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Short communication

No germline mutations in the dimerization domain of *MXI1* in prostate cancer clusters

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Summary There is evidence that predisposition to cancer has a genetic component. Genetic models have suggested that there is at least one highly penetrant gene predisposing to this disease. The oncogene *MXI1* on chromosome band 10q24–25 is mutated in a proportion of prostate tumours and loss of heterozygosity occurs at this site, suggesting the location of a tumour suppressor in this region. To investigate the possibility that *MXI1* may be involved in inherited susceptibility to prostate cancer, we have sequenced the HLH and ZIP regions of the gene in 38 families with either three cases of prostate cancer or two affected siblings both diagnosed below the age of 67 years. These are the areas within which mutations have been described in some sporadic prostate cancers. No mutations were found in these two important coding regions and we therefore conclude that *MXI1* does not make a major contribution to prostate cancer susceptibility.

Keywords: prostate cancer; gene *MXI1*; susceptibility

Prostate cancer is the second commonest cause of cancer mortality in men in the UK (OPCS figures, 1993). Its incidence is increasing by more than 10% every 5 years (Coleman et al, 1993), even when the effect of screening is taken into account, and approximately 13% of cases occur in men under 65 years. There is increasing evidence that there is an inherited component to many of the common cancers (Easton and Peto, 1990), and prostate cancer is no exception. There are several lines of evidence for this: familial clustering of prostate cancer has been observed, most dramatically in the large prostate cancer kindreds described in Utah, USA (Eeles and Cannon Albright, 1996); relatives of cases have an increased relative risk of developing the disease compared with relatives of control subjects in case–control studies (reviewed in Eeles, 1995), and this has been confirmed in two cohort studies (Goldgar et al, 1994; Grönberg et al, 1996). This relative risk increases markedly when the age of the index case decreases or the number of affected subjects in a cluster increases, which is evidence that this increase in risk has a genetic component. Segregation analysis has led to the proposed model of at least one highly penetrant gene (88% of the gene carriers would develop prostate cancer by age 85 years), which accounts for 43% of cases diagnosed at less than 55 years (Carter et al, 1992). When prostate cancer susceptibility genes are located, men at increased risk of the disease, particularly at a younger age, will be able to be identified. A prostate cancer susceptibility locus has recently been reported on 1q24–25 (Smith et al, 1996); however, this would only account for 34% of families, and further susceptibility loci remain to be identified.

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To date, with the exception of MEN2 due to *RET* (Mulligan et al, 1994), all high-risk cancer predisposition genes are tumour suppressors; one allele is inherited in a mutated form and tumour development occurs when the remaining allele at the cancer predisposition locus is inactivated by loss or mutation (Knudson, 1985). If prostate cancer follows the same model, candidates for susceptibility genes would reside in the areas of loss of heterozygosity (LOH) observed in prostate tumours.

The long arm of chromosome 10 is the fourth commonest area demonstrating LOH in sporadic prostate cancers (reviewed in Eeles, 1995). In a study of 42 informative tumours, LOH at 10qter was observed in 19% of cases (Steinberg et al, 1990) and, more recently, Eagle et al (1995) documented mutation at the non-deleted *MXI1* locus, which is in this region, in four out of ten prostate cancers that had LOH at 10q24–25. Furthermore, in one sample with no cytogenetic abnormality, the *MXI1* gene was shown to be absent. The mutations are in the non-deleted HLH and ZIP exons, which are the parts of the gene that code for the helix-loop-helix and leucine zipper regions involved in protein dimerization. This is needed for specificity of *MXI1* action. However, Gray et al (1995) subsequently failed to find any mutations in *MXI1* in tumour DNA from 37 prostate cancers.

The oncogene, *MYC*, has been shown to be overexpressed in higher-grade prostate cancers (Buttyan et al, 1987) and the MXI1 (MAX interactor factor 1) protein coded by the *MXI1* gene negatively regulates MYC activity. MXI1, MAX and MAD are all members of a family of proteins involved in the transcriptional control of MYC proteins. All three, together with MYC, are members of a larger family of proteins called helix-loop-helix leucine zipper (HLH-ZIP) transcription factors. Dimerization of proteins within this family permits subsequent DNA binding, a

*All collaborators are at the same position in this paper. The collaborators are listed at the end of the paper.

Table 1 Primers

Primer location	Primer sequence	PCR product size (bp)
MXI1 ZIP exon	Forward 5′-CGC AAG CTT TGT TTG TAC TGG ACT ATA CAC Reverse 5′-CGC GAA TTC ATG TTT AGT ATT TCA TTA GAG AAG	280
MXI1 HLH exon	Forward 5′-CGC AAG CTT TAA CCA GAC TGT GCT GAT TTG Reverse 5′-CGC GAA TTC ACC AGA ACT GAG GGA ATT GTG	250

function mediated by a highly basic region adjacent to the HLH-ZIP motif (Murre et al, 1989). MYC also has distinct transcriptional activation domains, which modulate gene expression (Kato et al, 1990). MAX forms heterodimers with MXI1 (Zervos et al, 1993) and this inhibits MYC function in two ways: first, by sequestering MAX (preventing the formation of MAX–MYC heterodimers); and, secondly, by competing with MAX–MYC heterodimers for binding to target sites (Zervos et al, 1993). Taken together these observations indicate that *MXI1* is a good candidate for a prostate cancer susceptibility gene.

The CRC/BPG UK Familial Prostate Cancer Study aims to investigate the role of genetic susceptibility to prostate cancer. The contribution of both low- and high-penetrance genes is being studied. As part of the study of high-penetrance genes, prostate cancer cases with an increased chance of harbouring a prostate cancer susceptibility gene are being collected. Those clusters with a relative risk of developing prostate cancer of greater than or equal to four are targeted for collection. We have, therefore, concentrated on collecting clusters of ≥ 3 prostate cancers at any age or related pairs, preferably where one is less than 65 years at diagnosis. The first 38 of these clusters were analysed in this study and *MXI1* was sequenced from germline DNA as a candidate for a prostate cancer susceptibility gene.

MATERIALS AND METHODS

DNA extraction

Samples (10 ml) of blood were collected from individuals and stored in EDTA at –70°C until required. For DNA extraction, the method of Kunkel et al (1977) was used with the following modification. Four volumes of cold water were initially added to whole blood. The phenol–chloroform extraction step was omitted and the 'salting out' procedure of Miller et al (1988) was used to clean and retrieve the DNA. The DNA was washed in 70% ethanol and dried briefly, dissolved in 0.2–0.3 ml of water and stored at –20°C.

Polymerase chain reaction (PCR)

Sample DNA (200 ng) was added to a reaction mixture consisting of 1 × PCR buffer [Applied Biosystems; 10 mM Tris (pH 8.3), 50 mM potassium chloride], 3.8 mM magnesium chloride (Applied Biosystems), 0.16 mM each dNTP (0.64 mM total; Stratagene), 0.2 μg (approximately 22 pmol) of each appropriate primer (Table 1) and 0.75 units of *Taq* polymerase (Applied Biosystems). The total reaction mixture was made up with water (BDH) to a volume of 50 μl. The tubes were topped with approximately 40 μl of mineral oil (Sigma) and cycled in a Biometra or Hybaid thermocycler. Thermocycling was programmed for a 'touchdown' procedure as follows: initial denaturation step 94°C for 2 min followed by four cycles of 94°C for 1 min, 64°C for 30 s and 70°C for 1 min. The annealing temperature was reduced by 2°C every four cycles, until the annealing temperature was 56°C. Samples were then given 24 cycles of 94°C for 1 min, 54°C for 30 s and 70°C for 1 min, followed by a final polymerization of 70°C for 10 min. A 5-μl aliquot of PCR reaction mixture was run on a 2% agarose gel to check for the presence of the required product.

Table 2 Patient characteristics

Identifier number (individual tested)	Age at diagnosis of prostate cancer in individual analysed (years)	Number of affected relatives	Age of other relative(s) in family (years)
PR3380.201	49	4	73, 70, uk*, 73
PRS2036.201	65	4	69, 63, 56, 74
PR3658.201	43	3	87, 37, 72
PRS2015.205	65	3	70, 65, 67
PRS2018.201	67	3	69, 70, 67
PRS2051.201	72	3	60, 75, 77
PR3106.201	67	2	74, 81
PR3382.201	71	2	87, 62
PRS2016.201	64	2	73, 60
PRS2024.201	56	2	38, 87
PRS2025.202	71	2	75, 65
PRS2031.202	59	2	67, 80
PRS2039.201	66	2	uk*, uk*
PRS2045.201	71	2	86, 67
PRS2053.201	71	2	72, 77
PRS2059.201	76	2	71, 81
PRY1061.201	49	2	58, 61
PR3173.201	63	1	64
PR3222.201	58	1	82
PR3378.201	59	1	71
PR3498.201	61	1	64
PR3569.201	54	1	72
PRS2001.201	62	1	64
PRS2003.201	62	1	64
PRS2005.202	63	1	66
PRS2010.201	60	1	63
PRS2012.201	62	1	62
PRS2017.202	60	1	66
PRS2047.201	64	1	64
PRS2052.201	57	1	66
PRS2058.201	67	1	72
PRY1010.201	49	1	66
PRY1026.201	52	1	65
PRY1042.201	54	1	48
PRY1052.201	54	1	78
PRY1056.201	53	1	uk*
PRY1064.201	49	1	69
PRY1081.201	46	1	58

*Age unknown.

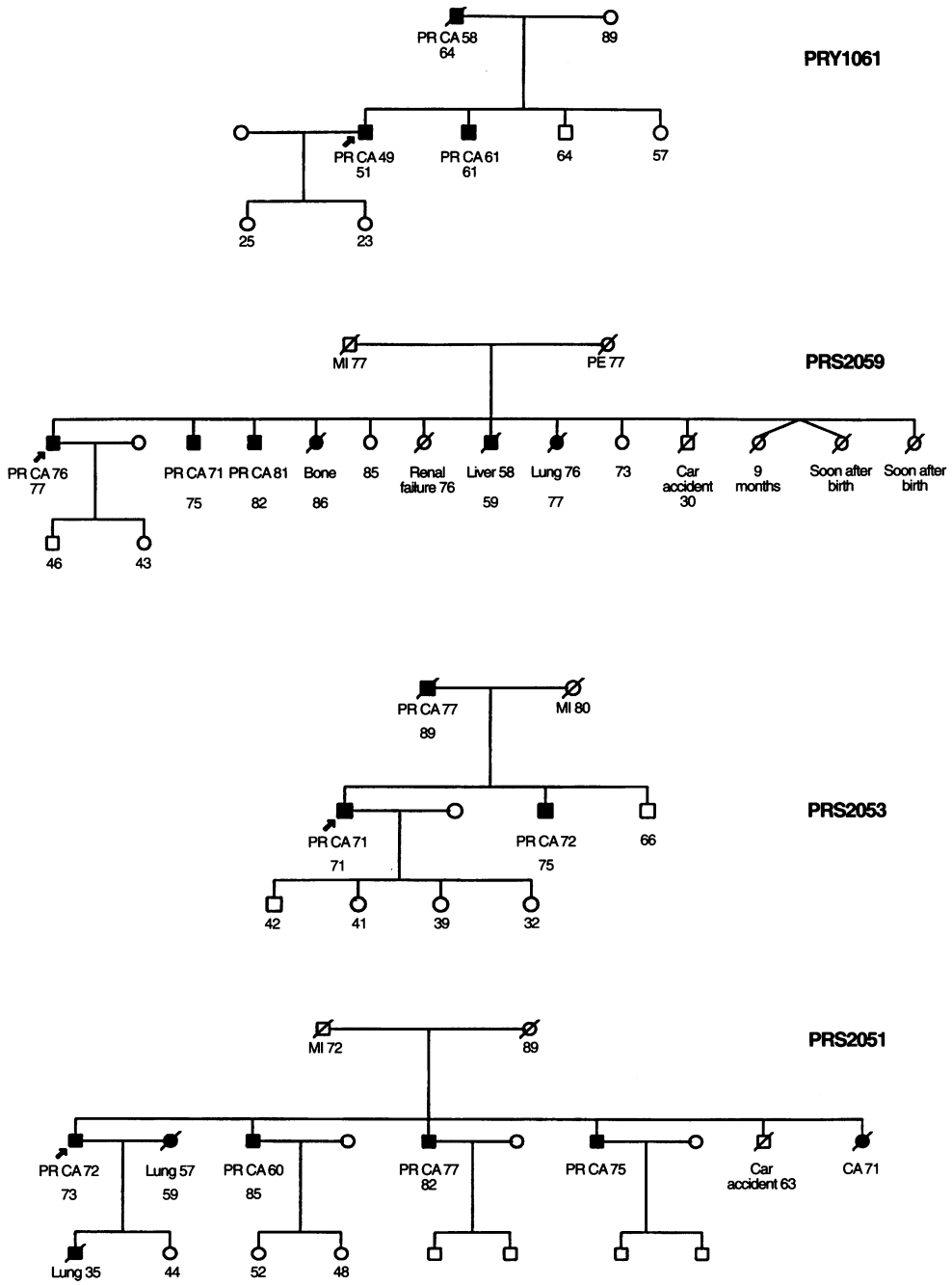

Figure 1 CRC/BPG UK Familial Prostate Cancer Study. Prostate family pedigrees with three or more cases of prostate cancer in this study. Ages shown are age at diagnosis and current age/age at death. PRCA, prostate cancer; MI, myocardial infarction; PE, pulmonary embolus; SCC, squamous cell carcinoma. Arrowed case: individual whose DNA was sequenced

PCR products were purified before dye terminator cycle sequencing according to Hamoudi et al (1996). The DNA was dissolved in 12–15 µl of water and 3–4 µl was run on a 2% agarose gel to check for the presence and purity of the product.

Cycle sequencing

PCR products were purified as above and sequenced directly using an ABI prism dye terminator cycle sequencing ready reaction kit (Perkin Elmer), as recommended in the instructions, with thermocycling as follows: 25 cycles of 96°C for 30 s, 50°C for 15 s and 60°C for 4 min. After thermocycling, the extension products were removed from beneath the oil and added to 2 µl of 3 M sodium acetate, pH 5.2, precipitated with 50 µl of absolute ethanol and centrifuged. The pellet was washed with 70% ethanol, dried, then stored at –20°C before automated sequencing.

Automated sequencing

The HLH and ZIP exons of the *MXI1* gene were sequenced both in the forward and reverse directions using the appropriate primer shown in Table 1. Sequencing was conducted on a 6%

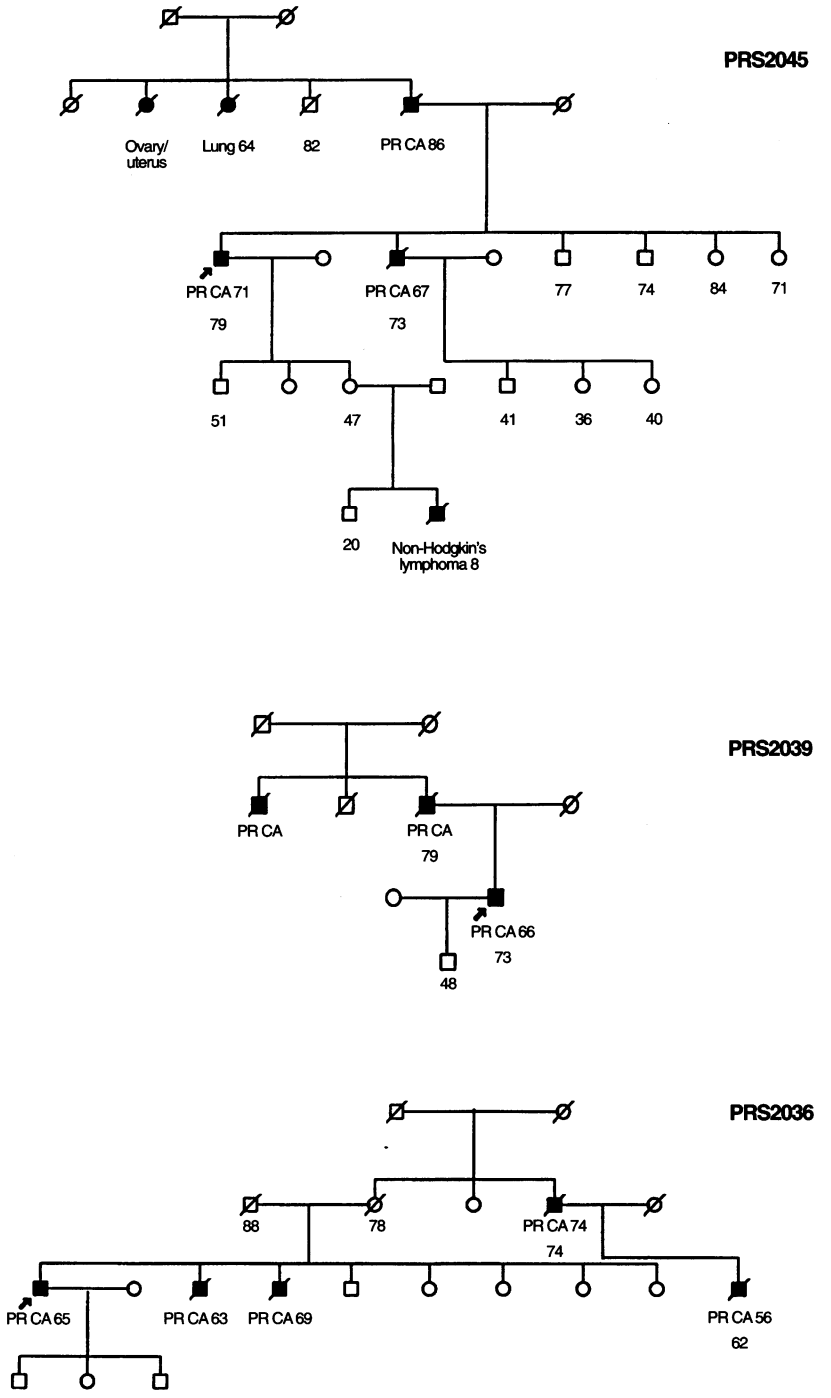

Figure 1 continued

polyacrylamide denaturing gel (Biorad) in a 1 × TBE (Tris borate buffer) using an ABI 373A automated fluorescent DNA sequencer. The DNA pellet was dissolved in 4 µl of formamide. This mix was denatured for 2 min at 92°C and loaded into each well. The gel was run for 10 h at 30 W, 40 mA and 2500 V. During electrophoresis, the fluorescence was detected in the laser scanner region using filter set A and data were collected and stored using the DNA Sequencing Analysis Software (v.1.2; ABI, CA, USA). On completion of the gel run, the data were analysed further using Factura and Sequence Navigator software (ABI).

PATIENTS, RESULTS AND DISCUSSION

Patients

Individuals with prostate cancer were identified by their urologist and referred to the CRC/BPG UK Familial Prostate Cancer Study.

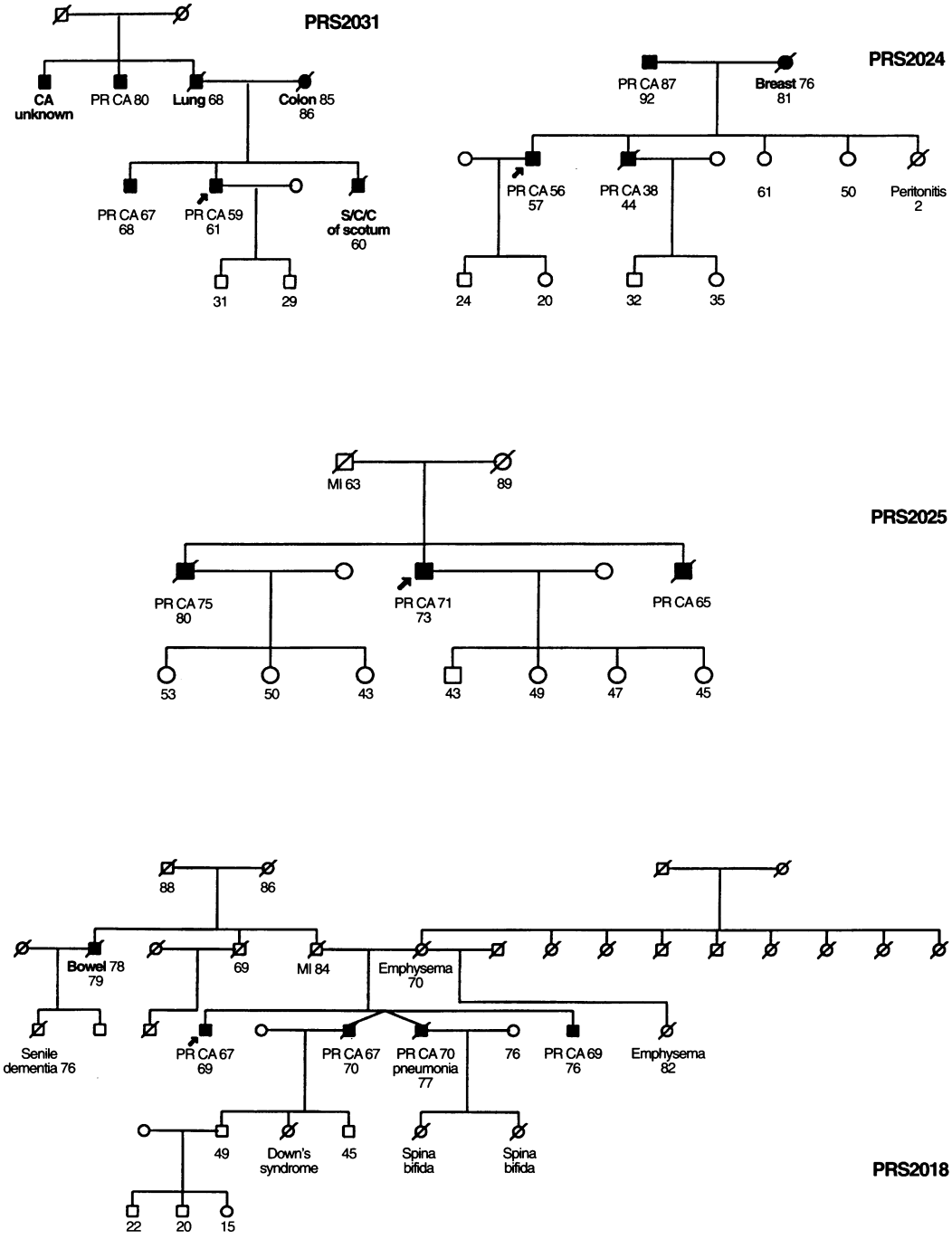

Figure 1 continued

Of these, 97% had disease that presented clinically; one presented as a result of PSA screening. Diagnoses were confirmed by pathology report or death certificate. A total of 38 patients with prostate cancer were studied; each patient had at least one other relative diagnosed with prostate cancer. The family member who was chosen for investigation was the youngest at diagnosis for whom DNA was available. The clusters were as follows: two families had five cases, four had four, 11 had three and 21 had two cases of prostate cancer. Those with two cases in all but one cluster had one affected at less than 65 years. Table 2 shows the details of each patient studied. Where there are more than two individuals with prostate cancer in a family, their family tree is shown in Figure 1.

The relative risk of prostate cancer to first-degree relatives of prostate cancer patients diagnosed below age 65 years is

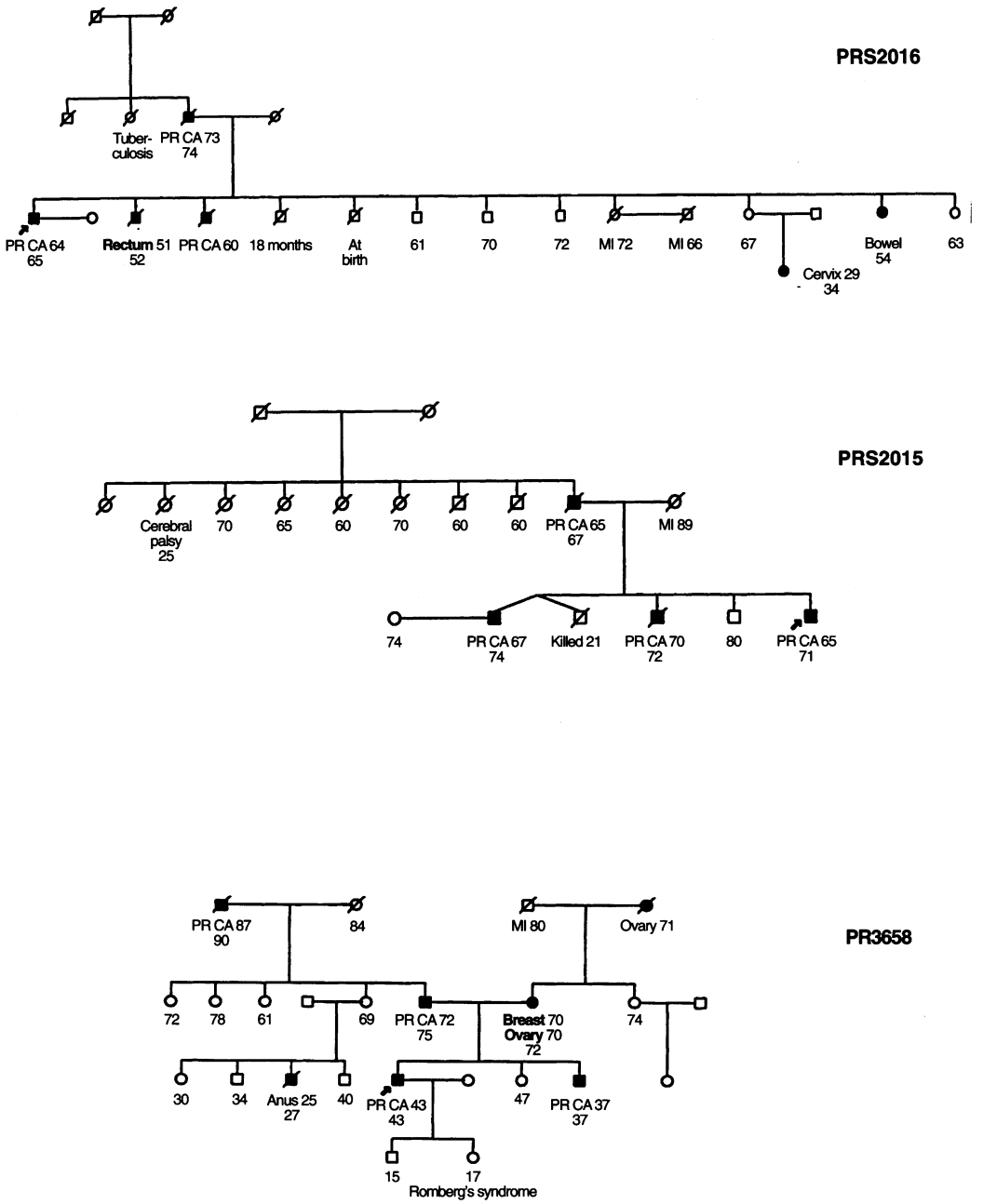

Figure 1 continued

approximately fourfold, i.e. the number of cases with an affected relative is four times the number that would be expected by chance. Thus, of the cases diagnosed below age 65 years with an affected relative, one-quarter of these relative pairs occur by chance (or, in general, 1/relative risk). Thus, among the 21 related pairs in which one case is diagnosed below age 65 years, approximately 25% will have occurred by chance and 75% will result from genetic or other familial factors. The 17 families with three or more cases of prostate cancer are less likely to have occurred by chance; therefore, at least 75% of cases must be caused by either genetic susceptibility or shared environmental risk factors.

The ZIP and HLH exons of *MXI1* were sequenced in both directions in all 38 samples No mutations were found in either region and the mutations reported by Eagle et al (1995), which included one intronic mutation, were not observed. On the basis of these observations, the upper 95% confidence limit for the proportion of families with *MXI1* mutations in ZIP and HLH would be 7.6% and therefore, assuming that familial prostate cancer is mediated by a single dominant gene as predicted by the Carter model, *MXI1* muta-

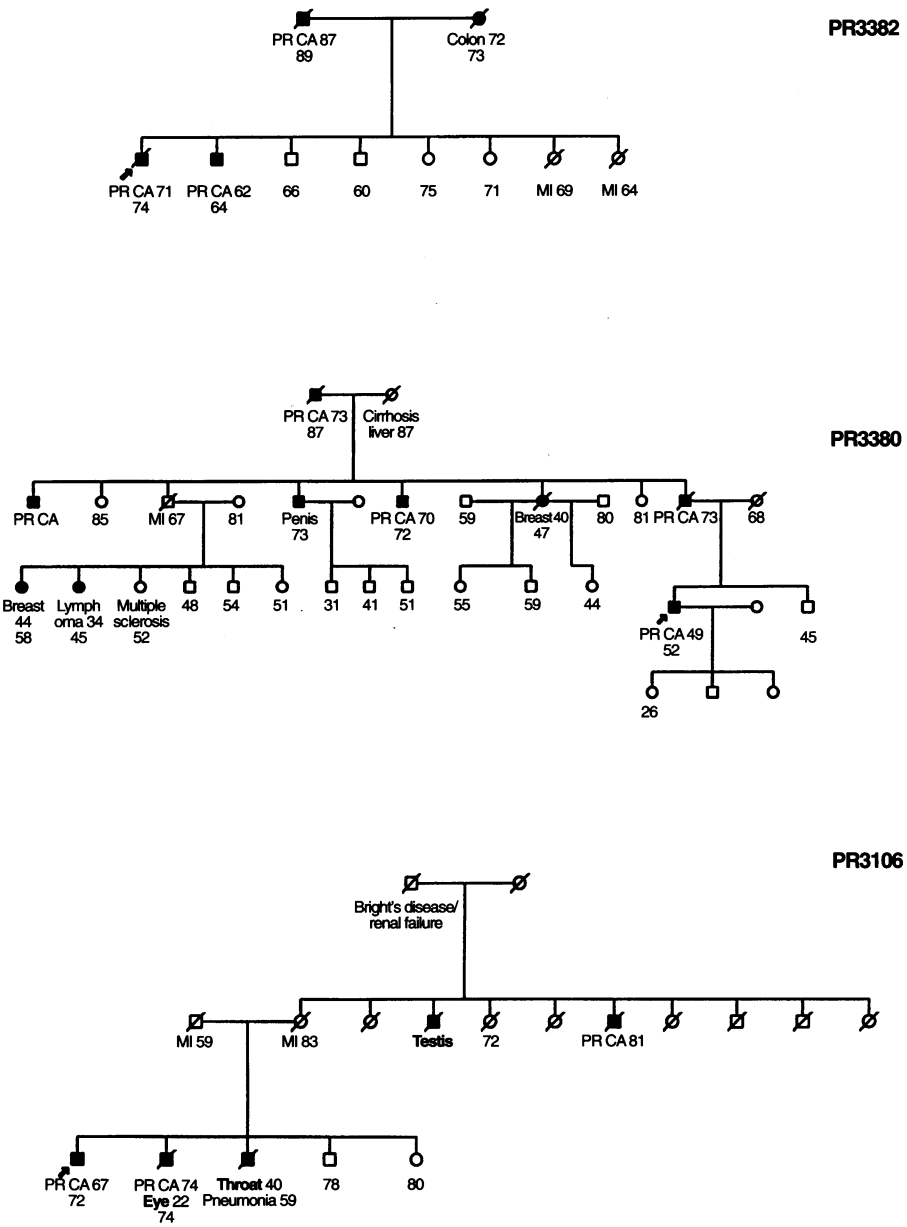

Figure 1 continued

tion in these regions could be responsible for at most 10% of high-risk families. These are the only regions that have been reported to be mutated in sporadic tumours. It is therefore very unlikely that *MXI1* is a prostate cancer susceptibility locus, *PRCA1*.

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The PCR machine used was donated by the Prostate Research Campaign, UK.

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APPENDIX: COLLABORATORS AS AT 14 OCTOBER 1996

Mr J Anderson	Royal Hallamshire Hospital, Sheffield
Mr J Archibold	Downe Hospital, Co Downe
Mr M Bailey	Epsom District Hospital, Surrey
Mr C Barker	Wharfedale Hospital, Otley
Mr J Bellringer	West Middlesex Hospital, Middlesex
Mr M Bishop	Nottingham City Hospital, Nottingham
Dr J Bolger	Weston Park Hospital, Sheffield
Mr J Boyd	St Helier Hospital, Carshalton
Mr D Budd	Horton Hospital, Oxford
Mr M Butler	Meath Hospital, Dublin
Mr R Brookstein	Queen Elizabeth Military Hospital
Mr C Charig	Epsom Health Care Trust, Epsom
Prof GD Chisholm	Western General Hospital, Edinburgh*
Mr I Conn	Aberdeen Royal Hospital, Aberdeen
Mr C Cranston	Churchill Hospital, Oxford
Mr M Crundwell	Queen Elizabeth Hospital, Birmingham
Mr G Das	Mayday University Hospital, Surrey
Mr A Doble	Addenbrooke's Hospital, Cambridge
Prof W Duncan	Western General Hospital, Edinburgh
Dr J Duchesne	Middlesex Hospital, London
Dr D Eccles	Southampton General Hospital, Southampton
Mr D Fawcett	Royal Berkshire Hospital, Reading
Dr C Fisher	Royal Marsden NHS Trust, London
Mr M Fletcher	St Luke's Hospital, Guildford
Mr JW Fowler	Western General Hospital, Edinburgh
Mr C Gallegos	Royal United Bath Hospital, Bath
Mr A Ghaznavi	St Helier Hospital, Surrey
Dr J Glaholm	Queen Elizabeth Hospital, Birmingham
Ms E Gordon	St George's Hospital, London
Mr S Hampson	St George's Hospital, London
Mr DC Hanbury	Lister Hospital, North Herts NHS Trust Hospital
Mr T Hargreave	Western General Hospital, Edinburgh
Dr S Harland	Middlesex Hospital, London
Mr GS Harrison	Royal Hampshire County Hospital, Hampshire
Mr NW Harrison	Brighton General Hospital, Sussex
Mr JL Hart	Llwynpia Hospital, Tonypandy, Wales
Mr M Hehir	Stirling Royal Infirmary, Stirling
Mr W Hendry	Royal Marsden NHS Trust, London and St Bartholomew's Hospital, London
Mr A Higgins	Hinchingbrook Hospital, Huntington, Cambs
Dr J Hopper	University of Melbourne, Australia
Mr M Hughes	Queen Elizabeth Hospital, Birmingham
Dr N James	Queen Elizabeth Hospital, Birmingham
Cdr IL Jenkins	Royal Naval Hospital, Haslar
Mr C Jones	St Helier Hospital, Surrey
Mr A Kaisary	Royal Free Hospital, London
Mr R Kirby	St George's Hospital, London
Mr D Kirk	Western Infirmary, Edinburgh
Mr J Lee	Noble's Isle of Man Hospital
Mr R Lemberger	Kings Mill Hospital, Nottinghamshire
Mr S Lloyd	Stirling Royal Infirmary, Stirling
Mr M Lynch	Kettering General Hospital, Kettering
Dr J Mansi	St George's Hospital, London
Prof M Mason	Velindre NHS Trust, Cardiff
Dr AB McEwan	Royal Infirmary, Blackburn
Mr TA McNicholas	Lister Hospital, North Herts NHS Trust
Mr LEF Moffat	Aberdeen Royal Infirmary, Aberdeen
Mr RJ Morgan	Royal Free Hospital, London
Mr G Muir	Royal Surrey Hospital, Surrey
Mr KW Munson	Derbyshire Royal Infirmary, Derby
Mr K Murray	Kent and Canterbury Hospital, Kent
Dr H Newman	Bristol Royal Infirmary, Bristol
Dr V Murday	St George's Hospital, London
Mr PJ O'Boyle	Taunton and Somerset Hospital, Somerset
Mr E O'Donoghue	Middlesex Hospital, London
Mr RG Notley	Royal Surrey County Hospital, Surrey
Mr A Pengelly	Battle Hospital, Oxford
Mr T Philp	Whipps Cross Hospital, London
Mr R Plail	Conquest Hospital, East Sussex
Mr C Powell	Leighton Hospital, Crewe
Dr J Russell	Beatson Oncology Centre, Glasgow

*Now deceased

Dr G Read	Christie Hospital, Manchester
Mr PJ Reddy	Somerfield Hospital, Maidstone
Mr W Richmond	Royal Albert Edward Infirmary, Wigan
Mr T Roberts	Newcastle General Hospital, Newcastle
Dr K Rowley	Velindre NHS Trust, Cardiff
Dr AD Rouse	Wordsley Hospital, Birmingham
Mr P Ryan	Nuffield Hospital, Birmingham
Dr L Senanayake	Royal Free Hospital, London
Mr D Sandhu	Leicester General Hospital, Leicester
Mr P Shridhar	King George Hospital, Goodmayes
Mr R Shweitzer	Royal Surrey County Hospital, Surrey
Mr R Shearer	Royal Marsden NHS Trust, London
Mr J Smith	Mater Hospital, Dublin
Mr P Smith	St James' University Hospital, Leeds
Dr A Stockdale	Solihull Hospital, West Midlands
Mr M Stower	York District Hospital, York
Mr P Thomas	Brighton General Hospital, Sussex
Mr T Terry	Leicester General Hospital, Leicester
Mr A Thurston	Doncaster Royal Infirmary, Doncaster
Cdr D Tullock	Royal Naval Hospital, Hassar
Dr G Turner	St James' University Hospital, Leeds
Mr M Wallace	Queen Elizabeth Hospital, Birmingham
Mr P Weston	Pinderfields General Hospital, Yorks
Mr P Whelan	St James' Hospital, Leeds
Dr D Whillis	Raigmore Hospital, Inverness
Mr R Wilson	Furness General Hospital, Cumbria
Mr G Williams	Hammersmith Hospital, London
Mr C Woodhouse	Royal Marsden NHS Trust, London