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Analysis of *PTEN/MMAC1* Alterations in Aerodigestive Tract Tumors[1]

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ABSTRACT

***PTEN/MMAC1* is a candidate tumor suppressor gene recently identified at chromosomal band 10q23. It is mutated in sporadic brain, breast, and prostate cancer and in the germ line of patients with hereditary Cowden disease. We searched for genetic alterations of the *PTEN/MMAC1* gene in 39 primary head and neck cancers (HNSCCs), 42 primary non-small cell lung cancers (NSCLCs), 80 pancreatic cancer xenografts, and 37 cell lines and xenografts from colon, lung, and gastric cancers. Microsatellite analysis revealed loss of heterozygosity at markers near the gene in 41% of primary HNSCCs, 50% of NSCLCs, and 39% of the pancreatic cancers. Three cases of HNSCCs displayed homozygous deletion involving the gene. We sequenced the entire coding region of the *PTEN/MMAC1* gene in the remaining tumors displaying loss of heterozygosity and found one terminating mutation in a HNSCC sample. Thus, a second inactivation event was observed in 4 of 39 primary HNSCC cases. By use of a protein truncation assay, one terminating mutation was also identified in one of eight NSCLC cell lines. Our results suggest that *PTEN/MMAC1* gene inactivation plays a role in the genesis of some tumor types.**

INTRODUCTION

Genetic alteration of tumor suppressor genes is an important step in carcinogenesis. Chromosomal alterations in several cancers have been investigated, and LOH[3] at chromosome 10q occurs at high frequency in a variety of human tumors (1). Previously, chromosome 10q loss was reported in 20% of HNSCCs (2), 20–27% of NSCLCs (3, 4), and 50% of pancreatic cancers (5). However, no tumor suppressor gene was identified in this region.

Recently, *PTEN/MMAC1* was identified at chromosome 10q23 and found to be mutated in several types of cancers, including glioblastoma, breast, and prostate (1, 6). This gene was also found mutated in the germ line of patients with Cowden disease (7). Using several methods, we screened for inactivating mutations of the *PTEN/MMAC1* gene in primary HNSCCs, NSCLCs, and pancreatic cancer, as well as cell lines and xenografts of different cancers. LOH was found in nearly one-half of the primary tumors (39–50%); three homozygous deletions and two inactivating mutations were also detected. These results suggest that *PTEN/MMAC1* inactivation may be tissue specific. Although it is altered in over 10% of primary HNSCCs, it is involved to a much lesser degree in other neoplasms.

MATERIALS AND METHODS

DNA Extraction. Thirty-nine HNSCCs and 42 NSCLCs were collected following surgical resection with prior consent from Johns Hopkins Hospital patients. The histopathological diagnosis of NSCLC was as follows: adenocarcinoma, 17; squamous cell carcinoma, 17; large cell carcinoma, 7; and adenosquamous carcinoma, 1. Specimens were fresh frozen and microdissected on a cryostat so that the tumor samples contained greater than 70% neoplastic cells. DNA from tumor sections was digested with SDS/proteinase K, extracted by phenol-chloroform, and ethanol precipitated as described previously (8, 9). Normal control DNA was obtained from peripheral lymphocytes or normal tissues and processed in the same manner as the tumor samples. Eighty pancreatic xenografts were established from fresh cancer tissue, and DNA was obtained as described previously (5, 10).

Microsatellite Analysis. DNA from tumor and normal control was examined for LOH by PCR-based microsatellite analysis. For HNSCC and NSCLC samples, two markers (*D10S215* and *D10S541*) flanking the *PTEN/MMAC1* gene were chosen (1), and one dinucleotide microsatellite marker *D10S2491* was isolated from the bacterial artificial chromosome containing the *PTEN/MMAC1* gene (11). Five additional markers (*D10S537, D10S1744, D10S579, D10S185,* and *D10S221*) were used for some cases to determine the border of LOH or to determine the presence of a homozygous deletion. For pancreatic cancer, *D10S579* and *D10S541* were used for microsatellite analysis. PCR conditions and criteria for LOH and homozygous deletion were described previously (9, 12). For informative cases, LOH was scored if one allele was decreased greater than 30% in tumor when compared to the same allele in normal control DNA. Determination of homozygous deletion was based on the presence of one or more markers demonstrating apparent retention flanked by markers showing clear LOH. This apparent retention results from amplification of a small amount of normal cells within the tumor without any amplification from the homozygously deleted region from the tumor cells (12).

Sequence Analysis. A majority of the primary tumors showing LOH at 10q23 were analyzed for mutation by direct sequencing. All nine exons of the *PTEN/MMAC1* gene were amplified and sequenced according to the method described previously (1, 13). The PCR product was sequenced using the [γ-^{33}P]ATP 5′ end-labeled sequencing primer and the AmpliCycle sequencing kit (Perkin-Elmer, Roche Molecular Systems Inc., Branchburg, NJ). Nucleotide substitutions in the samples were confirmed by a separate amplification and sequencing of the tumor DNA and the matched normal DNA sample.

For tumor cell lines, the *in vitro* synthesized protein assay (14) was used to detect truncated proteins resulted from internal deletions or terminating mutations. The entire coding region of *PTEN/MMAC1* was amplified in one PCR reaction using randomly primed tumor cDNA as a template. Primers used to amplify the coding sequence were: 5′-GGA TCC TAA TAC GAC TCA CTA TAG GGA GAC CAC CAT GGA GTC GCC TGT CAC CAT TTC-3′ and 5′-TTT TTT CAT GGT GTT TTA TCC CTC-3′. The T7 promotor and the Kozak consensus sequences shown for the longer forward primer allowed efficient *in vitro* transcription and translation. PCR products were transcribed and translated *in vitro*, and the resultant proteins were separated by SDS-PAGE. The cell lines and xenografts examined included 24 colorectal cancers, 8 NSCLCs, and 5 gastric and 5 esophageal cancers. To determine the nature of the genetic alteration in the NSCLC sample showing a truncated protein product, the cDNA product was cloned into a TA vector (Stratagene, San Diego, CA), and the individual clones were sequenced.

RESULTS AND DISCUSSION

Microsatellite Analysis. Sixteen of 39 HNSCC (41%) and 21 of 42 NSCLC (50%) cases displayed LOH with known markers near *PTEN/MMAC1*. In pancreatic cancer, 31 of 80 cases (39%) had LOH. Previously, using a whole-genome scanning approach, chromosome 10q loss was detected in 20% of primary HNSCCs (2), in 20–27% of NSCLCs (3, 4), and in 50% of pancreatic cancers (5). The eight

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[3] The abbreviations used are: LOH, loss of heterozygosity; NSCLC, non-small cell lung cancer; HNSCC, head and neck squamous cell cancer.

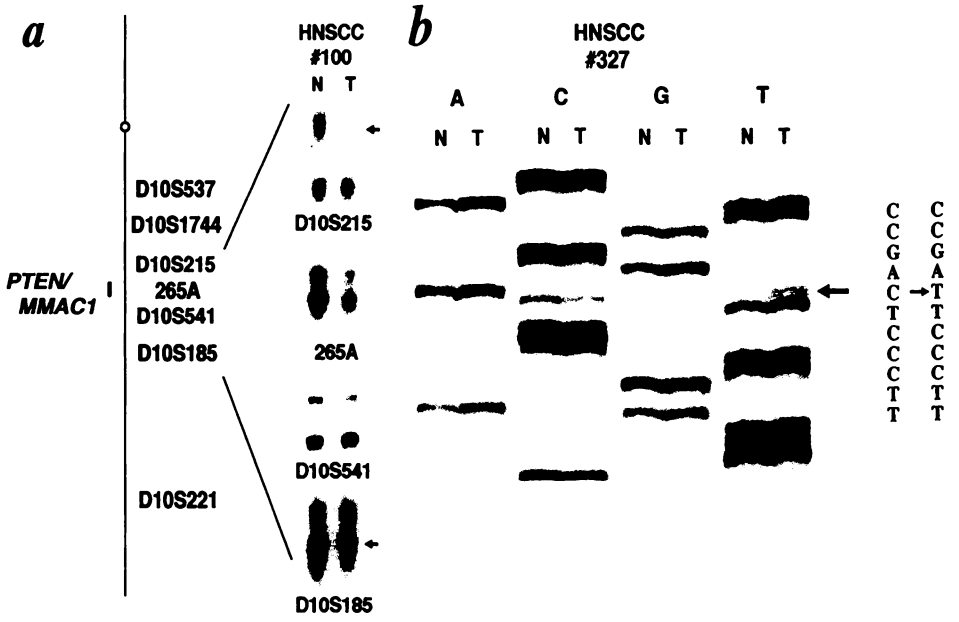

Fig. 1. *a*, homozygous deletion of *PTEN/MMAC1* by microsatellite analysis. Microsatellite markers including the newly cloned *D10S2491* (265A) are listed vertically to the *left*. HNSCC case 100 showed apparent retention of heterozygosity at *D10S2491* (265A) and *D10S541* flanked by LOH at *D10S215* and *D10S185*, indicative of a homozygous deletion (see text). *Arrows*, the lost allele. *N*, normal; *T*, tumor. *b*, mutation of the *PTEN/MMAC1* gene in HNSCC. *Arrow*, a mutation from C to T resulting in an amino acid change from glycine to a stop codon (codon 245) in exon 7. *N*, normal; *T*, tumor.

markers we used were located very near *PTEN/MMAC1* (on chromosome 10q) and may have contributed to the generally higher rate of LOH observed.

Homozygous deletion of the *PTEN/MMAC1* gene has been reported in some tumor cell lines (1, 6). We screened for homozygous deletion at the *PTEN/MMAC1* gene locus using the newly isolated *D10S2491* marker, which is located on the same bacterial artificial chromosome (120 kb) with *PTEN/MMAC1*. Three cases of HNSCC displayed apparent retention at *D10S2491* extending to *D10S1744*, *D10S541*, or *D10S215*, indicative of a homozygous deletion (Fig 1*a*). In primary prostate tumors, several cases of homozygous deletion of *PTEN/MMAC1* were identified by the same approach and confirmed by fluorescence *in situ* hybridization analysis (11). No homozygous deletion was identified in primary NSCLCs. Although the *PTEN/MMAC1* gene is closely flanked by two microsatellite markers, *D10S215* and *D10S541*, small homozygous deletions could still be missed by microsatellite analysis.

Sequence Analysis. The high incidence of LOH in HNSCCs, NSCLCs, and pancreatic cancers at this locus suggested the presence of a tumor suppressor gene in the region. We then sequenced the entire coding region of the *PTEN/MMAC1* gene (excluding the three HNSCCs with homozygous deletion) including the intron/exon boundaries. The results of our sequence analysis are summarized in Table 1. In one HNSCC sample, a substitution mutation from C to T at codon 245 in exon 7 resulted in an amino acid change from glycine to a stop codon (Fig. 1*b*). This alteration was tumor specific because it was not found in the matching normal DNA. Thus, a second inactivating event was found in 4 of 16 (25%) primary HNSCCs with 10q loss (three homozygous deletions and one point mutation).

We also screened for coding sequence changes that would result in an early termination of the protein in cancer cell lines by a protein truncation assay (14). cDNA samples from a total of 37 cell lines and xenografts derived from NSCLCs, gastric, and colorectal tumors were examined. A NSCLC cell line, H1155, was found to have a shortened protein product in the absence of a wild-type band, suggesting the loss of the wild-type allele and mutational inactivation of the other. The PCR-amplified cDNA products from all eight NSCLC cell lines were sequenced. The result showed that there was a substitution from C to T, resulting in an amino acid change from an arginine to a stop codon at codon 233 of exon 7 (Fig. 2*a*) and a very faint deletion of exon 6. Sequencing of the individually cloned alleles in sample H1155 revealed that about 30% of the cloned cDNA transcripts also contained a skipped exon 6 in the same allele (Fig. 2*b*). Although this skipped exon would have resulted in a frame shift of the coding sequence, the presence of a stop codon in the same allele suggests that it is probably of minor significance *in vivo*. Furthermore, due to the preferential PCR amplification and the preferential cloning of smaller products, the relative abundance of the aberrant transcript may be an overestimate.

Additionally, one primary HNSCC and one NSCLC also had a single bp insertion 31 bp up stream of exon 5 in intron 4. Another primary HNSCC had a 39-bp deletion in intron 7, 45 bp up stream of exon 8. These changes are not expected to alter the exon splicing pattern in the tumors because of their distance from intron/exon boundaries. Furthermore, the matching normal DNA from the patients harbored the change, suggesting that they are normal polymorphisms. No mutations were observed in any of the pancreatic cancer xenografts and other tumor cell lines.

Although occurring frequently in glioblastoma and prostate cancers, *PTEN/MMAC1* gene mutation is a relatively rare event in the cancers we tested here. In a previous report, *PTEN/MMAC1* was more likely to be mutated in advanced tumors (1, 6). In our study, the primary HNSCCs were generally more advanced cases than the primary NSCLCs and pancreatic cancers taken only from resectable cases. Although clinical and genetic stages of cancers cannot readily be compared between different cancer types, cancer stage at resection may explain the generally higher frequency of *PTEN/MMAC1* inactivation (4 of 39; 10%) in the primary HNSCC tumors. Alternatively, this difference may also reflect the tissue specificity of *PTEN/MMAC1* in the tumorigenesis of various cancer types. Our results

Table 1. *Genetic changes of PTEN/MMAC1 in primary cancers and NSCLC cell lines*

Tumor type	No. of tumors sequenced	Exon/ Intron	Codon	Nucleotide alteration	Predicted effect
HNSCC	16[a]	Exon 7	245	CAG to TAG	GLY to stop
		Intron 4		One-base insertion	Polymorphism
		Intron 7		39-base insertion	Polymorphism
NSCLC (primary)	17	Intron 4		One-base insertion	Polymorphism
NSCLC (cell line)	8	Exon 7	233	CGA to TGA	ARG to stop
Pancreatic cancer	31	None			

[a] Three primary HNSCC tumors contained a homozygous deletion (see text).

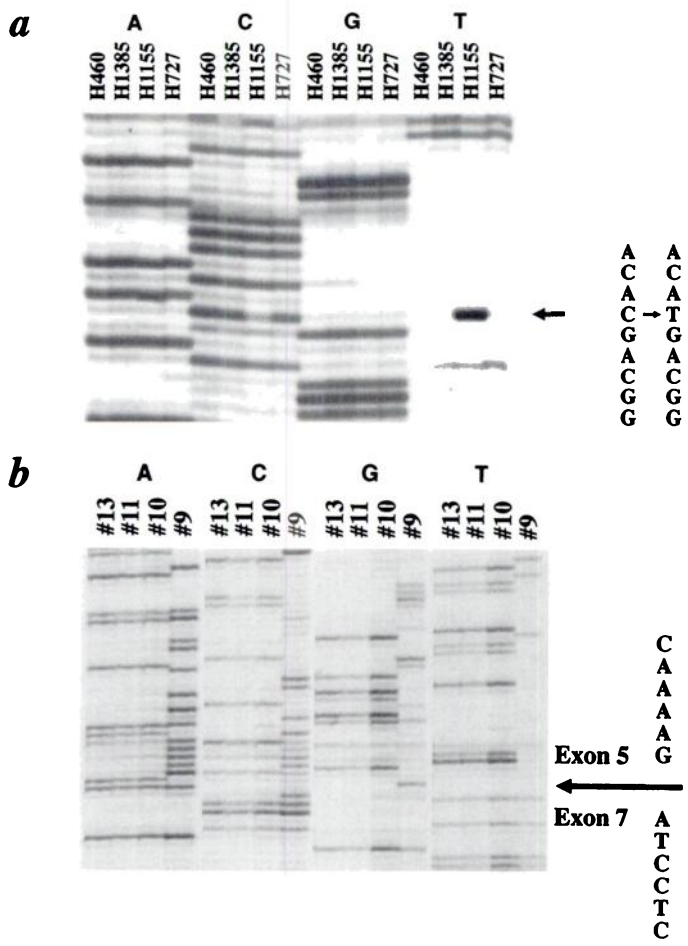

Fig. 2. *a*, sequence of the *PTEN/MMAC1* gene in NSCLC cell lines. Sequence of cDNA from H1155 shows a missense mutation with a substitution from C to T, resulting in an amino acid change from arginine to a stop codon at the codon 233 in exon 7 (*arrow*). *b*, a skipped exon 6 in the same allele as the stop codon in sample H1155 (*arrow*). Each number represents an individually cloned product.

suggest that *PTEN/MMAC1* is not a main target of inactivation in the tumorigenesis of NSCLCs or pancreatic or colon cancers. An alternative mechanism of tumor suppressor gene inactivation may lead to methylation of the 5′ CpG island within the promotor region (15, 16). However, no reports of *PTEN/MMAC1* loss of transcription secondary to methylation have been described.

The low frequency of *PTEN/MMAC1* gene mutation and homozygous deletion in NSCLCs lead us to believe that another tumor suppressor locus or loci may reside at chromosome 10q. In our study, 12 of 16 HNSCCs and 13 of 21 NSCLCs with LOH displayed loss extending to the telomeric marker (*D10S221*). Thus, other candidate tumor suppressor gene(s) at chromosome 10q remain to be identified.

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