Original articles

Polymorphisms in PTEN in breast cancer families

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Abstract

Germline mutations in PTEN are the underlying genetic defect in Cowden disease, which is associated with a lifetime risk of 25-50% of developing breast cancer. To investigate the role of PTEN in inherited breast cancer in the absence of manifestations of Cowden disease, we screened 177 unrelated subjects with breast cancer who also had a family history of breast cancer in at least one relative. We found no disease associated PTEN mutations in this cohort, supporting previous studies suggesting that PTEN mutations do not contribute to inherited susceptibility to breast cancer without associated manifestations of Cowden disease. We did identify an association between a common polymorphism in intron 4 and lower mean age of diagnosis of breast cancer. While preliminary, these findings suggest that further study is warranted to determine whether this allelic variant of PTEN could function as a low penetrance breast cancer susceptibility allele.

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PTEN/MMAC1/TEP1, located on chromosome 10q23, codes for a tyrosine phosphatase with putative tumour suppressing abilities.[1] Germline mutations of PTEN are thought to be responsible for Cowden disease, which is characterised by multiple benign and malignant neoplasms including breast cancer, thyroid cancer, and intestinal polyps.[2 3] Somatic mutations in PTEN have been implicated in the tumorigenesis of several types of cancers, including glioblastoma, breast cancer (in association with Cowden disease), advanced prostate cancer, thyroid cancer (in association with Cowden disease), and endometrial cancer.[4–7] Mutations of PTEN have been reported in 44 of 90 Cowden disease families described to date.[3 8–10] Mutations of PTEN have also been identified in families with Bannayan-Zonana syndrome,[10–12] a disorder that shares some clinical features with Cowden disease. The lifetime breast cancer risk in women with Cowden disease is estimated to be 25-50%.[10] Therefore, several groups have evaluated whether germline mutations in PTEN explain an inherited susceptibility to breast cancer in women.[8 9 13] PTEN mutations have been identified in women with breast cancer from Cowden disease families. However, no germline PTEN mutations have been reported in women with inherited susceptibility to breast cancer in the absence of other manifestations of Cowden disease. Nonetheless, the prevalence of breast cancer in Cowden disease families and the occurrence of somatic PTEN mutations in a small subset of sporadic breast tumours suggests that variants of PTEN could contribute to breast tumorigenesis, including inherited breast cancers.

To investigate the role of germline PTEN variants in inherited breast cancer, we screened 177 unrelated probands affected with breast cancer or ovarian cancer, who also had a family history of breast cancer, for PTEN mutations. Of these, 151 were women and 26 were men. All of the probands had been screened for, but did not have, detectable mutations in the hereditary breast cancer genes BRCA1 or BRCA2. Several tumour types, in addition to breast or ovarian cancer, that have been previously shown to be related to PTEN mutations or Cowden disease were diagnosed in some probands. These included three subjects with thyroid cancer, two with endometrial cancer, and one with a brain tumour. Thirty-five of the probands had a family history of male breast cancer, with 26 probands being affected males. All samples were screened for PTEN mutations by conformation sensitive gel electrophoresis (CSGE)[14] using primers as described by Steck *et al.*[15] A total of four different

Table 1 Polymorphisms seen in PTEN in patients with breast cancer and a family history of breast cancer

Exon/intron	*Variant*	*Observations*	*Frequency*	*Comments*
IVS1	82-126A>G	1/177	0.006	
IVS1	82-4A>G	1/177	0.006	No effect on splicing by in vitro and in vivo assays
IVS4	210+109ins5	66/177	0.37	Associated with decreased age of breast cancer diagnosis in insertion homozygotes
IVS9	1212+65insA	4/177	0.02	

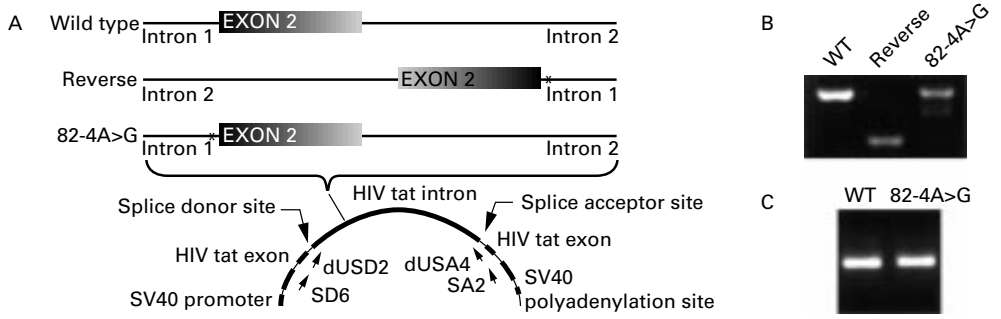

Figure 1 Construction of a splicing vector with the IVS1 82-4A>G mutation and analysis of RNA of an affected carrier. (A) A wild type sequence, the 82-4A>G sequence in reverse orientation, and the 82-4A>G sequence in the forward orientation were inserted into a pSPL3 exon trapping vector (GIBCO BRL). (B) RNA from cells transfected with the constructs were transcribed and amplified with dUSD2 and dUSA4 primers. (C) Randomly primed cDNA derived from peripheral blood lymphocytes was amplified with primers within exon 1 and exon 3.

variants were identified in nine of 177 subjects (table 1).

Two novel variants, IVS1, 82-126A>G and IVS 9, 1212+65insA, identified in five unrelated subjects, are presumed to be too distant from the PTEN exons to affect normal function. One is a transition from an A to G, positioned in intron 1, 126 bp upstream of exon 2 in one subject. The other is a single A inserted into a string of 10 As, positioned 65 bp downstream of exon 9 in the ninth intron in four subjects. Neither variant was associated with bilateral breast cancer, younger age of breast cancer diagnosis, nor with primary tumours at other sites associated with Cowden disease.

The third variant, IVS1, 82-4A>G, identified in one subject, was an A to G transition located in intron 1, 4 bp upstream of exon 2. This variant falls within a consensus splice acceptor site. However, this variant is at a non-conserved site within the consensus splice acceptor sequence, suggesting that a variant at this position would not affect splicing. In order to evaluate the possibility that this sequence change could disrupt splicing, we both constructed a splicing vector with this sequence and analysed PTEN RNA from the peripheral blood lymphocytes of an affected carrier of this variant.

Splicing vectors were created by inserting DNA containing exon 2 and a portion of the adjoining introns into a pSPL3 exon trapping vector (GIBCO BRL). The pSPL3 vector consists of two short exons with functional splice sites separated by an intronic segment that contains the multiple cloning site (fig 1A). When transfected into eukaryotic cells, these splice sites are functional and the resulting DNA fragment is transcribed. The resulting RNA is then analysed followed reverse transcription and PCR amplification. We amplified patient DNA using primers in introns 2 and 3, inserted the product into pCR2.1 cloning vectors, and then transferred the fragments into the pSPL3 vector to make three plasmids: (1) a wild type sequence, (2) the 82-4A>G sequence in the reverse orientation, and (3) the 82-4 A>G sequence in the forward orientation. The three constructs were transfected into Cos 7 cells according to the manufacturer's recommended protocols. At 24 hours, RNA was harvested and reverse transcribed. Primers (dUSD2 and dUSA4) within the two vector exons were used to amplify products from the three pools of cDNA. No alternatively spliced fragments resulting from the 82-4A>G (IVS1) variant were detected (fig 1B).

In addition, peripheral blood lymphocytes were obtained from the affected subject. Random primed cDNA was amplified with primers from exon 1 and exon 3 (5'GCCATCATCAAAGAGATCG3' and 5'CTAGCTGTGGTGGGTTATGG3'). The size of the resulting single band was consistent with the in vitro splicing data, suggesting that the 82-4 A>G (IVS1) variant did not disrupt splicing (fig 1C). Thus, it is likely that this variant is not a disease associated mutation.

The fourth variant, IVS4, 210+109ins5, identified in 37% of alleles (66 of 177 patients), is an ACTAA insertion 109 bp downstream of exon 4 in intron 4. As shown in fig 2, the mean age of diagnosis for homozygous normal and heterozygous subjects was 48.1 years, which was significantly different from the mean age of diagnosis of 42.7 years (χ^2_1=0.024) for subjects homozygous for the insertion variant. No effect was observed in the age of male breast cancer diagnosis, although the small sample size may have limited our ability to detect such a difference. While this polymorphism has been reported by three other groups,[7 16 17] no similar effect on age of diagnosis in any tumour types has been reported. The frequency of the 5 bp insertion allele is similar to the 40% of alleles reported in another study,[17] but is different from the 53% and 54% of alleles identified in the two reports of sporadic tumours.[7 16]

The potential functional role of the 5 bp insertion in the modification of age at breast cancer diagnosis is not clear. Of note, the frequency of mutations and the presence of the phosphatase core motif in the adjoining exon 5 have led to exon 5 being described as a "hot spot" for mutations in Cowden disease.[3 10] Whether 210+109ins5 (IVS4) disrupts splicing or expression is unknown. The region has no homology to consensus sites or lariat formation sites. The variant position, next to an exon that is a hot spot for Cowden disease mutations, suggests that a splicing error deleting exon 5 could have a potentially significant effect. However, no alternative splice forms were

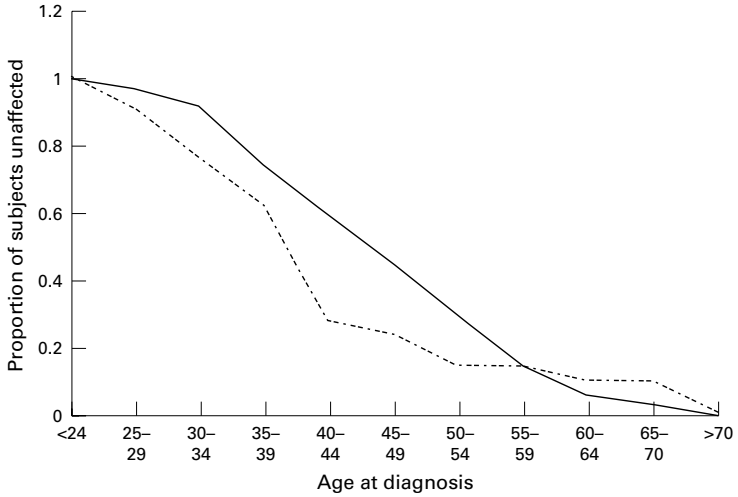

Figure 2 Age specific probability of breast cancer by genotype. The solid line represents the 113 homozygous wild type and heterozygous 210+109ins5 subjects. The dashed line represents the 20 subjects homozygous for the 210+109ins5 polymorphism.

identified in any of the three genotypes using RT-PCR across exon 5 (5'AGACATTATGACACCGCCA3' and 5'CAACAGTGCCACTGGTCTA3') (data not shown). Alternatively, this variant may be in linkage disequilibrium with a functional mutation in another gene. While the effect of the variant may be tissue specific and thus not detected by the methods used here, any functional effect remains speculative.

As in the two previous reports describing PTEN mutation studies in breast cancer families, there were no PTEN coding region mutations in any of the non-BRCA1, non-BRCA2 breast cancer families studied in this analysis. Based on our experience with CSGE for mutation analysis in other genes, we estimate the sensitivity of this heteroduplex detection technique to be in the range of 95-98%, far superior to single strand conformation polymorphism (SSCP) analysis in our hands.[18] These data add further support to the growing body of evidence that while there is convincing evidence for the role of PTEN in Cowden disease, which includes an increased risk for developing breast cancer, germline coding mutations in PTEN do not play a role in the inheritance of susceptibility for breast cancer in site specific breast cancer families. As Cowden disease is both rare and generally recognisable as a clinical entity, our data do not support clinical testing for PTEN mutations as part of the evaluation of familial breast cancer if other evidence of Cowden disease is lacking.

While germline coding region mutations in PTEN do not appear to explain a significant number of non-BRCA1, non-BRCA2 inherited breast cancer, the 210+109ins5 (IVS4) polymorphism is associated with earlier age of breast cancer diagnosis in our familial breast cancer cohort, and suggests that PTEN could be acting as a modifier gene in this setting. More detailed studies of the function of PTEN and the 5 bp insertion polymorphism may help elucidate the role of PTEN.

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