Identification of *PTEN* mutations in metastatic melanoma specimens

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Abstract

Context—*PTEN*, a tumour suppressor gene located on chromosome 10q23, develops somatic mutations in various tumours and tumour cell lines including brain, endometrium, prostate, breast, kidney, thyroid, liver, and melanoma.

Objectives—To investigate the mutational profile of this gene further, as well as its role in tumour progression in melanoma. **Design, Settings—**We examined 21 metastatic melanoma samples for 10q23 allelic losses and *PTEN* sequence alterations. Additionally, we screened these samples for mutations in *CDKN2A*, a gene in which alterations are well documented in primary melanoma as well as in the germline of familial melanoma.

Results—Loss of heterozygosity (LOH) at 10q23 was observed in 33% (7/21) of the samples tested. We identified four sequence alterations in *PTEN* (19%) and two in *CDKN2A* (9.5%). Of interest, only one case showed mutations in both genes.

Conclusions—These data support the notion that *PTEN* alterations occur in some metastatic melanomas, and that mutation of this gene plays a role in the progression of some forms of melanoma.

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A tumour suppressor gene, *PTEN* (also known as *MMAC1* or *TEP1*), was isolated by mapping homozygous deletions on human chromosome 10q23 from glioblastoma, prostate, and breast cancer cell lines.¹⁻³ Subsequently, a series of mutations in *PTEN* were identified in sporadic tumours and cancer cell lines from various tissues including brain, endometrium, prostate, breast, kidney, thyroid, liver, and melanoma.⁴⁻¹¹ Among all these tumours, *PTEN* is mutated with a high frequency in advanced stages of gliomas and prostate cancers, and in all stages of endometrial cancers.^{4 5 12 13} Furthermore, this tumour suppressor gene has been found to be the susceptibility gene for an inherited hamartoma syndrome with an increased risk of malignancy, Cowden syndrome (CS).¹⁴⁻¹⁸ Of interest, while breast and thyroid cancers are the most commonly observed tumours in CS, an increased risk of melanoma has not been documented in these patients.

PTEN is a phosphatase containing 403 amino acids. It is encoded by nine exons. The phosphatase catalytic domain is between the residues 122-132. Additionally, two potential phosphate acceptor sites are present at residues

233-240 and 308-315.² The sporadic and germline mutations in *PTEN* cluster within the presumptive catalytic domain, with many mutations altering residues required for enzymatic activity.¹⁹ Recent studies show that *PTEN* modulates cell cycle progression and cell survival by regulating phosphoinositide-3-kinase (PI3K) and the protein-Ser/Thr kinase (AKT) signalling pathway.²⁰⁻²²

Loss of heterozygosity (LOH) studies in melanoma have shown a high frequency of loss of 10q.²³⁻²⁶ Several studies suggested involvement of chromosome 10q22-10qter in melanoma,²⁷ as well as 10q24-26 in benign melanocytic proliferations, such as compound and dysplastic naevi.^{26 28} After the isolation of *PTEN* from cancer cell lines harbouring homozygous deletions in 10q23, melanoma cell lines and uncultured primary and metastatic melanoma samples were examined for deletions or mutations in *PTEN*. To date, most of the data showing sequence alterations of *PTEN* are from studies using melanoma cell lines and not primary tumour samples. The most common alterations identified are homozygous deletions.⁸ Of interest, the reported incidence of point mutations and deletions in *PTEN* is significantly low for uncultured melanomas (approximately 10%) when compared to tumour cell lines (approximately 29-43%).

In addition to 10q, LOH in melanoma has been observed in a number of different loci including 1p, 3p, 3q, 6q, 9p, 9q, 11q, 13q, 17p, 17q, and 22q.²³ Of these loci, 9p shows high frequency of allelic loss in melanoma. Alterations in *CDKN2A* located on 9p21 have been well documented in melanoma.^{29 30} Moreover, germline mutations in *CDKN2A* have been identified in 9p21 linked familial melanoma cases.^{31 32} *CDKN2A* regulates cell cycle at the G1 to S transition by inhibiting CDK4 and CDK6. Alterations in CDK4 have also been identified in melanoma, but appear to be rare.^{33 34}

In an attempt to investigate the role of somatic mutations of *PTEN* in metastatic melanoma and to understand its role in tumour progression further, we screened 21 sporadic metastatic melanoma samples for LOH at 10q23 and for mutations in the *PTEN* gene. All samples were subjected to direct sequencing analysis of the *PTEN* gene regardless of LOH data. In addition, the samples were also screened for LOH at 9p21 and for mutations in the *CDKN2A* gene to determine whether these two tumour suppressor genes act independently or synergistically in the tumour progression of melanomas.

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Figure 1 Loss of heterozygosity analyses of metastatic melanomas on chromosome 10q23. (A) Seven short tandem polymorphic repeat markers around the *PTEN* locus on 10q23 used in this study. (B) Allele losses of cases 1-6 on 10q23 are shown. N: normal tissue; T: tumour tissue.

Materials and methods

TUMOUR SPECIMENS AND DNA PREPARATION

A total of 21 metastatic melanoma samples in paraffin embedded blocks were obtained. Each tumour was examined histopathologically. All tumour samples contained greater than 70–80% tumour cells. A 15 µ section was cut for each sample to be tested. The normal and tumour tissues were dissected out and placed separately into 1.5 ml Eppendorf tubes. Total genomic DNA was purified from the deparaffinised section using a QIAamp Tissue Kit (Qiagen, Stanford, CA).

LOH ANALYSIS

LOH analysis was performed as described previously.³⁵ The following seven polymorphic short tandem repeat microsatellite markers located on 10q, in the interval known to contain the *PTEN* gene and its flanking regions, were used in this study: D10S219, D10S551, D10S215, D10S1765, D10S541, D10S1735, D10S17564, and D10S536 (fig 1A). Additionally, to look for LOH on 9p, in the region containing the *CDKN2A* gene and adjacent flanking regions, the following five microsatellite markers were used: D9S169, D9S171, D9S52, D9S178, and D9S492. LOH was assessed by quantitatively comparing polymorphic marker amplicons generated from tumour and normal DNA of each subject tested.

MUTATION SCREENING FOR *PTEN* AND *CDKN2A*

Nested PCR was performed for *PTEN* as described.² For *CDKN2A*, we used the following primers flanking the coding sequence and the splicing sites. The forward (f) and reverse (r) primers used for amplification of *CDKN2A* were as follows. For exon 1, e1f1: 5'GAA GAAAGAGGAGGGGCT3', e1r1: 5'GCGC TACCTGATTCCAATTC3', e1f2: 5'GGC

TGGTCACCAGAGGGTGG3', e1r2: 5' AG AGTCGCCCCGCCATCCCCCT 3'. For exon 2, e2f1: 5'GGAAATTGGAAGCTGGAAGC3', e2r1: 5'TTTGGAAGCTCTCAGGGTAC3', e2f2: 5'TGGCTCTGACCATTCTGTTC3', e2r2: 5'TCAGATCATCAGTCCTCACC3'. For exon 3, e3f1: 5'CCGGTAGGGACG GCAAGAGA3', e3r1: 5'CTGTAGGACCCT CGGTGACTGATGA3'.

PCR products were sequenced using an Applied Biosystem 310 automated sequencing system. Sequence alterations were verified by sequencing the reverse strand, as well as by sequencing of a second DNA sample.

Results

DNA from 21 metastatic melanomas was screened for LOH using seven microsatellite markers on chromosome 10 near the *PTEN* locus (table 1). Regardless of LOH data, all tumour samples were then amplified with primers flanking the nine exons of the *PTEN* gene and sequenced to detect coding sequence or splice site variations. LOH and sequencing data are summarised in table 2. Using this panel of markers, we observed LOH in seven cases (7/21, 33%). All tumours, except case 7, were informative for at least three markers in this region. No hemizygosity was observed in case 7 with all seven markers used. Of the seven samples with LOH, five showed *PTEN* sequence alterations. The mutations consisted of a nonsense mutation in exon 6 (633C>A) and two missense mutations in exons 1 (D19N) and 7 (V217I). Cases 4 and 5 showed LOH with at least two markers, but mutations by direct sequencing were not observed. In addition, two putative splice site changes in IVS1 (79+14 G>A) and IVS2 (165-13 G>A) were observed in cases 6 and 7. These intronic sequence alterations have not been identified in 100 control samples. Overall, six of 21 (28.5%) samples showed sequence

Table 1 LOH analyses on chromosome 10q23 in metastatic melanoma

Case No	Sample No	Microsatellite markers (centromere→telomere)						
		D10S219	D10S551	D10S215	D10S1765	D10S541	D10S1735	D10S536
1	14 456	o	o	o	-	+	-	+
2	14 126	-	+	o	+	+	-	o
3	20 855	o	+	o	-	+	+	-
4	15 047	o	-	-	+	o	-	-
5	14 161	+	-	o	-	+	-	o
6	5914	o	+	o	-	+	o	+
7	4890	o	o	o	o	o	o	o
8	23 807	-	-	o	-	o	-	-
9	359	-	-	o	o	-	-	o
10	17 974	o	-	-	o	-	-	-
11	3260	o	-	o	o	-	-	-
12	19 529	o	-	o	-	-	-	o
13	12 917	o	-	o	-	-	-	-
14	4789	ND	o	-	-	-	o	-
15	8523	ND	o	-	-	o	o	-
16	15 374	ND	o	-	NA	o	o	-
17	17 146	ND	-	-	-	o	-	o
18	5469	ND	-	-	-	o	o	-
19	12 714	ND	NA	-	-	o	-	-
20	23 151	ND	o	-	-	o	-	-
21	24 148	ND	o	o	-	o	-	-

ND, not done; NA, no amplification; o, uninformative.

alterations when analysed by direct sequencing. Of interest, one tumour sample, case 8, in which a missense mutation in exon 2 (154 G>A) was identified, did not show LOH with the panel of markers used.

Additionally, all samples were also tested for mutations in *CDKN2A* by direct sequencing. We identified two missense mutations in cases 2 and 5 (2/21, 9.5%), both in exon 2 of *CDKN2A*. These two cases also show LOH at 9p21. Of these, case 2 showed sequencing variations both in *PTEN* and *CDKN2A*, whereas case 9 showed a nucleotide change in *CDKN2A* only (table 2).

Discussion

To date, the information on mutational profile of *PTEN* in melanoma has been gathered primarily from studies using tumour cell lines and not primary tumour samples. These data show somatic mutations and deletions in 29–43% of the samples.^{8–10} Teng *et al*⁸ showed 48% LOH at 10q23 in primary melanomas, in which only one missense mutation in *PTEN* (10%) was identified. In the same study, they showed 50% LOH in melanoma cell lines, and found four homozygous deletions (28%). Guldberg *et al*⁹ studied melanoma cell lines and reported similar incidence of alterations (43%) in *PTEN*. Of interest, in this study they showed that for three specimens identical alterations found in the cultured cell lines also existed in the uncultured tumour specimens. Finally,

Tsao *et al*¹⁰ described a mutation rate of 29% in melanoma cell lines, and 6% in uncultured metastatic melanomas. Of the uncultured metastatic melanomas they examined, only one showed a 7 bp duplication in exon 7 leading to a premature stop codon. We have investigated uncultured metastatic melanomas for LOH at 10q23, and for alterations in *PTEN* and *CDKN2A*. In this set of 21 metastatic tumour samples, we detected 33% (7/21) LOH at 10q23 and 28.5% (6/21) sequence alterations in *PTEN* by direct sequencing. *CDKN2A* mutations were encountered in 9.5% (2/21) of the samples tested. Only one case showed sequencing variations, both in *PTEN* and *CDKN2A*. These data support the notion that chromosomal alteration involving 10q23 and *PTEN* occur in metastatic melanoma.

The mutations of *PTEN*, both germline and somatic, have been reported in all nine exons of the gene. However, mutations in *PTEN* tend to cluster in exon 5, which contains the phosphatase catalytic domain. Aside from exon 5, a significant number of mutations have been observed in exons 6, 7, and 8. Of the 21 samples analysed, we found one nonsense mutation in exon 6 and three missense mutations in exons 1, 2, and 7 in *PTEN*. All of the mutations identified in this study are novel somatic mutations in *PTEN*. Of interest, we observed the exon 6 mutation, C211X, in the germline of a family with Cowden syndrome (unpublished data). Additionally, we noted

Table 2 Summary of LOH on 10q23 and mutations in *PTEN* compared to *CDKN2A* in metastatic melanoma

Case No	Sample No	<i>PTEN</i>			Exon or IVS	<i>CDKN2A</i>			
		LOH	Mutation/sequence alteration	Predicted effect		LOH	Mutation/sequence alteration	Predicted effect	Exon or IVS
1	14 456	+	633 C>A	C211X	6	-	-		
2	14 126	+	55 G>A	D19N	1	+	193 C>T	L65F	2
3	20 855	+	649 G>A	V217I	7	ND	-		
4	15 047	+	-			ND	-		
5	14 161	+	-			+	220 G>A	D74N	2
6	5914	+	IVS1+14 G>A	Putative splice site or polymorphism	IVS1	ND	-		
7	4890	+	IVS2-13 G>A	Putative splice site or polymorphism	IVS1	ND	-		
8	23 807	-	154 G>A	D52N	2	ND	-		

ND, not done.

sequence alterations in *PTEN* in IVS1 (+14 G>A) and IVS2 (-13 G>A) in samples 6 and 7, which both also showed allelic loss of *PTEN*. These variations were not observed in 100 control sequences, thus suggesting that these intronic alterations may be either splice site changes resulting in exon skipping and thus a non-functional protein or rare polymorphisms in *PTEN*. To date, all of our studies of germline splice site alterations in CS have resulted in exon skipping, which suggests that this is likely to be the case here as well.¹⁸

It has been noted that mutations, both intra-genic and homozygous deletions, in uncultured tumour tissue are detected with less sensitivity than in cell lines, because of heterogeneity within the sample, as well as normal stromal contamination.⁸⁻⁹ Most tumours are predicted to contain 10-40% normal cell contamination. Even 5% normal DNA within a tumour can prevent identification of homozygous deletions using gel visualisation after PCR⁸ and thus homozygous deletions of uncultured specimens in *PTEN* have not been documented to date. Tumour tissue is also heterogeneous, so it is possible that only the most malignant cancer cells within a tumour may have detectable mutations. Similarly, mutations in tumour cell lines may be detected more easily because of the selection bias conferred in cells grown in cultures through multiple passages. For the reasons listed above, our results may be an under-representation of *PTEN* and *CDKN2A* mutations in metastatic melanoma. Despite the possibility of underestimation of mutations, our results and those reported previously suggest that *PTEN* and *CDKN2A* play a role in tumour progression in some, but not all, metastatic melanomas.

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