Effect of loss of heterozygosity of the *c-kit* gene on prognosis after hepatectomy for metastatic liver gastrointestinal stromal tumors

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The authors have previously reported that loss of heterozygosity (LOH) of the *c-kit* gene could be responsible for the gain in high proliferative activity in some gastrointestinal stromal tumors (GIST), resulting in enhanced metastatic potential. In the present study, an attempt was made to identify the factors that might predict the postoperative prognosis of patients with metastatic liver GIST. The clinicopathologic or genetic features of resected liver GIST in 14 patients who had undergone a hepatectomy for metachronous liver metastases and who had not received adjuvant imatinib treatment were examined. LOH of the *c-kit* gene was observed in seven of 12 metastatic liver GIST (58.3%), of which DNA suitable for testing could be extracted. Ten patients had recurrence after hepatectomy and four had none. The median post-recurrent disease-free survival (PRDFS) after hepatectomy was 27.5 months (range 8–104). The tumor-specific PRDFS was examined using clinicopathologic features, *c-kit* mutation and LOH of the *c-kit* gene. No single clinicopathologic or genetic finding was significantly associated with PRDFS. However, patients with 'Ki67 labeling index <5% and LOH(–)' had a significantly longer PRDFS than those with 'Ki67 ≥5% or LOH(+)' (*P* = 0.032), and there was no correlation between the presence of LOH of the *c-kit* gene and the Ki67 labeling index. LOH of the *c-kit* gene in metastatic liver seems to be a common event, and LOH of the *c-kit* gene in resected liver GIST may be a helpful factor in the prediction of the post-recurrent prognosis of patients with liver metastasis. (*Cancer Sci* 2007; 98: 1734–1739)

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal tumors of the gastrointestinal tract that express the KIT receptor tyrosine kinase. Most GIST have a gain-of-function mutation in the *c-kit* gene, resulting in ligand-independent KIT receptor activation.[1] Most *c-kit* mutations are located in exon 11, which encodes the KIT receptor juxtamembrane domain; other mutations are located in exon 9, 13 or 17.[2–4] In the small subset of GIST without the *c-kit* mutation, alternative oncogenic activating mutations (for example, in exon 12 or 18 of the *PDGRFA* gene) may be involved.[5,6]

Surgery is still regarded as the most effective and reliable modality for primary GIST; however, once postoperative recurrence or metastasis occurs, curing patients with only surgical treatment is difficult.[7–9] The orally bioactive tyrosine kinase inhibitor, imatinib mesylate (Glivec, Gleevec, STI571; Novartis, Basel, Switzerland), which was designed as a Bcr/Abl protein inhibitor for the treatment of chronic myeloid leukemia, also specifically inhibits KIT and PDGFR.[10] The safety and efficacy of imatinib treatment in patients with metastatic GIST has been confirmed by the results of phase I/II trials.[11,12] The National Comprehensive Cancer Network (NCCN) practice guidelines recommend imatinib treatment for recurrent GIST.[13] Although a high response rate of imatinib has been reported in GIST patients, late resistance to imatinib was observed in most patients during imatinib treatment, and a high incidence of adverse effects due to imatinib has also been reported.[11,12,14–20] These issues will remain a matter of vital importance to recurrent GIST patients until effective new therapeutic agents are generalized.

Metastatic GIST are found most commonly in the liver, followed by the mesentery.[7] There are some retrospective studies of treatment for the metastatic liver GIST in which the possibility of an effective hepatectomy is described when complete gross resection is possible,[21,22] Unfortunately, many patients with metastatic liver GIST are unresectable or inoperable because of multiple hepatic lesions or extrahepatic recurrence, but hepatectomy has been recognized as an efficacious treatment for patients with recurrent GIST when they have only one to several hepatic metastasis. However, it is necessary and quite important to determine the indication of imatinib treatment after hepatectomy based on a clinicopathologic or genetic analysis of resected specimens.

The authors have previously reported that loss of heterozygosity (LOH) of the *c-kit* gene could be responsible for the gain in high proliferative activity, resulting in an enhanced metastatic potential in a portion of GIST.[23] However, it remains unclear whether LOH of the *c-kit* gene is a common event in metastatic liver GIST, and whether it has an impact on patients' prognosis after hepatectomy. In the present study, 14 metastatic liver GIST were analyzed immunohistochemically and genetically to address these questions and to clarify the factors that might predict postoperative prognosis in patients with metastatic liver GIST.

Materials and Methods

Patients. Metastatic liver tumors from 14 patients who had undergone a hepatectomy for metachronous liver metastases of GIST at the Second Department of Surgery, Hamamatsu University School of Medicine, the Department of Surgery, Hamamatsu Medical Center, the Department of Surgery, Shizuoka Saiseikai General Hospital or the Division of Digestive and General Surgery, Niigata University Graduate School of Medical and Dental Sciences, were evaluated. Complete resection of both the primary and the metastatic liver GIST was confirmed clinically and pathologically in all 14 patients. Neither chemotherapy nor

radiotherapy was indicated in any of the patients throughout the study and none of them received imatinib after hepatectomy until postoperative recurrence occurred.

Immunohistochemistry. Formalin-fixed paraffin-embedded tissues were used for conventional HE staining and for immuno-histochemical examination as described previously.[24] A rabbit polyclonal antibody against human KIT (CD117, Dako, Kyoto, Japan, ×50) or against bovine S-100 protein (Dako, ×300), and a mouse monoclonal antibody against human CD34 (QB end10, Dako, ×50), human desmin (D33, Dako, ×50), SMA (1A4, Dako, ×50), vimentin (Dako, ×1), or Ki67 (MIB-1, Dako, ×400) were used as the primary antibodies, at the indicated dilutions. Polymer-conjugated secondary antibodies were from the Histofine MAX kit (Nichirei, Osaka, Japan) and visualization was performed according to the manufacturer's instructions.

DNA isolation. Genomic DNA from the metastatic liver GIST of the 14 patients was extracted from formalin-fixed, paraffin-embedded tissues using DEXPAT (Takara Bio, Shiga, Japan) or the Pinpoint Slide DNA Isolation System (Zymo Research, Orange, CA, USA). Normal control genomic DNA was isolated from normal liver tissue adjacent to the tumors.

Polymerase chain reaction and direct sequencing for *c-kit*. Exons 9 and 11 of the *c-kit* gene were amplified by polymerase chain reaction (PCR) using the following oligonucleotide primer pairs: for exon 9, 5′-ATTTATTTTCCTAGAGTAAGCCAGGG-3′/5′-ATCATGATATGGTAGACAGAGC-3′ and for exon 11, 5′-CTCCAGAGTGCTCTAATGACTGAGAC-3′/5′-GTCACTG-TTATGTGTACCCAAAAAGG-3′. PCR was performed in a reaction volume of 20 μL containing DNA extracted as described above. PCR products were electrophoresed through 3.0% agarose gels containing ethidium bromide. Each band was excised from the gel and extracted using a Geneclean Spin Kit (Q BIO gene, Irvine, CA, USA). Direct sequencing of the DNA extracted from the gel was carried out using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Bioscience, Piscataway, NJ, USA) and a 3100 DNA Analyzer (PE/Applied Biosystems, Foster, City, CA, USA).

Microsatellite analysis. LOH at the CA repeat, HK8810, which is located near the *c-kit* gene and is described in the authors' previous report,[23] was evaluated using PCR of tumor and normal specimens using the following oligonucleotide primer pair: 5′-TCAAGAGACAGAGAGACAGAAAG-3′/5′-TTCCTGAG-CACATATCTAACCAC-3′. PCR products were electrophoresed through 5.0% polyacrylamide gels and stained with ethidium bromide.

Statistical analysis. Patients' post-recurrent disease-free survival (PRDFS) rates were calculated using the Kaplan–Meier method, and statistically significant differences in PRDFS were identified using the log-rank test. The Student's *t*-test, Fisher's exact probability test or Mann–Whitney's *U*-test for LOH of the *c-kit* gene and other clinicopathologic factors were used for statistical comparison.

Results

Patient and tumor characteristics. The clinicopathologic and genetic findings in the 14 patients are shown in Table 1. The patient group consisted of nine men and five women, with a mean age of 63.1 years (range, 47–76) when hepatectomy was performed. The distribution of the primary tumor location included 10 in the stomach, two in the ileum, and one in each of the duodenum and the omentum. The median disease-free interval (DFI) between the initial surgery for resection of the primary tumor and the first evidence of liver metastases was 102.5 months (range, 10–159). The size of the metastatic liver tumor was <3 cm in five patients and ≥3 cm in nine (mean 7.8 cm, range 2–18). The number of liver tumors was one in nine patients and two in five patients.

Table 1. Clinicopathologic and genetic findings in 14 cases of metastatic liver gastrointestinal stromal tumors

Case no.	Age (years)[†]	Sex	Site of primary tumor	DFI[‡] (months)	Liver tumor size (cm)/no.	c-kit	CD34	SMA	Desmin	S-100	Vimentin	Ki67 labeling index (%)	c-kit mutation	LOH of the c-kit gene	PRDFS (months)	Recurrence (site)	Imatinib (dose)	Disease outcome	PSRS (month)
1	63	M	Stomach	10	6/1	+	+	+	–	–	+	23	exon 11	+	15	Liver	300 mg/day	AWD	19
2	63	M	Stomach	90	2.4/2	+	+	–	–	–	+	20	exon 11	+	10	Local	300 mg/day	DOD	33
3	68	F	Stomach	37	2/2	+	+	–	–	–	+	10	exon 11	+	20	Liver	–	AWD	15
4	65	F	Ileum	108	18/1	+	–	+	–	–	+	2	exon 11	+	8	Bone	–	DOD	2
5	76	M	Stomach	132	11/1	+	+	–	–	–	+	7	exon 9	+	14	Liver	–	DOD	4
6	53	M	Stomach	48	2.7/1	+	+	–	–	–	+	6.5	exon 11	+	66	–	–	NED	–
7	73	M	Stomach	108	3.5/1	+	+	+	–	–	+	1	exon 9	–	84	Liver	–	DOD	20
8	47	F	Ileum	108	15/2	+	+	+	–	–	+	6	exon 11	–	27	Lung	–	DOD	4
9	72	M	Stomach	159	5.5/1	+	–	+	–	–	–	4.3	exon 11	–	32	–	–	NED	–
10	63	M	Omentum	156	15.5/1	+	+	–	–	–	+	3.5	–	–	66	–	–	NED	–
11	71	F	Stomach	113	17/2	+	–	+	–	–	+	1	exon 11	+	70	Peritoneum	–	DOD	39
12	53	M	Duodenum	97	6.5/1	+	+	–	–	–	+	7.3	exon 9	–	43	Liver	400 mg/day	AWD	19
13	48	F	Stomach	89	2.2/2	+	+	+	–	–	+	1.1	N/D	N/D	13	Peritoneum	400 mg/day	AWD	73
14	69	M	Stomach	79	2/1	+	+	–	–	–	+	4.7	N/D	N/D	104	–	–	NED	–

[†]Age at hepatectomy. [‡]Disease-free interval (DFI) between the initial surgery for resection of the primary tumor and the first evidence of recurrence. AWD, alive with disease; DOD, died of disease; F, female; LOH, loss of heterozygosity; M, male; N/D, not detected; NED, no evidence of disease; PRDFS, post-recurrent disease-free survival; PSRS, post-second recurrent survival; SMA, smooth muscle actin.
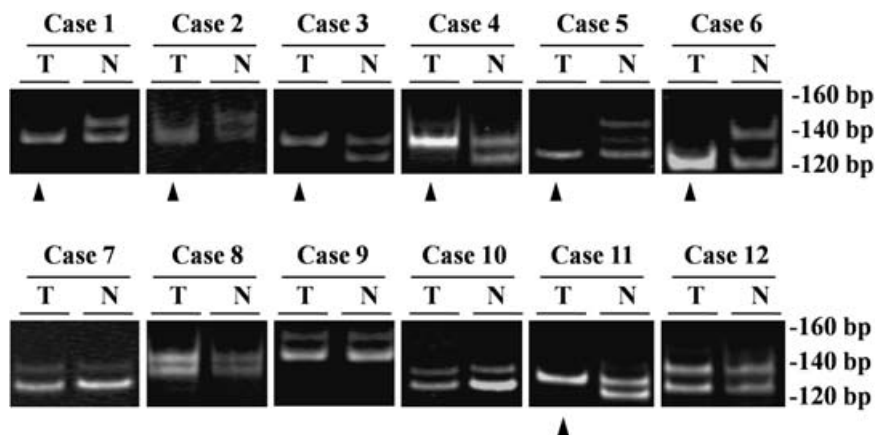
Fig. 1. Analysis of microsatellite markers near the *c-kit* gene. N, normal liver; T, metastatic liver tumor. Arrowheads indicate loss of heterozygosity of the *c-kit* gene.

Immunohistochemical findings. Resected liver tumors in 14 patients were diagnosed as GIST based on positive immunoreactivity for KIT. A positive reaction for CD34 was obtained in 11 patients (78.6%), four (28.6%) were positive for smooth muscle actin (SMA) and 13 (92.9%) were positive for vimentin. No immunoreactivity for desmin or S-100 was seen in any of the 14 tumors. The intensity of Ki67 staining was observed at various levels in the 14 tumors (mean 7.0%, range 1–23; Table 1).

Genetic findings. A mutation analysis of exons 9 and 11 of the *c-kit* gene in the metastatic liver GIST was performed. PCR products for *c-kit* could not be obtained from two of the 14 tumors due to the poor quality of the extracted DNA. Somatic mutations of exon 9 and 11 were observed in numbers 3 and 8, respectively, of the 12 GIST patients tested; neither mutation was detected in the remaining tumor tested.

To clarify the frequency of LOH of the *c-kit* gene in metastatic liver GIST and to investigate whether LOH of the *c-kit* gene contributed to post-recurrent prognosis of patients with metastatic liver GIST, an analysis of microsatellite markers near the *c-kit* gene was performed using PCR of tumor tissues and normal specimens. Microsatellite analysis revealed LOH of the *c-kit* gene in the metastatic liver tumors of seven of the 12 cases (58.3%; Table 1 and Fig. 1; cases 1–6, 11). In the other five cases, a double band that originated from the two different alleles of chromosome 4 was observed in the metastatic liver tumors as well as in the normal liver tissue (Fig. 1; cases 7–10, 12).

Post-recurrent disease-free survival. Of the 14 patients, six died from recurrent disease, one from another disease without recurrence, four were alive with disease, and three were alive without recurrence. The remnant liver was the most common recurrent site after hepatectomy: five of 10 (50%) recurrent cases were located there. The median PRDFS after hepatectomy was 27.5 months (range 8–104; Table 2, Fig. 2a). Of the 10 patients who had second recurrence after hepatectomy, four received imatinib treatment. Post-second recurrent survival and patients' status are shown in Table 1.

The relationships between the clinicopathologic and genetic findings, including LOH of *c-kit*, in the patients and their PRDFS were evaluated using univariate analysis to clarify factors that might predict postoperative prognosis in patients with metastatic liver GIST. As shown in Table 2, no single clinicopathologic or genetic finding was significantly associated with PRDFS. However, there was a trend toward a correlation between a low Ki67 labeling index (<5%) or LOH of the *c-kit* gene (–) and a longer PRDFS, although this trend was not statistically significant ($P = 0.11$: Table 2, Fig. 2b; and $P = 0.087$: Table 2, Fig. 2c, respectively). To clarify whether the Ki67 labeling index and LOH of the *c-kit* gene have an effect on PRDFS, patients were divided into two groups as follows: 'Ki67 labeling index <5% and LOH(–)' and 'Ki67 ≥5% or LOH(+)'. The group with 'Ki67

Table 2. Determinants of post-recurrence survival

Determinant	No.	Median PRDFS	*P*-value[‡]
All patients	14	27.5	
Age (years)			0.45
<65	7	27	
≥65	7	28	
Sex			0.084
Male	9	43	
Female	5	20	
Site of primary tumor			0.63
Stomach	10	24	
Other organ	4	35	
DFI (months)[†]			0.82
<100	7	15	
≥100	7	28	
Liver tumor size (cm)			0.79
<3	5	20	
≥3	9	32	
Liver tumor number			0.097
1	9	43	
2	5	20	
CD34			0.96
–	3	43	
+	11	27	
SMA			0.30
–	10	54.5	
+	4	21	
Ki67 labeling index (%)			0.11
<5	7	66	
≥5	7	20	
c-kit mutation			0.33
exon 11	8	23.5	
others	4	54.5	
LOH of the *c-kit* gene			0.087
–	5	43	
+	7	15	

[†]Disease-free interval (DFI) between the initial surgery for resection of the primary tumor and the first evidence of recurrence. [‡]Significance was estimated using the log-rank test. LOH, loss of heterozygosity; PRDFS, post-recurrent disease-free survival; SMA, smooth muscle actin.

labeling index <5% and LOH(–)' had a significantly longer PRDFS ($P = 0.032$; Fig. 2d).

Clinicopathologic and genetic features of LOH-negative and -positive GIST. We also examined whether the presence of LOH of the *c-kit* gene could be associated with other clinicopathologic or genetic features of GIST. The presence of LOH of the *c-kit*
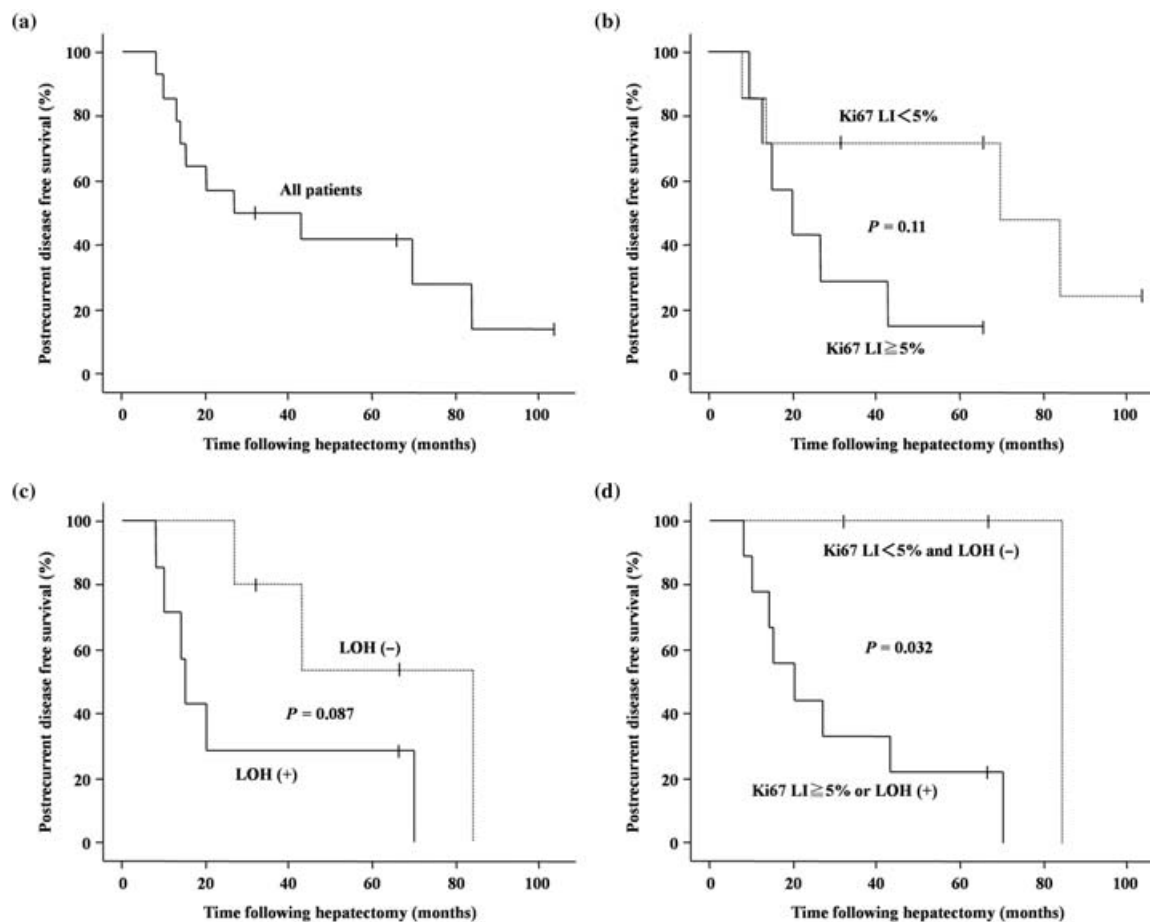
Fig. 2. Disease-free survival after recurrence (post-recurrent disease-free survival [PRDFS]) in (a) all patients, (b) by Ki67 labeling index or (c) the loss of heterozygosity (LOH) of the *c-kit* gene of the metastatic liver tumor, and (d) by the classification of 'Ki67 labeling index <5% and LOH of the *c-kit* gene (–)' and 'Ki67 labeling index ≥5% or LOH of the *c-kit* gene (+)'. Significance was estimated using the log-rank test.

gene was not associated with any other clinicopathologic or genetic features, including the Ki67 labeling index (Table 3).

Discussion

The NCCN practice guidelines note that surgery does not cure recurrent GIST and further recommend imatinib treatment for recurrence.[13] However, we must also bear in mind the fact that the complete response (CR) ratios obtained by imatinib treatment in any randomized clinical trial (RCT) are extremely limited and that imatinib is reported to become ineffective at a certain period after the initial treatment, even when imatinib has been effective initially.[14–19] In the present series, four of the 14 patients had not had a recurrence after hepatectomy and did not have imatinib treatment; three of these four patients were alive without disease more than 5 years after hepatectomy. Considering these results, in this imatinib era, it seems important to re-evaluate the indication of surgery as a first-line treatment for patients with recurrent GIST if all metastatic lesions are completely resectable. Furthermore, it is also important to determine the indication of imatinib treatment after surgery: that is, we should select those patients who have long PRDFS or post-recurrent survival (PRS) without imatinib treatment. It also seems to be beneficial for patients to avoid the adverse effects of imatinib and the medical costs involved. In this retrospective study, resected metastatic liver GIST were analyzed to examine the correlation between clinicopathologic or genetic factors and PRDFS instead of PRS. This was done because most of the recent patients have received imatinib intervention, which has

Table 3. Clinicopathologic features of LOH-negative and -positive metastatic liver gastrointestinal stromal tumors

LOH of the c-kit gene	Negative (–)	Positive (+)	P-value
No.	5	7	
Age (years)	61.6	65.6	0.48[‡]
Sex			0.58[§]
Male	4	4	
Female	1	3	
Site of primary			0.22[§]
Stomach	2	6	
Other organ	3	1	
DFI (months)[†]	125.6	76.6	0.063[‡]
Liver tumor size (cm)	9.5	8.4	0.79[‡]
Liver tumor number	1.2	1.4	0.52[¶]
Ki67 labeling index (%)	4.4	9.9	0.19[‡]
c-kit mutation			0.22[§]
exon 11	2	6	
Other	3	1	

[†]Disease-free interval (DFI) between the initial surgery for resection of the primary tumor and the first evidence of recurrence. [‡]Significance was estimated using Student's *t*-test. [§]Significance was estimated using Fisher's exact probability test. [¶]Significance was estimated using the Mann–Whitney *U*-test. LOH, loss of heterozygosity.

an effect on patient survival after a second recurrence. In fact in the present cases, three of four patients (75%) in the 'post-imatinib era' have survived for more than 19 months after their second recurrence, whereas four of six patients (67%) in the

'pre-imatinib era' died of a second recurrence within 15 months (Table 1).

As a result, no clinicopathologic or genetic feature was demonstrated to be significantly associated with PRDFS. It is reported that a high Ki67 labeling index (≥10%) is an independent indicator of poor prognosis in primary GIST.[25,26] In the present series, patients were classified according to Ki67 labeling index ≥5% or <5% instead of 10%, in order to select those patients with a long PRDFS and, in the same way, long DFI (100 months) and small tumor size (3 cm) were used. There was a trend toward a correlation between a low Ki67 labeling index (<5%) and a longer PRDFS, but this trend was not statistically significant ($P = 0.11$; Fig. 2b). As well as the Ki67 labeling index, the presence of LOH of the *c-kit* gene also could not predict patients with long PRDFS ($P = 0.087$; Fig. 2c). However, the patient group with 'Ki67 labeling index <5% and LOH(–)' had significantly longer PRDFS than those with 'Ki67 ≥5% or LOH(+)' ($P = 0.032$; Fig. 2d). There was no correlation between the presence of LOH of the *c-kit* gene and Ki67 labeling index ($P = 0.19$; Table 3). These results suggested that several mechanisms could be associated with patient prognoses. Thus a combination analysis of clinicopathologic and genetic factors may be helpful to select patients who have long PRDFS without imatinib treatment after hepatectomy.

In an earlier report by the authors, a highly suggestive case who had received neoadjuvant imatinib before gastrectomy was shown, in this case a small portion of the primary gastric GIST with LOH of the *c-kit* gene metastasized to the liver.[23] Neoadjuvant imatinib therapy might have caused the appearance of this heterogeneity in the primary GIST. There might be difficulty in predicting recurrence or prognosis from pathological and genetic analyses of primary GIST because of this heterozygosity. The authors propose the hypothesis that the features of metastatic liver tumors, which seem to be more homogeneous, are more predictive of post-recurrent prognosis than those of primary GIST. Although the clinicopathologic and genetic features of primary GIST have been well reported in many previous studies, those of liver metastases have not been satisfactorily assessed.[27–29] The present report is the first to detail predictive factors for PRDFS based on immunohistochemical and genetic analyses of metastatic liver GIST.

In the present study it was reported that seven of 12 patients (58.3%) had LOH of the *c-kit* gene in metastatic liver GIST. In previous reports, fluorescence *in situ* hybridization (FISH) or comparative genomic hybridization (CGH) analysis has revealed that the loss of chromosome 4 or the *c-kit* locus was observed in 0–3.3% of primary tumors and 40% of metastatic liver GIST.[30,31] These findings suggest that LOH of the *c-kit* gene in the metastatic liver is a more common event than that in primary GIST. However it is still unclear whether or how LOH of the *c-kit* gene causes metastatic progression of a GIST. There may be some conceivable mechanisms by which LOH of the *c-kit* gene can change the biological behavior of a GIST. First, loss of the wild allele of *c-kit* might cause relatively increased expression of the mutant KIT protein, which has 'gain of function mutation', resulting in enhanced downstream signaling and increased cell proliferation. Second, LOH of the *c-kit* gene might induce some secondary genetic changes such as an impaired function of the tumor suppresser gene in chromosome 4, resulting in enhanced metastatic potential. Unexpectedly, some important genes for tumor progression such as vascular endothelial growth factor receptor (VEGFR), PDGFR and the CXC chemokine family ligand near the *c-kit* locus in chromosome 4 might be activated by unknown mechanisms. However, advanced biological *in vitro* and *in vivo* studies will be necessary to verify any such mechanism. Moreover, LOH of the *c-kit* gene might be only partly responsible for GIST acquiring a highly malignant potential. In fact, other changes, such as loss of 1p, 14q or 22q or gene amplifications on 8q or 17q in accordance with *c-kit* alteration, have been reported in metastatic GIST.[30,32] However, as LOH of the *c-kit* gene was frequently observed in metastatic liver GIST, it seems to be plausible that LOH of the *c-kit* gene or a second genetic change around the *c-kit* locus has an effect on the metastatic potential of GIST.

In conclusion, LOH of the *c-kit* gene was frequently observed (58.3%) in metastatic liver GIST, and those patients with 'Ki67 labeling index <5% and LOH(–)' had a significantly longer PRDFS than those with 'Ki67 ≥5% or LOH(+)'. Although more extensive or intervention studies are necessary, LOH of the *c-kit* gene in resected liver GIST may be a helpful factor with which to predict the post-recurrent prognosis of patients with liver metastasis.

Acknowledgments

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