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Distinct Regions of Allelic Loss on 13q in Prostate Cancer[1]

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ABSTRACT

Loss of heterozygosity (LOH) involving the long arm of chromosome 13 has been reported to occur in as many as one third of primary prostate cancers. Candidate tumor suppressor genes on 13q that may be important in the development of prostate cancer include the retinoblastoma susceptibility gene (*RB1*) and a gene associated with inherited breast cancer (*BRCA2*). To define the pattern of allelic loss of 13q in prostate cancer, LOH analysis was performed using nine mapped polymorphic markers spanning the entire chromosomal arm. Nineteen (48%) of 40 prostate cancer cases obtained following radical prostatectomy demonstrated allelic loss with at least one marker. Furthermore, 13 (33%) of 40 cases had evidence of allelic loss involving a region of 13q14 containing *RB1*. To test the hypothesis that *RB1* is the targeted tumor suppressor gene in this region, 37 of 40 cases were assessed for expression of pRB, the protein product of *RB1* using immunohistochemical techniques. By this analysis, 8 (22%) of 37 prostate tumors demonstrated no pRB expression. However, allelic loss at *RB1*, assessed with an intragenic marker, did not correlate with absent pRB expression (Fisher's exact test, $P = 0.375$). Taken together, these data confirm that allelic loss of a common region of 13q14 occurs in approximately one third of prostate cancers. Lack of correlation of LOH at *RB1* with absent pRB expression suggests the existence of another tumor suppressor gene in this region important in prostate cancer.

INTRODUCTION

Prostate cancer is the most common cancer in men, with a projected 244,000 new cases to be diagnosed in 1995 (1). The molecular events leading to the development of this very common neoplasm have not been well characterized. To gain insight into the molecular genetics of prostate cancer, LOH[3] studies have been used to identify chromosomal regions containing putative tumor suppressor genes. Inactivation of both copies of a tumor suppressor gene by mechanisms such as deletion, mutation, or mitotic recombination can result in its loss of function (2) and regions of chromosomes 8p, 10q, 16q, and 18q have been implicated by this type of analysis (3). LOH on 13q has been noted less frequently (9–23% of cases; Refs. 4 and 5). Using the technique of comparative genomic hybridization, however, 32% of primary and 56% of recurrent prostate tumors were shown to have allelic loss of 13q (6). One of the candidate genes on chromosome 13 that may be important in prostate cancer is the retinoblastoma susceptibility gene *RB1* located at 13q14 (7). Bookstein *et al.* (8) previously demonstrated absent expression of pRB, the protein product of the *RB1* gene, in one of seven prostate cancer specimens. Sequence analysis of this metastatic prostate cancer revealed a 103-bp deletion in the promoter of one *RB1* allele and loss of the other normal *RB1* allele, supporting the role of *RB1* as a tumor suppressor gene in a subset of prostate cancers. Sarkar *et al.* (9), however, were unable to identify specific *RB1* molecular defects in any of 23 primary prostate cancers examined. Others have described *RB1* allelic loss in 27–67% of prostate cancers using intragenic polymorphisms (10–12).

Several epidemiological studies have described coaggregation of breast and prostate cancer (13–15). Linkage analysis of families with hereditary breast cancer has identified at least two loci associated with this disease: *BRCA1*, located at 17q21, responsible for a syndrome of familial breast and ovarian cancer, and *BRCA2*, located at 13q12–13, responsible for familial female and male breast cancer (16). Notably, carriers of an affected *BRCA1* allele have been shown to be at increased risk for both colon (estimated relative risk, 4.11, with 95% confidence interval 2.36–7.15) and prostate cancer (estimated relative risk, 3.33, with 95% confidence interval 1.78–6.20; Ref. 17).

There are data to suggest that there may be tumor suppressor genes other than *RB1* and *BRCA2* present on 13q. Studies of several other common cancers including head and neck (18), bladder (19), breast (20), and ovarian cancer (21, 22), as well as pituitary tumors (23) have demonstrated lack of correlation between LOH at *RB1* and loss of pRB expression. These experiments have been used to support the hypothesis that LOH involving 13q may be due to a tumor suppressor gene(s) other than *RB1*. Furthermore, there is strong evidence for a tumor suppressor gene telomeric to *RB1* in B-CLL (24–26). To determine the pattern of allelic loss of 13q in prostate cancer, we have performed LOH analysis of 40 prostate tumors using 9 polymorphic markers spanning the chromosomal arm (Fig. 1). Although the majority of the deletions included the *RB1* intragenic marker *D13S153*, no correlation was observed between LOH at *RB1* and loss of pRB expression determined by immunohistochemistry.

MATERIALS AND METHODS

Patient Material. Paired normal and tumor samples were obtained from 40 radical prostatectomies performed at the University of Michigan Medical Center. Patient data and tumor characteristics are listed in Table 1. Radical prostatectomy cases were staged using the standard TNM criteria according to the American Joint Commission on Cancer (27). All tissue was frozen in liquid nitrogen and stored at −70°C. Six 6–12-μm sections were prepared from both normal and tumor tissue for each case. One slide was stained with hematoxylin and eosin and examined by a pathologist (K. J. W.); regions containing at least 70% normal or tumor nuclei were outlined and used as a template for subsequent microdissection. Normal and tumor tissue were excised using a single-edged razor blade and digested with proteinase K.

LOH Analysis. DNA was amplified by PCR using a panel of microsatellite markers spanning the length of 13q in the following linkage order (centromere to telomere, see Fig. 1): *D13S221*, *D13S260*, *D13S267*, *D13S263*, and *D13S153* (28); *D13S133*; *D13S144*; and *D13S121* (29); *D13S173* (28). Primer sequences were obtained from Genome Data Base. Markers *D13S260* and *D13S267* are within the *BRCA2* candidate region (16) and *D13S153* is located in intron 2 of *RB1* (30). For PCR, one primer of each primer pair was 5′ end labeled with ^{32}P using T4 polynucleotide kinase. PCR products were electrophoresed on 4.9% denaturing polyacrylamide gels at 65 W. Gels were exposed to film at −80°C for 2 to 20 h. Each PCR reaction was performed twice and scored visually for LOH (defined as an approximately 50% loss of one tumor allele) by three independent observers (K. A. C., S. D. M., J. A. M.).

Immunohistochemistry. One normal and one tumor slide from each case was stained using mouse monoclonal antibody PMG3–245 (PharMingen) with

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[3] The abbreviations used are: LOH, loss of heterozygosity; B-CLL, B cell chronic lymphocytic leukemia.

Table 1 *Patient and tumor characteristics and summary data on allelic loss at RB1 and pRB expression*

Clinical data on the 40 prostate cancer patients used are depicted in the first five columns. The top 19 tumors demonstrated allelic loss for at least one marker and correspond to the 19 tumors depicted in Fig. 1. The last two columns list the results of allelic loss at *RB1* and pRB expression studies. Tumor 376 is a prostate cancer resected from a patient with a concurrent renal cell carcinoma.

Tumor	Age (yr)	Gleason	Stage	Comment	LOH at *RB1*	Absent pRB expression
402	69	8	$T_{3a}N_0M_x$[a]		No	No
368	74	6	$T_{3c}N_0M_x$	SV^+	Yes	No
378	60	7	$T_{2c}N_0M_x$		Yes	No
384	63	7	$T_{3c}N_0M_x$	SV^+	Yes	No
424	71	8	$T_{2c}N_0M_x$		Yes	Yes
370	66	7	$T_{3a}N_0M_x$		Yes	No
410	53	7	$T_{2c}N_0M_x$		Yes	No
408	64	7	$T_{3a}N_0M_x$		Yes	Yes
442	74	9	$T_{3c}N_1M_x$	SV^+	Yes	ND
382	70	7	$T_{3c}N_0M_x$	SV^+	Yes	Yes
394	68	7	$T_{3a}N_0M_x$		Yes	No
434	73	7	$T_{3a}N_0M_x$		ND	No
440	52	9	$T_{3c}N_xM_x$	SV^+	Yes	No
390	66	7	$T_{3b}N_0M_x$		Yes	No
426	67	7	$T_{3a}N_0M_x$		NI	Yes
366	69	7	$T_{2c}N_0M_x$		No	No
388	55	8	$T_{3c}N_0M_x$	SV^+	No	ND
396	64	7	$T_{2a}N_0M_x$		No	No
404	73	7	$T_{2c}N_0M_x$		No	No
364	64	7	$T_{2a}N_0M_x$		No	No
372	57	7	$T_{3c}N_0M_x$	SV^+	No	No
374	61	7	$T_{2c}N_0M_x$		No	Yes
376	62	6	$T_{2a}N_0M_x$	Renal cell	No	No
380	63	9	$T_{4a}N_2M_x$	SV^+	No	Yes
386	73	6	$T_{3c}N_0M_x$	SV^+	No	No
392	69	7	$T_{3a}N_0M_x$		No	No
398	62	6	$T_{2c}N_0M_x$		NI	Yes
400	68	7	$T_{3a}N_0M_x$		No	No
406	52	7	$T_{3a}N_0M_x$		No	Yes
412	55	7	$T_{2a}N_0M_x$		No	No
414	66	6	$T_{2c}N_0M_x$		No	No
416	60	6	$T_{2a}N_xM_x$		No	No
418	51	7	$T_{3a}N_0M_x$		No	No
420	60	7	$T_{3a}N_0M_x$		No	No
422	59	7	$T_{2c}N_0M_x$		No	No
428	71	7	$T_{2c}N_0M_x$		No	ND
430	59	7	$T_{3a}N_2M_x$		No	No
432	53	6	$T_{3b}N_0M_x$		No	No
436	73	7	$T_{3c}N_0M_x$	SV^+	No	No
438	71	7	$T_{3c}N_0M_x$	SV^+	NI	No

[a] x, undetermined; SV^+, seminal vesicle involvement; ND, not determined; NI, noninformative.

an adaptation of the protocol of Presti *et al.* (31). The primary antibody, which recognizes an epitope of pRB located between amino acids 300–380, was used at a 1:25 dilution, and incubation was allowed to proceed for 1 h at room temperature. Diaminobenzidine was used as the final chromogen, and hematoxylin was used as the counterstain. Each normal and tumor pair was scored for the presence of nuclear staining in the region outlined for microdissection. All slides were reviewed by two independent observers (T. P. S., K. J. W.) without knowledge of the results of the allelotype analysis.

Statistical Analysis. LOH at *RB1* was compared to pRB expression using the Fisher exact test. LOH at *RB1*, loss of any 13q locus, and absent pRB expression were compared to Gleason grade (grades 6, 7, 8–9), American Joint Committee on Cancer pathological stage, and seminal vesicle involvement using χ^2 analysis. The mean age was compared using the Student *t* test. A $P < 0.05$ was interpreted as significant.

RESULTS

Allelic Loss of Chromosome 13 in Prostate Cancer. Forty prostate tumors were studied for evidence of LOH using nine polymorphic microsatellite markers spanning chromosomal arm 13q. By this analysis, 19 (48%) of 40 tumors had evidence of allelic loss at one or more markers (Fig. 1). Although one tumor (specimen 402) demonstrated loss of a region centromeric to *RB1* and five tumors (specimens 426, 366, 388, 396, and 404) demonstrated loss of a region telomeric to *RB1*, the majority of tumors (13/19) had evidence of allelic loss of a segment of DNA containing *RB1*. The minimum overlapping region of loss extends from *D13S153*, which is intragenic to *RB1*, to *D13S133*, which is approximately 4 cM distal to the *RB1* gene.

Fig. 2 demonstrates distinct patterns of allelic loss in three prostate tumors. Tumor 402 demonstrates LOH using marker *D13S267* with retention of both alleles proximally and distally. Although the marker *D13S267* is in the *BRCA2* candidate region (16), this patient has no family history of breast cancer or other malignancies. Tumor 442 reveals the most common pattern of 13q allelic loss centered at chromosomal band 13q14. Tumor 388 represents the third pattern of allelic loss with LOH distal to *RB1* and retention of both alleles at *RB1*.

Immunohistochemical Evaluation of pRB Expression. To determine whether allelic loss of *RB1* corresponded to lack of pRB expression, 37 of 40 tumors were assayed using immunohistochemical techniques. All normal prostate cases examined ($n = 36$) had heterogeneous pRB nuclear staining. In the prostate tumors, pRB expression was scored as absent if there was no nuclear staining in the region outlined for tumor DNA microdissection. By this analysis, 3 (27%) of 11 evaluable prostate tumors that demonstrated LOH at *RB1* showed corresponding absent pRB expression (Table 1). However, an additional three tumors without allelic loss at *RB1* (using intragenic marker *D13S153*) and two tumors that were not informative at that locus did not express pRB. In summary, 8 (22%) of 37 prostate tumors did not demonstrate pRB expression, and LOH at *RB1* did not correlate with absent pRB expression ($P = 0.375$).

Correlation of Chromosome 13 Allelic Loss or Absent pRB Expression with Clinical Parameters. The pathological stage and Gleason grade of the 40 prostate cancers, as well as the patient age at diagnosis, are displayed in Table 1. Deletion at any 13q locus was positively associated with higher Gleason grade tumors ($P = 0.044$), but LOH at the *RB1* locus did not correlate significantly with grade. Loss at any 13q locus did not correlate with patient age, pathological stage, or seminal vesicle involvement. LOH at *RB1* or absent pRB expression failed to correlate with any of these same clinical parameters including Gleason grade.

DISCUSSION

Although allelic loss involving 13q has been observed to occur in prostate cancer, the precise loci involved have not been well defined. Previous studies have suggested that the tumor suppressor gene *RB1* may be important in prostate cancer. Much of this work has relied upon the use of *RB1* intragenic polymorphisms to determine the LOH frequency of this gene (10–12). However, tumor suppressor genes may be inactivated by a variety of genetic events other than interstitial deletions, including point mutations and deletions of large chromosomal regions. In our study, the allelotype analysis of 40 prostate cancers using 9 polymorphic markers spanning 13q reveals that 13 (33%) of 40 tumors have evidence of LOH at more than one contiguous marker. The minimal region of chromosomal loss detected by our analysis, however, contains the *RB1* gene. It is therefore reasonable to ask whether *RB1* is the targeted tumor suppressor gene on 13q, or alternatively, whether there is a second yet unidentified tumor suppressor gene that is inactivated by the relatively large chromosomal deletions in prostate cancer.

Immunohistochemical analysis of 37 prostate cancer cases in our study shows no correlation between genetic LOH at *RB1* and absent pRB staining. This same observation has been made in a variety of other common solid tumors including breast (20), head and neck (18), bladder (19), and ovarian cancer (21, 22). Immunohistochemical detection of pRB may be more difficult in prostate cancer than in

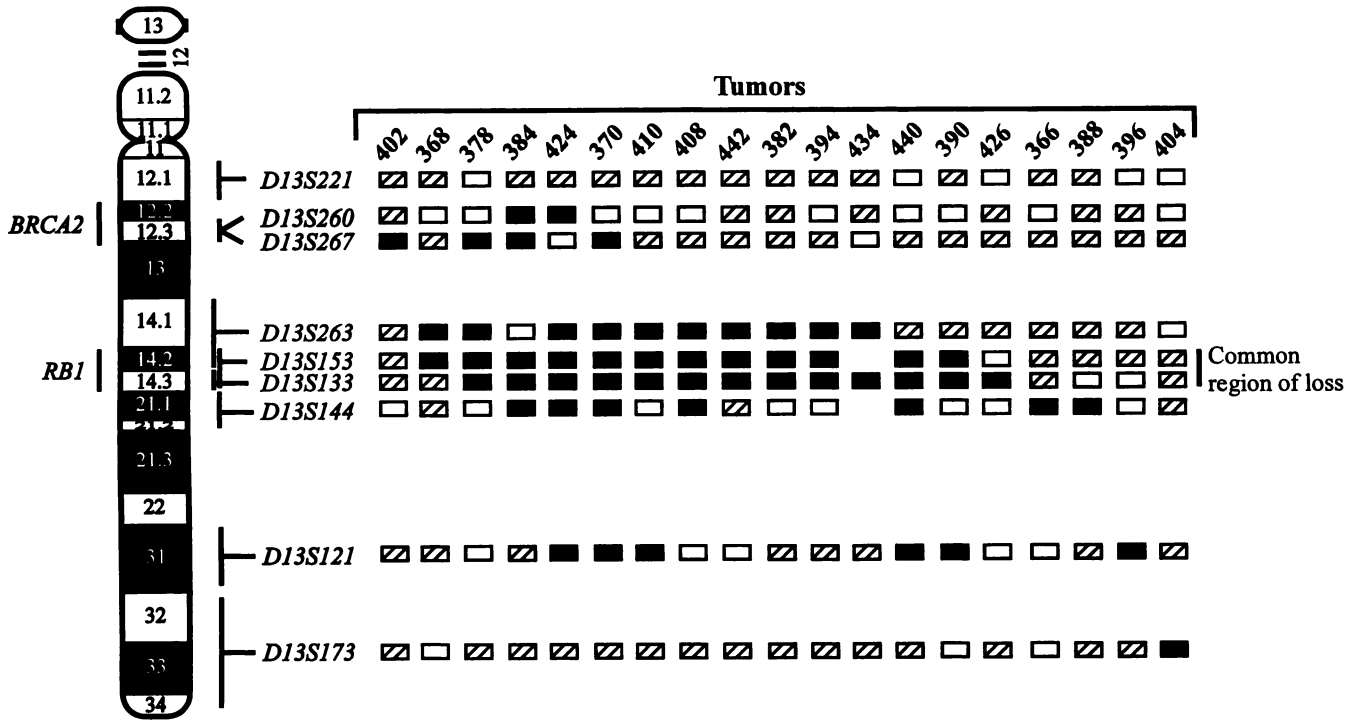

Fig. 1. 13q loss in human prostate cancer. Nine linked markers spanning chromosomal arm 13q were used to study 40 prostate cancers for evidence of allelic loss. The relative locations of tumor suppressor genes *RB1* and *BRCA2* are also depicted. Nineteen of the 40 tumors, shown here, demonstrated LOH for at least one marker. By this analysis, the minimal region of deletion extends from *D13S153* to *D13S133*, which is approximately 4 cM. ■, LOH; ▨, retained; □, noninformative.

other tumor types due to the observed variable staining in normal prostate. For example, atrophic glands often have increased nuclear staining compared to hyperplastic glands, consistent with the role of pRB in cell cycle regulation (32). Despite this observation, nuclear pRB staining was detected in all normal prostate tissues examined. Prostate tumors were scored for pRB expression only in regions prepared for microdissection to control for potential tumor heterogeneity. Our finding that 8 (22%) of 37 prostate cancers have no pRB expression as detected by a mouse monoclonal antibody directed against the amino acids 300–380 of pRB is consistent with the observations of Bookstein *et al.* (8), who describe absent pRB expression in 1 (14%) of 7 primary and/or metastatic prostate cancers studied using a rabbit polyclonal antibody. In that report, the tumor with absent pRB staining was shown to have a 103-bp deletion in one allele with LOH involving the second allele resulting in loss of pRB expression. Phillips *et al.* (11), however, reported that 7 (78%) of 9

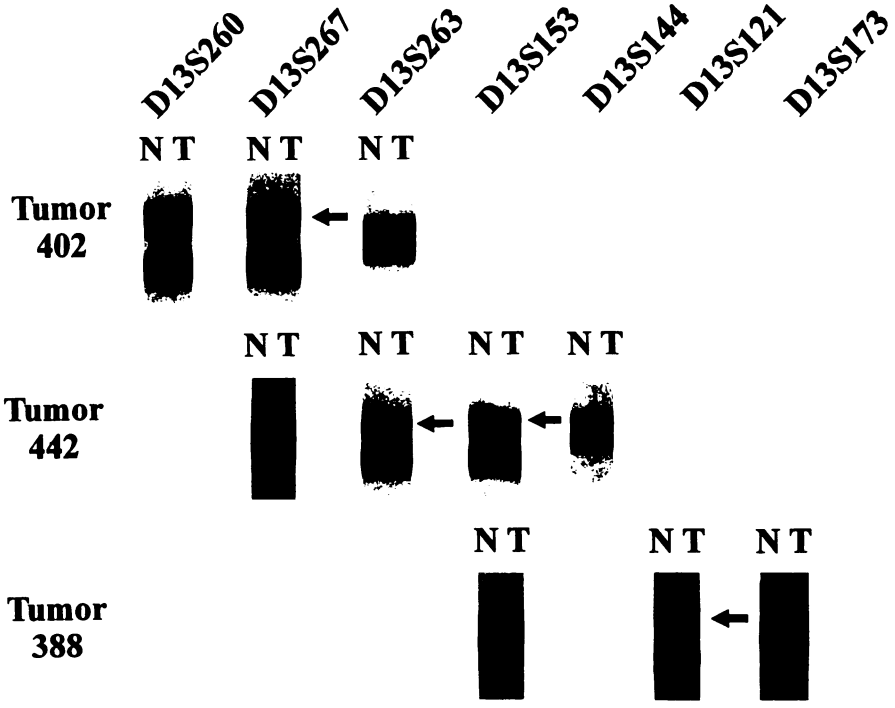

Fig. 2. Three patterns of 13q loss in prostate cancer. Tumor 402 shows evidence of allelic loss only at the proximal marker *D13S267*. Tumor 442 is representative of the 13 tumors with allelic loss centering around 13q14. Tumor 388 is one of five tumors with evidence of allelic loss at only one chromosome 13q marker distal to *RB1*. *N*, normal tissue; *T*, tumor; *arrows*, allelic loss.

primary prostate tumors had absent pRB nuclear staining using a commercially available monoclonal antibody different from the one used in our current study. Immunohistochemical assessment of protein expression is dependent on the detecting antibody as well as unique tissue staining characteristics, and these facts may account for the variation in reported loss of pRB expression in prostate cancer.

Three of eight tumors with absent pRB expression in our study demonstrated LOH at *RB1* consistent with the hypothesis that RB1 is the targeted tumor suppressor in this region. In five additional tumors demonstrating decreased expression of pRB without apparent LOH, sequence analysis may reveal inactivating pRB mutations. However, eight additional tumors with LOH at *RB1* express pRB. In these tumors, the minimal region of deletion extends from *D13S153*, intragenic to *RB1*, to *D13S133*. These data suggest that another tumor suppressor gene(s) may lie within this region that is important in the development of prostate cancer.

Analysis of B-CLL has identified a new tumor suppressor locus, *DBM* (disrupted in B-cell malignancy), located on chromosome 13q14 (26). Several lines of evidence have demonstrated that *DBM* is distinct from *RB1*. Liu *et al.* (25) confirmed the presence of at least one functional *RB1* allele in seven of eight B-CLL cases with evidence of allelic loss at 13q14 studied using immunofluorescence, reverse transcription-PCR, and single-strand conformation polymorphism analysis. Fine mapping experiments using additional markers in this region have identified 10 cases of B-CLL with small deletions centered around *D13S25*, located approximately 1.6 cM from *RB1*, with retention of both alleles demonstrated near *RB1*. The evidence for a tumor suppressor gene near *RB1* in solid tumors is less complete. Two studies of ovarian cancer report LOH using 13q14 markers in 52% of all epithelial tumors (21) and 50% of high-grade tumors (22) analyzed. However, the majority (>90%) of ovarian cancers, including tumors with allelic loss of 13q, have normal patterns of pRB nuclear staining. Similarly, 94% of head and neck cancers were shown to have LOH at *D13S133*, yet only 19% of tumors with allelic loss had loss of pRB expression. The results of our study add prostate cancer to the growing list of tumor types in which evidence of genetic loss of 13q14 including *RB1* does not correspond to absent pRB expression. Finer mapping of this region in prostate cancer with additional markers will narrow the critical region of deletion, and may help to identify a tumor suppressor gene(s) in addition to *RB1* that is important in prostate tumorigenesis. The intriguing correlation between 13q loss and Gleason grade found in this study suggests that 13q loss may be a marker of malignant potential. Identification of tumor suppressor genes on 13q may therefore have important diagnostic, prognostic, and therapeutic implications for the clinical management of prostate cancer.

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