Mutational analysis of the PTEN/MMAC1 gene in primary oesophageal squamous cell carcinomas

Y C Hu, K Y Lam, J C O Tang, G Srivastava

Abstract

***Aim*—To investigate whether PTEN/MMAC1 mutations play a role in the carcinogenesis of oesophageal squamous cell carcinoma.**

***Methods*—A panel of 33 primary oesophageal squamous cell carcinoma tumour samples and 20 corresponding morphologically normal tissues was examined for mutations in all nine exons of the PTEN/MMAC1 gene by means of polymerase chain reaction single strand conformational polymorphism analysis (PCR–SSCP) and direct DNA sequencing methods.**

***Results*—Only one of 33 oesophageal squamous cell carcinomas showed an aberrant SSCP band. Further sequencing analysis of this sample revealed an 802 −29 T → C substitution in intron 7. PTEN/MMAC1 mutations were not found in the mutational "hot spot" in exon 5, even after direct sequencing of six oesophageal squamous cell carcinoma samples and three normal tissues. However, a deletion of one nucleotide T at position 492 +8 in intron 5 was seen in all samples.**

***Conclusion*—These results suggest that PTEN/MMAC1 mutations do not play a major role in the carcinogenesis of oesophageal squamous cell carcinoma.**

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Keywords: oesophageal carcinoma; mutation; PTEN/MMAC1; polymerase chain reaction single strand conformational polymorphism analysis

A novel candidate tumour suppressor gene, the PTEN/MMAC1 (phosphatase and tensin homologue deleted on chromosome ten/mutated in multiple advanced cancers 1) gene, also known as TEP1 (transforming growth factor β regulated and epithelial cell enriched phosphatase), located at the chromosome 10q23 region, has been isolated recently by three independent research groups.[1–3] The PTEN/MMAC1 gene encodes a 403 amino acid dual specific phosphatase and shows sequence homology with the cytoskeletal proteins (tensin and auxilin).[1–4] Somatic mutations of the PTEN/MMAC1 gene were initially detected in a variety of human cancer cell lines and primary tumours of brain, prostate, breast, and kidney.[1 2] Germline mutations of the PTEN/MMAC1 gene have also been found in patients with Cowden disease[5 6] and Bannayan-Zonana syndrome.[5 7] Although subsequent studies revealed PTEN/MMAC1 mutations in a wide variety of tumour types, some studies uncovered no PTEN/MMAC1 mutations in other tumours,[8–11] indicating that the PTEN/MMAC1 gene might play a role in the tumorigenesis of some cancers but not others.

Oesophageal squamous cell carcinoma is one of the most common cancers worldwide, with a particularly high frequency in Chinese patients.[12] Despite its prevalence, the exact molecular pathogenesis of oesophageal squamous cell carcinoma is still uncertain. To date, mutational analysis of the PTEN/MMAC1 gene in oesophageal squamous cell carcinomas has not been reported. Therefore, to determine whether PTEN/MMAC1 mutations play a role in the pathogenesis of oesophageal squamous cell carcinoma, we analysed 33 oesophageal squamous cell carcinoma tumour samples and 20 corresponding normal oesophageal tissues by polymerase chain reaction single strand conformational polymorphism analysis (PCR–SSCP) and direct DNA sequencing methods. Our data suggest that PTEN/MMAC1 mutations do not play a major role in the carcinogenesis of oesophageal squamous cell carcinoma.

Materials and methods

COLLECTION OF TISSUE AND PATHOLOGICAL DATA

Matched normal and tumour samples were collected prospectively from 20 Chinese patients who underwent surgery for oesophageal squamous cell carcinoma during 1997 at Queen Mary Hospital, the University of Hong Kong. Another 13 oesophageal squamous cell carcinoma tumour samples were collected in 1997 from the First University Hospital, West China University of Medical Sciences (Chengdu, China). Altogether, there were 26 men and seven women. The patient ages ranged from 46 to 82 years, with a mean age of 58 years. The tumours were found in the upper (n = 4), middle (n = 21), and lower (n = 8) third of the oesophagus. The median length of the tumours was 5 cm (range, 1 to 19.5). The histology of the carcinomas was reviewed according to the criteria described previously.[13] The squamous carcinomas were well differentiated in five cases, moderately differentiated in 23, and poorly differentiated in five. The tumours were staged according to the TNM classification.[14] Most tumours were stage III (n = 19); of the remainder, one was stage I, nine were stage II, and four were stage IV.

DNA EXTRACTION

Excised fresh tissues from the tumours and the corresponding non-tumour samples were snap frozen in liquid nitrogen after surgical resection and stored at −70°C before DNA extraction. Cryostat sections were prepared from these fresh frozen tissues. They were checked micro-

Department of Pathology, University Pathology Building, Queen Mary Hospital, Pokfulam Road, Hong Kong
Y C Hu
K Y Lam
J C O Tang
G Srivastava

Correspondence to:
Dr Srivastava
email: sgopesh@hkucc.hku.hk

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Table 1 Primers used for mutation analysis of PTEN/MMAC1 gene

Exon	*Forward primer sequence (5'–3')*	*Reverse primer sequence (5'–3')*	*Ref*
1	AGT CGC CTG TCA CCA TTT C	ACT ACG GAC ATT TTC GCA TC	6
2	GTT TGA TTG CTG CAT ATT TCA G	GGC TTA GAA ATC TTT TCT AAA TG	16
3	AAT GAC ATG ATT ACT ACT CTA	TTA ATC GGT TTA GGA ATA CAA	16
4	CAT TAT AAA GAT TCA GGC AAT G	GAC AGT AAG ATA CAG TCT ATC	16
5	ACC TGT TAA GTT TGT ATG CAA C	TCC AGG AAG AGG AAA GGA AA	6
6	CAT AGC AAT TTA GTG AAA TAA CT	GAT ATG GTT AAG AAA ACT GTT C	6
7	TGA CAG TTT GAC AGT TAA AGG	GGA TAT TTC TCC CAA TGA AAG	6
8	CTC AGA TTG CCT TAT AAT AGT C	TCA TGT TAC TGC TAC GTA AAC	6
9	AAG GCC TCT TAA AAG ATC ATG	TTT TCA TGG TGT TTT ATC CCT C	6

scopically to determine their suitability for DNA analysis. The selection criterion for use of the tumour block was the extent of tumour present (surface area ≥ 4 cm^2 and $> 70\%$ of the area occupied by the tumour). DNA was then extracted from selected tissues according to the method described previously.[15]

PCR–SSCP ANALYSIS

All samples were screened for mutations in all nine exons of the PTEN/MMAC1 gene by SSCP analysis. The nine exons were amplified using the previously described intronic primers.[6 16] Table 1 lists the sequences of these primers. Samples with alterations were analysed at least twice.

All the primers used in our study were commercially synthesised by Integrated DNA Technologies Inc (Coralville, Iowa, USA). The PCR reaction mixture consisted of 1× PCR buffer, 1.5 or 2 mM $MgCl_2$, 0.25 μM primers, 200 μM dNTPs, 1 μCi of [α-^{32}P]dCTP (Amersham, Aylesbury, UK), 0.5 U Taq polymerase (Life Technologies Inc, Gaithersburg, Maryland, USA), and 20 ng of genomic DNA in a total 25 μl reaction volume. All exons were amplified with the following PCR conditions: pretreatment at 94°C for four minutes, 35 cycles of amplification, and a single 10 minute final extension procedure. Each of these 35 cycles consisted of a denaturing step at 94°C for one minute, an annealing step of one minute (55°C for exon 1; 54°C for exons 2–5, 8, and 9; and 53°C for exons 6 and 7), and an extension step at 72°C for one minute. The PCR products were mixed with an equal volume of SSCP dye mixture (98% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol FF, and 20 mM NaOH), heat denatured at 97°C for seven minutes, and rapidly placed on ice water for more than five minutes. A 5 μl aliquot of each denatured mixture was loaded on to an 8% polyacrylamide gel containing 10% formamide and electrophoresed at 4–6 W for 16 hours at room temperature with a cool air conditioner blowing on it. The gel was lifted with Whatman 3MM paper (Whatman International Ltd, Maidstone, Kent, UK) and dried at 80°C for 90 minutes. This was followed by autoradiography with Fuji-RX films (Tokyo, Japan) at −70°C for one to three days.

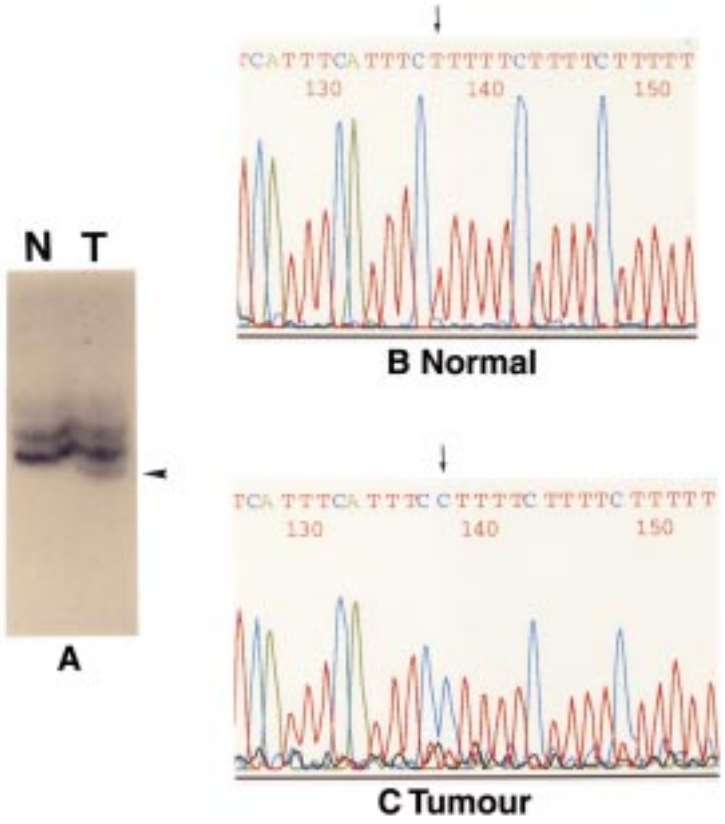

Figure 1 (A) Single strand conformational polymorphism (SSCP) analysis of PTEN/MMAC1 exon 8. The arrowhead shows an extra SSCP band in the tumour sample. N, DNA from corresponding normal tissue; T, tumour tissue DNA. (B) DNA sequencing of the corresponding normal tissue DNA. (C) DNA sequencing of the tumour DNA revealed a T → C substitution at the position 802 − 29 in intron 7 (indicated by an arrow).

DNA SEQUENCING

The abnormally migrating bands were excised from gels after autoradiography and suspended in deionised water overnight. The samples were then reamplified using the same conditions as in SSCP analysis except omitting the [α-^{32}P]dCTP. The PCR products were subjected to electrophoresis in 2% low melting point agarose gel. The amplified band was cut out and purified using the Wizard PCR preparations DNA purification system (Promega, Madison, Wisconsin, USA). The purified PCR products were sequenced on an ABI 377 DNA automated sequencer using a dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Norwalk, Connecticut, USA). The sequencing primers were the same as those used for the PCR–SSCP procedure. The DNA sequencing procedure was performed twice in each case to confirm the findings.

In addition, six randomly chosen tumour samples and three corresponding morphologically normal tissues with normal SSCP patterns were used to sequence exon 5 and its flanking regions after PCR product purification. The sequencing primers were the same as those used for the PCR–SSCP procedure.

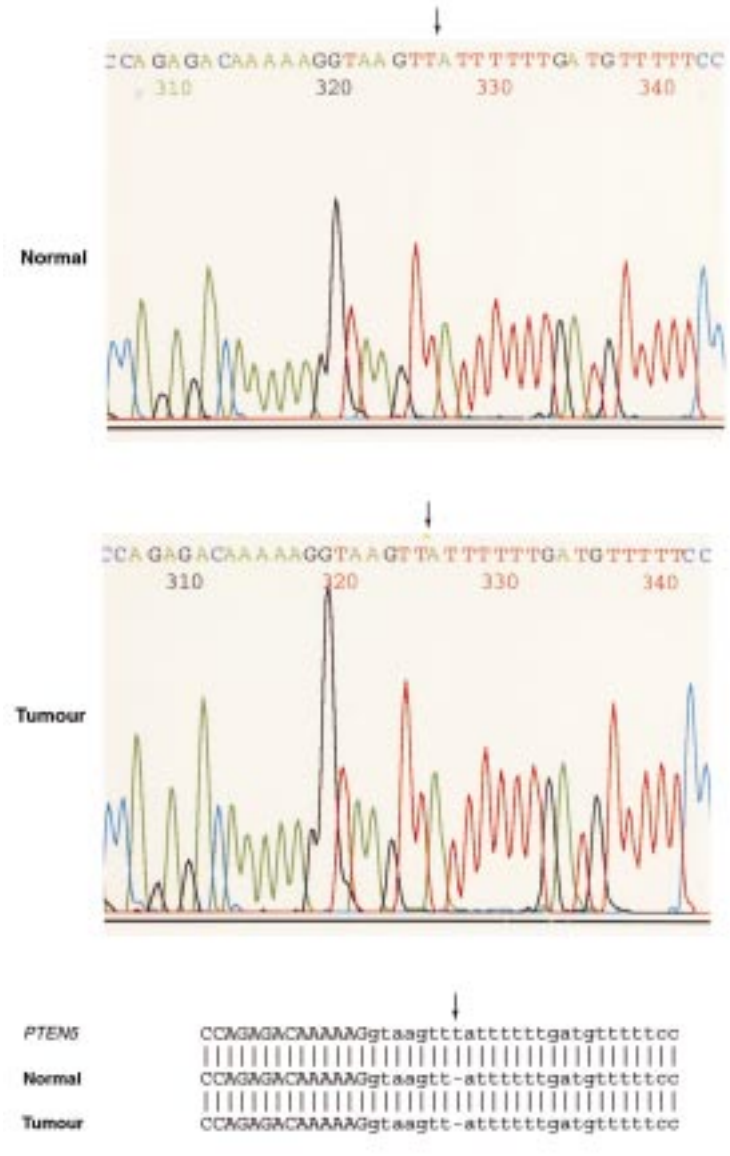

Figure 2 DNA sequence analysis of PTEN/MMAC1 gene exon 5 (GenBank accession number, AF000730). Alignment of sequences from corresponding normal tissue and tumour tissue showing a deletion of one nucleotide T at position 492 +8 in intron 5 in both normal and tumour tissues (small letters represent intron 5).

Results

Only one of the 33 oesophageal squamous cell carcinoma samples showed an aberrant SSCP shift in exon 8. Further DNA sequencing analysis of this sample revealed an 802 − 29 T → C substitution in intron 7 (fig 1). No PTEN/MMAC1 mutations were found in the mutational "hot spot" in exon 5 by means of direct sequencing of six tumour samples and three corresponding morphologically normal tissues with a normal SSCP migration pattern. However, a deletion of one nucleotide T at the position 492 +8 in intron 5 was seen in all samples, including tumours and the corresponding morphologically normal tissues (fig 2).

Discussion

Our study is the first report of mutational analysis of the recently discovered PTEN/MMAC1 tumour suppressor gene in primary oesophageal squamous cell carcinomas. We detected only one mutation in intron 7 among the nine exons of the PTEN/MMAC1 gene in 33 primary oesophageal squamous cell carcinoma samples. PCR–SSCP analysis is a method with high sensitivity and is the most widely used method for detecting gene mutations. Thus, the relative absence of mutations in the PTEN/MMAC1 coding region in the 33 primary oesophageal squamous cell carcinoma samples analysed in our study strongly indicated that PTEN/MMAC1 gene mutation is an extremely rare event in oesophageal carcinogenesis. Nevertheless, other mechanisms of inactivation of the PTEN/MMAC1 gene, such as homozygous deletion, hypermethylation, or protein phosphorylation, have still to be investigated. However, they are not known to be important mechanisms for PTEN/MMAC1 inactivation in other tumours.

Analysis of the PTEN/MMAC1 gene in squamous cell carcinomas from other sites also found that PTEN/MMAC1 gene mutation does not contribute greatly to the formation of squamous cell carcinoma of lung,[10] cervix,[17] skin,[18] or head and neck.[9 19] Petersen *et al* failed to detect any mutation in the PTEN/MMAC1 gene in 25 lung squamous cell carcinomas.[10] Tashiro *et al* did not find any mutation of PTEN/MMAC1 in 10 cervical squamous carcinomas.[17] Okami and colleagues[9] described only four mutations of PTEN/MMAC1 in 39 primary head and neck squamous cell carcinomas, and Henderson and colleagues[19] did not find any mutations of PTEN/MMAC1 in 10 head and neck squamous cell carcinoma cell lines and biopsy tumour samples. In addition, Kubo *et al* did not detect any mutations of PTEN/MMAC1 in 21 skin squamous cell carcinomas.[18] These observations of a lack of PTEN/MMAC1 mutation in squamous cell carcinoma from other sites are in concurrence with our observations from oesophageal squamous cell carcinoma. These findings indicate that the mutation of the PTEN/MMAC1 gene is a rare event in the carcinogenesis of squamous cell carcinoma.

PTEN/MMAC1 mutations were frequently found in cancers arising from endometrium,[8 17 20] brain,[1 2 16 21–24] and prostate.[25–27] As cited above, they are rarely seen in carcinomas arising from the head and neck region (including oesophagus),[9 19] lung,[10] cervix,[17] and skin,[18] or in adenocarcinomas arising from colorectum,[8] pancreas,[8 9 28] ovary,[11 17 28] and kidney.[28] These findings suggest that the tumorigenic effects of the PTEN/MMAC1 gene are tissue specific. In other words, the PTEN/MMAC1 gene plays a role in the tumorigenesis of some cancers but not in others.

In our study, we found a mutation in intron 7 in one of 33 oesophageal squamous cell carcinomas. The corresponding morphologically normal tissue did not show this abnormality. The importance of this mutation is unclear, but it is unlikely that this mutation plays a major role in the carcinogenesis of oesophageal squamous cell carcinoma. This is because this mutation is not in the coding region of PTEN/MMAC1 and is 29 base pairs away from the exon–intron junction. However, whether this

mutation in the intron affects transcriptional or post-transcriptional modulation remains to be elucidated.

Most reports of mutations found in PTEN/MMAC1 are localised to exon 5,[6 16 22 24] the sequence encoding the putative phosphatase domain. Thus, we randomly chose six oesophageal squamous cell carcinoma samples and the three corresponding morphologically normal tissues with normal SSCP patterns to sequence the hot spot exon 5 and its flanking regions. Although we could not find a cancer linked abnormality in exon 5, we detected a mutation in intron 5 in both tumours and corresponding morphologically normal tissues. Because all the six tumours and three corresponding normal tissues had a deletion of one nucleotide T at position 492 +8 in intron 5, it is unlikely that this is a germline mutation. The importance of this deletion in intron 5 remains unclear. It is possible that this might be the result of allelic variation of the PTEN/MMAC1 gene in different ethnic groups.

The exact molecular mechanism(s) by which PTEN/MMAC1 functions in tumorigenesis or growth inhibition remains unclear. Based on the sequence homologies of the PTEN/MMAC1 protein and protein tyrosine phosphatases, the PTEN/MMAC1 protein was defined as a dual specificity phosphatase with a high degree of substrate specificity.[4] More recent studies showed that the PTEN/MMAC1 protein dephosphorylates the 3 position of phosphatidylinositol 3,4,5-trisphosphate to reverse the reactions catalysed by phosphoinositide 3-kinase.[29–31] This indicates that PTEN/MMAC1 inhibits the phosphoinositide 3-kinase signalling pathway for regulation of cell growth and survival. Furthermore, Davies *et al* showed that the PTEN/MMAC1 protein inhibits Akt activation and induces anoikis, a kind of apoptosis.[32] This implies that loss of PTEN/MMAC1 increases cellular proliferation and augments a cell's survival potential during cellular processes that are associated with malignant transformation. In addition, Tamura *et al* suggested that the PTEN/MMAC1 protein disrupts cell spreading and migration by dephosphorylating focal adhesion kinase.[33] Thus, mutation of PTEN/MMAC1 will enhance tumour spreading.

In conclusion, our results suggest that PTEN/MMAC1 gene mutations do not play an important role in the carcinogenesis of oesophageal squamous cell carcinoma.

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