Letters to the Editor

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No evidence of germline *PTEN* mutations in familial prostate cancer

EDITOR—Prostate cancer is the second most common cause of male cancer mortality in the UK.[1] Current indications are that like many common cancers, prostate cancer has an inherited component.[2] Segregation analysis has led to the proposed model of at least one highly penetrant, dominant gene (with an estimated 88% penetrance for prostate cancer by the age of 85 in the highly susceptible population). Such a gene or genes would account for an estimated 43% of cases diagnosed at less than 55 years.[2]

One prostate cancer susceptibility locus (*HPC1*) has been reported on 1q24-25[3] and confirmed by Cooney *et al*[4] and Gronberg *et al.*[5] Latest estimates suggest that this locus would only account for 4% of families overall in the UK (upper 95% confidence interval (CI) limit of 31%).[6] Another locus has been reported on 1q42.2-43 after a genome wide search of 47 French and German families.[7] This locus is estimated to explain 50% of these families and appears to be distinct from the *HPC1* locus as the two are estimated to be 60 cM apart. Confirmatory studies of this second locus have not yet been reported. A third locus has been reported. This locus, situated on the X chromosome, is estimated to explain approximately 16% of the families studied (including the families which were first typed to map the 1q24 locus).[8] The heterogeneity lod score for linkage to this locus is 3.85 with the strongest evidence being a locus in proximity to the markers DXS297 and DXS1200.

While linkage studies have not identified chromosome 10 as the site of a predisposing gene, the long arm of chromosome 10 is the fourth commonest region showing loss of heterozygosity (LOH) in sporadic prostate cancers after 7q, 8p, and 16q.[9] Deletion mapping studies have identified 10q23 to be the minimal region of loss.[10–12] One candidate gene which maps adjacent to this region, *MXI1*, has been assessed for a role in familial prostate cancer susceptibility but no germline mutations were identified.[13] *PTEN*/*MMAC1* (Phosphatase and Tensin homologue deleted on chromosome Ten/Mutated in Multiple Advanced Cancers 1), a tumour suppressor gene, has recently been identified at 10q23 through mapping of homozygous deletions in tumour cell lines.[14–16] Li *et al*[14 15] and Steck *et al*[16] found *PTEN* mutations in four out of four and one out of three prostate cancer cell lines respectively, suggesting a role in prostate carcinogenesis.[15 16] Cairns *et al*[17] found LOH at 10q in 23 of 80 prostate tumours. Sequencing identified a mutation in *PTEN* in 10 of these 23 tumours (43%).[17] More recently, Wang *et al*[18] found that of 60 prostate adenocarcinomas, 10-15% of primary stage B prostate carcinomas had *PTEN* inactivation by homozygous deletion. A number of studies have examined the frequency of somatic mutations in this gene. For instance, somatic mutations have been found in glioblastomas, melanomas, and breast and prostate carcinomas.[15 16] Germline mutations in *PTEN* have been shown to be the cause of Cowden disease,[19 20] while *PTEN* deficient mice (*PTEN* +/-) show hyperplastic and dysplastic changes in the prostate and indeed develop prostate cancer.[21 22]

We hypothesised that germline *PTEN* mutations could be important in familial prostate cancer for the following three reasons: somatic mutations have been found in *PTEN* in prostate tumours; germline mutations in Cowden disease produce a phenotype (although with no evidence of an associated susceptibility to prostate cancer); and *PTEN* deficient mice exhibit prostate abnormalities. We have therefore screened the Cancer Research Campaign/British Prostate Group (CRC/BPG) UK Familial Prostate Cancer Study samples for evidence of *PTEN* mutations.

The CRC/BPG UK Familial Prostate Cancer Study has collected lymphocyte DNA from 188 subjects from 50 prostate cancer families. These families were chosen because each contained three or more cases of prostate cancer at any age or related sib pairs where at least one man was less than 67 (original criterion was 65) years old at diagnosis. In fact, the majority of the clusters consist of affected sib pairs, with DNA often only available from cases. Twenty eight of the families had two affected males, 10 had three affected, nine had four affected, and three had five affected; the average age of onset was 66.9 years. Sample family pedigrees and DNA extraction protocols are described in Edwards *et al.*[13] These families were previously analysed for linkage to *HPC1* and showed no evidence for linkage.[6] DNA extracted from a known Cowden disease patient was used as a positive control for mutational analysis. The study was approved by the Royal Marsden NHS Trust Local Research Ethics Committee.

Linkage analysis was performed after genotyping using three polymorphic DNA markers flanking *PTEN* (D10S541, D10S1765, and D10S2491). Lod scores for linkage to *PTEN* were calculated under the assumption that prostate cancer was caused by an autosomal dominant gene with Carter's estimate of penetrance[2] using the GENEHUNTER software[23] and assuming the marker order of D10S1765/D10S2491 - 20.5 kb - *PTEN* - 0.2 cM - D10S541. Under this model of inheritance, there is an assumed lifetime penetrance (to 85 years of age) of 88% for prostate cancer in the highly susceptible population and with 0.6% of the general population carrying such a predisposition. The risk to age 85 in the non-susceptible male UK population was assumed to be 6.5%. Linkage analysis was performed under a model of homogeneity with *PTEN* being the only high penetrance predisposition gene for prostate cancer and also under a model of heterogeneity in which other high penetrance predispositions were assumed. For D10S1765 and D10S541, the allele frequencies were estimated from the family data, while for D10S2491 the alleles were assumed to be equally frequent as limited typing precluded estimation (at the time this was a new marker with no published allele frequencies). Non-parametric analysis of linkage was also performed using the NPL statistic of GENEHUNTER.[23]

Pairwise and multipoint linkage analysis showed no evidence of linkage to the *PTEN* region. Indeed, under homogeneity and tight linkage there was strong evidence against the hypothesis of a gene in the region of *PTEN*, which approached the conventional limit for exclusion mapping; the multipoint lod score was −1.96 close to the cut off of −2.0. Under heterogeneity, the overall heterogeneity lod score at the *PTEN* locus was 0.18 with an estimated 29% of the families being the result of *PTEN* with a 95% confidence interval of 0% to 79%. Non-parametric linkage analysis using the NPL statistic of GENEHUNTER was also performed to guard against a misspecified mode of inheritance; again, this showed some evidence for allele

sharing which did not reach formal significance in the region of *PTEN* (NPL=0.88, p=0.19). Further, there was no difference in age of onset in those families consistent with linkage versus those against linkage to *PTEN* and there was no trend in terms of the proportion of linked families by number of affected males (data not shown).

Linkage analysis of common cancers is complicated by the presence of heterogeneity. As the majority of our families consist only of sib pairs, the opportunity of confirming identity by descent among the affected brothers is extremely limited, thus compromising the ability of linkage analysis to extract convincing evidence of cosegregation. While there is no formal evidence for linkage, even under multipoint analysis for a familial prostate cancer gene in the *PTEN* region of 10q23, the point estimates for the proportion of families linked is 29% (95% CI (0.00-0.79)) showing lack of discrimination from the family material. With these data, and given the putative role of *PTEN* in prostate cancer carcinogenesis, we decided to pursue *PTEN* further by mutation testing in families in which the brothers appeared to share at least one parental haplotype around *PTEN*.

All nine exons of the *PTEN* gene of the youngest diagnosed member from the remaining 37 families for whom DNA was available and a Cowden disease positive control were sequenced in both directions using dRhodamine labelled ddNTPs (Perkin Elmer). Primers were designed to amplify all coding sequence and at least 15 flanking base pairs.

The IVS8 +32T>G polymorphism in intron 8 reported by Wang *et al*[24] was found in 27 (73%) of the 37 cases. Forty five percent of the alleles were the "wild type" G, while the remaining 55% of alleles were T. Fourteen (38%) of the cases were TT homozygotes, 13 (35%) were TG heterozygotes, and 10 (27%) were GG homozygotes; these figures are consistent with Hardy-Weinberg equilibrium (χ^2 with 2 df=3.1, p=0.21). No association was found between age of onset of prostate cancer and genotype (TT *v* TG *v* GG) when the cases were divided into two categories depending on age of onset being less than or equal to 60 years of age *v* 61 years of age or higher (χ^2 with 2 df=1.0, p=0.61).

A novel polymorphism (IVS4 −31insT) was found in intron 4 in one patient, but not in the patient's brother who also had prostate cancer, suggesting that it is not important in prostate cancer susceptibility.

While a missense mutation, c.494G>A (p.165G>E), was found in exon 6 of the Cowden disease positive control used, no coding mutations were found in the prostate cancer families. In some cases sequencing only extended 15 bp into non-coding sequence, so non-coding mutations which may possibly influence RNA splicing or stability may have gone undetected. The promoter and regulator regions of *PTEN* are also unknown at present, so any mutations in these regions were also not detected. The *HPC1* locus in 1q24-25 has been shown to be preferentially involved in families with four or more affected cases[6] and this might also be the case for *PTEN*.

Our linkage results suggest that it is unlikely that *PTEN* or any gene nearby in the 10q23.3 region plays any significant part as a high penetrance susceptibility gene for prostate cancer. This is consistent with the concept that somatic mutations in *PTEN* are a late event in carcinogenesis, as suggested by Feilotter *et al*,[25] who found very few *PTEN* mutations in primary prostate cancer tumours, and the work of Cairns *et al*,[17] who found a positive correlation between high prostate tumour grade and presence of *PTEN* mutation.

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Mutation analysis of *H19* and *NAP1L4* (*hNAP2*) candidate genes and *IGF2* DMR2 in Beckwith-Wiedemann syndrome

EDITOR—Beckwith-Wiedemann syndrome (BWS) is a human overgrowth disorder with a variable phenotype and genetic heterogeneity. Recent data indicate that the BWS locus is subject to genomic imprinting and current evidence shows that in many patients the disease is associated with epigenetic lesions of genes on 11p15.5. BWS is characterised by pre- and postnatal overgrowth, macroglossia, and anterior abdominal wall defects. Additional, but variable complications include organomegaly, hypoglycaemia, hemihypertrophy, genitourinary abnormalities, and a predisposition to embryonal tumours in about 5% of patients.[1] The genetics of BWS are complex, but parent of origin effects, suggesting genomic imprinting, have been implicated in the pathogenesis of three major groups of patients[2]: (1) for patients (~2%) with chromosome 11p15.5 abnormalities, duplications are of paternal origin and balanced translocations or inversion breakpoints of maternal origin; (2) in familial cases (~15% of all cases) which exhibit more complete penetrance with maternal transmission; and (3) approximately 20% of sporadic cases have uniparental disomy (paternal isodisomy) for chromosome 11p15.5. Cloning of genes in the vicinity of BWSCR1, the most distal breakpoint cluster associated with BWS balanced cytogenetic anomalies, and within the area of minimal disomy present in cases of paternal isodisomy, has led to the identification of a group of genes as potential candidates in the aetiopathology of BWS. Thus, multiple imprinted genes bounded centromerically by *NAP1L4* and telomerically by L23mrp (*RPL23L*) have been identified.[3–6]

For a gene to be a good candidate to account for a significant number of BWS cases, it should map to this region and constitute either a paternally expressed growth promoter or a maternally expressed growth inhibitor. However, because factors involved in maintaining or modifying genomic imprints may affect the epigenotype and therefore expression status of imprinted genes, it is possible that an underlying lesion in a non-imprinted gene may manifest itself as an imprinted trait. Extensive characterisation of *IGF2*, *CDKN1C*, and *KVLQT1* in BWS patients has been undertaken already. With the exception of the involvement of *KVLQT1* in some balanced translocations and inversions,[7] and *IGF2* in paternal duplications,[8] the only mutations within the coding sequence of a candidate gene described are for *CDKN1C*.[9–14] In our series, germline *CDKN1C* mutations accounted for ~40% of familial cases and 5% of sporadic cases.[14] This suggests that further BWS genes remain to be identified. Most sporadic BWS patients show LOI of *IGF2*[15 16] and a candidate BWS gene might cause BWS by influencing *IGF2* imprinting status.

The *H19* gene maps approximately 200 kb telomeric of *IGF2* in humans; synteny is conserved in the mouse.[17] Data from human tumours, BWS, and experimental manipulation of the mouse genome indicate that the regulation of *H19* and *IGF2* expression is closely and reciprocally linked.[18] For some BWS patients with *IGF2* LOI, biallelic *IGF2* expression is associated with suppression of *H19* expression and reversal of the normal (unmethylated) maternal allele methylation patterns, so that both parental *IGF2* and *H19* alleles display a paternal methylation pattern.[19 20] However, in other cases with biallelic *IGF2* expression, *H19* and *IGF2* allelic methylation is normal.[16] A possible explanation for these observations is that the maternal *H19* RNA is functionally inactivated, not affecting its own imprinting status, but leading to loss of repression of the maternal *IGF2* allele. This hypothesis is consistent with data from the mouse.[21]

The most centromeric imprinted gene in the 11p15.5 imprinted region is the candidate tumour suppressor gene *TSSC3*.[22 23] The *NAP1L4* gene lies 15 kb 5' of *TSSC3* and encodes a chaperone protein associated with chromatin assembly and has been shown to bind to core and linker histones facilitating transfer to the DNA template.[24 25] Although *NAP1L4* has not been shown to be imprinted to date, this has not been extensively investigated and tissue specific or developmentally regulated imprinting cannot be excluded. *NAP1L4* lies within the interval associated with loss of heterozygosity in Wilms tumour (WT2) and centromeric to the BWSCR1 breakpoint cluster. It therefore fulfils one of the criteria for a BWS candidate gene. The possibility that chromatin structure affects the activity and imprinting status of genes is very strong[26] and it is possible that mutations in *NAP1L4* might appear to have an allele specific effect even if *NAP1L4* is not itself imprinted. Recent studies on a BWS family with a maternally inherited inversion of 11p15.5 suggest that relaxation of *IGF2* imprinting may result from an *H19* independent pathway. In the family reported by Brown *et al*,[27] a BWSCR1 breakpoint in the region of *NAP1L4* was associated with *IGF2* LOI and normal *H19* expression. Importantly, *NAP1L4* is expressed in normal kidney and some Wilms tumours (WT) lacking *NAP1L4* expression show *IGF2* LOI (Munroe *et al*, unpublished observations). While mutations in *NAP1L4* have not been found in sporadic Wilms tumours,[25] those associated with a genetic predisposition have not yet been examined, leaving open the possibility that *NAP1L4* mutations in the germline or somatic mutations early in development may predispose to the changes seen in BWS and familial WT through an effect on the imprinting status of key genes such as *IGF2*.

Overexpression of Igf2 in mouse development mimics many features of BWS,[28] further implicating *IGF2* in BWS. The *IGF2* gene has a conserved differentially methylated region (DMR) in exon 9. This region has been shown in mice to be consistently methylated on the expressed paternal allele. It has been postulated that the DMR is a methylation sensitive site for silencer binding. Hence, *IGF2* LOI