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SHORT REPORT

Point mutation and homozygous deletion of *PTEN/MMAC1* in primary bladder cancers

Paul Cairns[1], Ella Evron[1], Kenji Okami[1], Naomi Halachmi[1], Manel Esteller[2], James G Herman[2], Shikha Bose[3], Steven I Wang[3], Ramon Parsons[3] and David Sidransky[1]

[1]*Head and Neck Cancer Research, Department of Otolaryngology,* [2]*Oncology Center, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205 and* [3]*Department of Pathology and Medicine, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA*

A new tumor suppressor gene *PTEN/MMAC1* was recently isolated at chromosome 10q23 and found to be inactivated by point mutation or homozygous deletion in glioma, prostate and breast cancer. *PTEN/MMAC1* was also identified as the gene predisposing to Cowden disease, an autosomal dominant cancer predisposition syndrome associated with an increased risk of breast, skin and thyroid tumors and occasional cases of other cancers including bladder and renal cell carcinoma. We screened 345 urinary tract cancers by microsatellite analysis and found chromosome 10q to be deleted in 65 of 285 (23%) bladder and 15 of 60 (25%) renal cell cancers. We then screened the entire *PTEN/MMAC1* coding region for mutation in 25 bladder and 15 renal cell primary tumors with deletion of chromosome 10q. Two somatic point mutations, a frameshift and a splicing variant, were found in the panel of bladder tumors while no mutation was observed in the renal cell carcinomas. To screen for homozygous deletion, we isolated two polymorphic microsatellite repeats from genomic BAC clones containing the *PTEN/MMAC1* gene. Using these new informative markers, we identified apparent retention at the gene locus indicative of homozygous deletion of *PTEN/MMAC1* in four of 65 bladder and 0 of 15 renal cell tumors with LOH through chromosome 10q. Identification of the second inactivation event in six bladder tumors with LOH of 10q implies that the *PTEN/MMAC1* gene is occasionally involved in bladder tumorigenesis. However, the low frequency of biallelic inactivation suggests that either *PTEN/MMAC1* is inactivated by other mechanisms or it is not the only target of chromosome 10q deletion in primary bladder and renal cell cancer.

Keywords: PTEN/MMAC1; bladder cancer; renal cell cancer; chromosome 10q; homozygous deletion

Adult sporadic cancers are known to arise through the accumulation of multiple genetic events (Fearon and Vogelstein 1990). Several of these genetic events have been identified in bladder and renal cell cancer while others remain to be elucidated (Cairns *et al.*, 1991, 1995; Ishikawa *et al.*, 1991; Sidransky *et al.*, 1991; Reiter *et al.*, 1993; Latif *et al.*, 1993; Gnarra *et al.*, 1994). The *Rb* gene on chromosomal arm 13q (Cairns *et al.*, 1991; Ishikawa *et al.*, 1991), the *p53* gene on chromosomal arm 17p (Sidransky *et al.*, 1991; Reiter *et al.*, 1993) and the *CDKN2a* gene on chromosomal arm 9p (Cairns *et al.*, 1995) are known to be involved in both of these tumor types while the *VHL* gene is frequently inactivated in renal cell carcinomas (Latif *et al.*, 1993; Gnarra *et al.*, 1994). The identification of other areas of chromosomal deletion suggest that other suppressor loci important in bladder and renal cell carcinogenesis remain to be discovered (Knowles *et al.*, 1994; Morita *et al.*, 1991).

Recently, a tumor suppressor gene on chromosome 10q23, *PTEN/MMAC1*, was cloned and somatic mutations were identified in glioma, breast and prostate cancer (Li *et al.*, 1997; Steck *et al.*, 1997). *PTEN/MMAC1* has also been identified as the gene predisposing to Cowden disease (Liaw *et al.*, 1997), an autosomal dominant cancer predisposition syndrome associated with an increased risk of breast, skin and thyroid tumors and occasional cases of other cancers (Eng *et al.*, 1994; Starink *et al.*, 1986) including bladder (Starink *et al.*, 1986) and renal cell carcinoma (Haibach *et al.*, 1992). Deletion of chromosome 10 has been observed in cytogenetic studies of bladder cancer (Sandberg 1994), mostly as monosomy but in some cases as specific deletion of 10q. Loss of heterozygosity on chromosome 10q has previously been reported in 5–7% of bladder tumors (Knowles *et al.*, 1994; Habuchi *et al.*, 1993) and 29% of renal tumors in allelotype studies (Morita *et al.*, 1991a).

To further elucidate the role of chromosome 10 in the progression of sporadic bladder and renal cancer and to identify tumors for eventual sequence analysis, we screened a large representative series of 285 primary bladder tumors and 60 primary renal cell tumors with a panel of microsatellite markers (Figure 1) spanning chromosome 10. We found LOH in 65 of 285 (23%) bladder tumors and 15 of 60 (25%) renal cell tumors at one or more markers on 10q. There were 15 cases of deletion of the q arm only in the bladder tumors suggesting the existence of a tumor suppressor locus on the q arm. The renal cell tumors all had monosomic loss of chromosome 10 with the exception of one tumor that displayed loss only at *D10S215*. A previous deletion mapping study of renal cell carcinoma has suggested that the target of deletion is on 10q (Morita *et al.*, 1991b). We found a higher level of deletion of chromosome 10q in bladder tumors, and a similar level of LOH in our panel of sporadic renal cell tumors, to that previously reported.

Correspondence: D Sidransky
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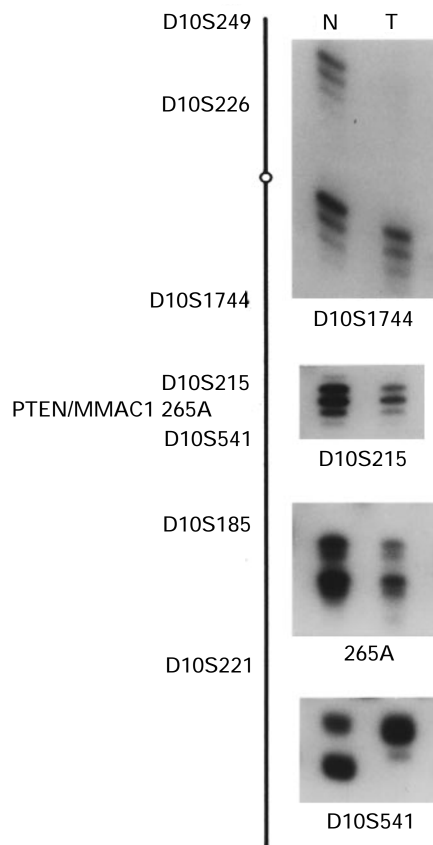

Figure 1 Approximate map location of chromosome 10 markers and homozygous deletion of *PTEN/MMAC1* by microsatellite analysis. The previously mapped 10p markers *D10S249* and *D10S226* and 10q markers *D10S1744, D10S215, D10S541, D10S185* and *D10S221* are indicated together with the newly cloned *PETN/MMAC1* polymorphic marker 265A (*D10S2491*). To the right is a bladder tumor showing apparent retention of heterozygosity indicating homozygous deletion at D10S215 and 265A (*D10S2491*), flanked by LOH indicated by loss of the upper allele at *D10S1744* and loss of the lower allele at *D10S541* in the tumor (T) lane. Primary bladder tumor specimens were obtained by transurethral resection or cystectomy and primary renal tumor specimens after nephrectomy. All primary tumor samples were frozen immediately and were of representative grade and stage at presentation. Peripheral blood from each patient was collected in EDTA as a normal control. Macroscopically pure tumor was dissected from the frozen biopsies and leukocytes were pelleted from blood samples before extraction and purification of DNA (Sambrook *et al*., 1989). For PCR amplification and LOH analysis, DNA from tumor and venous blood was analysed for LOH by amplification of dinucleotide repeat containing sequences using PCR and the conditions previously described (van der Riet *et al*., 1994). For informative cases, allelic loss was scored if the intensity of signal from one allele was significantly reduced (>30%) in the tumor DNA when compared to the normal DNA. Primer sequences for *D10S249, D10S226, D10S1744, D10S215, D10S541, D10S185* and *D10S221* are available from Research Genetics (Huntsville, AL) or the Genome Database (JHU, MD). To isolate new microsatellite markers, the human genomic BAC clones 265 and 60 (Li *et al*., 1997) containing the *PTEN/MMAC1* gene were subcloned into Bluescript and plated. Colonies were lifted onto nylon membranes and screened with the microsatellite repeat oligomer, $(GT)_{10}$. Two of the microsatellite blocks isolated, designated *D10S2491* and *D10S2492*, were found to be polymorphic. The primer sequences used for PCR amplification are as follows; D10S2491 F 5′ GTTAGATAGAGTACCTG CACTC 3′, D10S2491 R 5′ TTATAAGGACTGAGTGAGG GA 3′, D10S2492 F 5′ TGCAGTGAGCTGTGAAGATG 3′, D10S2492 R 5′ TGTTTCTCTTACTACCTATGTGA 3′. Both markers have alleles in the size range of 120 160 base pairs and amplify well at an annealing temperature of 55°C. *D10S2491* was informative in 82% of cases and *D10S2492* in 20% of cases. Microsatellite marker *D10S2492* was only used on cases non informative for *D10S2491*.

Because the initial reports of *PTEN/MMAC1* also described frequent homozygous deletion of the gene in tumor cell lines (Li *et al*., 1997; Steck *et al*., 1997) we wanted to identify homozygous deletion in primary tumors before selecting tumors for *PTEN/MMAC1* sequence analysis. Although we screened the bladder and renal tumors with the closest mapped flanking markers to *PTEN/MMAC1*, *D10S215* and *D10S541*, in the initial reports approximately half of the homozygous deletions did not extend to these flanking markers (Li *et al*., 1997; Steck *et al*., 1997). Furthermore, we have previously shown at the *CDKN2a* tumor suppressor locus on chromosome 9p21 that the frequency of homozygous deletion increases when markers near or within the gene are used since homozygous deletions are nested in size around the target gene (Cairns *et al*., 1995). The marker *WG9* (Li *et al*., 1997), located between *D10S215* and *D10S541* and within *PTEN/MMAC1*, is practically non-informative (<5%). A non-polymorphic marker can be used to detect homozygous deletion by simple presence or absence of signal in tumor cell lines which are composed of tumor cells only. In primary tumor specimens, normal cells complicate or render impossible this method of detecting homozygous deletions. To reliably detect homozygous deletion, we prefer to assess apparent retention of heterozygosity at the gene of interest in tumors with LOH of flanking markers (Cairns *et al*., 1995). The apparent retention of

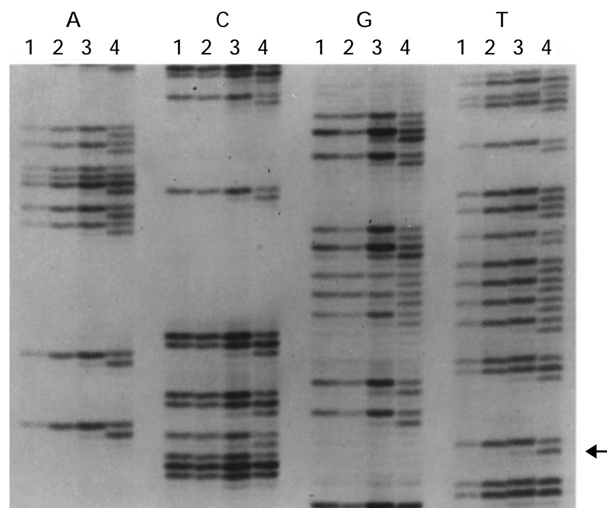

Figure 2 Sequencing gel showing a frameshift mutation of *PTEN/MMAC1*. Autoradiograph of a sequencing gel of exon 7 of *PTEN/MMAC1* in primary bladder tumors. Lanes 1 and 2 show tumor of DNAs with wild type sequence. Lane 3 is normal DNA from patient 140 and lane 4 is tumor 140 DNA showing a one base pair deletion resulting in a frameshift (arrow). The mutation was confirmed by reamplification and resequencing. For PCR amplification and cycle sequencing of *PTEN/MMAC1*, 50 nanograms of genomic template DNA was amplified with primers for exons 1 9 of *PTEN/MMAC1* at 95°C for 30 s, 50 58°C for 1 min and 72°C for 1 min for 30 35 cycles with a final extension step at 72°C for 5 min. The resulting PCR product was cycle sequenced according to the manufacturer's instructions (Perkin Elmer, Roche Molecular Systems Inc., Branchburg, NJ) and run on a 6% acrylamide gel. The primer sequences used for amplification and sequencing of the gene were as described in Liaw *et al*. (1997) and Wang *et al*. 1997). Sequence changes were confirmed by reamplification and resequencing of the tumor DNA and normal DNA.

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heterozygosity is due to amplification of DNA from normal cells contaminating the tumor biopsy and correlates with homozygous deletion assessment by Southern and FISH analysis (Cairns *et al*., 1995).

We therefore obtained two overlapping BACs that together contained the entire genomic *PTEN/MMAC1* gene (Li *et al*., 1997) and screened for microsatellite blocks with a GT oligomer. We isolated several microsatellite blocks, two of which were found to be polymorphic. These markers must map within or immediately adjacent to *PTEN/MMAC1* and are nearer to the gene than *D10S215* and *D10S541* which are not included on the BAC clones (Li *et al*., 1997). We tested the bladder and renal tumors with 10q LOH for *PTEN/MMAC1* homozygous deletion with the new markers *D10S2491* and *D10S2492*. These markers recently detected *PTEN/MMAC1* homozygous deletions in primary prostate tumors confirmed by FISH analysis (Cairns *et al*., 1997).

We found four cases of homozygous deletion of *PTEN/MMAC1* in the 65 primary bladder tumors with LOH of 10q (Figure 1). Two of the four cases were grade II, T1 tumors and two were grade III, T2 tumors. After exclusion of tumors with homozygous deletion, we then proceeded with complete sequence analysis of the coding region of *PTEN/MMAC1* and the intron/exon boundaries. In the 25 bladder tumors sequenced we found one frameshift mutation in exon 7 (Figure 2), predicted to result in a truncated protein in tumor 140 (grade III, T2) and a somatic mutation in the 5′ UTR near the start of exon 1, probably representing a splicing variant (Senapathy *et al*., 1990) in tumor 115 (grade III, T4). Tumor suppressor genes in general and *CDKN2a* in particular, can be inactivated by epigenetic methylation of the promoter resulting in complete blocking of transcription (Merlo *et al*., 1995). We investigated promoter methylation as a possible inactivation mechanism of the retained allele of *PTEN/MMAC1* in tumors with 10q LOH but without homozygous deletion or point mutation. However, using methylation-specific PCR (Herman *et al*., 1996) with appropriate controls, we found no evidence of *PTEN/MMAC1* promoter methylation in 12 bladder and 12 renal cell tumors (data not shown).

Thus, we detected the second inactivation event at *PTEN/MMAC1* in two of 25 (8%) bladder tumors by sequence analysis and four of 65 (6%) by homozygous deletion. Overall, 14% of bladder tumors with LOH of 10q analysed for both point mutation and homozygous deletion had a second mutation of *PTEN/MMAC1*. No somatic mutation or homozygous deletion was observed in the renal cell tumors with LOH through *PTEN/MMAC1* despite a mutation reported in one of four primary renal cell tumors with 10q LOH sequenced by Steck *et al*., (1997) and our finding of one tumor with a localized LOH at *D10S215*.

Previous reports suggested that mutation of *PTEN/MMAC1* is associated with advanced cancers (Li *et al*., 1997; Steck *et al*., 1997). LOH of chromosome 10 was seen in renal cell carcinomas of all stages. For bladder cancer, we observed LOH of 10q in 8% of Ta, 20% of T1 and 29% of T2 tumors. The six tumors with deletion and point mutation were all grade II or III with stromal (T1) or muscle (T2) invasion. It appears then that *PTEN/MMAC1* is mutated and inactivated in approximately 14% of primary bladder tumors with 10q LOH. Moreover, as in other cancer types, tumors of high grade and stage are more likely to harbor 10q loss and *PTEN/MMAC1* mutations. This result is likely to be an underestimation since we did not search for mutations in the promoter or regulatory regions, did not sequence tumors without LOH (potentially harboring point mutations of both alleles), and almost certainly missed some small homozygous deletions. However, the relatively infrequent detection of the second inactivating event leads us to conclude that other mechanisms of inactivation may exist for *PTEN/MMAC1* or that another tumor suppressor locus may be an additional target of 10q deletion in bladder and renal cancer.

Abbreviations

LOH, Loss of Heterozygosity; PCR, Polymerase Chain Reaction; CDKN2a, Cyclin Dependent Kinase 4 Inhibitor/p16/MTS1; PTEN/MMAC1, Phosphatase and Tensin homolog deleted on chromosome Ten/Mutated in Multiple Advanced Cancers 1; BAC, Bacterial Artificial Chromosome; UTR, Untranslated Region.

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