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Frequent Splicing Aberration of the Base Excision Repair Gene *hMYH* in Human Gastric Cancer

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Abstract. *Background: Missense mutation of hMYH, which prevents transversion mutations induced by oxidative DNA damage, is reportedly associated with the development of gastric and colon cancer. We investigated whether deficiency or mutation of hMYH is associated with gastric carcinogenesis. Patients and Methods: Thirty patients with gastric carcinoma, three gastric cancer cell lines and lymphocytes from three healthy volunteers were investigated. Reverse transcription-polymerase chain reaction (RT-PCR) was performed for hMYH, and the full-length sequence of hMYH mRNA was analysed. Results: A silent mutation at codon 473 was seen in two tumours. Single nucleotide polymorphism at codon 345 was observed in 14 patients. These two base substitutions had no pathogenic effect. Seven splice variants were observed and two aberrant transcripts were detected more frequently in cancer specimens (67%) than in normal mucosa (10%). Conclusion: The high frequency of splicing aberration in cancer tissues suggests that aberrant transcripts may be involved in gastric carcinogenesis and cancer development.*

Gastric cancer is one of the most common forms of cancer in the world. The development and evolution of malignant tumours is generally accelerated by the stepwise accumulation of genetic mutations in various genes, including proto-oncogenes and tumour suppressor genes (1). However, the mechanisms underlying mutation in these cancer-associated genes remain unclear.

The gastric mucosa can be exposed to various stressors, such as sodium chloride, *Helicobacter pylori* infection and smoking and these may induce several types of DNA damage and cause repetitive inflammation and the development of gastric cancer (2-4). Oxidative DNA damage can be assessed by means of the levels of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxoG), a stable product of oxidative DNA damage (5). Several reports have indicated that elevated 8-oxoG levels are observed in lung, breast and renal carcinomas (6-8). Intriguingly, *H. pylori*-associated gastritis also shows high levels of 8-oxoG (3). This strongly suggests that the accumulation of 8-oxoG due to oxidative DNA damage is associated with the pathogenesis of carcinomas. Since 8-oxoG can pair with both adenine and cytosine, this potent mutagen leads to an increase in base substitutions, such as G:C-to-T:A transversion mutations. In normal cells, however, such transversion mutations are kept at low levels by the synergic function of DNA repair enzymes (5). Mechanisms protecting against the mutagenic effect of 8-oxoG are well-characterised in *Escherichia coli* (9). The genes *mutM* and *mutY* encode DNA glycosylase, and mutM excises 8-oxoG in the template strand of the genome. If 8-oxoG is not removed prior to replication, DNA synthesis can form an 8-oxoG:A mispair. The misincoporated adenine is removed by MutY-DNA glycosylase and replaced by cytosine in a subsequent base excision repair pathway, enabling MutM protein to repair the remaining 8-oxoG in the template strand. Although 8-oxoG can be incorporated into the genome to pair with adenine, as well as cytosine during replication, the product of the *mutT* gene is responsible for preventing the mispair by hydrolysing 8-oxoG in the nucleotide pool. Synergic repair systems have evolved from bacteria to humans, and mammalian counterparts of these *E. coli* genes are well-known, including *hMYH* (human *mutY* homologue), *hOgg1* (human *mutM* homologue) and *hMTH1* (human mutT homologue) (10-12). In *E. coli*, *mutY* and *mutM* mutant strains manifest as an increased frequency of G:C-to-T:A transversions (13). Deficiencies in *Myh* and *Ogg1* in

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mammals also reportedly predispose more than half of mice to tumours such as lung and ovarian carcinomas (14). In these mice, G-to-T transversion mutations have been observed frequently at codon 12 of the K-*ras* oncogene. Oxidative damage repair genes thus play important roles in preventing mutagenesis and tumour formation.

Bi-allelic germline mutations of the *hMYH* gene have recently been reported to induce elevated somatic G:C-to-T:A transversion mutations in the adenomatous polyposis coli (*APC*) gene (15). The *APC* gene has a strong association with familial adenomatous polyposis and colorectal carcinomas, and inactivating mutations of *APC* following *hMYH* malfunction lead to manifest autosomal recessive colorectal adenomatous polyposis and a high risk of colorectal cancer (16). Likewise, somatic mutations and allelic loss of the *hMYH* gene reportedly contribute to the development of gastric carcinomas, although associations between loss of *hMYH* expression and gastric cancer development have not yet been clarified (17). The present study screened for expression of the *hMYH* gene to investigate associations with gastric carcinogenesis. Somatic mutations in *hMYH* from gastric cancer patients were also investigated.

Patients and Methods

Cell lines. Three gastric cancer cell lines (MKN1, MKN45 and MKN72) were obtained from the Health Science Research Resources Bank of the Japan Health Sciences Foundation. The cell lines were cultured in RPMI 1640 medium supplemented with 10% of foetal calf serum.

Patients. Thirty patients (23 men, 7 women) with different stages of gastric cancer, who had been treated surgically between 2003 and 2004 in the Department of Surgery at Jikei University Hospital, Japan, were included. Their mean age at the time of surgery was 61 years (range, 34-85 years). Familial cancer syndromes were negative based on family histories. All the tumours were reviewed pathologically and classified according to the criteria of the Japanese Research Society for Gastric Cancer. Informed consent was obtained from each patient, and all the study protocols were approved by the Institutional Review Board of Jikei University School of Medicine.

In addition, *hMYH* mRNAs from the three gastric cancer cell lines (MKN1, MKN45 and MKN72) and lymphocytes from three healthy volunteers (1 man, 2 women) were also investigated as positive and negative controls, respectively. The mean age of the healthy volunteers at the time of sampling was 28 years (range, 24-35 years).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). The gastric carcinomas and corresponding normal (non-cancer) mucosae were obtained immediately after gastric resection. Total RNA was extracted from the tissue specimens, the blood samples of healthy donors and the cell lines using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and converted to first-strand cDNA with a First-strand cDNA Synthesis kit (Roche Applied Science, Penzberg, Germany) for RT-PCR according to the instructions of the manufacturer. The PCR reaction was performed using six pairs of oligonucleotide primers as follows: MYH-1 primers (sense: gagcgcagaggctttgaagg; antisense: ggtaggtcccgtttctcttggtc); MYH-2 primers (sense: ggtattgcaggcctctgtct; anti-sense: cagggtctct gctgtacgtg); MYH-3 primers (sense: tgggctggcctgggctactattct; anti-sense: gctgttcctgctccactctc); MYH-4 primers (sense: agcccggc caggagatttcaa; anti-sense: cctggtggtacggtggtc); MYH-5 primers (sense: aggcccaactcaggtct; anti-sense: aagcactttactaacaacagga); and β-actin primers (sense: cttctacaatgagctgcgtg; anti-sense: tcatgaggt agtcagtcagg). One microlitre of cDNA was amplified using a High-Fidelity PCR Master mix (Roche) in a 50-μl reaction mixture containing 2x reaction buffer, 0.4 mM of each deoxyribonucleoside triphosphate (dNTP), and 300 nM of each primer. The PCR amplifications were performed for 35 cycles consisting of: denaturation for 1 min at 95˚C; annealing for 1 min at 48-55˚C; extension for 2 min at 72˚C; and a final extension for 10 min at 72˚C, using a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems, Foster City, CA, USA). The PCR products amplified with MYH5 primer and β-actin primer were loaded on 2.0% agarose gels containing ethidium bromide, and the gels were scanned by a CCD (Charge coupled device) camera to analyse the levels of *hMYH* gene expression.

DNA sequencing. DNA sequencing of all the PCR products was performed using an ABI Prism 3700 Genetic Analyzer (Applied Biosystems). For each PCR product, the same primers were used as the sequencing primers. Labelled dideoxynucleotide terminator cycle sequencing reactions were performed using a BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems). The amplified PCR products with MYH1 primer were subcloned into pCRII-TOPO® vectors (Invitrogen, Carlsbad, CA, USA). Ten clones were randomly collected for each patient and sequenced using a TOPO TA cloning® kit (Invitrogen). Every sequence result was confirmed by reverse sequencing, and alterations were designated by comparison with data deposited in the National Center for Biotechnology Information (NCBI) database sequence.

Statistics. Differences between groups were determined using the Chi-square test. Statistical differences in the present study were assessed using Microsoft Excel software, and values of $p<0.05$ were considered statistically significant.

Results

In the panel of 30 gastric cancer patients, mRNA expression and nucleotide sequence of the *hMYH* gene was determined using the RT-PCR products. All the cancer specimens and normal mucosae showed similar levels of *hMYH* expression to the lymphocytes from healthy donors and gastric cancer cell lines (Figure 1a), indicating that loss of *hMYH* expression is not associated with gastric carcinogenesis.

A base substitution at the third nucleotide of codon 473 (ACG to ACC), which results in no amino acid change, was observed in two cancer tissues (#21 and #41) (Figure 1b). A single nucleotide polymorphism (SNP) at the second nucleotide of codon 345 was identified in 14 patients (Figure 1c). Base substitution from guanine to cytosine results in an amino acid change from serine (S345) to

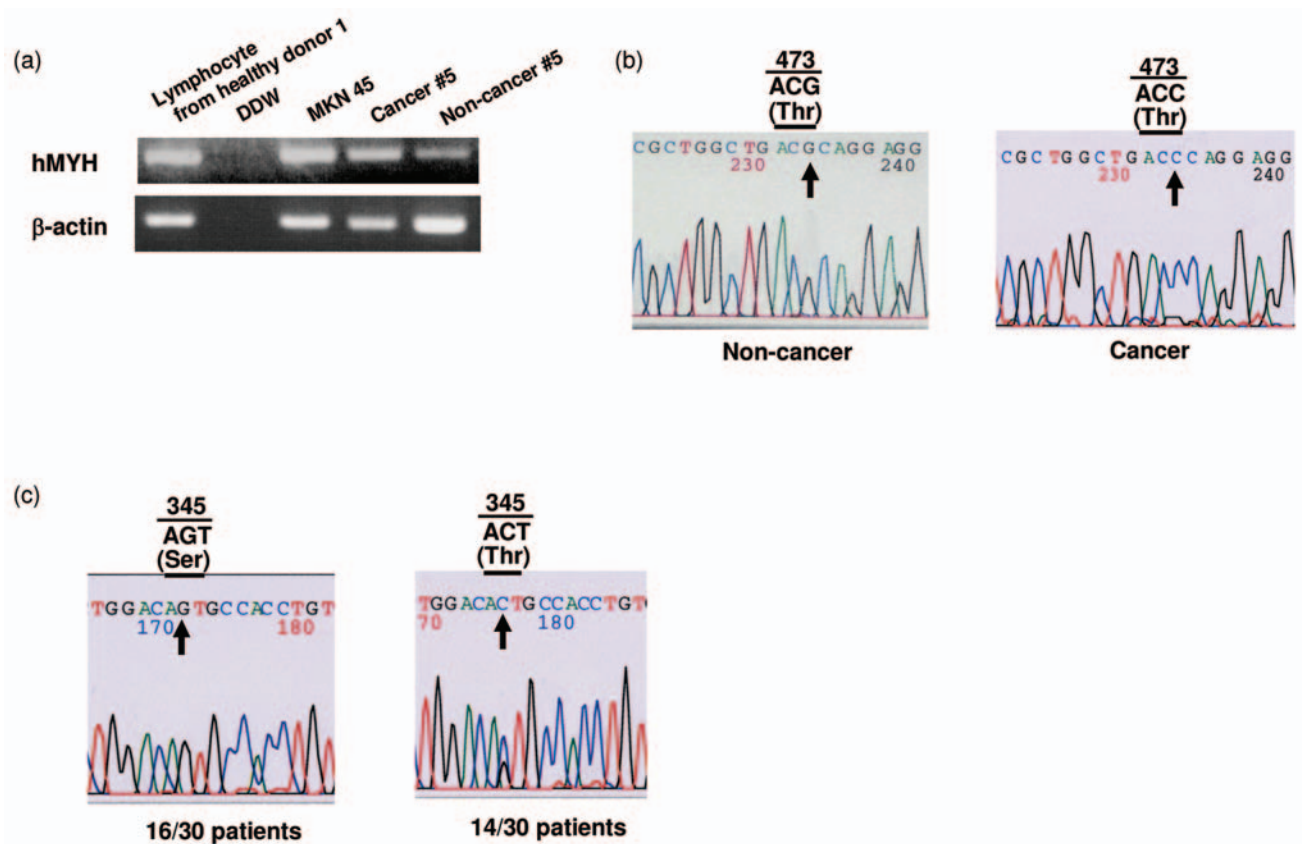


Figure 1. *Expression of hMYH mRNA and observed mutations in gastric cancer patients. (a) Expression of hMYH mRNA in both cancer and non-cancerous tissues, with β-actin visualised as internal control. DDW: Distilled water, MKN45: gastric cancer cell line. (b) Silent mutation at codon 473. Guanine at the 3rd nucleotide in codon 473 changes to cytosine. However, both sequences code threonine. (c) Single nucleotide polymorphism at codon 345. Allele frequency for G/C in the present study was 0.53/0.47.*

threonine (T345). The sequence of codon 345 was conserved between normal mucosa and cancer tissue in these 14 patients. The allele frequencies for S345 and T345 in the present study were 0.53 and 0.47, respectively.

Since direct sequencing analysis of the *hMYH* cDNAs demonstrated heterogeneity in the 5' region, subcloning followed by sequencing analysis was performed. Eight sequence types were detected from all the samples, including cancerous and normal specimens, cell lines and healthy donors. Of the 8 sequence types, 6 had been registered as *hMYH* transcript variants in the NCBI database, α1, α2, α3, β1, γ2 and γ4. Type α3 was considered as a major transcript in a previous report, and likewise in our study, as type α3 was observed most frequently in the normal tissues (Table I). Type α2 had an insertion sequence, CAG, in α3 and type α1 had a 33-bp insertion sequence with the CAG between exons 2 and 3, respectively (Figure 2). Types β1 and γ2 also displayed the same 3-bp and 33-bp insertion sequences as α2 and α1, respectively. In addition, a 4-bp deletion (32delGTGG) was seen in exon 1 (Figure 3), resulting in a shift of the initiation codon from 1 to 15, since another open reading frame initiates at an AUG codon in codon 15 of α3 according to a previous report (19). In type γ4, the first 64 bp of exon 3 were missing (Figure 2), resulting in a truncated protein. In the present study, two new variants were obtained, designated as insertion 1 (IS1) and insertion 2 (IS2). Type IS1 displayed a 42-bp insertion sequence between exons 2 and 3 (Figure 2). These insertion sequences were derived from the intron 2 sequence just in front of exon 3 without frame shift. However, type INS2 showed a 17-bp insertion, derived from intron 1, just behind exon 1 (Figure 3), leading to frameshift and a truncated protein. In healthy individuals, four forms of transcripts (β1, γ2, α2 and 3) were observed, indicating that these transcripts can be considered normal and have no pathogenic effect (Table I). Conversely, type γ4 and IS2 encode truncated proteins. It is suggested that incorrect splice-outs appeared to occur in these two variants.

All three gastric cancer cell lines showed some of these aberrant transcripts, type γ4 and IS2 (Table II).

Table I. *Distribution of hMYH transcripts in normal specimens (mucosa and lymphocytes).*

	#2	#4	#5	#6	#7	#8	#9	#10	#11	#13	#21	#22	#23	#25	#26	#38	#39
Clone1	α3	α3	α2	β1	α1	α3	α3	γ2	β1	α3	α2	α3	α3	α2	α3	α3	α3
Clone2	α3	α3	α3	α3	γ2	α3	α2	α3	α3	α3	α3	α2	α3	α2	α2	α3	β1
Clone3	α3	β1	α2	α3	α2	α2	α3	γ2	α3	α3	**γ4**	α3	α3	α3	β1	α3	β1
Clone4	β1	β1	α3	α3	α3	α2	α3	α2	β1	α3	α2	α1	α3	α2	α3	β1	α3
Clone5	α3	β1	α3	α1	β1	α3	α3	β1	α2	α3	α2	α3	α3	α3	α3	α3	α3
Clone6	α3	β1	α3	α2	α3	α3	α3	α2	α2	α3	α2	α3	β1	α3	α3	α3	α3
Clone7	α2	β1	α3	α3	α3	α2	α2	α2	α2	α2	α2	α2	α3	α2	α3	α3	α3
Clone8	α2	**IS2**	α3	γ2	α3	α2	α3	α3	α3	α3	α2	β1	α3	α2	α3	α3	α3
Clone9	α3	α3	α2	α3	α3	α3	α2	γ2	β1	α3	α3	α3	α3	α3	α3	α2	α3
Clone10	α3	β1	α3	α3	α2	α3	α3	γ2	α3	α3	α3	α3	α3	α3	α2	α3	α3
	#40	**#41**	**#43**	**#44**	**#45**	**#50**	**#51**	**#52**	**#53**	**#54**	**#57**	**#58**	**#59**		**HV1**	**HV2**	**HV3**
Clone1	α2	α3	α3	α2	α3	α3	α3	α3	α3	α3	α2	α3	α3		α3	β1	α3
Clone2	α1	α3	α2	α2	α3	α3	α2	α2	α3	α3	α3	α3	α3		α3	α3	α3
Clone3	α1	α3	α3	α3	α3	α3	β1	α2	α3	α3	α2	α3	α3		α3	α3	α3
Clone4	α3	α3	α3	α3	α3	**γ4**	α3	α3	α1	α2	α3	α3	α3		α3	α3	α3
Clone5	α3	α3	α2	α3	α3	α3	α3	α3	α3	α3	α3	α1	α3		γ2	α3	α3
Clone6	α3	α3	α3	α3	α3	α3	α3	α3	α3	α3	α3	γ2	α3		α3	α2	α3
Clone7	α3	α3	α2	α2	α3	α3	α3	α3	α2	α3	α3	α3	α3		α3	α3	α3
Clone8	α3	α3	α3	β1	α3	α3	α3	α3	α2	α3	α3	α3	α3		α3	α3	α3
Clone9	α3	α3	α1	α3	α3	β1	β1	α3	α3	α3	α3	α3	α3		α3	α3	α3
Clone10	α3	α3	α3	α3	α3	α3	α3	α2	α3	α3	α2	β1	α3		α2	α3	α3

Bold capital indicates aberrant transcripts coding truncated proteins. HV: Healthy volunteer.

Table II. *Distribution of hMYH transcripts in cancer specimens.*

	#2	#4	#5	#6	#7	#8	#9	#10	#11	#13	#21	#22	#23	#25	#26	#38	#39
Clone1	α3	α3	α3	α3	α1	γ2	α3	γ2	β1	β1	α3	α1	α3	α3	α3	**IS2**	γ2
Clone2	α3	α3	α3	α2	α2	α3	α3	γ2	β1	α3	α3	α3	α3	α2	α3	α3	α3
Clone3	α2	**γ4**	α1	α3	β1	**γ4**	α3	IS1	α3	α3	**γ4**	α3	β1	α2	α3	α3	**IS2**
Clone4	α3	β1	α2	α3	α3	α3	α2	α3	α3	α3	**IS2**	β1	**γ4**	**IS2**	α3	α3	α2
Clone5	β1	α3	α3	α3	α3	β1	**γ4**	α3	α3	α3	α3	α1	α3	α2	α3	α2	β1
Clone6	α3	**IS2**	α3	α2	α3	α2	α3	α3	α3	**IS2**	α2	β3	α3	α2	α2	β1	**γ4**
Clone7	α3	β1	α2	**γ4**	**γ4**	**γ4**	α3	α3	β1	α3	IS1	α2	**IS2**	α3	α3	**IS2**	α3
Clone8	β1	α2	α3	α3	α3	α1	α2	γ2	α2	α2	α2	α3	**γ4**	α2	α3	β1	α3
Clone9	α3	**γ4**	α1	α3	γ2	β1	α3	α3	α2	α3	α3	β1	α3	β1	α2	α2	β1
Clone10	α2	**γ4**	γ2	α3	**γ4**	α3	α3	IS1	α3	α3	IS1	α3	α3	α3	α3	IS1	α1
	#40	**#41**	**#43**	**#44**	**#45**	**#50**	**#51**	**#52**	**#53**	**#54**	**#57**	**#58**	**#59**		**MKN1**	**MKN45**	**MKN72**
Clone1	**IS2**	α3	α3	α3	β1	**γ4**	α2	α3	α3	β1	α1	β1	**γ4**		α2	α2	α2
Clone2	β1	α3	α3	α1	α3	α1	α2	β1	α3	**γ4**	α3	α3	α3		α3	**γ4**	β1
Clone3	α3	α3	α3	α3	α1	α3	α2	**γ4**	**γ4**	α3	γ2	α3	α2		α3	α3	α2
Clone4	α3	α3	α3	α3	α1	**γ4**	γ2	**γ4**	α3	α3	α3	α1	α3		**IS2**	β1	IS1
Clone5	α3	α3	α3	α3	α3	α3	β1	α3	α3	α3	α3	α1	β1		α3	α3	α2
Clone6	α1	α3	α3	α3	α2	α3	α3	γ2	**γ4**	α3	α3	β1	α3		β1	α3	α2
Clone7	α3	α3	**γ4**	**γ4**	**IS2**	α2	α3	α2	α2	α3	β1	α3	α2		α3	α3	**γ4**
Clone8	α3	α3	α3	α3	α3	α3	α2	α3	α3	**γ4**	α3	α2	α3		**IS2**	**γ4**	α2
Clone9	α3	α3	α3	α2	α1	α1	α3	α3	α3	α3	α3	α3	α2		α3	**γ4**	β1
Clone10	α3	α3	IS1	α3	α3	α1	α3	β1	α3	α3	α3	α3	α2		**γ4**	α3	β1

Bold capital indicates aberrant transcripts coding truncated proteins.

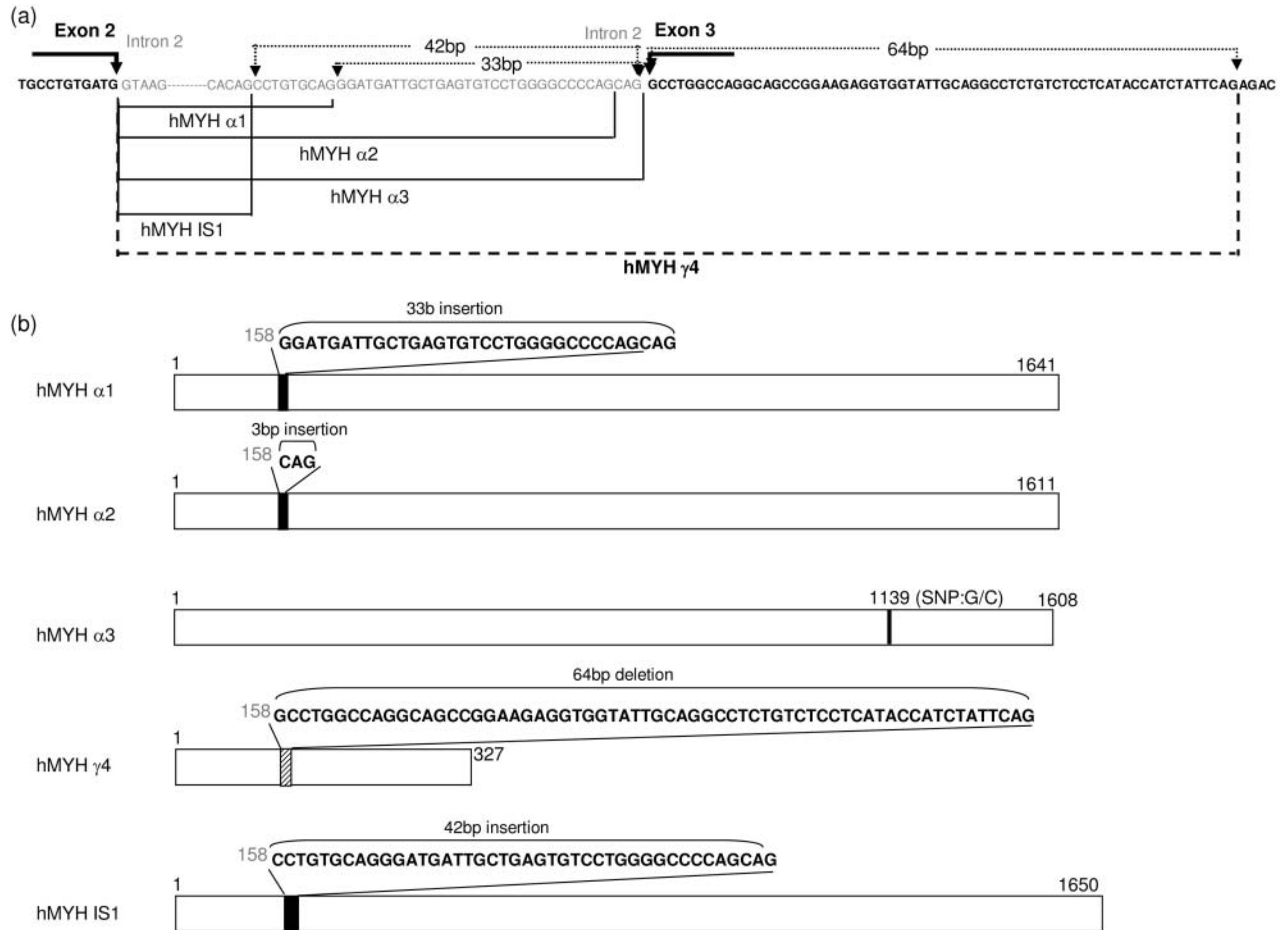


Figure 2. *Variant splicing of intron 2. (a) Bold and grey letters represent the exon and intron, respectively. Frames show sequences spliced out in each variant. Dot frames indicate the splicing out resulting in transcripts coding truncated proteins. (b) Predicted transcripts according to the various splicings of intron 2. Filled and hatched boxes show insertion and deletion of sequences, respectively.*

Conversely, no aberrant transcripts were observed in any of the three healthy controls. More significantly, 20 cancer specimens contained γ4 and IS2 (67%), while only three normal mucosae (10%) showed these splicing aberrations. Aberrant splicing was significantly more frequent in cancer specimens than in normal mucosae ($p<0.01$) (Table III).

Discussion

As reported elsewhere, *hMYH* expression was observed in the gastric cancer cell lines and lymphocytes from healthy volunteers (19). All the gastric cancer specimens and the paired normal mucosa from surgically treated patients also showed *hMYH* expression in this study. These observations indicate that the loss of *hMYH* expression is not involved in the carcinogenesis of gastric cancer.

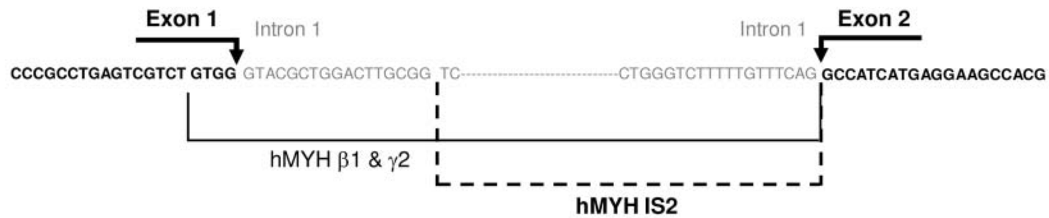
Table III. *Statistical analysis of aberrant transcript frequency.*

	Patients with aberrant transcript (%)	Patients without aberrant transcript (%)
Cancer specimens	20/30 (67%)	10/30 (33%)*
Normal mucosae	3/30 (10%)	27/30 (90%)

*$p=0.01$.

In our study, mutations reported by other investigators, such as Y165C, G382D, P391S and Q400R (15, 17), were not observed in the gastric cancer patients or the cell lines. A silent mutation at codon 473 was seen in two out of the 30 gastric cancer specimens. A SNP at codon 345 was identified in both cancer and paired normal specimens for

(a)



(b)

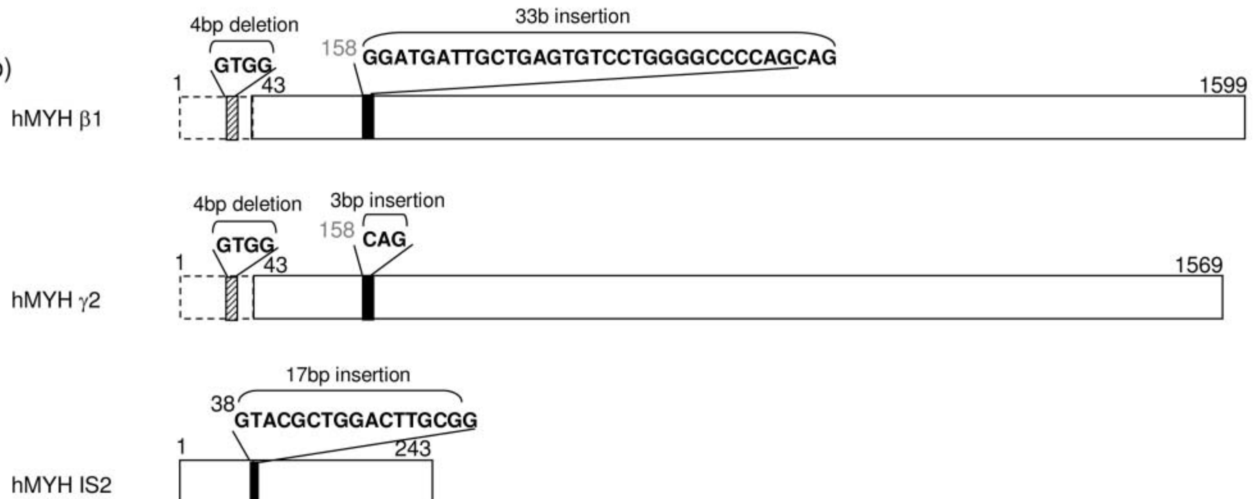


Figure 3. *Variant splicing of intron 1. (a) Bold and grey letters represent the exon and intron, respectively. Frames show the sequences spliced out in each variant. Dot frames indicate the splicing out resulting in transcripts coding truncated proteins. (b) Predicted transcripts by various splicings of intron 1. Filled and hatched boxes show insertion and deletion of sequences, respectively.*

14 patients, and the allele frequencies for S345 and T345 were close to those reported elsewhere for healthy individuals (18). The mutation and SNP observed in our study thus do not appear to have any pathogenic effect. Kim *et al*. (17) reported that two out of 95 gastric cancer patients displayed pathogenic missense mutations in exon 13 of *hMYH*. They suggested that patients with these mutations might have dysfunction of the base excision repair system contributing to gastric carcinomas. Given the present results, we conclude that either loss of *hMYH* expression or missense mutation in *hMYH* is not a frequent event in gastric cancer.

A total of eight splicing variants of *hMYH* were observed in the present study. According to our observations, two aberrant transcripts were presumed not to produce functioning proteins, and the frequency of aberrant *hMYH* transcripts was significantly higher in the cancer lesions than in the normal tissues. This indicated a connection between aberrant transcripts of *hMYH* and gastric tumorigenesis or cancer development, which suggests two possibilities. First, aberrant transcripts of *hMYH* may play a significant pathogenic role in gastric carcinogenesis. Alternatively, aberrant transcripts of *hMYH* may have no pathogenic effect, but instead represent a relaxation of RNA splicing fidelity in cancer cells. Some studies have documented that 35-59% of human genes have at least one alternative splicing isoform (20, 21). In addition, several studies have shown that alternative RNA splicing and aberrant transcripts occur more frequently in human cancer cells than in normal tissues, although the causes of compromised splicing fidelity are not well known (22-24). As the cancer specimens in the present study contained functioning mRNA at similar levels to normal tissues, frequent transcript aberration appears to reflect relaxation of *hMYH* mRNA splicing, rather than a direct contribution to stomach cancer development. The relaxed status of *hMYH* mRNA splicing may be critical for cells, since the aberrant transcript of *hMYH* can reportedly reduce translation efficiency (25). In addition, the observation that compromised splicing fidelity occurs in normal tissues supports the idea that genetic changes required for gastric tumorigenesis take place even in normal mucosae in the stomach (26, 27).

Somatic mutation and loss of expression of *hMYH* do not seem to be involved in gastric carcinogenesis. However, aberrant transcripts are frequently present in cancer cells, suggesting that compromised splicing fidelity of the *hMYH* gene may play some role in the development of gastric cancer.

Acknowledgements

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