Pergamon

European Journal of Cancer, Vol. 35, No. 4, pp. 647–651, 1999

Printed in Great Britain
0959-8049/99/$ - see front matter

PII: S0959-8049(98)00411-0

Original Paper

Mutation Analysis of the *PTEN/MMAC1* Gene in Cancers of the Digestive Tract

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The 10q23.3 gene *PTEN* (phosphatase and Tensin homologue deleted on chromosome 10) or *MMAC1* (mutated in multiple advanced cancers 1) was recently reported to undergo frequent mutation, including mutations and deletions in multiple advanced cancers. This study showed that the aberrant transcripts of this gene are frequently found in cancers of the digestive tract, paired non-cancerous tissues and normal peripheral mononuclear cells. Sequence analysis of the aberrant transcripts revealed three types of deletions: (i) a deletion junction with a splicing-like donor or acceptor sequence; (ii) several-base homology near or between the donor acceptor site at the deletion junction; and (iii) deletion with insertion. From these results, it is suggested that aberrant transcripts of *PTEN/MMAC1* found by nested reverse transcription-polymerase chain reaction are a common (or natural) phenomenon unrelated to oncogenesis. The mechanism producing these aberrant transcripts needs further investigation. Using single-strand conformation polymorphism and direct sequencing to analyse for small base changes of the genomic DNA of the *PTEN/MMAC1* gene revealed no point mutations or small base changes.

Key words: mutation analysis, *PTEN/MMAC1*, digestive tract cancer, aberrant transcript
Eur J Cancer, Vol. 35, No. 4, pp. 647–651, 1999

INTRODUCTION

THE GENE *PTEN* (phosphatase and tensin homologue deleted on chromosome 10) or *MMAC1* (mutated in multiple advanced cancers 1) was isolated from the homozygous deletion region of chromosome 10q23.3 in human glioma cell lines and has been identified as a candidate tumour suppressor gene [1, 2]. The gene was found to be deleted or to have point mutations in glioma, breast, prostate and kidney cancers. The predicted PTEN/MMAC1 protein contains sequence motifs with significant homology to the catalytic domain of a protein phosphatase and to the cytoskeletal proteins tensin and auxilin [1, 2]. The *in vivo* function of this enzyme is not yet known. Recently, two autosomal dominant cancer predisposition syndromes, Cowden disease and Bannayan–Zonana syndrome, also revealed *PTEN* germline mutations [3, 4]. These results further confirmed that *PTEN/MMAC1* is a tumour suppressor gene.

Several studies have demonstrated alterations of the chromosome 10q22–25 in several tumours [5–7]. Recently, two candidate tumour suppressor genes (*FHIT* and *TSG101*) were proposed based on the presence of large intragenic deletions in several cancers, suggesting that the deletions play some roles in the oncogenesis of these tumours [8, 9]. In this study, using a similar approach, *PTEN/MMAC1* mutations were analysed in various gastrointestinal tract cancers at the cDNA level by nested reverse transcription-polymerase chain reaction (RT-PCR) and at the level of genomic DNA by single-strand conformation polymorphism (SSCP) and direct sequencing.

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Received 30 Jul. 1998; accepted 14 Oct. 1998.

MATERIALS AND METHODS

Isolation of DNA and RNA from tissues and peripheral mononuclear cells

Tumour tissues from 21 colon cancers, 15 gastric cancers and 10 oesophageal cancers, along with matched normal tissues from the same cases, and peripheral mononuclear cells of a normal control were obtained from Mackay Memorial Hospital and Taipei Municipal Jen-Ai Hospital. The tissues were frozen immediately after surgical resection and stored in liquid nitrogen until extraction of DNA or RNA. DNA extraction was performed as described previously [10]. Total RNA was extracted using a commercial kit (RNAzolTMB, Biotecx Laboratories, Houston, Texas, U.S.A.). RNA was stored as a pellet in ethanol or solubilised in RNase-free water and kept at -70°C. Reverse transcription was performed as described previously [11, 12].

Reverse transcription-polymerase chain reaction and sequencing of cDNA

The nested RT-PCR conditions were as described previously with some modifications [11, 12]. In brief, 1 µl of cDNA was used for the first PCR amplification in a volume of 100 µl containing 100 ng each of primers (P1-5′-1: 5′-AGAGCCATTTCCATCCTGCA-3′ and P1-3′-1: 5′-GTGTCAAAACCCTGTGGATG-3′), 50 µM of each dNTP, 1× PCR buffer, and 2.5 units Taq polymerase. The PCR condition was 35 cycles of 1.5 min at 94°C for denaturation, 1.5 min at 56°C for annealing, and 2.5 min at 72°C for extension, with a final extension for 5 min at 72°C, using a Perkin-Elmer Cetus PCR Thermocycler. Then 1–10 µl of the first PCR products were subjected to a second round of PCR amplification using nested primers (P2-5′-2: 5′-CTCCTCCTTTTTCTTCAGCC-3′ and P2-3′-2: 5′TGACACAATGTCCTATTGCC-3′) for 35 cycles under the above conditions. The PCR products were electrophoresed and analysed using agarose gel. All abnormal bands were subjected to isolation and sequencing analysis. For the isolation of fragments from the PCR products, the products were electrophoresed in a 3% low melting agarose gel and the desired fragments were excised after separation. They were then purified using a commercial kit (Qiaex II gel extraction kit, Qiagen, Chatsworth, California, U.S.A.) and the purified fragments were sequenced by the cycling sequencing method with an ABI 310 automatic sequencer.

Allele deletion analysis (loss of heterozygosity)

The following microsatellite markers were analysed in the cancerous and matched non-cancerous tissues: *D10S541*, *D10S215*, *D10S579* and *AFM280*. PCR and gel analyses were performed as described, previously [2].

Single-strand conformation polymorphism analysis of genomic DNA and direct sequencing

Since point mutations, small insertions or small deletions of the *PTEN/MMAC1* gene have been reported in several cancers [1, 2], a non-isotope SSCP analysis was designed and performed to screen for such changes. Details of the method have been previously described [13]. In brief, nine sets of primers were digested to cover the full coding region of nine exons of the *PTEN/MMAC1* gene (Table 1). PCR amplification was performed with each set of primers for 35 cycles in a Perkin–Elmer Cetus DNA Thermocycler (Foster City, California, U.S.A.), with each cycle including denaturation at 94°C for 1 min, annealing for 1 min at temperatures shown in Table 1 and extension at 72°C for 1 min. For exons with an abnormal SSCP pattern, the same PCR product was subjected to gel purification and direct sequencing analysis.

RESULTS

Using primers flanking the *PTEN/MMAC1* coding region, in addition to the normal-sized transcript, one or two bands of smaller size were detected in 6 of the 21 colorectal cancers and 1 of their matched non-cancerous tissues, 5 of the 15 gastric cancers and 2 of their matched non-cancerous tissues, 2 of the 10 oesophageal cancers and 2 of their matched non-cancerous tissues (Figure 1). Sequence analysis confirmed that these smaller RT-PCR products represented aberrant *PTEN/MMAC1* transcripts. A description of these aberrant transcripts is summarised in Figures 2 and 3, and Table 2. In brief, 24 different aberrant transcripts with loss of different segments of the normal *PTEN/MMAC1* transcripts were observed (Figure 2). All the deletions encompassed all the

Table 1. *Oligonucleotide primers for the study of the* PTEN/MMAC1 *gene*

Primer	Sequence	Annealing temperature (°C)
Exon 1	Upstream primer: 5′-CTCCTCCTTTTTCTTCAGCC-3′ Downstream primer: 5′-ATATGACCTAGCAACCTGAACCA-3′	58
Exon 2	Upstream primer: 5′-TGACCACCTTTTATTACTCCA-3′ Downstream primer: 5′-TAGTATCTTTTTCTGTGTGGCTTA-3′	56
Exon 3	Upstream primer: 5′-ATAGAAGGGGTATTTGTTGGA-3′ Downstream primer: 5′-TCCTCACTCTAACAAGCAGATA-3′	56
Exon 4	Upstream primer: 5′-TTCAGGCAATGTTTGTTA-3′ Downstream primer: 5′-TTCGATAATCTGGATGACTCA-3′	46
Exon 5	Upstream primer: 5′-GCAACATTTCTAAAGTTACCTA-3′ Downstream primer: 5′-TCTGTTTTCCAATAAATTCTCA-3′	52
Exon 6	Upstream primer: 5′-GAGTAACTATTCCCAGTCAGA-3′ Downstream primer: 5′-TAATTTGTTCAAATGCTTCAGA-3′	52
Exon 7	Upstream primer: 5′-ATCGTTTTTGACAGTTTG-3′ Downstream primer: 5′-ATCGTTTTTGACAGTTTG-3′	46
Exon 8	Upstream primer: 5′-AGGTGACAGATTTTCTTTTTTA-3′ Downstream primer: 5′-TAGCTGTACTCCTAGAATTA-3′	52
Exon 9	Upstream primer: 5′-GTTCATCTGCAAAATGGA-3′ Downstream primer: 5′-TGGTAATCTGACACAATGTCCTA-3′	47

phosphatase core motif and/or the potential tyrosine phosphate acceptor motifs [1, 2]. Therefore, they may not be able to encode a functional PTEN protein.

One oesophageal cancer patient had aberrant transcripts in both tumorous and non-tumorous tissues, but their sizes were not identical. 4 cases of matched normal tissues revealed aberrant transcripts, but no aberrant transcripts were found in their matched tumour tissues (2 gastric cancers, 1 oesophageal cancer and 1 colon cancer). 12 cases (6 colorectal cancers, 5 gastric cancers and 1 oesophageal cancer) had aberrant transcripts in cancerous tissues only (Figure 2).

Analysis of the deletion regions showed that at least 3 groups of deletion could be found. Group 1 was similar to that found previously [14], and may be due to relaxation of

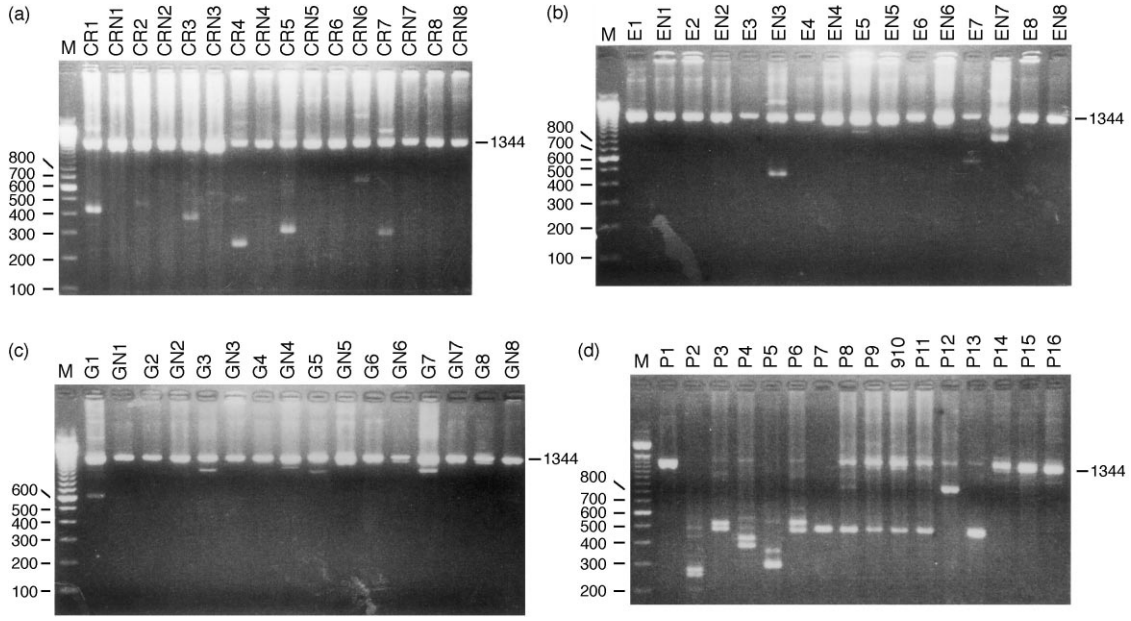

Figure 1. Representative nested reverse transcription-polymerase chain reaction results for matched colorectal cancers (CR), oesophageal cancers (E), gastric cancers (G) and their matched non-cancerous tissues (CRN, EN and GN, respectively) and normal peripheral mononuclear cells (P). In addition to a 1344 base-pair major band, one or two small bands were presented in the cancerous tissues, non-cancerous tissue and normal peripheral mononuclear cells.

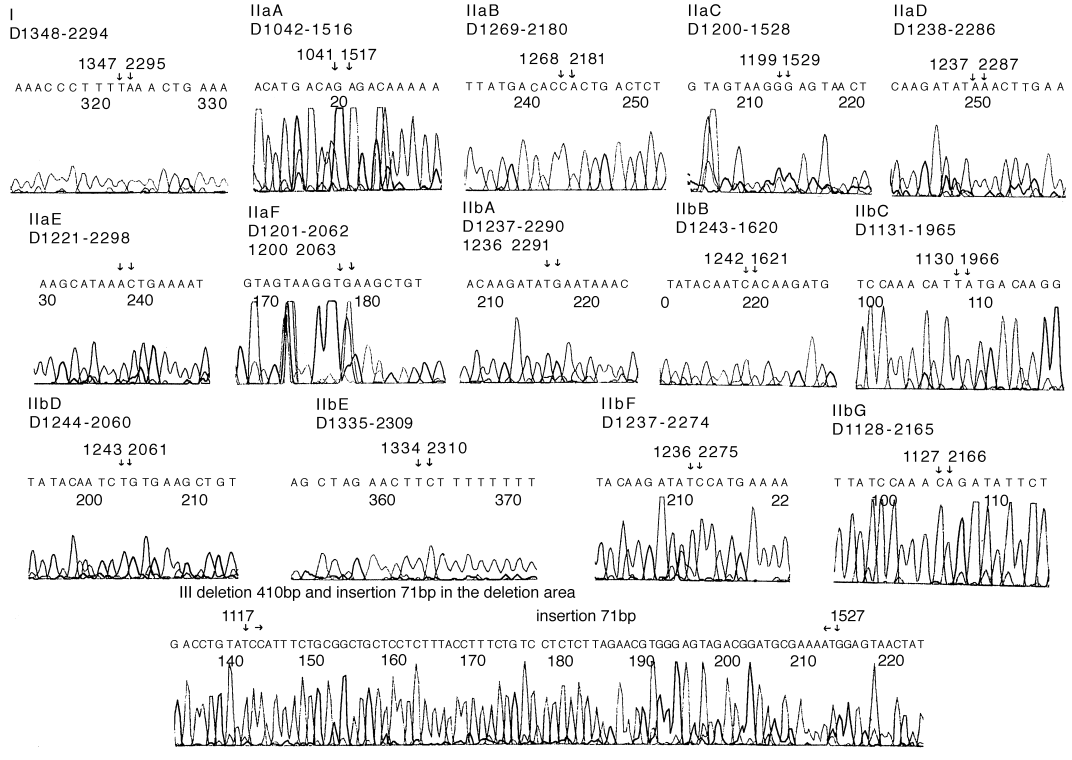

Figure 2. Sequencing of the isolated fragments from abnormal transcripts of *PTEN/MMAC1*. D, deletion bases. I, IIa, IIb and III represent different deletion types.

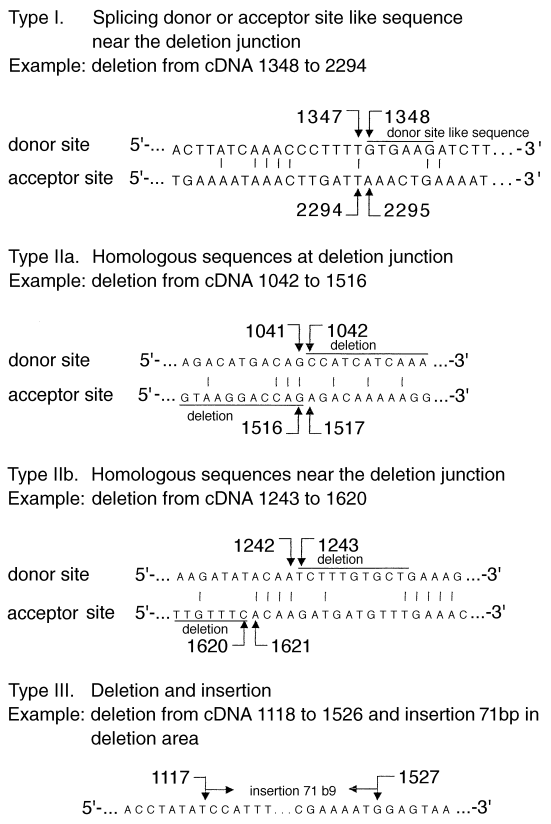

Figure 3. Types of deletion pattern. Type I: the deletion junction had splicing-like donor or acceptor sequences; type IIa: several bases had homology between the donor and acceptor sites at the deletion junction; type IIb: several bases had homology near the deletion junction but not the donor or acceptor site; type III: base deletion and base insertion in the deletion area.

the splicing mechanism (Figure 3, type I). Group 2 had a common homologous core sequence between the donor and acceptor area (type IIa) or a highly homologous sequence near the deletion junction regions (type IIb) (Figure 3, type II). Group 3 had both a deletion and an insertion (Figure 3, type III).

Allelic deletion [loss of heterozygosity (LOH)] was also analysed using microsatellite markers *D10S541*, *D10S215*, *D10S579* and *AFM 280*. No LOH was found in these paired tissues (data not shown). Therefore, it seems that the *PTEN/MMAC1* gene does not undergo large rearrangements, despite the appearance of aberrant transcripts.

Analysis of the *PTEN/MMAC1* gene in normal peripheral mononuclear cells showed that 12 of the 16 samples had aberrant transcripts (Figure 1d; Table 2), with most of the aberrant transcripts belonging to the above-mentioned types. From these results, it is suggested that the aberrant transcripts of *PTEN/MMAC1* gene found using nested RT-PCR were not related to oncogenesis of these tumours or other cancers. Rather, they are a common phenomenon for both cancerous and normal tissues.

SSCP and direct sequencing methods were also used to analyse small base changes in the *PTEN/MMAC1* gene. No point mutations or other small base changes were found. These results suggest that the *PTEN/MMAC1* gene plays no role in the oncogenesis of sporadic digestive tract cancers.

Table 2. Aberrant transcripts of the PTEN/MMAC1 *gene in cancers of the digestive tract and normal blood of representative cases*

Case No.	Pathological finding	Abnormal transcripts: Tumour	Abnormal transcripts: Non-tumour
CR1	Colon cancer	IIa B	—
CR2		IIa F	—
CR3		I	—
CR4		IIa E, IIb C	—
CR5		IIa D	—
CR6		—	IIb D
CR7		IIb G	—
E3	Oesophageal cancer	—	IIa C
E5		III	—
E7		IIb D	IIa A
G1	Gastric cancer	IIb D	—
G3		IIb B	—
G4		—	IIa C
G5		IIb B	—
G7		—	III
P2	Normal blood mononuclear cells	—	IIa E, IIb A
P3		—	IIa F, IIb C
P4		—	IIa B, I
P5		—	IIb F, IIb E
P6		—	IIb C, IIa F
P7,P8, P9, P10, P11, P13		—	IIa F
P12		—	IIa A

CR, colorectal cancer; E, oesophageal cancer; G, gastric cancer; P, normal peripheral mononuclear cells.

DISCUSSION

PTEN/MMAC1 is a tumour suppressor gene recently identified at chromosome 10q23.3, which contains 9 exons and encodes a 403 amino acid protein [1, 2]. The *PTEN/MMAC1* protein is a dual-specificity phosphatase containing a protein tyrosine phosphatase (PTPase) catalytic domain and a region homologous with the cytoskeletal protein tensin [1, 15]. PTPases have been thought to play a role in tumour suppression in view of their antagonising activity to protein tyrosine kinase. Somatic deletions and mutations of the *PTEN/MMAC1* gene have been identified in several cancer lines and primary cancers [1–3, 16–24]. The loss of function of *PTEN/MMAC1* appears to occur during the progression of multiple human cancers.

The nested RT-PCR method has been used for the detection of deletions of *FHIT*, *p16* and *TSG101* genes [8, 9, 11, 12, 14, 25]. In the present study, a similar approach was used to study the deletions in the *PTEN/MMAC1* gene in several types of digestive tract cancers. Truncated transcripts of *PTEN/MMAC1* occurred both in these cancers and in matched normal non-cancerous tissues and normal peripheral mononuclear cells, and were caused by aberrant RNA splicing. These results suggest that aberrant transcripts of *PTEN/MMAC1* are a common phenomenon in cancers, including abnormal expression of *TSG101* and *FHIT* [11, 12, 14, 25].

There is no evidence to support that this phenomenon is involved in oncogenesis. The deletion of a tumour suppressor gene by the nested RT-PCR method should be reconfirmed at the genomic DNA level by Southern blot analysis or pulse field gel analysis.

In this study 0.1–0.01% of the first-stage PCR products were used as a template for the second PCR and some aberrant transcripts were too weak to be detected. In order to increase the sensitivity of detection, 35 cycles were used in both the first and second stages and 1–10% of the first-stage PCR products were used as templates for the second stage PCR. Several negative controls from the first PCR to nested PCR were also used to increase the specificity of detection. Although some non-specific fragments were presented, they were all ruled out after sequencing analysis.

The deletion area of *PTEN/MMAC1* was also analysed and at least three groups of deletion were found. These results support the idea that aberrant transcripts are rare natural splicing products of imperfect spliceosome products [14]. The point mutations or small base changes in several tumours [1, 2, 16–19] is a result not found in the digestive tract cancers in the present study. It is suggested that *PTEN/MMAC1* plays no role in the development of sporadic gastrointestinal cancers.

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Acknowledgements—This study was supported in part by a grant from Mackay Memorial Hospital (MMH8714) and a grant from China Medical College Hospital (DMR-88-044).