shock 90 kDa protein 1, alpha, class A member 2
HSP90AB1	Heat-shock 90 kDa protein 1, alpha, class B member 1
IGF1	Insulin-like growth factor 1 (somatomedin C)
IGF1R	Insulin-like growth factor 1 receptor
ILK	Integrin-linked kinase
INPP5D	Inositol polyphosphate-5-phosphatase, 145 kDa
INPPL1	Inositol polyphosphate phosphatase-like 1
IRAK1	Interleukin-1 receptor-associated kinase 1
IRS1	Insulin receptor substrate 1
ITGB1	Integrin, beta 1 (fibronectin receptor, antigen CD29 includes MDF2, MSK12)
JUN	V-Jun sarcoma virus 17 oncogene homolog (avian)
LBP	Lipopolysaccharide binding protein
LEF1	Lymphoid enhancer-binding factor 1
LY96	Lymphocyte antigen 96

Table III. *continued*

Table III. *continued*

MAP2K1	Mitogen-activated protein kinase kinase 1
MAPK1	Mitogen-activated protein kinase 1
MAPK14	Mitogen-activated protein kinase 14
MAPK3	Mitogen-activated protein kinase 3
MAPK8	Mitogen-activated protein kinase 8
MAPK8IP1	Mitogen-activated protein kinase 8 interacting protein 1
MKNK1	MAP kinase-interacting serine/threonine kinase 1
MLLT7	Myeloid/lymphoid or mixed-lineage leukemia
MTCP1	Mature T-cell proliferation 1
MYD88	Myeloid differentiation primary response gene (88)
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
PABPC1	Poly(A) binding protein, cytoplasmic 1
PAK1	P21/Cdc42/Rac1-activated kinase 1
PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide
PDK1	Pyruvate dehydrogenase kinase, isoenzyme 1
PDK2	Pyruvate dehydrogenase kinase, isoenzyme 2
PDPK1	3-Phosphoinositide dependent protein kinase-1
PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide
PIK3CB	Phosphoinositide-3-kinase, catalytic, beta polypeptide
PIK3CG	Phosphoinositide-3-kinase, catalytic, gamma polypeptide
PIK3R1	Phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)
PIK3R2	Phosphoinositide-3-kinase, regulatory subunit, polypeptide 2 (p85 beta)
PIK3R3	Phosphoinositide-3-kinase, regulatory subunit, polypeptide 3 (p55, gamma)
PPP2R3B	Protein phosphatase 2 (formerly 2A), regulatory subunit B", beta
PRKAR1B	Protein kinase, cAMP-dependent, regulatory, type I, beta
PRKCA	Protein kinase C, alpha
PRKCB1	Protein kinase C, beta 1
PRKCZ	Protein kinase C, zeta (PRKCZ), mRNA
PTEN	Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)
PTK2	PTK2 protein tyrosine kinase 2
PTPN11	Protein tyrosine phosphatase, non-receptor type 11 (Noonan syndrome 1)
RAC1	Ras-related C3 botulinum toxin substrate 1 (small GTP binding protein Rac1)
RAF1	V-Raf-1 murine leukemia viral oncogene homolog 1
RASA1	RAS p21 protein activator (GTPase activating protein) 1
RBL2	Retinoblastoma-like 2 (p130)
RHEB	Ras homolog enriched in brain
RHOA	Ras homolog gene family, member A
RPS6	Ribosomal protein S6
RPS6KA1	Ribosomal protein S6 kinase, 90 kDa, polypeptide 1
RPS6KB1	Ribosomal protein S6 kinase, 70 kDa, polypeptide 1
SHC1	SHC (Src homology 2 domain containing) transforming protein 1
SOS1	Son of sevenless homolog 1
SRF	Serum response factor (c-*fos* serum response element-binding transcription factor)
TCL1A	T-Cell leukemia/lymphoma 1A
TCL1B	T-Cell leukemia/lymphoma 1B
TIRAP	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLR4	Toll-like receptor 4
TOLLIP	Toll interacting protein
TSC1	Tuberous sclerosis 1
TSC2	Tuberous sclerosis 2
WASL	Wiskott-Aldrich syndrome-like
WNT1	Wingless-type MMTV integration site family, member 1
YWHAH	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta
ZFYVE21	Zinc finger, FYVE domain containing 21
PUC18	PUC18 Plasmid DNA
AS1R2	Artificial sequence 1 related 2 (80% identity)(48/60)
AS1R1	Artificial sequence 1 related 1 (90% identity)(56/60)
AS1	Artificial sequence 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
B2M	Beta-2-microglobulin
HSP90AB1	Heat-shock 90 kDa protein 1, beta class B member 1
ACTB	Beta-actin

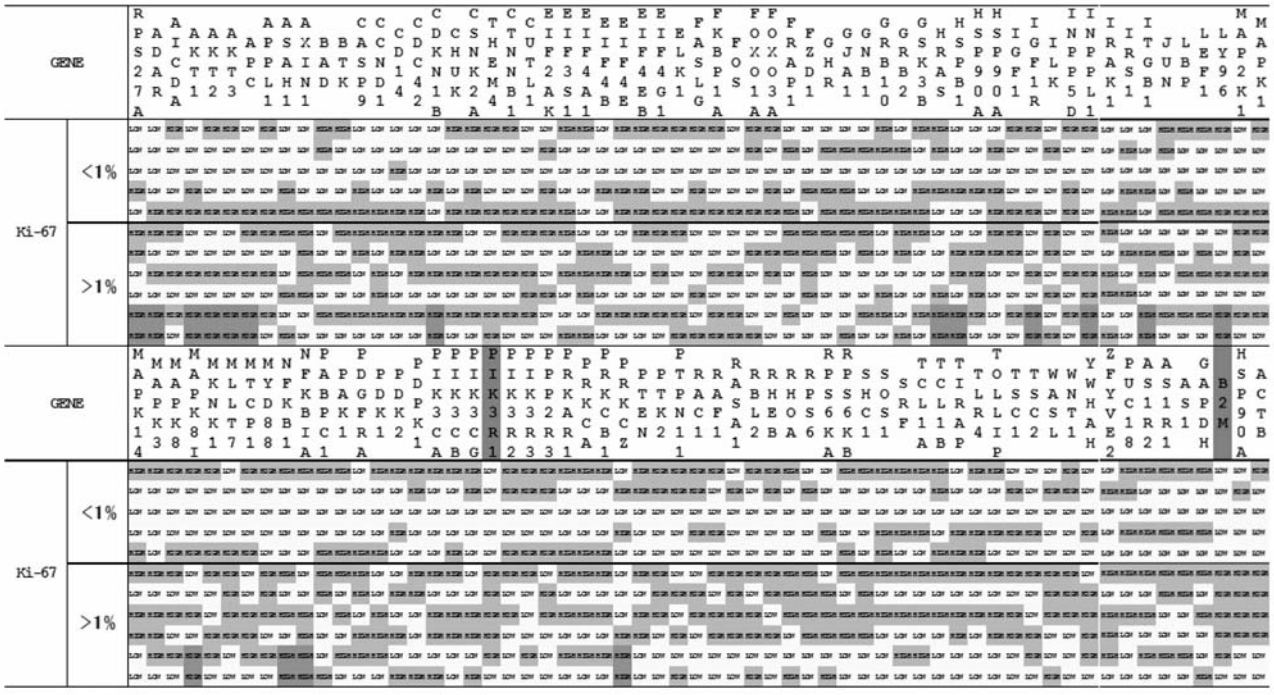

Figure 1. *Ki-67 labeling indices and gene expressions using the Oligo GEArray. The cases were divided into two groups with respect to their Ki-67 labeling indices (cutoff value, 1%). The expressions of the following 30 genes were higher in the >1% Ki-67 group: RPS27A, ADAR, APC, AXIN1, CCND1, CDC42, CDKN1B, CUTL1, EIF4B, FOS, FZD1, HSPB, HSP90AA2, ILK, INPPL1, ITGB1, MAP2K1, MYD88, NFKB1, NFKBIA, PIK3R1, RBL2, RHOA, RPS6KA1, TSC2, YWHAH, ZFYVE21, B2M, HSP90AB1, and ACTB. Among these 30 genes, PIK3RI and B2M were underexpressed in the ≤1% Ki-67 group.*

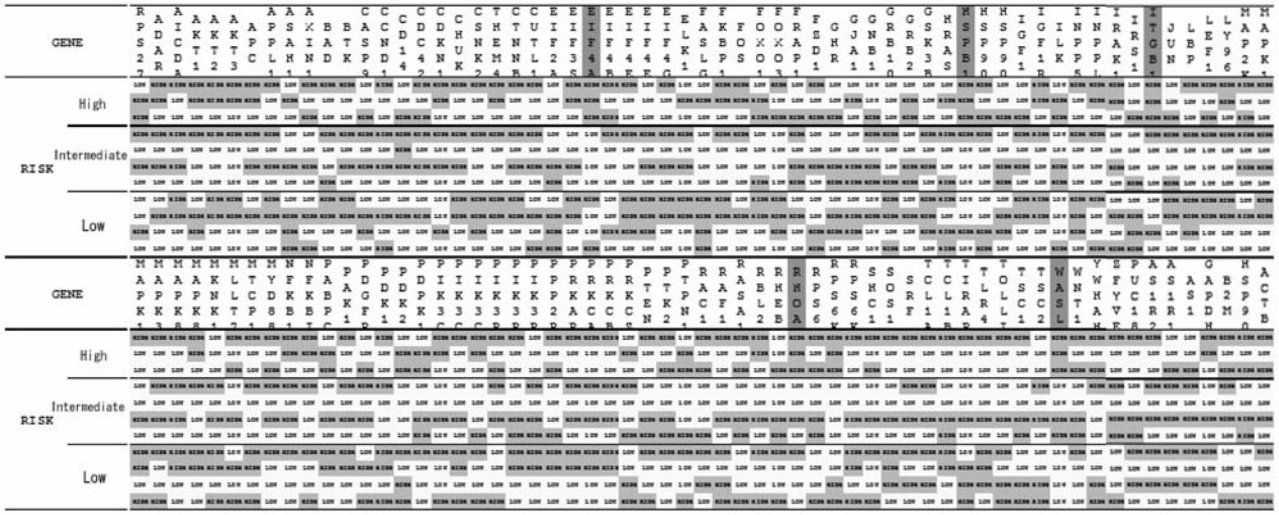

Figure 2. *Risk classifications and gene expression levels using the Oligo GEArray. The following 5 genes were overexpressed in the three high-risk patients: EIF4A1, HSPB, ITGB, RHOA, and WASL. Among these 5 genes, only WASL was underexpressed in the low-risk patients.*

differentiation, apoptosis and cytoskeleton reconstruction (15). The risk classification system devised by Fletcher *et al*. (8) takes into account the tumor diameter and mitosis. We found that *WASL* was overexpressed in the high-risk group and underexpressed in the low-risk group. *WASL* is a gene involved in cellular structure and movement, and may be useful in risk-assessment criteria (16). In terms of the primary lesion sites, *SHC1* was overexpressed in stomach lesions and underexpressed in other lesions. *SHC1* is a gene that facilitates MAPK activation and cellular

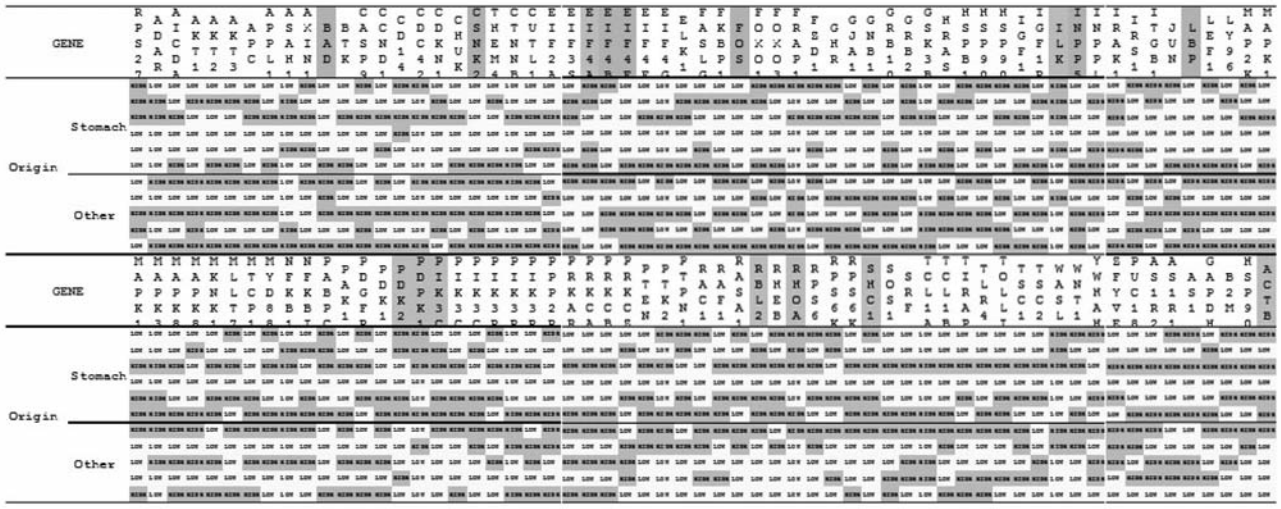

Figure 3. *Origin and gene expressions using the Oligo GEArray. Regarding the primary lesion sites, the following 6 genes were overexpressed in tumors in the stomach: RBL2, RHOA, SHC1, HSP90AB1, ACTB, and BAS2C .*

Table IV. *Patients' characteristics, risks, gene alterations, TUNEL indices and Ki-67 indices.*

Case	Age (years)	Gender	Tumor size (cm)	Origin	Mitotic count	Risk	Gene alteration	TUNEL	Ki-67
1	74	Female	0.5×0.7	Stomach	<5	Very low	Exon 11	<1%	<1%
2	59	Female	2.0×1.6	Stomach	30-50	High	Exon 11	<1%	6.49%
3	55	Female	2.5×2.0	Stomach	<5	Low	ND	<1%	3.37%
4	64	Female	3.5×2.3	Duodenum	2	Low	Exon 11	<1%	<1%
5	44	Male	4.5×3.0	Intestine	<5	Low	Exon 13	<1%	<1%
6	25	Female	4.5×4.0	Stomach	5	Low	Exon 11	<1%	<1%
7	68	Female	5.0×4.0	Mesentery	<10	Intermediate	Exon 11	<1%	<1%
8	69	Male	5.5×5.5	Stomach	<5	Intermediate	Exon 11	<1%	1.63%
9	49	Male	5.8×4.0	Stomach	6	Intermediate		<1%	5.77%
10	51	Female	6.0×8.0	Stomach	10	High	Exon 11	<1%	2.22%
11	80	Female	7.5×4.0	Intestine	5	Intermediate		<1%	5.77%
12	58	Male	15.0×15.0	Stomach	100-150	High	Exon 11	<1%	10.57%
13	70	Male	16.0×16.0	Rectum		High	Exon 11	<1%	2.42%
14	71	Male	8.0×8.5	Liver		High	Exons 11, 17	<1%	3.59%
15	52	Male	21.0×5.0	Mesentery	100-150	High	Exon 11	<1%	6.95%

ND: Not detected.

proliferation regulation (17). In the present study, the kinase activities varied between the stomach and other regions of the gastrointestinal tract, and differences in malignancy and therapy may exist with respect to the location of the primary lesion.

By comparing and analyzing various factors and microarray gene analyses, new indicators for malignancy may be identified in the future. Since the present analyses were based on mRNA, further studies are required to analyze the protein expressions.

In conclusion, gene analysis is currently only useful for diagnostic assessment and predicting therapeutic effects. However, new malignancy-related factors may be identified by comparing and investigating gene expression levels and other factors using such analyses.

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