Loss of the retinoblastoma susceptibility gene (RB1) is a frequent and early event in prostatic tumorigenesis

S.M.A. Phillips¹, C.M. Barton², S.J. Lee³, D.G. Morton⁴, D.M.A. Wallace⁵, N.R. Lemoine² & J.P. Neoptolemos⁶

¹Department of Urology, Queen Elizabeth Hospital, Edgbaston, Birmingham B15 2TH; ²ICRF Oncology Unit, RPMS Hammersmith Hospital, London W12 0NN; ³Department of Pathology, The Medical School, University of Birmingham B15 2TT; ⁴Academic Surgical Unit, Dudley Road Hospital, Birmingham B18 7QH, UK.

Summary Loss of the RB1 gene is an important event in the initiation and progression of many tumours. Prostatic tumours from 43 patients with prostate cancers and ten with benign prostatic hypertrophy (BPH) were studied for loss of heterozygosity of the RB1 gene. Four intragenic polymorphic loci were studied with two techniques. These were restriction fragment length polymorphism (RFLP), Southern blotting and hybridisation with the p123ml.8 and p68RS2.0 probes (to introns 1 and 17 respectively) and also the polymerase chain reaction (PCR) to amplify loci within introns 17 and 20. Protein product (pRB) expression was determined by immunohistochemistry using the NCL-RB antibody in nine patients with cancer and four patients with BPH. Loss of heterozygosity was found in 24 out of 40 (60%) informative patients with cancer. Loss of RB1 occurred with a similar frequency in early-stage and low-grade cancers as in more advanced cancers. Loss of RB1 was also found in one patient with BPH. Expression of pRB was completely absent from seven cancers and markedly reduced in the other two, while nuclear pRB staining was always present in areas of BPH, whether alongside cancer-containing tissue or with BPH alone. We conclude that loss of RB1 is an early event in prostatic tumorigenesis.

Correspondence: S.M.A. Phillips.
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LOH by restriction fragment length polymorphism (RFLP) analysis

Restriction enzyme digest (RsaI and BamHI, Boehringer Mannheim) of 10 µg samples of DNA was followed by Southern blotting and hybridisation of the resulting filters (Hybond N, Amersham UK) with radiolabelled probes. Twenty-five ng of probe was labelled with [α-32P]dCTP by the random primer technique (Feinberg & Vogelstein, 1984). Filters were hybridised overnight at 65°C in 0.23 M disodium hydrogen phosphate in 7% SDS. Somatic human placental DNA was added as a blocking agent. They were then washed with solutions of increasing stringency and exposed to autoradiographic film. Two intragenic probes were used. Probe p68RS2.0 reveals a RsaI polymorphism (eight alleles between 1.3 and 2.0 kb long) within a variable number tandem repeat (VNTR) sequence in intron 17 of the RBl gene (Wiggs et al., 1988). Probe p123m1.8 reveals a BamHI polymorphism (two alleles, 4.5 and 2.2/2.3 kb long) to the 5' end of the RBl gene within intron 1 (Greger et al., 1989).

LOH using polymerase chain reaction (PCR)

Primers to RBl introns 17 (Greenwald et al., 1992) and 20 (Onadim et al., 1992) were used in PCR reactions to amplify across polymorphic regions. PCR to intron 17 reveals a Xbal polymorphism (two alleles, 190 and 123/55/12 bp long). PCR to intron 20 reveals a VNTR (26 alleles, from 300 to 350 bp long). Conditions were optimised for each set of primers used (Table I). By mixing DNA from homozygotes in different parents, loss of heterozygosity with varying degrees of 'non-stringency' could be simulated, as described by McDaniel et al. (1991). Using this model we were able to check in preliminary studies that the PCR conditions used could detect LOH, even with significant benign tissue contamination.

A 100 ng sample of DNA was amplified, typical conditions being: reaction volume, 25 µl; Taq polymerase, 2 units (HT Technologies); primers, 60 ng each; and total NTPs, 0.4 mM. For intron 17, 23 cycles of amplification were used; for intron 20, 35 cycles. All PCR reactions were carried out in the presence of [α-32P]dCTP, which was incorporated into the PCR products as described by Onadim et al. (1992). This technique allowed greater incorporation of radioactivity into the PCR products, enabling quicker visualisation of results. This method of labelling was also preferable to primer end labelling, as PCR followed by restriction enzyme digestion would have given labelled restriction enzyme fragments of such disparate size that visual and densitometric analysis of results would have been difficult.

For intron 20, the PCR products were loaded directly into the wells. For intron 1 a 5 µl aliquot was digested overnight at 37°C with 20 units of XbaI and the resulting digest fragments loaded. Fragments were separated by electrophoresis in 6% non-denaturing polyacrylamide gels incorporating 10% glycerol (similar to those used for single-strand conformation polymorphism analysis). Gels were run at low power so that no heating occurred and the DNA strands did not melt. Table II patients showing loss of heterozygosity for each locus examined

| Patient no. | Intron 1 (p123m1.8) | Intron 17 (PCR) | Intron 17 (p68RS2.0) | Intron 20 (PCR) |
|-------------|---------------------|-----------------|---------------------|-----------------|
| BENIGN (BPH) |                     |                 |                     |                 |
| A           | –                   | –               | –                   | –               |
| B           | –                   | –               | –                   | –               |
| C           | np                  | inf             | inf                 | inf             |
| D           | np                  | inf             | inf                 | inf             |
| E           | DEL                 | DEL             | inf                 | ns              |
| F           | inf                 | np              | inf                 | ns              |
| G           | inf                 | np              | inf                 | inf             |
| H           | –                   | np              | inf                 | inf             |
| I           | inf                 | np              | inf                 | inf             |
| J           | –                   | np              | inf                 | ns              |
| TUMOURS     |                     |                 |                     |                 |
| T1M0        |                     |                 |                     |                 |
| 1           | –                   | –               | –                   | DEL             |
| T2M0        |                     |                 |                     |                 |
| 2           | –                   | inf             | –                   | ns              |
| 3           | –                   | inf             | –                   | inf             |
| 4           | inf                 | np              | inf                 | inf             |
| 5           | –                   | np              | –                   | DEL             |
| 6           | –                   | –               | –                   | DEL             |
| 7           | –                   | np              | –                   | DEL             |
| T3M0        |                     |                 |                     |                 |
| 13          | –                   | inf             | –                   | REAR            |
| 14          | –                   | –               | –                   | –               |
| 15          | –                   | –               | –                   | –               |
| 16          | –                   | –               | –                   | –               |
| 17          | –                   | –               | –                   | –               |
| 18          | –                   | –               | –                   | –               |
| T3M1        |                     |                 |                     |                 |
| 19          | DEL                 | np              | –                   | DEL             |
| 20          | inf                 | np              | inf                 | inf             |
| 21          | inf                 | np              | inf                 | inf             |
| 22          | inf                 | np              | DEL                 | ns              |
| 23          | –                   | np              | inf                 | inf             |
| 24          | –                   | DEL             | –                   | ns              |
| 25          | –                   | –               | –                   | DEL             |
| 26          | –                   | –               | –                   | DEL             |
| 27          | –                   | –               | –                   | –               |
| 28          | –                   | –               | –                   | inf             |
| 29          | inf                 | np              | inf                 | inf             |
| 30          | –                   | np              | –                   | DEL             |
| T4M0        |                     |                 |                     |                 |
| 31          | –                   | –               | –                   | inf             |
| 32          | –                   | –               | –                   | –               |
| 33          | –                   | –               | –                   | –               |
| 34          | inf                 | DEL             | DEL                 | ns              |
| 35          | inf                 | np              | inf                 | inf             |
| T4M1        |                     |                 |                     |                 |
| 36          | DEL                 | DEL             | DEL                 | ns              |
| 37          | inf                 | np              | inf                 | inf             |
| 38          | –                   | np              | inf                 | inf             |
| 39          | inf                 | np              | DEL                 | inf             |
| 40          | DEL                 | DEL             | DEL                 | ns              |
| 41          | –                   | –               | –                   | DEL             |
| 42          | –                   | –               | –                   | DEL             |
| 43          | –                   | –               | –                   | inf             |

Table I Details of polymerase chain reaction

| RB intron 17 | Primer sequences 3' | S' | Cycles |
|--------------|---------------------|---|---------|
| 5'           | CTGCAGTCCCCACCCTGAGCTCTAGTAGA | 3' | 'Hot start', reaction heated to 95°C for 10 min before DNA added. Then: 1 x 95°C for 10 min, 62°C for 1 min, 72°C for 1 min |
|              | GGATCCGCAGCTCTAGACTAATCCCAGCAC |   | 23 x 95°C for 1 min, 62°C for 1 min, 72°C for 1 min |
|              |                     |   | 1 x 95°C for 10 min, 62°C for 1 min, 72°C for 1 min |
|              |                     |   | 23 x 95°C for 1 min, 62°C for 1 min, 72°C for 1 min |

| RB intron 20 | Primer sequences 3' | S' | Cycles |
|--------------|---------------------|---|---------|
| 5'           | GTTAGAAGCTCATGAGCAAGCCGAT | 3' | 1 x 95°C for 15 min, 30 x 95°C for 20 s, 59°C for 20 s, 72°C for 60 s |
|              | AATTTACAAAGGTTGGTGTTACAGC |   |        |

| Polymorphism | Allele sizes | Cycles |
|--------------|--------------|--------|
| Xbal RFLP    | Two alleles, 190 and 123/55/12 base pairs | |
| VNTR         | 26 alleles, 300–350 base pairs long. | |
|              | 1 x 95°C for 15 min, 30 x 95°C for 20 s, 59°C for 20 s, 72°C for 60 s | |

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DEL, deletion; inf, informative, no LOH; –, non-informative; ns, PCR amplification to RB intron 17 was not performed; ns, PCR reaction was not successful in amplifying both tumour and normal pair for RB intron 20; REAR, rearrangement with novel allele.
not separate. After electrophoresis the gels were dried and applied to autoradiographic film. Results were assessed visually and then checked using densitometry.

All tumour and BPH samples were hybridised with probes p123m1.8 and p68RS2.0 and amplified using PCR to intron 20. Patients who were non-informative to all of these three loci, were informative but showed no loss of heterozygosity, or were cases of special interest were additionally examined using PCR to intron 17. Cases showing deletions had the RFLP and/or PCR reactions repeated to confirm the result. Particular care was taken to confirm results in those patients in whom just one locus showed a deletion. All cases of LOH were confirmed using densitometry.

**Immunohistochemistry**

Nine patients with cancer and four with BPH were selected at random, and paraffin sections cut at 3 μm from their original histology blocks. The streptavidin–biotin complex method was used to demonstrate Rb protein. Sections were mounted on Vectabond (Vector laboratories) treated glass slides, dewaxed in xylene, then rehydrated through graded alcohols to water. The sections were then placed in a citrate buffer bath (pH 6) and microwaved (750 W) for 30 min (Norton, 1993). They were then rinsed with water, bathed in 1% hydrogen peroxide in methanol, rinsed in water and incubated with the mouse monoclonal antibody NCL-RB (NovoCastra Laboratories), diluted to optimum with 0.01 M PBS. After 1 hour the sections were washed in 0.01 M PBS and the primary antiserum labelled with streptavidin–biotin complex–horseradish peroxidase (Duet Kit; K492, Dako). The horseradish peroxidase was visualised with 3,3' diaminobenzidine tetrahydrochloride as a substrate and the nuclei counterstained for interpretation (Cattoretti et al., 1992). Immunohistochemical staining showed pRB to be within the cell nucleus. In common with another immunohistochemical study of pRB using a different antibody (RB1-Ab20; Varley et al., 1989), epithelial cells (tumour and benign), monocytes and endothelial cells may stain positively, but stromal cells do not. In those cells showing loss of nuclear pRB, some cytoplasmic staining was apparent. Benign epithelial cells staining positively within the nucleus sometimes also showed punctate staining within the cytoplasm. The sections stained for pRB by immunohistochemistry were assessed by the intensity of cell nuclear and cytoplasmic pRB staining and the percentage of cells showing loss of staining in these compartments for tumour, BPH, stroma and other benign tissue. Staining intensity was recorded as weak (+), moderate (+ +) and strong (+ + +). The percentage of cells with pRB nuclear or cytoplasmic staining was estimated as follows: loss in 95–100% (0), loss in 75–94% (1), loss in 50–74% (2) and loss in 0–49% (3).

**Results**

**Patients and tumour stage and grade**

The age range of the 43 patients with prostate cancer was 55–88 (mean 72.8) years and of the ten with BPH was 61 to 80 (mean 71.9) years. The stages of the tumours were T1M0 (n = 1), T2M0 (n = 6), T2M1 (n = 5), T3M0 (n = 6), T3M1 (n = 12), T4M0 (n = 5 and T4M1 (n = 8). The tumours were graded as well (n = 5), moderately (n = 14) and poorly differentiated (n = 24).

**Loss of heterozygosity in cancer tissue**

Samples from 40 of 43 cancer patients were informative to one or more DNA probes/PCR sequences (Table II). Overall 24 (60%) out of 40 tumours showed loss of heterozygosity at one or more loci examined. Of these 24 patients, 4 (27%) out of 15 informative cases showed loss with probe p123m1.8, 11 (48%) out of 23 informative cases showed loss with probe p68RS2.0 and 13 (54%) out of 24 informative cases showed loss with PCR to RB1 intron 20 (Figure 1).

Twelve out of 24 cancers showing LOH exhibited a deletion at the only intron at which the case was informative.

**Figure 1** Examples of loss of heterozygosity. A. LOH in a prostate cancer using probe p123m1.8. B. LOH in cases of prostate cancer using probe p68RS2.0. C. LOH in cases of prostate cancer by PCR of intron 20. D. LOH in patient E with BPH only and also a case of prostate cancer by PCR of intron 17. N, normal DNA; T, tumour DNA; BPH, benign prostatic hypertrophy; bp, base pairs.
Seven cases showing LOH had losses at both of the two informative introns for that patient. The remaining five patients showed interstitial loss with preservation of part of the gene. Loss of the 5' region with preservation of the 3' end was shown by one tumour (number 10). Loss of the 3' region with preservation of the 5' end was found in three tumours. Loss of intron 17 with preservation of the flanking 5' and 3' ends was seen in one case (number 39). Examination of gel loading and comparison with the allele strength in informative cases gave no indication of any chromosomal duplications. One tumour (number 13) showed a rearrangement with novel allele formation on PCR to intron 20 (Figure 2). This PCR reaction was repeated with consistent results. All of the tumour/blood DNA samples have been examined at 22 different chromosomal loci (unpublished data), mostly VNTR polymorphisms. None of them, including tumour number 13, showed any disparity between the allele size displayed by tumour or blood DNA at these other loci, indicating that there had been no error in sample labelling during paired normal and tumour DNA extraction.

Prostate cancer is a diffusely infiltrating tumour, and the densitometry readings were used to assess the degree of background benign tissue contamination. Of those tumours showing LOH, 15 had a 70% or greater reduction in the signal strength of the deleted allele, six showed a 50–69% reduction and three showed a 30–40% reduction in the deleted allele. The last three cancers had a high percentage of benign tissue contamination despite tumour microdissection.

Loss of heterozygosity in BPH tissue

Of the nine informative benign (BPH) prostates, one showed LOH to intron 1 (BamHI polymorphism) using probe p123m1.8 and intron 17 (XhoI polymorphism) as revealed by PCR (Figure 1d). This tissue showed retention of the 3' locus within intron 17 as revealed by probe p6R52.0. Intron 17 is large and the XhoI and VNTR polymorphism are 20 kb apart (Wiggs et al., 1988). Densitometry showed a greater than 60% reduction in the size of the deleted allele from the BPH tissue when compared with its counterpart derived from blood.

Examination of the two loci showing loss in this patient was repeated five times with consistent results. All of the tissue resected from this 79-year-old patient (E) showed benign hyperplasia with both epithelial and stromal elements. There was no evidence of malignancy on histological examination, including the frozen section material used for DNA extraction. Despite the benign histology, this patient had a nodular prostate on rectal examination. A bone scan was performed, which was normal, and the prostate-specific antigen (PSA) was marginally elevated (20 ng/ml). The nodularity of the prostate and the elevated PSA strongly suggest an occult prostatic cancer. After reviewing the overall clinical picture in this patient, further biopsy of the prostate gland was not considered to be ethical, but he remains under careful clinical follow-up.

Loss of heterozygosity vs stage and grade

RBI loss was similar between different stages and grades of tumour. LOH within tumours confined to the prostate (T1 and T2) was 60% (6/10); loss within those with extracapsular spread of tumour (T3 and T4) was also 60% (18/30). The M0 tumours showed LOH in 56% (9/16) cases; those with bone metastases (M1) showed loss in 62% (15/24) cases. Well-differentiated tumours showed loss in 80% (4/5) cases, moderately differentiated tumours showed loss in 71% (10/14) of cases and the poorly differentiated tumours showed LOH in 48% (10/21) of cases. There was no correlation between loss of RBI and the grade or stage of tumour.

Immunohistochemistry

Of the nine tumour patients examined, four had shown a deletion of RBI, four were informative without LOH and one was non informative to all introns examined. Seven showed complete loss of pRB and two showed marked reduction of tumour nuclear staining (Table III). Seven of the tumours also contained areas of BPH within the sections examined, and a higher percentage and a greater intensity of nuclear staining was seen in the BPH cells than in adjacent tumour cell nuclei (Figure 3). All four cases with BPH alone showed greater staining than the tumours. There was no relationship between the proportion of nuclei showing loss of pRB staining and the reduction of the allele signal (as assessed by densitometry) in those showing LOH. This may have been because the material used for immunohistochemistry was from different prostatic curettings to those used for microdissection and DNA extraction.

Discussion

Loss of heterozygosity within the RBI gene was identified in 60% of prostate cancers, which is higher than has previously been reported (Carter et al., 1990; Macoska et al., 1992;

![Table III Immunohistochemistry results indicating percentage of cells showing loss of pRB staining](image)

| Patient no. | Tumour nucleus | Tumour cytoplasm | BPH nucleus | BPH cytoplasm | Stroma |
|-------------|----------------|------------------|-------------|---------------|-------|
| 8           | 1++           | 3+++            | 3++         | 1+            | 0     |
| 9           | 0             | 3+++            | 3++         | 1+            | 0     |
| 18          | 0             | 0               | 1++         | 1+            | 0     |
| 21          | 1+++          | 2+              | NA          | NA            | 1++   |
| 22          | 0             | 3++             | NA          | NA            | 0     |
| 31          | 0             | 3++             | 2++         | 1+            | 0     |
| 33          | 0             | 0               | 1++         | 0             | 0     |
| 35          | 0             | 0               | 1+++        | 0             | 0     |
| 42          | 0             | 2+              | 2+++        | 3+b           | 0     |

BPH

A: NA, NA: 3+++ 1++ 1+a 0
C: NA, NA: 2+++ 2+ 0
I: NA, NA: 2+++ 2++ 1++ b
J: NA, NA: 3+++ 1+ 0

*Endothelial, inflammatory and transitional epithelium cells with strong nuclear pRB staining. Percentage of cells showing loss of staining: 0, 95–100%; 1, 75–94%; 2, 50–74%; 3, 0–49%. Intensity of staining: +, weak; ++, moderate; ++++, strong. NA, not applicable; BPH, benign prostatic hyperplasia.
Figure 3 Demonstration of NCL-RB immunohistochemistry, showing an area of benign glands with retention of nuclear pRB (top) and an area of prostate tumour that has lost nuclear pRB expressions (bottom).

Sarkar et al. (1992; Brooks et al., 1993) and is similar to that for retinoblastoma tumours (Zhu et al., 1992). Point mutations, not usually identified by RFLP studies, have been identified as the somatic mutation in retinoblastomas (Dunn et al., 1988). In non-hereditary retinoblastoma, after excluding those with gross gene alterations by Southern blotting, point mutations of RB1 were found in all seven tumours examined (Yandell et al., 1989).

Carter et al. (1990) reported losses of RB1 in 3 out of 13 prostate cancers when combining the results of the intragenic probe p68RS2.0 and a more distant probe to 13q. Results were not given independently for the intragenic and chromosomal arm probes. Brooks et al. (1993) showed 27% loss of RB1 in prostate cancer when examining RB1 intron 20 alone.

Sarkar et al. (1992) looked for deletions within the DNA of the RB1 promoter region and within exon 21 mRNA. They found an abnormal short-sized mRNA transcript of RB1 exon 21 extracted from a pure population of cells in tissue culture (cell line DU 145), but not in their seven cancer cases. No deletions within the promoter region were detected. The techniques used, however, would not have detected deletions lying outside the two loci studied or deletions of the whole of the promoter or exon 21 loci. If the whole of these loci had been lost in the cases they studied, DNA or mRNA from benign tissue contamination would have been amplified to give a normal-sized band. Macoska et al. (1992) used only a single chromosome 13q marker and accepted a high degree of benign tissue contamination of tumour DNA. They found no losses in 19 informative cases, possibly because of benign DNA contamination and the fact that the probe used would not detect the small intragenic deletions that we and other workers have found within RB1.

The RB1 gene consists of 27 exons spread over 200 kb of genomic DNA (Hong et al., 1989), which poses problems for the detection of small deletions. The cancer samples in this study were informative at one or more loci studied in 93% (40/43) of cases. Combining the results of all four loci gave a higher rate of allelic loss than the use of any single locus alone. There were 12 cases in which the only informative locus showed the allele loss. Small interstitial deletions of the RB1 gene have been widely reported in a number of different tumours, including bone and soft-tissue sarcomas (Wunder et al., 1991), breast cancer (T'Ang et al., 1988) and bladder cancer (Cairns et al., 1991).

In seven of the nine tumours immunohistochemistry showed pRB loss in 95–99% of tumour nuclei. In the other two cases loss was seen in 75–94% of tumour nuclei. The presence of some pRB nuclear staining could be explained by the presence of mutant pRB which may be functionally inactive or by the retention a small clone of cells without pRB loss. This may suggest that loss of RB1, though early, is not the initiating event in carcinogenesis (Benedict et al., 1990). Another possibility is that there is limited expression of the retained RB1 allele (possibly mutant), subject to activity of controlling genes.

One patient with BPH showed loss of heterozygosity of RB1, which may represent a premalignant field change occurring within the gland. de Vere White et al. (1992) noted abnormal RB1 mRNA expression in 1 of 13 benign prostates. Our case may be similar to this, though whether alteration of RB1 is representative of a premalignant change or has a role in prostatic adenoma or hyperplasia is unclear.

This study has shown that loss of RB1 gene is a frequent and possibly early event in prostatic tumorigenesis.

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