Bladder Cancer: Allelic Deletions at and around the Retinoblastoma Tumor Suppressor Gene in Relation to Stage and Grade

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ABSTRACT

Inhibition of the retinoblastoma tumor suppressor gene (RB) is probably important in the pathogenesis of urinary bladder cancer. Little information is available concerning allelic loss on 13q11 to 13q32 and its relation to grade and stage. In a population-based study, freshly frozen tissue was collected from all new cases of urinary bladder cancer in the Stockholm region during 1995–1996. Here we report the occurrence of loss of heterozygosity (LOH) at seven sites in 13q11 to 13q32 as analyzed in 236 cases by a fluorescent multiplex PCR-based on tumor DNA and peripheral blood. For each site, about 30% of the cases were not informative because of homozygosity. Replication errors were detected in 4% (17 cases). LOH was found in 21 (at 13q11–12.1) to 32% (at 13q14.3 in RB) of the informative cases. A correlation was found between the prevalence of LOH at all observed loci and stage and grade, respectively, and it was statistically significant for 13q14.3. LOH at RB was found in Ta as well as grade 1 tumors. Also, a statistically significant correlation was found between the number of loci with LOH at 13q and tumor stage and grade, respectively. Typically an altered RB function is related to the expected clinical course of urinary bladder cancer, but allelic loss including the gene also occurs in low grade and low stage tumors. An altered RB function probably is not necessary for a malignant transformation of urothelial cells. The causal direction of the relation between the quantity of the deleted DNA and tumor aggressiveness is not clear.

INTRODUCTION

An altered function of one or several of the tumor suppressor genes p16, p53, and RB is part of the pathogenesis in many cases of urinary bladder cancer; allelic loss, mutation, or methylation of at least one of these genes has been found in all series investigated (1–5). The micropathology of the disease is further outlined by reports of allelic loss at a number of sites other than 9p (p16), 13q (RB), and 17p (p53), including chromosomes 3p, 4p, 8p, 9q, and 11p (6–10). It has been argued that the observation of all these changes indicates that p16, p53, and RB constitute just a few of a multitude genes that are important, but also—that many changes purely reflect a genetic instability of malignant cells. Any suggested pathogenic pathway rests on sparse data. Although the simplicity of the “two-hit” model for tumor development built on inherited RB mutations is attractive, more events than two typically are suggested as important when tumor formation is modeled (11).

The increased risk of the disease in families with a germ-line RB mutation and the occurrence of genetic changes corresponding to RB (detected by varying techniques) display the relevance of RB inhibition to urinary bladder cancer pathogenesis (4, 5, 12–15). Advanced stage has been related to genetic changes indicating RB inhibition, but it is unclear if the tumor aggressiveness depends on the biology of the gene by itself or, for example, concurrent genetic changes. RB codes for a 110,000 nuclear phosphoprotein that probably regulates the cell cycle at the G1-S transition (16–19). Apart from retinoblastoma and urinary bladder cancer, RB seems to be important in breast, colorectal, and prostate cancer (20–22).

Possibly a large unselected population-based cohort can add validity when one elucidates pathogenesis. In Stockholm, we have collected a consecutive population-based series of more than 600 newly diagnosed cases of bladder cancer during 1995–1996. In the present analysis of 236 patients, we describe allelic loss at different loci between 13q11 and 13q32, that is, from a broad region adjacent to, and including, RB.

MATERIALS AND METHODS

Patients and Tissue. Through a collaboration between urologists in Stockholm, all newly diagnosed cases of urinary bladder cancer were identified during 1995–1996. Before transurethral resection, four tissue samples were taken by cold-cup biopsy and freshly frozen at −80°C. The frozen tissue was cut in to about 5-µm-thick sections, and the first and last sections were stained and examined for tumor content. A sample with at least 70% tumor content was required for inclusion in the present analysis. Tumor DNA was extracted by a previously described method (23).

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† The abbreviations used are: RB, retinoblastoma tumor suppressor gene; LOH, loss of heterozygosity.
Blood. Venous blood from each patient was collected in EDTA tubes and frozen. Leukocyte DNA was prepared from 200 μl of whole blood, adding 800 μl freshly 170 mM ammonium chloride for 20 min of vortex mixing. White cells were collected by centrifugation at 3000 rpm for 2 min. The pellet was washed with 300 μl of 10 mM NaCl/10 mM EDTA three times to remove hemoglobin. The pellet was resuspended in 500 μl of 50 mM NaOH and incubated for 20 min at 100°C. Afterward, 100 μl of 1 M Tris-HCl (pH 7.5) was added to adjust the pH to be neutral. Cell debris was removed from the solution by centrifugation, and leukocyte DNA was stored at −20 or −80°C.

PCR Amplification. We used seven microsatellites with 3- or 4-bp repeat motifs. Primer sequences for these markers were obtained from the Genome Database.4 RB1.20, located 54 bp from the 3’ end of exon 20 at 13q14.3, was used as an internal RB gene marker (24). PCR were carried out using Perkin-Elmer DNA cycler (Norwalk, CT) in a 10-μl reaction volume containing 0.2 mM of each primer (one of the primers labeled with colors FAM, HEX, or TET), 2.00 mM MgCl₂, 0.20 mM dNTP, 1 unit of Taq polymerase, and about 10 ng of genomic DNA. The DNA was amplified for 32–36 cycles at 94°C during 45 s, at 55°C during 60 s, and at 72°C during 45 s.

Multiplex PCR. The Multiplex PCR method was the same as described above with the exception that 2–7 primer pairs were added in the same PCR tube. Primers were selected on the basis that the resultant PCR products could be distinguished by color and by size range without overlapping fragments.

PAGE. PCR products were analyzed on 4% polyacrylamide denaturing gels in 1× TBE buffer in a model 377 automated fluorescent DNA sequencer (Applied Biosystems, Foster City, CA), which has four-color detection systems. One micro-liter of each PCR product was resuspended using 3.5 μl of loading solution (2.5 μl formamide, 0.5 μl Blue Dextran (50 mM EDTA with 50 mg/ml Blue Dextran), and 0.5 μl GeneScan Size Standard: GS500). This mix was denatured at 95°C during 5 min and 1.5 μl was loaded into each well. The gel was run for 2 h at 40 W and 52°C.

Data Analysis. The fluorescent gel data were collected during the run by online laser detection. The obtained results were analyzed using Gene Scan Analysis Software. For each fluorescent fragment the size, height and area of the peak was determined.

Calculation of Allele Ratios. In case of heterozygosity, peak areas of the two alleles—in paired blood and tumor samples—were calculated by Gene Scan program. For the detection of LOH, we required that one allele from the tumor sample was reduced by more than 50%.

Stage and Grade. Stage was assessed prospectively with a modification of the TNM system; the distinction between muscle invasive tumors was based on tumor palpation before transurethral resection (25). T₂ tumors were confined to the mucosa, T₃ to the submucosa, muscle-invasive tumors without a palpable mass were regarded as T₄; a mobile palpable mass defined T₃ tumors; a mass judged to be invading the prostate, rectum, vagina, or uterus categorized the tumor as T₄a and fixation to the pelvic wall as T₄c. Grading was done with a modification of the WHO system, which distinguishes grade 1a, 2 tumors from grade 2b ones according to the basic tissue order (26).

Statistical Analysis. The χ² test for trend was used to assess the correlation between the frequency of LOH and pathological information. The relation between the number of LOH and pathological information was analyzed by Spearman’s rank correlation test.

RESULTS

We examined 236 transitional cell carcinomas of the urinary bladder using seven microsatellite markers for chromosome 13 with tri- or tetranucleotide repeats.

Background. Table 1 shows some characteristics of the bladder cancer patients.

Heterozygosities. Table 2 shows heterozygosity for the observed proportions of cases with each marker. It ranged between 0.68 and 0.87, which were close to the reported rates from the Genome Database.4

Deletion Mapping of Chromosome 13. Table 3 shows the prevalence of loss of heterozygosity in relation to stage. In two regions LOH was prevalent; one was at 13q12.3–14.2 (D13S894). In Ta tumors, seven cases (Fig. 1: K181, S44, S66, S154, S144, S192, K123) had LOH at 13q12.3–14.2 together with allelic retention at 13q14.3. The second frequently changed region was at 13q14.3, which is within the RB gene. Six tumors (Fig. 2: K110, S27, K120, S30, H25, K146) in stage T₂ had lost a single locus at 13q14.3, but the deleted loci comprised less than three alleles alongside each other.

LOH and Replication Error on Chromosome 13. Of the 236 tumors, 131 (56%) had LOH at 1 or more loci on chromosome 13 and 17 (4%) had a replication error. LOH was detected in 36 (21%) of 168 informative cases by D13S877, 41 (25%) of 161 by D13S894, 45 (22%) of 202 by D13S325, 54 (32%) of 170 by RB1.20, 41 (24%) of 173 by D13S800, 39 (26%) of 151 by D13S793, and 35 (22%) of 161 by D13S779 (data not shown).

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LOH According to Tumor Stage and Grade at 13q14.3.

Table 3 shows the relation between LOH of each microsatellite and tumor stage. In each marker, LOH was more prevalent for higher stages, but a statistically significant trend was found for 13q14.3 only. The relation between LOH and tumor grade is presented in Table 4. Again, a statistically significant trend was found for 13q14.3 only. We also analyzed a collapsed variable: LOH at least one of the seven regions included in the study. This
variable was related to grade in a statistically significant way. In grade 4, anaplastic tumors, 5 of 5 (100%) informative cases had LOH at one or more loci.

A restricted analysis was done concerning \textit{RB}; we selected the cases that did not have any LOH at any of the six investigated loci other than at 13q14.3. Among them, we documented the correlation between LOH and tumor grade and stage, respectively. Four (8%) of 41 Ta cases had the defined change, none (0%) of 11 cases with T1 tumors, 5 (50%) of 10 with T2, 3 of 6 (50%) at T3, and 1 of 1 (100%) at T4. The corresponding figures for grade 1 was 1 of 9 (11%), grade 2, 3 of 33 (9%), grade 2, 0 of 14 (0%), grade 3, 7 of 22 (32%), and grade 4, 2 of 2 (100%). A statistically significant relation was found with regard to tumor stage ($P < 0.001$) as well as grade ($P = 0.020$; data not shown).

DISCUSSION

Our study adds further evidence to the notion that altered \textit{RB} function is important for the development and clinical behavior of some urinary bladder cancer cases. Clearly, there is a
relation between tumor grade and stage and the prevalence of RB-related changes. We also showed, however, that inhibition of RB alone is not sufficient to determine the tumor as aggressive. LOH at 13q14.3 was found in 11 of 59 (19%) tumors classified as Ta G 2A or less, that is, in tumors that almost never progress to being metastatic. Finally, we found an association between grade and stage, respectively, and the number of loci at 13q with LOH. Which biological phenomenon this finding reflects, if any, remains to be elucidated.

No previous study on bladder cancer has addressed associations with the variable "number of loci with LOH at 13q," and our findings must be substantiated before any biological significance is looked for. The variable may simply be a marker for genomic instability caused, in turn, by previous genetic changes, e.g., by the genetic events that initially formed the malignant cell. Nevertheless, it may also be true that nonspecific loss of normal cellular function contributes to degree of malignancy. Our data, together with cytogenetic studies showing deletions in a large number of areas outside of those harboring p15, p16, p19, p53, and RB (1–5), may imply that altered expression of several additional genes is important for urinary bladder cancer growth.

Our study covered LOH within a broad area on 13q. We did find LOH at all loci, and in 131 of 236 (56%) cases at least one locus was measured as being deleted. These observations cannot only be explained by frequent deletion of a large allele including RB: we restricted an analysis to cases with no LOH at 13q14.3, and still 42% of the cases had LOH at at least one locus. Previous evidence for deletion at 13q outside of RB has been presented by Habuchi et al. (14). In 39 informative cases, they found a prevalence of LOH of 30% at 13q22, of 25% at 13q31, and of 18% at 13q34. These figures correspond to ours.

### Table 5: Losses of heterozygosity at seven investigated loci in relation to stage and grade

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<th>Stage&lt;sup&gt;b&lt;/sup&gt;</th>
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<sup>a</sup> Values are number of occurrences, with percentage in parentheses.  
<sup>b</sup> P = 0.034; Spearman’s rank correlation test.  
<sup>c</sup> P = 0.005; Spearman’s rank correlation test.

Fig. 3 Average number of loss of heterozygosity at seven investigated loci in relation to stage and grade.
pathogenesis of the bladder tumor provides one explanation to the data. The altered function also may just be random events resulting from the malignant transformation.

In 170 informative cases, we found that the LOH prevalence of an allele within RB varies with stage and grade, respectively. Our findings corroborate those of Cairns et al. from 162 patients, of Knowles et al. analyzing 83 tumors and by Miyamoto, based on 45 observations (4, 5, 13). The combined evidence leaves no doubt that typically there is an association between the possibility of a fatal course of a case with transitional cell carcinoma and an altered RB function. However, it is also clear that LOH occurs at 13q14.3 in bladder tumors with a low stage and grade. Cairns reported for T\textsubscript{1} and T\textsubscript{2} cases 2 LOH in 48 tumors, for T\textsubscript{2}+ , 26 LOH in 46 tumors, for grade 1, 2 LOH in 31 tumors, for grade 2, 8 LOH in 25 tumors, and for grade 3, 18 LOH in 38 tumors (4). We can conclude that inhibition of RB is not sufficient cause for a urinary bladder tumor to be potentially lethal. Studying the suggested interaction between RB and p16 in human tumors could possibly expand our knowledge in this regard.

It is also clear from the combined data that inhibition of RB is not a necessary cause for a transitional cell to become malignant. We could not detect LOH on the markers within RB in 68% of the informative cases. The corresponding figures for Cairns are 70%, for Knowles, 85% and, for Miyamoto, 77% (4, 5, 13). Thus, reasonably at least two pathogenetic pathways operate in the disease. Suggested models include one in which inhibition of p53 is central and another with RB. As the evidence accumulates concerning mutation, methylation, and LOH of, for example, p16, p19arf, p53, p21, p27, and RB in relation to stage, grade, and clinical course (for various treatment approaches), a better understanding of the relevant genetic events for urinary bladder cancer will probably be achieved. Unselected series might possibly be important for the pursuit; we will continue to analyze inhibition of tumor suppression genes in this population-based series.

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REFERENCES

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