ELECTRONIC LETTER

A variant form of *hMTH1*, a human homologue of the *E coli mutT* gene, correlates with somatic mutation in the *p53* tumour suppressor gene in gastric cancer patients

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Oxidative damage to diverse physiological molecules, including proteins, lipids, and nucleic acids, is an inevitable outcome of various cellular activities in living organisms. In particular, some oxidised forms of nucleotides cause miscoding of genetic information, and have therefore been present as a major threat for cells. Multigene systems to counteract such oxidative damage have evolved in diverse organisms. In *E coli*, several mutants designated 'mutator' have been isolated, and in these cells the mutation rate is significantly elevated, due to disruption of genes regulating the spontaneous mutation rate on the genome. Previous studies using these mutators have identified three genes that function in the system to counteract mutagenic oxidative damage. The *mutT*⁻ strain is one of the mutators that exhibit the highest spontaneous mutation rate. Maki H *et al* have shown that the product of the *mutT* gene hydrolyses an oxidised form of guanine nucleotides, 8-oxo-2'-deoxyguanosine 5'-triphosphate (8-oxo-dGTP).¹ 8-oxo-dGTP incorporated into the genome stably pairs with adenine as well as cytosine in the template strand, accumulation of this oxidised form of guanine nucleotides leads to an increase in base substitution mutations—that is, A:T to G:C and G:C to T:A transversions. In the *mutT*⁻ strain, the rate for A:T to C:G transversion is indeed elevated 1000 fold over the wild type level.² The other two genes that function in cooperation with *mutT* are *mutM* (*fpg*) and *mutY*, both of which encode a DNA glycosylase to excise deleterious bases on the genome.³ The former excises 8-oxo-guanine in the opposite site of cytosine on the genome, the latter removing adenine that pairs with 8-oxo-guanine. Thus, even in cells lacking *MUT*, G:C to T:A transversions are suppressed low.⁴ Multiplicity of cellular anti-mutagenic systems guarantees the spontaneous mutation rate on the genome at an extremely low level.

Several mammalian counterparts of these *E coli* genes are now known. *MTH1* is the first identified mammalian homologue of *E coli mutT*.⁵ The human *MTH1* gene, *hMTH1*, has been described in detail.⁶ Altered function of *hMTH1* and consequent elevation of the mutation rate may be an attractive hypothesis for various human diseases, particularly cancer. In several human diseases, including some common malignancies, the nucleotide sequence of *hMTH1* has been explored.⁶ However, no apparent mutations were found. Instead, a single nucleotide polymorphism (SNP) at the first nucleotide of codon 83, which results in amino acid change from valine (V83: GTG) to methionine (M83: ATG), has been found, and the incidence of the M83 allele is relatively frequent in healthy controls (allele frequency = 0.09).⁷ In mice, an *mutT* homologue has been identified and designated *Mth1*. *MTH1*-null mice, in which *Mth1* is homozygously disrupted, have been reported.⁸ Although the estimated increase in the mutation rate was only twice that in wild type counterparts, relatively frequent tumorigenesis was observed in the liver, lung, and stomach of the animals.⁹ In

Key points

- *hMTH1* is a human homologue of the *E coli mutT* gene, which encodes a polypeptide that hydrolyses a potent mutagenic substrate for DNA replication, 8-oxo-dGTP. Altered function of *hMTH1* has been suspected in various pathological states.
- In some common malignancies, the nucleotide sequence of *hMTH1* has been explored. However, no mutations have been found. Instead, G:C to A:T transition at the first nucleotide of codon 83, GTG(Val) to ATG(Met), has been reported as a polymorphism.
- We show here that this polymorphic variation (V83M) is significantly more frequent in gastric cancer patients than in healthy controls, and correlates with mutation in the *p53* tumour suppressor gene.
- V83M variation of *hMTH1* correlated with mutation in *p53* suppressor gene. The frequency of *p53* mutation is significantly higher in tumours harbouring the M83: ATG allele (6/14, 42.9%) than those without this variant allele (9/58, 15.5%).
- Connection between variant *hMTH1* and mutation in a common tumour suppressor gene may suggest a possible mechanism for a mutator phenotype underlying the pathogenesis of tumours.

patients with liver and lung cancer, sequence of *hMTH1* has been determined.⁷ However, no relevant sequence alterations were found, and the M83 allele is not also frequent in patients with these malignancies.

Gastric cancer is one of the most common malignancies in human populations, and in this cancer mutations are frequently found in various genes, including proto-oncogenes and tumour suppressor genes, as in other cancers.¹⁰ It still remains controversial whether increase in mutation rate plays an important role in tumourigenesis, and the sources of mutations in cancer are unknown.

Increased occurrence of gastric tumours in *MTH1*-null mice prompted us to explore the structure of this gene in a patient population. Here we report that the minor polymorphic base alteration at codon 83 is significantly more frequent in gastric cancer patients than in healthy controls, and correlates with mutation in *p53* tumour suppressor gene.

MATERIALS AND METHODS

Patients and tissue specimens

Samples were collected from 72 gastric cancer patients who underwent surgery in our department from January 1990 to December 1997. Men and women numbered 47 and 25,

respectively; age ranged from 35 to 86 years, with a mean of 64 years. Written informed consent for studies using the tissues was obtained from each patient. Ethical approval was obtained from the IRB of Kyushu University. Specimens were taken immediately after resection and placed in liquid nitrogen.

Reverse transcription-polymerase chain reaction (RT-PCR) for *hMTH1* gene

Total RNA was extracted from cancerous and corresponding normal tissue specimens using ISOGEN (Nippon Gene, Toyama, Japan), according to the manufacturer's instructions. The first strand of cDNA was synthesised using 2 µg of total RNA, random hexadeoxynucleotide primer (TaKaRa, Tokyo, Japan), and RAV-2 reverse transcriptase (TaKaRa). After the first strand synthesis, PCR was performed using a GeneAmp 9600 thermal cycler (Applied Biosystems, Foster City, CA, USA.). A 50 µl reaction mixture contained 0.2 µM oligonucleotide primer, 25 ng cDNA, 1 X PCR buffer (TaKaRa), 250 µM dNTP, and 2.5 U *ExTaq* polymerase (TaKaRa). The oligonucleotide primers used to amplify the *hMTH1* sequence were 5'-ACCTGCCCCACCAATTACA-3' (forward), and 5'-GCCTCTTGTAAGACTGGTT-3' (reverse) (fig 1). The thermal conditions were as follows: 35 cycles at 95°C for three seconds, 55°C for 45 seconds, and 72°C for 60 seconds; and one cycle at 72°C for five minutes. The PCR products were purified using a microcon-100 microconcentrator (Amicon, Beverly, MA, USA.).

Genomic PCR for *p53* gene

Genomic DNA was extracted from tumour specimens. Tissue was ground in liquid nitrogen and lysed in digestion buffer (10 mM Tris.Cl, pH 8.0; 0.1 M EDTA, pH 8.0; 0.5% SDS; and 20 µg/ml pancreatic RNase). After treatment with proteinase K and extraction with phenol, DNA was precipitated with ethanol, and then dissolved in 1 X TE (10 mM Tris-Cl, pH 7.5; and 1 mM EDTA). To amplify the sequences corresponding to *p53* exon 5 to exon 9, PCR was carried out using a GeneAmp 2400 thermal cycler (Applied Biosystems). A 100 µl of reaction mixture contained 100 ng genomic DNA, 10 µM of each primer, 200 µM dNTP, and 2.5 U *Taq* DNA polymerase (TaKaRa). The primer sequences were for exon 5: 5'-TCTGTTCACCTGTGCCCTGAC-3' (forward), and 5'-ATCAGTGAGGAATCAGAGGCC-3' (reverse); for exon 6: 5'-GCGCTGCTCAGATAGCGATG-3' (forward), and 5'-GGAGGGCCACTGACAACCA-3' (reverse); for exon 7: 5'-TGCCACAGGTCTCCCCAAGG-3' (forward), and 5'-GCACAGCAGGCCA GTGTGCA-3' (reverse); for exons 8 and 9: 5'-TTGGGAGTAGTGGAGCCT-3' (forward), and 5'-AGTGTAGACTGGAAA CTTT-3' (reverse). The thermal conditions were as follows: one cycle at 95°C for 10 minutes; 35 cycles at 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 45 seconds. PCR products were purified using a microcon-100 microconcentrator (Amicon).

DNA sequencing

Labelled dideoxynucleotide terminator cycle sequencing reactions were performed using ABI Prism Dye Terminator Sequencing Kits (Applied Biosystems) and an ABI Prism 310 Genetic Analyser (Applied Biosystems). For each of PCR products, the same primers were used as a sequencing primer. Data were analysed using the ABI sequence software, DNA Sequencing Analysis, ver. 3.0 (Applied Biosystems). In each analysis, electropherograms were checked visually, not to misread minute sequence alterations. Sequence alterations were designated by comparison with the data deposited in the NCBI database sequence alterations found in one PCR product were verified by reverse sequencing and finally

Figure 1 Schematic representation of *hMTH1* gene structure and sequencing strategy. Genomic and cDNA structures are shown. The position of codon 83 is indicated by a vertical arrow. The region corresponding to the 'phosphohydrolase module' is shown by a hatched box on mRNA. The 333-bp region, including codon 10–107, was amplified by RT-PCR. PCR primers are indicated by horizontal arrows. Another polymorphism in the 5' untranslated region is also shown (asterisk).²⁸

confirmed in more than two independently amplified PCR products.

RESULTS

A variant form of *hMTH1* (M83) is more frequent in gastric cancer patients

In a panel of 72 gastric cancer patients, the nucleotide sequence of the *hMTH1* gene was determined using reverse transcript-polymerase chain reaction (RT-PCR) products. The 333-bp cDNA fragment that encompasses a region coding the 'phosphohydrolase module', the catalytic domain of oxidised purine nucleoside triphosphatase, was amplified and sequenced (fig 1). In agreement with previous reports,⁶ the minor M83 allele that encodes methionin (ATG), instead of valine (GTG), at codon 83 was found in several patients. According to the sequence data, the subject population was divided into three allelotypes, V83: GTG/V83: GTG, V83: GTG/M83: ATG, and M83: ATG/M83: ATG (table 1). In gastric cancer patients, the M83 allele appeared more frequent than that in healthy controls (χ^2 test, $p = 0.007$). Among 72 gastric cancer patients, 14 (19.5%) carried the M83 allele. Four patients (5.6%) were homozygous for this variant allele. The subject population was next divided into two subgroups, one homozygous for this variant allele and the other. Another χ^2 test revealed that the allelotype homozygous for the variant *hMTH1* allele (M83) was significantly more frequent in gastric cancer patients than in healthy controls ($p = 0.01$) (table 1). Thus, we conclude that the variant form of *hMTH1* (M83) is more frequent in gastric cancer patients. These sequence alterations were also confirmed by sequencing PCR products of genomic DNA extracted from tumour specimens and corresponding normal tissues (data not shown). No other sequence alterations were found within the region examined.

We statistically tested whether this V83M variation of *hMTH1* correlates with clinicopathological features of the patients. However, no statistically significant correlation was found between the presence of this variant allele and any of the common clinicopathological features of patients (data not shown).

V83M variation of *hMTH1* correlates with mutation in *p53* gene

hMTH1 encodes an enzyme that hydrolyses mutagenic oxidised purine nucleoside triphosphates such as 8-oxo-dGTP. Variation of a cellular component functioning in the

Table 1 The frequency of single nucleotide polymorphism at codon 83 in the *hMTH1* gene: a comparison between gastric cancer patients and healthy controls

<i>hMTH1</i> alleleotype	Gastric cancer (n=72)	Controls (n=400) ⁷	
V83: GTG/V83: GTG	58(80.5%)	330(83.5%) ⁷	
V83: GTG/M83: ATG	10(13.9%)	67(16.8%) ⁷	
M83: ATG/M83: ATG	4(5.6%)	3(0.8%) ⁷	p=0.0073
V83: GTG/V83: GTG, V83: GTG/M83: ATG M83: ATG/M83: ATG	68(94.4%) 4(5.6%)	397(99.2%) ⁷ 3(0.8%) ⁷	p=0.01

anti-mutagenic systems may affect the sequence stability maintained throughout the genome. Carcinogenesis is thought to comprise stepwise alterations in proto-oncogenes or tumour suppressor genes. We therefore examined the relationship between *hMTH1* variation and mutation in *p53* gene, a tumour suppressor gene most commonly mutated in various human malignancies. In our panel of 72 patients with gastric cancer, *p53* mutations resulting in an amino acid substitution were detected in 15 tumours (20.8%). The mutations were predominantly G:C to A:T transitions in codons, including the acknowledged hot spots (data not shown). Intriguingly, *p53* mutation correlated with the variant form of *hMTH1* (table 2). The frequency of *p53* mutation was significantly higher in tumours harbouring at least one M83 allele than in those without M83 allele (Fisher's exact test, $p = 0.034$). However, in a comparison between tumours homozygous for M83 allele and others, difference in the incidence of *p53* mutation was not statistically confirmed, probably due to a paucity of M83-homozygous tumours. Among nine *p53*-mutant tumours without the M83 allele, G:C to A:T and A:T to G:C transitions were noted in three (3/9, 33.3%) and two (2/9, 22.2%), respectively. G:C to T:A transversion was also shown in three tumours (3/9, 33.3%) (table 3). On the other hand, among six tumours with *p53* mutations that possessed at least the M83 allele, G:C to A:T transitions were observed in four (66.7%) (table 3). However, this partiality of mutation to G:C to A:T transition in tumours harbouring a M83 allele was not statistically confirmed. A:T to C:G transversion was not found in neither.

DISCUSSION

Altered function of *hMTH1* has been suspected in various pathological states, particularly in tumourigenesis, in human bodies. However, in spite of efforts to explore *hMTH1* gene in various human diseases,⁶ no mutations have been found. Single nucleotide polymorphism (SNP) at codon 83, V83: GTG to M83: ATG, was not clearly associated with lung or liver cancers.⁷ However, in the present study, we have shown that in gastric cancer patients this polymorphic variation of *hMTH1* is significantly more frequent than in healthy

controls. In this context, this is the first report that has shown a connection between V83M variation of *hMTH1* and human malignancies.

Mutations are found in various genes in tumours. One may presume a state with an elevated mutation rate in some steps of tumourigenesis. However, it still remains controversial whether a state with an elevated mutation rate plays an important role in tumourigenesis. Bodmer and colleagues¹¹⁻¹² pointed out that tumour cells that harbour mutations in tumour suppressor genes or proto-oncogenes can be selected merely by phenotypical advantage, without an elevated mutation rate. On the other hand, a line of evidences suggests an existence of a state with an elevated mutation rate in tumours, and this state is now referred to as 'mutator phenotype'. The critical role of mutator phenotype in tumourigenesis has been emphasised by Loeb and colleagues.¹³⁻¹⁴ Spontaneous mutation rate on the genome is invariably regulated. Previous studies using *E coli* mutators suggest that there are several cellular systems, the failure of which will lead to a significant increase in the mutation rate.

The first example of mutator phenotype derived from a disruption of such cellular systems is defective DNA mismatch repair in a cancer prone syndrome, hereditary non-polyposis colorectal cancer (HNPCC).¹⁵⁻¹⁶ Deficiency in mismatch repair is also associated with other human malignancies.¹⁷ One may expect that the spontaneous mutation rate is elevated in mismatch repair defective cells. Indeed, in mismatch repair gene knock out mouse cells, the mutation rate was 15-fold higher than in wild type cells.¹⁸ However, mutations in representative tumourigenic genes, such as *p53* and *ras*, were not found in mismatch repair defective human tumours. Instead, insertion/deletion mutation in repetitive sequences such as microsatellites was observed. This phenomenon is now referred to as 'microsatellite instability' (MSI), and the cellular phenotype with MSI has been designated 'microsatellite mutator phenotype' (MMP).¹⁹⁻²⁰ The discrepancy between knock out animals and

Table 2 Relationship between *p53* gene status and V83M variation of *hMTH1*

<i>hMTH1</i> alleleotype	<i>p53</i> gene status	
	wt (n=57)	m (n=15)
V83: GTG/V83: GTG	49(84.5%)	9(15.5%)
V83:GTG/M83:ATG, M83: ATG/M83: ATG	8(57.1%)	6(42.9%)
		p=0.034

Table 3 Spectra of *p53* mutations in gastric tumours with and without the M83 *hMTH1* allele

<i>p53</i> mutation	V83:GTG/ V83:GTG (n=9)	V83:GTG/M83:ATG, M83:ATG/M83:ATG (n=6)
Transition		
G:C → A:T	3(33.3%)	4(67.4%)
A:T → G:C	2(22.2%)	0
Transversion		
G:C→T:A	3(33.3%)	1(16.7%)
G:C→C:G	0	0
A:T→T:A	1(11.1%)	1(16.7%)
A:T→C:G	0	0

human tumours may suggest an oversimplification in our discussions.

The second example is failure in the systems counteracting mutagenic oxidative damage in cells. In *E coli*, MutT, MutY, and MutM cooperatively function to minimise the effects of an oxidised form of guanine nucleotides, 8-oxo-dGTP, in the pool and on the genome.⁴ Mammalian homologues of these proteins and mice in which their genes are homozygously disrupted have been reported.^{9 21–23} As mentioned above, the failure in this system that is clearly associated with human diseases has not been found. However, in *MTH1*-null ES cells, the mutation rate is elevated twofold higher, compared with the wild type counterparts.⁹ Altered function of *hMTH1* may also lead to accumulated mutations in various genes in human cells.

Connection between V83M variation and mutation in other genes, particularly proto-oncogenes or tumour suppressor genes, has not thus far been addressed. This is the first report of a connection between the variant *hMTH1* and *p53* mutation.

E coli MutT hydrolyses an oxidised nucleotide, 8-oxo-dGTP, into its monophosphate form.¹ 8-oxo-dGTP incorporated in the genome pairs with adenine, as well as cytosine, which results in G:C to T:A or A:T to C:G transversions. In an *E coli* strain lacking MutT, the spontaneous occurrence of A:T to C:G transversion increases 1000-fold higher than in wild type strains.² In the *mutT*[−] strain, G:C to T:A transversions are suppressed low by compensatory activities of MutM and MutY glycosylases.⁴

Frequent variation of *hMTH1* in gastric cancer may suggest a biased spectrum in observed mutations. However, the majority of mutations found in *p53* was G:C to A:T transitions, albeit this bias has not been statistically confirmed (table 3). No A:T to C:G transversions were found. In fact, the biological significance of V83M variation has not been determined. Yakushiji H *et al*²⁴ reported that recombinant M83-variant *hMTH1* exhibited an increased heat lability in both structure and catalytic function; however, within a physiological range of temperature, difference in the activity to hydrolyse 8-oxo-dGTP was not confirmed.

Recently, other forms of oxidised nucleotides have been found as a mutagenic substrate for DNA replication. 2-hydroxy-2'-deoxyadenosine 5'-triphosphate (2-OH-dATP) is known to be formed in cells by an oxidative stress²⁵ and, once incorporated into the genome, pairs with various bases including cytosine.²⁶ Pairing of cytosine with an incorporated adenine nucleotide results in G:C to A:T transitions. Intriguingly, *hMTH1* efficiently hydrolyses this oxidised adenine nucleotide as well as 8-oxo-dGTP, whereas *E coli* MutT does only the latter.^{6 27} Difference in the activity to hydrolyse 2-OH-dATP between M83-variant *hMTH1* and its authentic form is now being approached in our laboratories.

V83M variation may conceal a previously unrecognised biological significance. H Oda *et al*²⁸ reported that the polymorphism at codon 83 is tightly linked with another SNP, exon2b(GT) to exon2b(GC) at the 5'-splicing site of exon2b segment that is an exonic intron and spliced out by alternative splicing (fig 1). In the exon2b(GC) allele, an alternative translation initiation occurs on *hMTH1* transcripts, resulting in *hMTH1*, a protein that possesses a functional mitochondrial targeting sequence (Sakai and Nakabeppu, in preparation). Although this polymorphism has not been addressed in this study, a tight linkage between M83 allele and the exon2b(GC) allele may suggest the existence of another molecular variety with different biological functions. This present study suggests that a functional alteration of *hMTH1* due to these polymorphisms may occur in vivo, and that an imbalance among these molecular varieties may relate to tumorigenesis, at least in the stomach.

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