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Allelic Loss of 14q and 22q, *NF2* Mutation, and Genetic Instability Occur Independently of c-*kit* Mutation in Gastrointestinal Stromal Tumor

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Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the gastrointestinal tract. Since c-*kit* mutation occurs only in one-third of GIST, there might be other molecular mechanisms. Loss of heterozygosity (LOH), microsatellite instability (MSI) and *NF2* gene mutation were investigated in 22 GISTs (9 low-risk and 13 high-risk tumors). LOH and MSI were evaluated using 41 markers on 21 chromosomal arms, and *NF2* gene mutation was examined by PCR-SSCP. High frequency of LOH was observed on 14q (9/19, 47%), and 22q (17/22, 77%). The frequencies were similar in low-risk and high-risk tumors, and were unrelated with gastric or intestinal origin. Two other abnormalities, additional LOH on other chromosomes and MSI at more than two loci, were characteristic of the high-risk tumors ($P<0.05$). *NF2* gene mutation was identified in two cases showing 22q-LOH (8 bp deletion on the splice donor site of exon 7, and 1 bp insertion at position 432 of exon 4, which resulted in nonsense mutation). There was no significant correlation between these results and c-*kit* gene mutation, which was observed in 8 of 22 tumors. Suppressor genes on 14q and 22q may be involved, independently of c-*kit* gene mutation, in the development of GIST. *NF2* contributes as a tumor suppressor in a small subset of GIST. These abnormalities are presumably followed by increased genetic instability.

Key words: Gastrointestinal stromal tumor — Allelic loss — Genetic instability — *NF2* — c-*kit*

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the human gastrointestinal (GI) tract.[1)] Morphologically, GIST consists of spindle or epithelioid cells with differentiation to smooth muscle, neuronal, or both types of cell. Although there has been controversy regarding the cellular origin of GIST, an unexpected clue has emerged from studies of GI-tract abnormality in W/W^v mice,[2)] a strain in which a proto-oncogenic receptor tyrosine kinase (KIT) is inactivated due to loss-of-function mutation of the c-*kit* gene.[3)] The studies demonstrated that KIT expression is indispensable for the development and maintenance in the interstitial cells of Cajal (ICC),[4,5)] a pacemaker for the periodic peristalsis of the GI tract.[6)] KIT expression was further demonstrated in most GISTs, leading to the assumption that GIST is a tumor of ICC.[7,8)] Additional findings supporting a link between GIST and ICC are the common expression of a surface marker for hematopoietic stem cell, CD34,[7–9)] and a non-smooth muscle (embryonic) type of heavy chain myosin isoform in both types of cell.[10)]

As for the genetic changes in GISTs, a gain-of-function mutation of c-*kit* was observed in some GISTs, and the mutated c-*kit* in a GIST exerted transformation activity when transfected to Ba/F3 murine lymphoid cell line.[7)] However, since c-*kit*-mutation was identified only in 30% of tumors,[11–14)] except for one report,[15)] there might be some other molecular mechanisms for the development and progression of GIST. In the present study, we evaluated loss of heterozygosity (LOH) and microsatellite instability (MSI) using 41 microsatellite markers on 21 chromosomes. We found a high frequency of LOH on chromosomes 1p, 14q, and 22q, and there were additional genetic abnormalities characteristic of the high-risk tumors. Since a further evaluation of the chromosome 22q demonstrated that the highest LOH was located at 22q12.1–2, a locus containing the *NF2* gene,[16,17)] we further evaluated *NF2* gene mutation by PCR-SSCP analysis. Then, these abnormalities were comparatively analyzed with c-*kit*-mutation to clarify how and to what extent they contribute to the development and progression of GIST.

MATERIALS AND METHODS

Patients and samples A total of 22 pairs of GIST and corresponding normal tissues were obtained from 22 patients (12 men and 10 women, age 33–80 years) at Jichi Medical School Hospital and Tokyo Metropolitan Koma-

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gome Hospital from 1988 to 1997. The clinicopathological features are presented in Table I together with the results of the percentage of Ki-67-positive nuclei (Ki-67 labeling index, LI) and immunophenotype analysis. Nineteen tumors were primary and three were metastatic. The metastatic liver tumors were derived from a duodenal GIST and a rectal GIST, and the peritoneal tumor from an ileal GIST. The samples were taken immediately after resection, frozen in dry ice-hexane, and stored at −80°C for DNA analysis. The remaining tissue was processed for histological and immunohistochemical analysis.

Based on Franquemont's criteria[1)] with slight modification, GISTs were classified into low- and high-risk tumors as reported previously.[18)] High-risk tumor was (a) size >5 cm and mitotic count >2/10 high power field (HPF), or (b) size >5 cm or mitotic count >2/10 HPF and Ki-67 LI >7%. On the other hand, low-risk tumor was (c) size <5cm and mitotic count <2/10 HPF, or (d) size >5 cm or mitotic count >2/10 HPF and Ki-67 LI <7%.

Immunohistochemistry Three-micrometer-thick paraffin sections were cut from formalin-fixed and paraffin-embedded specimens. The avidin-biotin-peroxidase complex (ABC) method was carried out with antibodies against c-*kit* gene product, KIT (MBL, Nagoya; polyclonal antibody, dilution for working solution 1:20) and CD34 (Becton Dickinson, San Jose, CA; monoclonal, 1:20). To further characterize the diverse cellular differentiation in GIST, antibodies against α-smooth muscle actin (α-SMA) (Dako, Glostrup, Denmark; monoclonal, 1:500) and S-100 protein (Dako; polyclonal, 1:1000) were used to evaluate muscle cell differentiation and neuronal cell differentiation, respectively.

Monoclonal antibody Ki-67 (clone MIB-1, Immunotech, Marseilles, France; monoclonal, 1:100) was used for the evaluation of proliferating cells in GIST. To recover the antigenicity of Ki-67, the sections were pretreated in a microwave oven before incubation with the primary antibody. Since regional variation in the mitotic rate is typical

Table I. Clinicopathological Findings and Immunophenotype in Gastrointestinal Stromal Tumors

Case	Age (year)	Sex[a)]	Tumor site[b)]	Size (cm)	Mitotic count (/10 HPF)	Ki-67 LI[c)] (%)	Immunophenotype			
							CD34	KIT	α-SMA	S-100
Low-risk primary										
1	61	f	g	4.5	2	2.0	+[d)]	+	−	−
2	48	f	g	14.0	1	6.1	+	+	−	−
3	62	m	g	6.0	1	1.8	+	+	−	−
4	58	m	g	3.5	3	1.5	+	+	−	−
5	62	f	g	3.5	2	3.6	+	+	−	−
6	55	m	g	4.0	1	2.9	+	+	−	−
7	47	f	g	11.0	2	3.9	+	−	−	−
8	51	f	j	5.0	1	1.5	+	+	+	−
9	64	m	j	2.0	1	5.1	−	+	+	−
High-risk primary										
10	58	m	g	15.0	10	7.8	+	+	+	−
11	59	f	g	13.0	8	14.8	+	+	−	+
12	57	m	g	17.0	10	4.2	+	−	−	−
13	56	m	g	10.0	15	16.3	+	+	−	−
14	70	f	g	10.0	2	14.9	+	+	−	−
15	66	f	g	3.5	3	8.6	+	+	−	−
16	62	m	g	5.5	5	7.2	+	−	−	−
17	52	m	g	7.0	5	4.0	+	+	−	−
18	60	m	d	7.0	20	15.1	+	+	+	−
19	33	m	c	13.0	6	21.1	−	−	−	−
High-risk metastatic										
20	80	f	d (liver)[e)]	17.0	2	23.8	−	+	−	−
21	80	m	i (peritoneum)	19.0	10	7.5	+	+	−	−
22	62	f	r (liver)	3.0	15	4.9	+	+	−	−

a) m: male, f: female.
b) g: gastric, d: duodenal, j: jejunal, i: ileal, c: colonic, r: rectal.
c) Ki-67 labeling index.
d) +: positive staining, −: no detectable staining.
e) Parenthesis: metastatic site.

of GISTs, the percentage of Ki-67-LI was estimated by selecting two or three high-power fields, which showed the highest Ki-67 positivity in each section and by counting 500 to 1000 nuclei.[17)]

Analysis using microsatellite markers DNA was isolated by proteinase K digestion and a phenol/chloroform extraction procedure. LOH was examined by PCR using 1–3 microsatellite markers per chromosome as listed in Table II. Eight microsatellite markers on chromosome 22q (D22S264, D22S446, D22S275, D22S280, D22S268,

Table II. Loss of Heterozygosity (LOH) and Microsatellite Instability (MSI) on 41 Microsatellite Loci of 21 Chromosomal Arms in Gastrointestinal Stromal Tumor

Chromosome marker		LOH[a)] (%)	MSI[b)]	Chromosome marker		LOH[a)] (%)	MSI[b)]
1p		**7/19 (37)**	**4**	**9p**		**0/9 (0)**	**0**
	D1S484	1/17	1		D9S171	0/9	0
	D1S209	4/6	2				
	D1S228	3/13	1	**10p**		**0/5 (0)**	**0**
					D10S197	0/5	0
1q		**0/3 (0)**	**2**				
	D1S447	0/3	2	**11q**		**2/12 (17)**	**0**
					D11S1356	2/12	0
2p		**2/15 (13)**	**1**				
	D2S123	2/15	1	**13q**		**0/16 (0)**	**0**
					D13S168	0/11	0
2q		**1/6 (17)**	**1**		D13S291	0/10	0
	D2S111	1/6	1				
				14q		**9/19 (47)**	**2**
3p		**1/17 (8)**	**2**		D14S281	5/15	2
	D3S1261	1/7	0		D14S254	6/14	0
	D3S1029	0/6	2				
	D3S11	0/11	0	**15q**		**2/17 (12)**	**0**
					D15S122	2/14	0
3q		**1/15 (7)**	**1**		D15S126	0/10	0
	D3S1262	1/10	1				
	D3S1265	1/9	0	**17p**		**1/18 (6)**	**2**
					TP53	1/18	2
4q		**2/18 (11)**	**1**				
	D4S398	1/15	1	**17q**		**1/6 (17)**	**0**
	D4S428	2/8	0		D17S250	1/6	0
5q		**0/10 (0)**	**0**	**22q**		**11/19 (58)**	**2**
	Mfd27	0/10	0		D22S275	9/17	2
					D22S280	4/6	0
6q		**1/14 (7)**	**1**				
	D6S262	0/11	1	**22q[c)]**		**15/22 (68)**	**2**
	Mfd47	1/9	0		D22S264	45/12	1
					D22S446	3/20	0
7q		**1/12 (9)**	**0**		D22S315	9/16	0
	D7S525	1/7	0		NF2CA3	8/15	1
	D7S522	0/7	0		D22S268	3/10	0
					D22S277	8/19	0
8p		**0/11 (0)**	**1**		D22S423	6/20	0
	D8S254	0/11	1		D22S274	3/17	0
8q		**0/16 (0)**	**0**				
	myc	0/16	0				

a) Number of cases showing LOH/number of informative cases.
b) Number of positive cases showing MSI.
c) Additional marker for deletion mapping on 22q.

D22S277, D22S423, and D22S274) were additionally used to further clarify the deletion map on 22q.

The PCR protocol was basically the same as that reported previously.[19, 20] The reaction mixture (6 μl) contained 200 ng of genomic DNA, the proper primer pair (0.2 μM), 25 mM of each deoxynucleotide triphosphate, 1× PCR buffer, *Taq* polymerase (5 U/μl) (Life Technology, Rockville, MD), and [α-^{32}P]dCTP. After heating at 94°C for 5 min, PCR was performed for 35 cycles of 1 min at 55°C for annealing, 1 min at 72°C for extension, and 1 min at 94°C for denaturing. Final extension was performed for 10 min at 72°C. The reaction product was then denatured and electrophoresed in 6% polyacrylamide gel containing 7 M urea. After electrophoresis, the gel was fixed on paper and exposed to X-ray film for 24–72 h.

The genetic alterations in the mobility and density of microsatellite markers were classified into two categories, MSI and LOH.[20] Alterations were judged as MSI when additional bands, which were not seen in the corresponding normal DNA, appeared in the tumor DNA. LOH was defined when a band corresponding to one allele of the normal DNA was lost in the tumor DNA. When MSI appeared in the tumor DNA, we judged it as not informative for LOH.

PCR-SSCP analysis of *NF2* gene *NF2* exons were amplified with primers, which were located within the surrounding intronic sequences. The primer sequences and PCR conditions were according to Jacoby *et al*.[16, 17] The amplified DNA was electrophoresed on 6% non-denaturing polyacrylamide gel containing 6% glycerol, and a single mutated band was then excised from the gel. Direct sequencing of the extracted DNA was carried out with an ABI PRISM TM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Chiba) and an ABI PRISM TM 377 DNA sequencer using the same primers as were used for amplification.

Statistical analysis Statistical analysis was performed using Fisher's exact test.

RESULTS

Subtype and phenotype of GIST According to the modified Franquemont's classification, nine primary GISTs belonged to the low-risk group and 10 primary and 3 metastatic tumors belonged to the high-risk group (Table I). Immunohistochemically, all tumors except one (case 19) showed the expression of KIT (18/22) or CD34 (19/22). The expression of either molecule was not correlated with high-risk morphology. In the additional differentiation, 4 of 22 GISTs showed weak and focal immunoreactivity for α-SMA and only one tumor showed weak and focal immunostaining for S-100 protein. The one case (case 19) negative for both KIT and CD34 expressed neither α-SMA nor S-100 protein.

Screening of LOH on 21 chromosomal arms LOH was evaluated using 1–3 microsatellite markers per chromosome arm (Table II): 1p, 1q, 2p, 2q, 3p, 3q, 4q, 5q, 6q, 7q, 8p, 8q, 9p, 10p, 11q, 13q, 14q, 15q, 17p, 17q, and 22q (Fig. 1). A high frequency of LOH (over 30%) was observed on three chromosomes, 1p (37%, 7/19), 14q

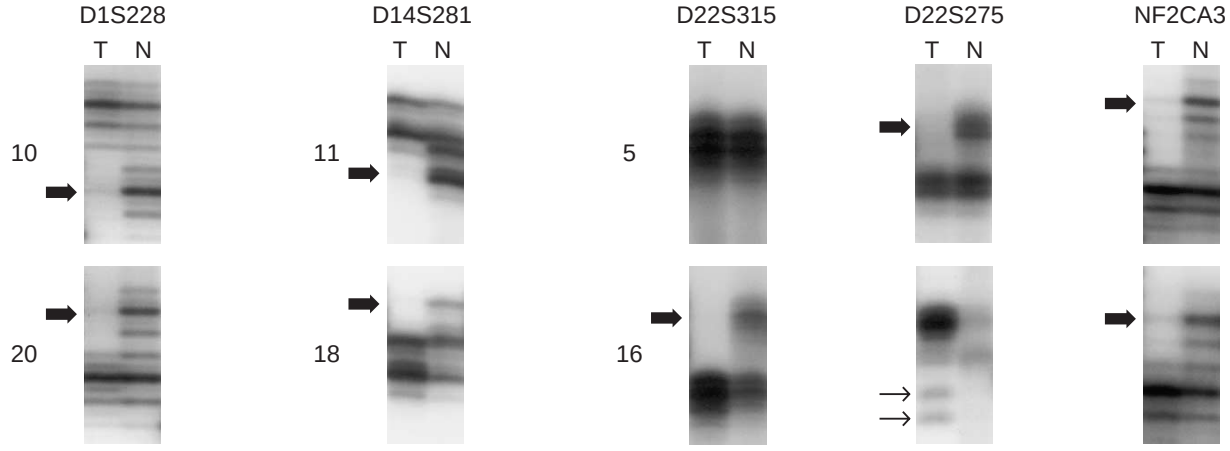

Fig. 1. Loss of heterozygosity (LOH) and microsatellite instability (MSI) in GIST. Genomic DNA extracted from tumors (T) and normal tissue (N) samples was amplified using primers at D1S228 on chromosome 1p, D14S281 on chromosome 14q and D22S315, D22S275 and NF2CA3 on chromosome 22q. LOH (thick arrows) and MSI (thin arrows) are shown as deletion of bands and additional bands, respectively, in the tumor compared to the bands of the normal tissue. Representative cases in D1S228 (cases 10 and 20) and D14S281 (cases 11 and 18) are high-risk GISTs. Representative cases in D22S315, D22S275 and NF2CA3 are low-risk GIST (case 5) and a high-risk GIST (case 16). LOH is commonly observed in cases 5 and 16 on NF2CA3.

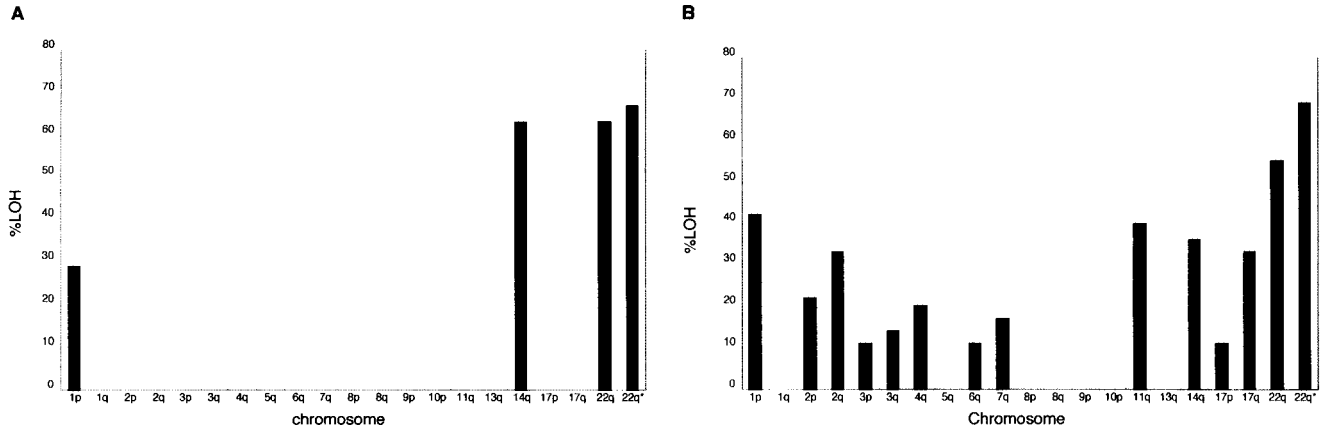

Fig. 2. The frequencies of LOH on 21 chromosomal arms in low- (A) and high-risk (B) GIST. %LOH is obtained as the number of cases showing LOH/the number of informative cases. Upper column: low-risk GIST, and lower column: high-risk GISTs. 22q* is % LOH on 22q which was obtained using additional eight markers for deletion mapping.

Table III. Loss of Heterozygosity (LOH) and Microsatellite Instability (MSI) in Relation to Tumor Grade and Origin of GIST

	n	LOH[a]				MSI[b]		
		1p	14q	22q	Other loci[c]	One locus	2 loci<	Total
Low-risk	9	2/7	5/8	7/9	1/9[e]	2/9	0/9[f]	2/9[h]
Gastric	7	0/5[d]	4/6	5/7	1/7	0/7	0/7[g]	0/7[j]
intestinal	2	2/2[d]	1/2	2/2	0/2	2/2	0/2	2/2[i]
High-risk	13	5/12	4/11	10/13	6/13[e]	3/13	6/13[f]	9/13[h]
Gastric	8	3/7	3/9	7/8	5/8	2/8	5/8[g]	7/8[j]
intestinal	5	2/5	1/2	3/5	1/5	1/5	1/5	2/5

a) Number of cases showing LOH/number of informative cases.
b) Number of cases showing MSI/number of examined cases.
c) Eighteen chromosomal loci (other than chromosomes 1p, 14q and 22q).
d– *i*) ($P<0.05$) and *j*) ($P<0.01$) Significant difference between pairs by Fisher's exact test.

(47%, 9/19), and 22q (58%, 15/22). The frequencies of 14q- and 22q-LOH were similarly high in low-risk and high-risk (primary or metastatic) tumors (Fig. 2, Table III), irrespective of gastric or intestinal origin. Although chromosome 1p-LOH was similarly observed in both low- and high-risk GISTs, no LOH was identified in low-risk GISTs of gastric origin. LOH on other chromosomes, such as 2p, 2q, 3p, 3q, 4q, 6q, 7q, 11q, 15q, 17p, 17q, were only observed in high-risk GISTs at 11–40% (Fig. 2).

LOH on 22q Since *NF2*, a known tumor suppressor gene, lies between D22S275 and D22S280, LOH on chromosome 22q was further examined using eight additional microsatellite markers, including NF2CA3, which is located at intron 1 of *NF2* on 22q12.2 (Fig. 3). The highest frequency of LOH (73%) was observed between D22S315 and D22S277, located on 22q12.1–13.1, while LOH was less frequent in the centromeric (D22S264 and D22S446 on 22q11.2) or telomeric region (D22S423 and D22S274 on 22q13.1–3). The deletions were centromeric to the NF2CA3 locus in four cases (cases 1, 2, 9, 12), but three cases (cases 3, 15, 19) exhibited only deletion on the NF2CA3 locus.

PCR-SSCP analysis of *NF2* in GIST All of 17 exons of *NF2* were evaluated in this study. Mobility shift was observed in two of 22 GISTs (cases 10 and 18, Fig. 4), both of which were high-risk tumors showing 22q-LOH (Table IV). Eight base pairs were deleted on the splice donor site of exon 7 in case 10. One base pair was inserted at position 432 of exon 4 in case 18, which resulted in nonsense mutation at codon 144 (TAC to TAA, Tyr144X).

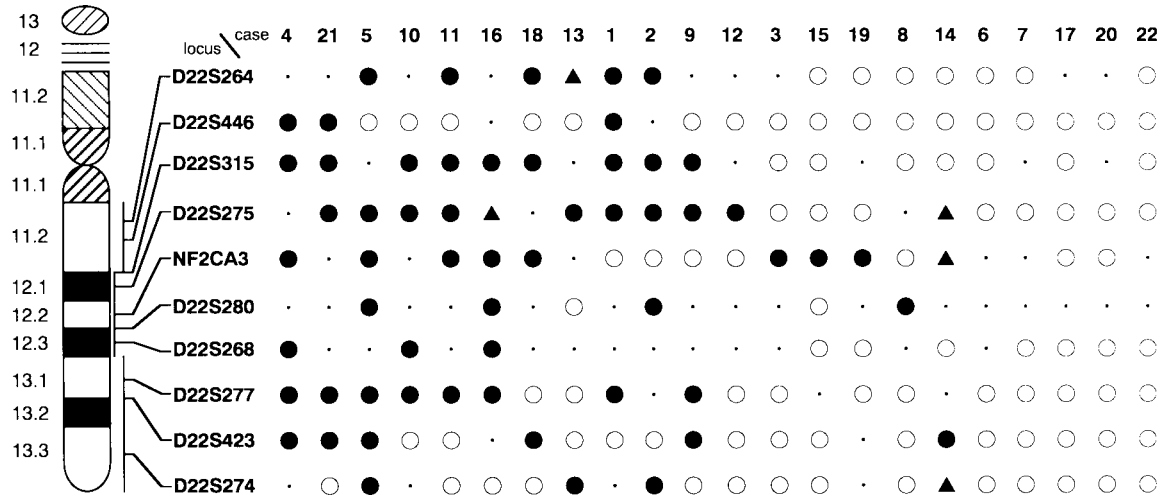

Fig. 3. Deletion map of chromosome 22q in GIST. ○: Constitutionally heterozygous with retention of both alleles in GIST DNA, ▲: homozygous/uninformative, ●: heterozygous with loss of one allele in GIST DNA.

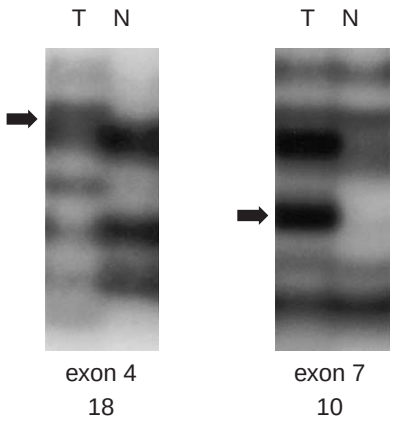

Fig. 4. PCR-SSCP analysis of *NF2* exons. DNA extracted from tumor (T) and DNA from the corresponding normal tissue (N) were comparatively analyzed. Band shift (arrowhead) is observed in exon 4 in case 18, and in exon 7 in case 18. The abnormal bands were excised for sequencing analysis.

MSI in GIST MSI was observed in 11 (50%) out of 22 cases (Tables II, III). In low-risk GIST, MSI was detected at a single locus in 2 of 9 tumors. On the other hand, it was observed in 69% (9/13) of high-risk tumors: 1 at a single locus, 5 at two loci, 1 at three loci, 1 at four loci, and 1 at five loci. There was no locus at which MSI preferentially occurred in GISTs.

Correlation of genetic alterations with tumor grade, site and differentiation When the results of LOH and MSI were evaluated according to the tumor grade and the site of origin (Table III), two features were characteristic of high-risk tumors: LOH on chromosomes other than 1p, 14q or 22q, and MSI on more than 2 loci ($P<0.05$). These features were apparent in gastric GISTs, compared to intestinal ones. *NF2* mutation was identified in two cases of high-risk GIST (cases 10 and 18), both of which showed α-SMA immunoreactivity (Table I), although its expression was only focal in the tumor.

Comparison of the genetic changes with c-*kit* gene mutation C-*kit* gene mutation had been identified in 8 of 22 cases (Table IV) as reported previously.[14] C-*kit* gene mutation was observed in 5 of 9 or two of 8 cases with or without 14q-LOH, respectively. It occurred in 6 of 17 or two of 5 cases with or without 22q12-LOH, respectively. These differences were not statistically significant. *NF2* mutation was identified in two cases, neither of which showed c-*kit* mutation. Thus, c-*kit* gene mutation was unrelated with any of the other abnormalities described above.

DISCUSSION

Gain-of-function mutation of c-*kit* is an important genetic alteration observed in GIST, but it has been identified only in 30% of tumors,[11–14] suggesting the involvement of other molecular mechanisms in the development and progression of GIST. Using 1–3 microsatellite markers per chromosome arm, we demonstrated LOH on chromosomes 1p, 14q and 22q in more than 30% of GISTs. The results indicate that these loci might contain tumor suppressor genes important for the development and progression of GIST. On three chromosomes, putative tumor

Table IV. C-*kit* Mutation and Other Genetic Abnormalities in Gastrointestinal Stromal Tumors

Case	14q LOH	22q LOH	Additional LOH	MSI >2 loci	*NF2* mutation[a]	C-*kit* mutation[b]
Low-risk						
1	n[c]	+[d]	–	–	–[e]	–
2	+	+	–	–	–	–
3	+	+	–	–	–	1701[f] del[g] 28 bp+ins[h] 1 bp
4	–	+	–	–	–	–
5	+	+	–	–	–	1669 del 6 bp
6	+	–	–	–	–	–
7	–	–	–	–	–	1676 del 9 bp
8	+	+	–	–	–	1703 del 24 bp+ins 3 bp
9	–	+	+	–	–	–
High-risk						
10	–	+	–	–	675+8[i] del 8 bp	–
11	+	+	+	+	–	1671 del 6 bp
12	–	+	+	+	–	–
13	+	+	+	–	–	1673 del 21 bp
14	–	+	–	+	–	–
15	n	+	–	–	–	–
16	–	+	+	+	–	–
17	+	–	+	–	–	–
18	+	+	–	–	432 ins 1 bp	–
19	–	+	–	–	–	–
Metastatic						
20	–	–	+	+	–	1737 del 3 bp
21	n	+	+	+	–	1656 del 15 bp
22	–	–	–	–	–	–

a) NF2 mutation: mutation on 17 exons in *NF2* gene.
b) C-*kit* mutation: mutation on exon 11 in c-*kit* gene.
c) n: uninformative.
d) +: LOH was detected.
e) –: LOH or mutation was not detected.
f) The first nucleotide number at which the mutation was detected.
g) del: deletion.
h) ins: insertion.
i) Intronic mutation is designated as + relative to the last nucleotide of the exon.

suppressor genes have been implicated in various kinds of neoplasm.[21–24] In the present study, the frequencies of 14q- and 22q-LOH were similar in low-risk and high-risk tumors, and were unrelated with gastric or intestinal origin. On the other hand, 1p-LOH occurred in both low- and high-risk GISTs of intestinal origin, but in only high-risk GISTs of gastric origin. Thus, LOH on 14q and 22q may be a common and primary abnormality responsible for the development of GIST. This result is compatible with findings from comparative genomic hybridization studies on GIST.[25, 26]

On chromosome 22q, LOH covered a relatively wide region, but the highest frequency of LOH was located in the region between D22S315 and NF2CA3, suggesting the involvement of the centromeric portion of the *NF2* gene. The *NF2* gene is responsible for neurofibromatosis 2 (NF2), encoding a 595-amino acid protein called schwannomin or merlin (moesin-ezrin-radixin-like protein), that exhibits significant homology to a highly conserved family of proteins that connect the cytoskeleton to components of the plasma membranes.[27, 28] The gene is considered to be a tumor suppressor gene and its mutations are detected in multiple tumor types related with NF2 disorder (schwannoma and meningioma)[16, 17, 28, 29] and in NF2-unrelated tumors (mesothelioma and colon cancer).[30–32] In the present study, PCR-SSCP analysis demonstrated *NF2* gene mutation in two cases, both of which were accompanied by 22q-LOH: 8 bp was deleted on the splice donor site of the exon 7 in one case, and a 1-bp insertion on exon 4 resulted in a premature stop codon in the other. Frameshift, nonsense and splice site alterations located in the N-terminal domain are the major types of *NF2* gene muta-

tions in schwannoma and meningioma.[16, 17, 28, 29] Thus, *NF2* behaves as a tumor suppressor gene in a subset of the GIST, and the fact that two cases of GIST with *NF2* mutation were high-risk tumors suggests that loss of *NF2* function may be related with more aggressive behavior of GIST. On the other hand, α-SMA expression in both tumors may be only coincidental, since the expression was only focal and was also observed in two cases of low-risk GIST.

With reference to the previous study focusing on exon 11 of the c-*kit* gene,[14] no correlation was observed between 22q12-LOH or 14q-LOH and the c-*kit* gene. The cases showing *NF2* gene mutation lacked c-*kit* gene mutation. Thus, these genetic alterations may independently contribute to the development of GIST. The differences of genetic changes in high-risk tumors compared to the low-risk ones were, first, the high frequency of LOH on chromosomes other than 1p, 14q and 22q, and second, the presence of MSI at more than two loci. Since there was no specific chromosome deleted or showing MSI, this phenomenon may represent a genetic instability in higher-grade neoplasms.[19, 20]

The percentage of malignant cases is generally higher in intestinal than gastric GISTs,[33] and all three metastatic tumors in the present study were intestinal in origin. In this context, several differences of genetic abnormalities in gastric and intestinal GISTs need further investigation. For example, the genetic instability described above was more remarkable in high-risk GISTs of the stomach, whereas 1p-LOH was observed earlier in low-risk GISTs of the intestine. Since the interstitial cell of Cajal shows several differences in ultrastructural morphology and expression profile of KIT and CD34 in relation to the GI-tract organs,[10] it is possible that the genetic changes underlying GIST may vary according to the micro-environment from which the tumor arises.

In conclusion, GIST is a distinct type of mesenchymal tumor of the GI tract, in which a tumor suppressor gene on 14q and 22q may be involved independently of c-*kit* mutation. Additional LOH and MSI may be related to the progression of GIST, which might occur preferentially in gastric GIST. *NF2* contributes as a tumor suppressor in a subset of GIST, but it may not be the critical tumor suppressor gene on chromosome 22q.

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REFERENCES

1) Franquemont, D. W. Differentiation and risk assessment of gastrointestinal stromal tumors. *Am. J. Clin. Pathol.*, **103**, 41–47 (1995).
2) Shimada, M., Kitamura, Y., Yokoyama, M., Miyano, Y., Maeyama, K., Yamatodani, A., Takahashi, Y. and Tatsuta, M. Spontaneous stomach ulcer in genetically mast-cell depleted W/W^v mice. *Nature*, **283**, 662–664 (1980).
3) Ward, S. M., Burns, A. J., Torihashi, S. and Sanders, K. M. Mutation of the proto-oncogene c-*kit* blocks development of interstitial cells and electrical rhythmicity in murine intestine. *J. Physiol.*, **480**, 91–97 (1994).
4) Maeda, H., Yamagata, A., Nishikawa, S., Yoshinaga, K., Kobayashi, S., Nishi, K. and Nishikawa, S. Requirement of c-*kit* for development of intestinal pacemaker system. *Development*, **116**, 369–375 (1992).
5) Isozaki, K., Hirota, S., Nakama, A., Miyagawa, J., Shinomura, Y., Xu, Z., Nomura, S. and Kitamura, Y. Distributed intestinal movement, bile reflux to the stomach, and deficiency of c-*kit*-expressing cells in Ws/Ws mutant rats. *Gastroenterology*, **109**, 456–464 (1995).
6) Huizinga, J. D., Thuneberg, L., Kluppel, M., Malysz, J., Mikkelsen, H. B. and Bernstein, A. W/*kit* gene required for interstitial cell of Cajal and for intestinal pacemaker activity. *Nature*, **373**, 347–349 (1995).
7) Hirota, S., Isozaki, K., Moriyama, Y., Hashimoto, K., Nishida, T., Ishiguro, S., Kawano, K., Hanada, M., Kurata, A., Takeda, M., Tunio, G. M., Matsuzawa, Y., Kanakura, Y., Shinomura, Y. and Kitamura, Y. Gain-of-function mutations of c-*kit* in human gastrointestinal stromal tumors. *Science*, **279**, 577–580 (1998).
8) Kindblom, L. G., Remotti, H. E., Aldenborg, F. and Meis-Kindblom, J. M. Gastrointestinal pacemaker cell tumor (GIPACT). Gastrointestinal stromal tumors show phenotypic characteristics of the interstitial cells of Cajal. *Am. J. Pathol.*, **152**, 1259–1269 (1998).
9) Miettinen, M., Virolainen, M. and Rikala, M. S. Gastrointestinal stromal tumors—value of CD34 antigen in their identification and separation from true leiomyomas and schwannomas. *Am. J. Surg. Pathol.*, **19**, 207–216 (1995).
10) Sakurai, S., Fukasawa, T., Chong, J.-M., Tanaka, A. and Fukayama, M. Embryonic form of smooth muscle myosin heavy chain (SMemb/MHC-B) in gastrointestinal stromal tumor and interstitial cells of Cajal. *Am. J. Pathol.*, **154**, 23–28 (1999).
11) Ernst, S. I., Hubbs, A. E., Przygodzki, R. M., Emory, T. S., Sobin, L. H. and OÅfLeary, T. J. KIT mutation portends poor prognosis in gastrointestinal stromal/smooth muscle tumors. *Lab. Invest.*, **78**, 1633–1636 (1998).
12) Lasota, J., Jasinski, M., Sarlomo-Rikala, M. and Miettinen, M. Mutations in exon 11 of c-kit occur preferentially in malignant *versus* benign gastrointestinal stromal tumors and

do not occur in leiomyomas or leiomyosarcomas. *Am. J. Pathol.*, **154**, 53–60 (1999).
13) Moskaluk, C. A., Tian, Q., Marshall, C. R., Rumpel, C. A., Franquemont, D. W. and Frierson, H. F., Jr. Mutations of c-*kit* JM domain are found in a minority of human gastrointestinal stromal tumors. *Oncogene*, **18**, 1897–1902 (1999).
14) Sakurai, S., Fukasawa, T., Chong, J.-M., Tanaka, A. and Fukayama, M. C-*kit* gene abnormalities in gastrointestinal stromal tumors (tumors of interstitial cell of Cajal). *Jpn. J. Cancer Res.*, **90**, 1321–1328 (1999).
15) Taniguchi, M., Nishida, T., Hirota, S., Isozaki, K., Ito, T., Nomura, T., Matsuda, H. and Kitamura, Y. Effect of c-kit mutation on prognosis of gastrointestinal stromal tumors. *Cancer Res.*, **59**, 4297–4300 (1999).
16) Jacoby, L. B., MacCollin, M., Louis, D. N., Mohney, T., Rubio, M. P., Pulaski, K., Trofatter, J. A., Kley, N., Seizinger, B., Ramesh, V. and Gusella, J. F. Exon scanning for mutation of the NF2 gene in schwannomas. *Hum. Mol. Genet.*, **3**, 413–419 (1994).
17) Jacoby, L. B., MacCollin, M., Barone, R., Ramesh, V. and Gusella, J. F. Frequency and distribution of NF2 mutations in schwannomas. *Genes Chromosom. Cancer*, **17**, 45–55 (1996).
18) Sakurai, S., Fukayama, M., Kaizaki, Y., Saito, K., Kanazawa, K., Kitamura, M., Iwasaki, Y., Hishima, T., Hayashi, Y. and Koike, M. Telomerase activity in gastrointestinal stromal tumor (GIST). *Cancer*, **83**, 2060–2066 (1998).
19) Chong, J.-M., Fukayama, M., Hayashi, Y., Takizawa, T., Koike, M., Konishi, M., Kikuchi-Yanoshita, R. and Miyaki, M. Microsatellite instability in the progression of gastric carcinoma. *Cancer Res.*, **54**, 4595–4597 (1994).
20) Chong, J.-M., Fukayama, M., Hayashi, Y., Hishima, T., Funata, N., Koike, M., Matsuya, S., Konishi, M. and Miyaki, M. Microsatellite instability and loss of heterozygosity in gastric lymphoma. *Lab. Invest.*, **77**, 639–645 (1997).
21) Tamura, G., Sakata, K., Nishizuka, S., Maesawa, C., Suzuki, Y., Terashima, M., Eda, Y. and Satodate, R. Allelotype of adenoma and differentiated adenocarcinoma of the stomach. *J. Pathol.*, **180**, 371–377 (1996).
22) Praml, C., Finke, L. H., Herfarth, C., Schlag, P., Schwab, M. and Amler, L. Deletion mapping defines different regions in 1p34.2–pter that may harbor genetic information related to human colorectal cancer. *Oncogene*, **11**, 1357–1362 (1995).
23) Schofield, D. E., Beckwith, J. B. and Sklar, J. Loss of heterozygosity at chromosome regions 22q11–12 and 11p15.5 in renal rhabdoid tumors. *Genes Chromosom. Cancer*, **15**, 10–17 (1996).
24) Simon, M., von Deimling, A., Larson, J. J., Wellenreuther, R., Kaskel, P., Waha, A., Warnick, R. E., Tew, J. M., Jr. and Menon, A. G. Allelic losses on chromosomes 14, 10, and 1 in atypical and malignant meningiomas: a genetic model of meningioma progression. *Cancer Res.*, **55**, 4696–4701 (1995).
25) Knuutila, S., Armengol, G., Bjorkqvist, A. M., El-Rifai, W., Larramendy, M. L., Monni, O. and Szymanska, J. Comparative genomic hybridization study on pooled DNAs from tumors of one clinical-pathological entity. *Cancer Genet. Cytogenet.*, **100**, 25–30 (1998).
26) Maarit, S. R., WaÅfel, E. R., Tomi, L., Leif, C. A., Markku, M. and Sakari, K. Different patterns of DNA copy number changes in gastrointestinal stromal tumors, leiomyomas, and shwannomas. *Hum. Pathol.*, **29**, 476–481 (1998).
27) Trofatter, J. A., MacCollin, N. N., Rutter, J. L., Murrell, J. R., Duyao, M. P., Parry, D. M., Eldridge, R., Kley, N., Menon, A. G., Pulaski, K., Haase, V., Ambrose, C. M., Munroe, D., Bove, C., Haine, J. L., Martuza, R. L., MacDonald, M. E., Seizinger, B. R., Short, M. P., Buckler, A. J. and Gusella, J. F. A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. *Cell*, **72**, 791–800 (1993).
28) Rouleau, G. A., Merel, P., Lutchman, M., Sanson, M., Zucman, J., Marineau, C., Hoang-Xuan, K., Demczuk, S., Desmaze, C., Plougastel, B., Pulst, S. M., Lenoir, G., Bijlsma, E., Fashold, R., Dumanski, J., de Jong, P., Parry, D., Eldrige, R., Aurias, A., Delattre, O. and Thomas, G. Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. *Nature*, **363**, 515–521 (1993).
29) De Vitis, L. R., Tedde, A., Vitelli, F., Bigozzi, U., Montali, E. and Papi, L. Screening for mutations in the neurofibromatosis type 2 (NF2) gene in sporadic meningiomas. *Hum. Genet.*, **97**, 632–637 (1996).
30) Sekido, Y., Pass, H. I., Bader, S., Mew, D. J., Christman, M. F., Gazdar, A. F. and Minna, J. D. Neurofibromatosis type 2 (NF2) gene is somatically mutated in mesothelioma but not in lung cancer. *Cancer Res.*, **55**, 1227–1231 (1995).
31) Rustgi, A. K., Xu, L., Pinney, D., Sterner, C., Beauchamp, R., Schmidt, S., Gusella, J. F. and Ramesh, V. Neurofibromatosis 2 gene in human colorectal cancer. *Cancer Genet. Cytogenet.*, **84**, 24–26 (1995).
32) Arakawa, H., Hayashi, N., Nagase, H., Ogawa, M. and Nakamura, Y. Alternative splicing of the NF2 gene and its mutation analysis of breast and colorectal cancers. *Hum. Mol. Genet.*, **3**, 565–568 (1994).
33) Ranchod, M. and Kempson, R. L. Smooth muscle tumors of the gastrointestinal tract and retroperitoneum. A pathologic analysis of 100 cases. *Cancer*, **39**, 255–262 (1977).