Forerunner genes contiguous to RB1 contribute to the development of *in situ* neoplasia

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Edited by Webster K. Cavenee, Ludwig Institute, University of California at San Diego, La Jolla, CA, and approved June 28, 2007 (received for review February 27, 2007)

We used human bladder cancer as a model system and the whole-organ histologic and genetic mapping strategy to identify clonal genetic hits associated with growth advantage, tracking the evolution of bladder cancer from intraurothelial precursor lesions. Six putative chromosomal regions critical for clonal expansion of intraurothelial neoplasia and development of bladder cancer were identified by using this approach. Focusing on one of the regions, which includes the model tumor suppressor *RB1*, we performed allelotyping of single-nucleotide polymorphic sites and identified a 1.34-Mb segment around *RB1* characterized by a loss of polymorphism associated with the initial expansion of *in situ* neoplasia. This segment contains several positional candidate genes referred to by us as forerunner genes that may contribute to such expansion. We subsequently concentrated our efforts on the two neighbor genes flanking *RB1*, namely *ITM2B* and *CHC1L*, as well as *P2RY5*, which is located inside *RB1*. Here, we report that *ITM2B* and *P2RY5* modulated cell survival and were silenced by methylation or point mutations, respectively, and thus by functional loss may contribute to the growth advantage of neoplasia. We also show that homozygous inactivation of *P2RY5* was antecedent to the loss of *RB1* during tumor development, and that nucleotide substitutions in *P2RY5* represent a cancer predisposing factor.

bladder cancer | single-nucleotide polymorphic sites | whole-organ histologic and genetic mapping

Many common epithelial cancers, including those arising in the bladder, begin as clonal *in situ* expansion of neoplastic cells, which show no or minimal deviation from the normal phenotype (1–4). Such lesions often form plaques involving large areas of the affected mucosa, and their expansion precedes the development of microscopically recognizable dysplasia or carcinoma *in situ* (4, 5). Identification of chromosomal regions associated with the initial expansion of neoplasia is a requisite for more specific studies of their positional candidate genes that may drive the initial clonal expansion of neoplasia.

We have used an approach referred to as whole-organ histologic and genetic mapping (WOHGM) to identify clonal hits associated with growth advantage, tracking the evolution of human bladder cancer from occult *in situ* lesions to invasive disease on a total genomic scale (6–8). Human bladder carcinoma was used as a model of common epithelial malignancy that develops by progression of microscopically recognizable intraurothelial preneoplastic lesions ranging from mild dysplasia to carcinoma *in situ* (5). Bladder cancer was also selected because it is close to an ideal model human tumor to study the early events of carcinogenesis due to simple anatomy of the organ and the ease in mapping preneoplastic conditions geographically across the entire mucosa of cystectomy specimens (9–11).

In this paper, we begin by presenting the identification of the six critical chromosomal regions that may contain genes driving the development of bladder cancer. Then, we focus on one of the regions, which contain the model tumor suppressor *RB1*, and by performing high-resolution mapping with SNPs, we identified alternative target genes subsequently referred to by us as forerunner (FR) genes that may contribute to the development of *in situ* urothelial neoplasia. Finally, we provide evidence that their loss of function is likely to be critical to the initial clonal expansion of *in situ* neoplastic lesions.

Results and Discussion

To identify chromosomal regions that may contain genes most relevant for the development of the initial clonal expansion of neoplasia, we performed the genome-wide WOHGM on human cystectomy specimens with transitional cell carcinoma (TCC) using 787 hypervariable DNA markers (mostly microsatellites) mapping to autosomes 1–22. By using this approach, we identified chromosomal regions characterized by clonal loss of heterozygosity (LOH) that had a geographic relationship to the distribution of two major types of intraurothelial precursor lesions [supporting information (SI) Fig. 6]. The first group consisted of alterations associated with expansion of a dominant clone with minimal or no phenotypic change that involved large areas of bladder mucosa. The second group consisted of alterations associated with the development of successive clones showing a fully transformed phenotype (i.e., those related to severe dysplasia/carcinoma *in situ* with subsequent progression to invasive cancer). We identified clonal LOH with both characteristics in four of the five tested bladders, with each one showing a unique combination of changes (SI Fig. 7). However, when constellations of putative critical losses from the individual bladders were analyzed, it became evident that six chromosomal

Author contributions: S.L., J.J., and T.M. contributed equally to this work; B.C. designed research; S.L., J.J., T.M., S.E.S., M.-S.K., T.T., H.B.G., J.-H.Z., L.S., J.B., S.S.A., S.S., P.S., X.W., J.-P.I., W.F.B., and B.C. performed research; D.M. and M.B.-E. contributed new reagents/analytic tools; S.L., T.M., S.E.S., K.S.T., K.B., L.S., J.B., X.W., S.F., D.M., M.B.-E., J.-P.I., and W.F.B. analyzed data; and B.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Abbreviations: WOHGM, whole-organ histologic and genetic mapping; FR, forerunner; TCC, transitional cell carcinoma; LOH, loss of heterozygosity; Mb, megabase; LOP, loss of polymorphism; BH3, Bcl-2 homology 3; LGIN, low-grade intraurothelial neoplasia; HGIN, high-grade intraurothelial neoplasia.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0701771104/DC1.

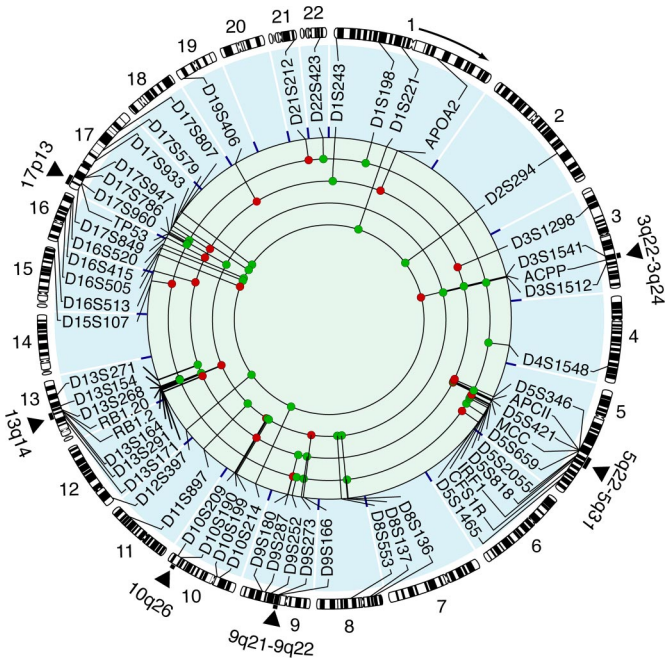

Fig. 1. Critical chromosomal regions involved in the development of bladder cancer. For details, see SI Fig. 8. The outer circle depicts the recombination-based map of chromosomes arranged clockwise from p to q arms. The four innermost circles represent maps of the informative cystectomies. Green dots designate markers with clonal LOH forming plaques involving microscopically normal appearing urothelium (NU), LGIN, HGIN, and TCC. Red dots designate markers with clonal LOH restricted to HGIN and TCC. Black arrows indicate six regions critical for the development of bladder cancer.

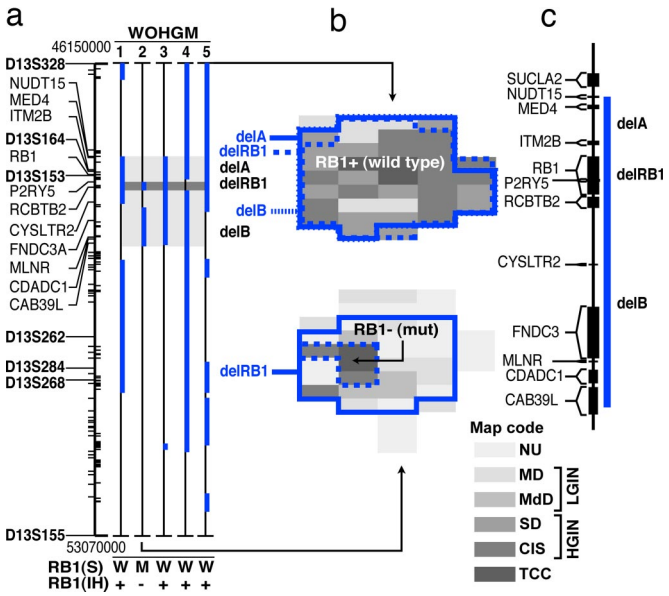

Fig. 2. Integration of LOH and LOP patterns identified in the 13q14 region with *RB1* sequencing data and RB protein expression implicating the involvement of FR genes in the intraurothelial expansion of a neoplastic clone. For details, see SI Fig. 10. (*a*) Regions of LOP associated with early clonal expansion identified by WOHGM with SNPs in five cystectomy specimens related to the status of *RB1* sequence, *RB1*(S), and RB protein expression revealed by immunohistochemistry [RB(IH)] are illustrated. The results of *RB1* sequencing and immunohistochemical studies for RB protein expression are tabulated below the maps of individual bladders. W, wild-type *RB1*. M, mutant *RB1*. The mutation in map 2 involved codon 556 of exon 17 consisting of CGA→TGA and resulting in the change of Arg to a stop codon. The presence of immunohistochemically detectable RB protein is designated by +. The absence of RB protein expression is designated by −, and its distribution pattern is shown in the lower image of *b*. The genome sequence map with the positions of hypervariable markers as well as known genes are designated by the bars on the left side of map. The regions of LOP in five cystectomies (maps 1–5) are depicted by the blue solid bars. The shadowed areas labeled delA and delB designate the regions of LOP flanking *RB1* involved in the incipient expansion of a neoplastic clone. The shaded area labeled del*RB1* designates the segment of LOP corresponding to the position of *RB1* on the sequence genome map. (*b*) The distribution of clonal LOP involving *RB1* and the same regions shown in *a* for map 5 (upper image) is depicted. The lower image shows the distribution of the segment with LOP in map 2 depicted in *a*. (*c*) Region of clonal LOP associated with growth advantage of *in situ* neoplasia identified by SNP-based mapping. The histologic map code is as follows: NU, normal urothelium; MD, mild dysplasia; MdD, moderate dysplasia; SD, severe dysplasia; CIS, carcinoma *in situ*; and TCC. For the purpose of statistical analysis, intraurothelial precursor conditions were classified into two groups: LGIN (mild to moderate dysplasia) and HGIN (severe dysplasia and carcinoma *in situ*). The immunohistochemical patterns of RB protein expression in representative mucosal samples of map 2 and map 5 are shown in SI Fig. 10.

regions, mapping to 3q22-3q24, 5q22-5q31, 9q21-9q22, 10q26, 13q14, and 17p13, were involved in more than one case and could represent six regions critical for the initial clonal expansion of urothelial neoplasia (Fig. 1). This observation was confirmed by testing multiple microsatellite markers mapping to the six chromosomal regions on voided urine and peripheral blood from 32 patients with clinically evident bladder cancer and from 31 patients with a history of bladder tumors who were disease free at the time of urine collection (SI Fig. 8 *b–d*). LOH affecting at least one of the regions was identified in 98% of these patients, and a similar frequency of LOH was present in patients with a history of TCC as well as those with clinically evident disease. In 82% of the cases, synchronous involvement of two to five chromosomal regions was present, and this confirmed that losses in these regions were frequent in bladder cancers and thus may contain genes critical for its development (SI Fig. 8*d*).

To identify prototypic genes that may drive such expansion, we concentrated our efforts on the 13q14 region, which contains the model tumor suppressor gene, *RB1* (12). We have previously reported that the patterns of *RB1* inactivation and LOH in this region suggest that *RB1* is not providing growth advantage for *in situ* urothelial neoplasia, whereas other genes in the region may drive the initial expansion of bladder neoplasia (13). This observation was based on low-resolution microsatellite and limited SNP mapping, which did not permit the precise identification of the involved region and its candidate genes. To map the positional candidate genes involved in the initial clonal expansion of urothelial neoplasia, we performed high-resolution whole-organ SNP-based allelotyping of the 27-megabase (Mb) segment around *RB1* (13, 14) (SI Fig. 9). When the integrated whole-organ maps of loss of polymorphism (LOP) identified by SNPs and maps of LOH identified by microsatellite markers were compared with *RB1* sequencing data and RB protein expression patterns, it became evident that LOH/LOP that affected *RB1* and its flanking regions was an early event in bladder neoplasia (Fig. 2 and SI Fig. 10). This loss was associated with initial clonal expansion that formed a plaque involving large areas of bladder mucosa and was never associated with the inactivation of the second *RB1* allele. In those cases in which loss of one *RB1* allele was associated with the mutational inactivation of the second allele, this inactivation was a later event corresponding to the onset of severe dysplasia/carcinoma *in situ* progressing to invasive carcinoma.

The patterns of integrated LOH and LOP identified by microsatellite and SNP markers from five cystectomy specimens defined the minimal region associated with clonal expansion of *in situ* neoplasia, which involved a 1.34-Mb segment around

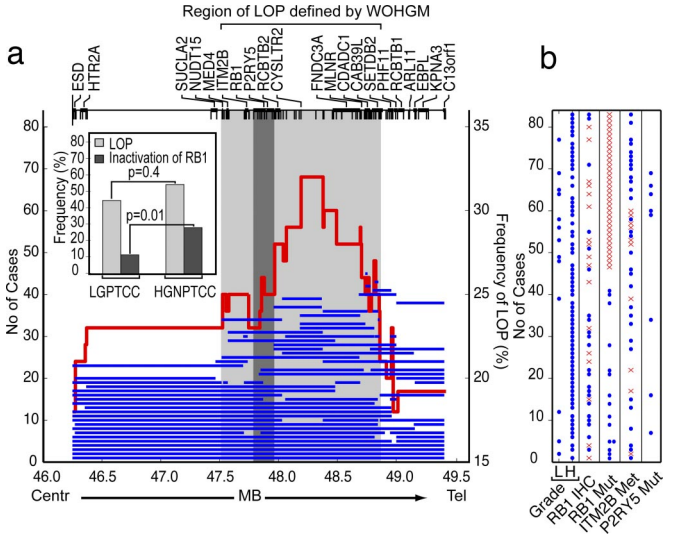

Fig. 3. Map of LOP within a 3.16-Mb segment around *RB1*. (*a*) Allelic losses were tested on 84 paired samples of bladder tumors and peripheral blood by using SNP multiplex technology. Predicted sizes of LOP are depicted as blue bars, and a continuous red line shows their frequency. The genomic map above the diagram shows positions of individual genes (solid black bars) and tested SNPs (thin black downward bars). (*Inset*) The analysis of LOP and inactivation of *RB1* documented by either immunohistochemical analysis or DNA sequencing is shown. LGPTCC, low-grade (grades 1 and 2) superficial (T_a–T_{1a}) papillary TCC; HGPTCC, high-grade (grade 3) nonpapillary invasive (T_{1b} and higher) TCC. (*b*) The allelic losses are related to histologic grade, RB protein expression, *RB1* mutation, methylation of *ITM2B*, and nucleotide substitutions of *P2RY5* summarized in a diagram on the right. Details of *RB1* sequencing are provided in SI Table 1. L, low grade (grades 1–2); H, high grade (grade 3); solid blue dots indicate the absence of RB protein expression, mutation of *RB1*, methylation of *ITM2B*, and nucleotide substitutions in *P2RY5*. Details of the analyses are provided in *SI Text*.

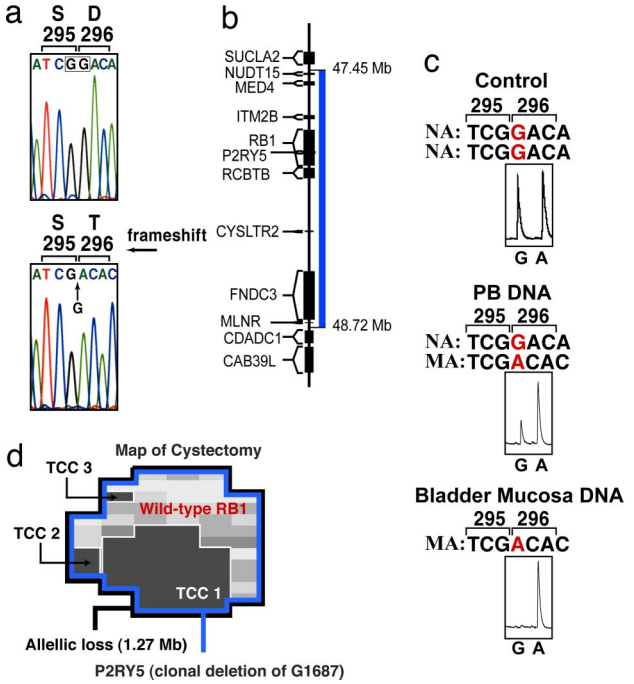

Fig. 4. Inactivation of *P2RY5* is associated with expansion of *in situ* neoplasia. For details, see SI Fig. 11. (*a Upper*) A wild-type sequence of *P2RY5*. (*a Lower*) A deletion of G at codon 296 after subcloning from paired peripheral blood (PB) DNA corresponding to the cystectomy shown in *d*. (*b*) A 1.27-Mb segment of LOP (blue bar) identified by mapping with SNPs. (*c Top*) A wild-type sequence from control DNA. (*c Middle and Bottom*) A G–A polymorphism at codon 296 in the PB DNA resulting from deletion of a G residue (*Middle*) and a loss of wild-type allele in bladder mucosa (*Bottom*) revealed by pyrosequencing. NA, wild-type allele; MA, mutant allele. (*d*) Inactivation of *P2RY5* by germ-line point mutation and loss of 1.27-Mb segment as it relates to precursor lesions and TCC in a cystectomy map (same case as shown in *a*–*c*). The black line depicts the area of LOP involving the 1.27-Mb segment; the blue line delineates the area with *P2RY5* showing the deletion at codon 296. TCC1–3 designate separate foci of carcinoma. The code for the histologic map is shown in Fig. 2.

RB1.[¶¶] Because this region was defined by WOHGM of only five cystectomies, we further investigated the frequency of its involvement on 84 paired samples of bladder tumors and peripheral blood DNA by allelotyping of 92 SNPs mapping to a 3.16-Mb segment around *RB1* (Fig. 3). The position of the segment with the most frequent LOP identified by this approach overlapped with the position of the region associated with clonal expansion initially found by WOHGM. (For details, see *SI Text*.) These data further supported the hypothesis that genes located near the model tumor suppressor, *RB1*, subsequently referred to by us as FR genes, may significantly contribute to the development of bladder cancer. By analyzing the predicted functions of their encoded proteins, we decided to concentrate our initial efforts on the two nearest neighbor genes flanking *RB1*, namely *ITM2B*, which encodes a mitochondrial membrane protein with a Bcl-2 homology 3 (BH3) domain (15), *CHC1L* (*RCBTB2*), which encodes a guanine nucleotide exchange factor protein for the ras-related GTPase (16), and *P2RY5* located within intron 17 of *RB1*, which encodes a G protein-coupled receptor (17).

To investigate the involvement of these candidate FR genes in bladder carcinogenesis, we initially analyzed their sequence and expression in 12 bladder cancer cell lines and 14 paired samples of adjacent urothelium and TCCs. The initial sequencing of bladder cancer cell lines as well as paired samples of adjacent urothelium and TCCs did not identify mutations within FR genes. However, by sequencing DNA from tumors and surface urothelium of the five resected bladders used for WOHGM as well as paired peripheral blood DNA, we found a germ-line deletion of G at codon 296 of the predicted P2RY5 protein (Fig. 4*a*); the resulting frameshift producing a stop signal at codon 302. In addition, this germ-line mutation was associated with LOP involving a 1.27-Mb segment in the surface urothelium of bladder mucosa (Fig. 4 *b*–*d* and SI Fig. 11). Consequently, this loss homozygously inactivated *P2RY5* in the urothelium (Fig. 4*c*), whereas the remaining alleles of *ITM2B*, *RB1*, and *CHC1L* were wild type. The homozygous inactivation of *P2RY5* was associated with expansion of an *in situ* neoplastic clone involving large areas of bladder mucosa and the subsequent development of multifocal TCC. Evidence was thus provided that *P2RY5* rather than *RB1* was a target for mutations within the 1.27-Mb segment in the 13q14 region and was likely involved in the initial expansion of the *in situ* neoplastic clone.

We subsequently found that the FR genes were down-regulated in bladder cancer cell lines with inactivated *RB1* as well as in those cell lines with wild-type *RB1* (SI Fig. 12*a*). Typically, more than one candidate FR gene was down-regulated, and the reduction of expression was often >4-fold (>75%). The FR genes were often down-regulated in both superficial and invasive TCCs and in corresponding samples of adjacent surface urothelium, again suggesting their involvement in early *in situ* phases of bladder neoplasia (SI Fig. 12*b*). To rule out the possibility of nonspecific down-regulation of the candidate FR genes in the

[¶¶]LOP by SNP mapping was identified in all five cystectomy specimens, including the specimen in which WOHGM mapping with microsatellites did not reveal LOH involving 13q14 region.

urothelium, the expression pattern of 29 genes around *RB1* was analyzed. These studies were performed on the same 12 bladder cancer cell lines and showed that the candidate FR genes were the most significantly and frequently down-regulated genes within the distance of ≈3 Mb around *RB1* (data not shown).

Because the 5′ regions of *ITM2B* and *CHC1L* have typical CpG islands, we further investigated whether methylation of their putative promoter regions was a mechanism responsible for silencing these genes (SI Fig. 13). Hypermethylation of the *ITM2B* promoter was identified in 6 of 12 bladder cancer cell lines and, in each instance, was associated with >2-fold (>50%) reduction in gene expression (SI Fig. 12*a*). Expression of *ITM2B* could be restored by treatment with the DNA methylation inhibitor 5-aza-2′-deoxycytidine, confirming that methylation was involved in silencing of this gene. The analysis of 14 paired samples of adjacent urothelium and TCC confirmed these observations and documented that methylation of *ITM2B* could be detected in 64% of tumor samples, which was also present in the adjacent urothelium (SI Fig. 12*b*). No abnormal pattern of methylation of *CHC1L* was identified.

To address how frequently the candidate FR genes are involved in bladder carcinogenesis, we performed methylation-specific PCR and sequencing analyses of *ITM2B* and *P2RY5* on an additional 195 TCCs and an equal number of controls matched for age, sex, and ethnicity. In addition, paired nontumor DNA from the same patient in 131 of the TCCs was also sequenced. Methylation of *ITM2B* followed the trend identified in bladder cancer cell lines or paired adjacent urothelium and tumor samples being detected in 40% of TCC. It was equally frequent in low-grade (grade 1–2) superficial (T_a–T_{1a}) as in high-grade (grade 3) invasive (T_{1b} and higher) TCCs. Missense point mutations of *P2RY5* were found in 7% of TCCs and, of particular importance, some of these were documented as germ-line alterations (Fig. 5*a* and SI Table 2). The mutations of *P2RY5* (somatic and germ-line) identified in bladder tumor samples and paired nontumor DNA from the same patient were not present in unaffected control samples or in the human genome SNP databases.

Although missense mutations in *P2RY5* were fairly rare, we also detected three polymorphic sites in *P2RY5*, namely 1386 T-C, 1602 A-C, and 1722 G-T. A 1722 G-T polymorphism resulting in the substitution of tryptophan for cysteine at position 307 was of potential importance because it was detected in several bladder tumors and nontumor DNA from the same patient. In addition, molecular models of P2RY5 protein developed by using its homology to rhodopsin suggest that this substitution involving the cytoplasmic domain of the protein may affect its interaction with the G protein complex and compromise its biological activity (18–23) (Fig. 5*b*). In fact, the loss of apoptotic activity for the 1722 T variant allele was documented by *in vitro* transfection assays (see below). Therefore, we extracted DNA from laser microdissected surface bladder urothelium with *in situ* neoplasia adjacent to invasive TCC from a patient with 1722 G-T polymorphism. By using allele-specific pyrosequencing, we showed that loss of a wild-type 1722 G *P2RY5* allele with retention of the variant 1722 T *P2RY5* allele occurred in *in situ* phase of bladder neoplasia and preceded the loss of RB protein expression (Fig. 5 *c* and *d*).

To further investigate the role of a 1722 G-T polymorphism of *P2RY5* in the development of bladder cancer, we conducted a case-control study on peripheral blood DNA from 790 patients with bladder cancer and 712 age- and sex-matched controls. The heterozygous 1722 G-T *P2RY5* genotype was identified in 2.78% of bladder cancer patients and 2.81% of controls. We did not identify any individuals with a homozygous 1722 T-T genotype. In addition, the presence of 1722 G-T genotype was not associated with an overall higher risk for bladder cancer (adjusted odds ratio, 1.07; 95% confidence interval, 0.57–2.03). However, it appeared that smoking modulates the effect of this polymorphism and all 1722 G-T carriers who were smokers developed bladder cancer (odds ratio not calculable). This strongly suggests that exposure to tobacco smoking-related carcinogens in the presence of a 1722 T *P2RY5* allele synergistically increases the risk for bladder cancer. Details concerning the frequency of LOP, *RB1*, and FR

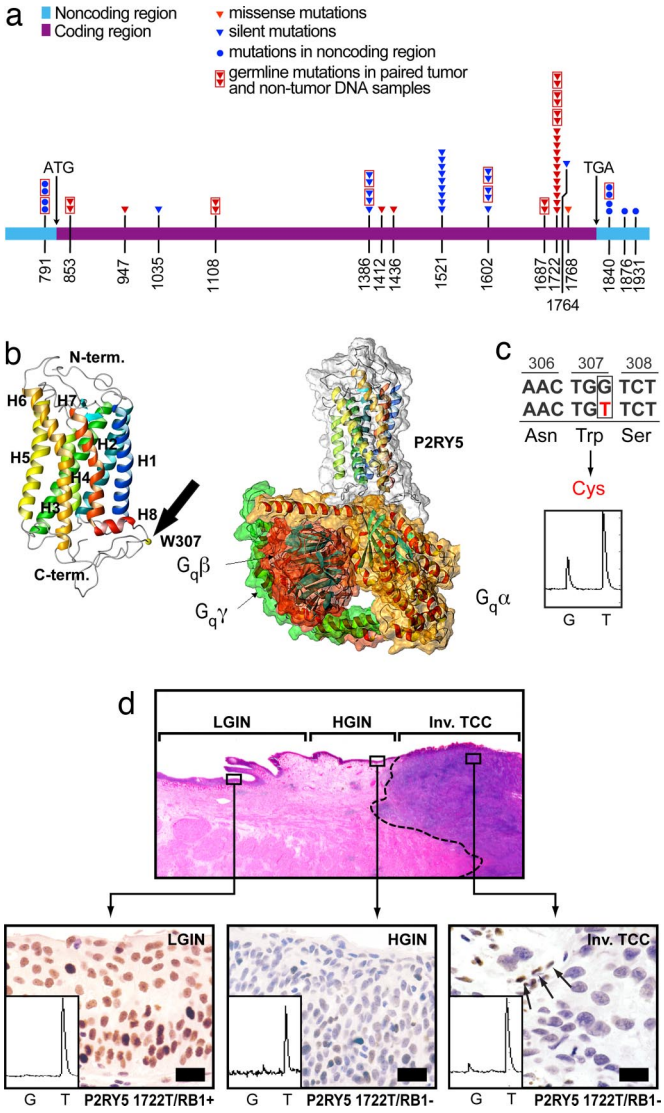

Fig. 5. Nucleotide substitutions of *P2RY5* in bladder cancers. (*a*) Summary of sequence analysis of *P2RY5*. The positions of nucleotide substitutions are shown on the full-length mRNA. For details, see SI Table 2. (*b*) A model of inactive P2RY5 containing seven-transmembrane (H1–H7) and one cytoplasmic (H8) helix structures showing the position of polymorphism in codon 307 located within the cytoplasmic domain of the protein (*Left*) that may affect its interaction with the $G_{\alpha\beta\gamma}$ trimeric protein complex (*Right*). (*c*) A G-T polymorphism at codon 307 in the PB DNA resulting in substitution of cysteine for tryptophan identified by pyrosequencing (same case as shown in *d*). (*d*) Sequential inactivation of *P2RY5* and *RB1* in the development of bladder cancer from *in situ* neoplasia. (*Upper*) Low-power view of invasive bladder cancer and adjacent LGIN and HGIN. (*Lower Left*) Microdissected DNA corresponding to LGIN shows loss of wild-type *P2RY5* allele and retention of normal RB expression pattern. (*Lower Center*) Microdissected DNA corresponding to HGIN shows similar loss of wild-type *P2RY5* allele and additional loss of RB protein expression. (*Lower Right*) Same loss of wild-type *P2RY5* allele and loss of RB protein expression is seen in invasive TCC. Arrows indicate retention of RB protein expression in endothelial cells adjacent to tumor. (Scale bars, 50 μm.)

genes inactivation in various pathogenetic subsets of TCC are provided in *SI Text*.

Collectively, our data strongly suggest that loss of FR gene function promotes early clonal expansion, presumably by directly regulating cell proliferation and/or cell death. Indeed, analysis of the ITM2B sequence identified a putative BH3 domain, a sequence motif that mediates the proapoptotic functions of an important family of cell-death regulators (24). To test this hypothesis, we transiently transfected *ITM2B* and *P2RY5* expression constructs into the ITM2B- and P2RY5-deficient UC6 bladder cancer cell line, which showed >4-fold down-regulation of both genes but has wild-type *RB1* (SI Fig. 14). Because human urothelial and bladder cancer cells express alternatively spliced long (L) and short (S) variants of *ITM2B* (data not shown), the experiments were performed with the constructs containing *ITM2B*L and *ITM2B*S inserts. Because ITM2BS was previously shown to be the apoptotically active form of ITM2B protein and this activity was attributed to an unmasked BH3 domain (15), we performed additional experiments with constructs containing inactivating mutations within its BH3 domain. These included constructs with: (*i*) substitutions involving two conserved amino acids at positions 35 and 40; (*ii*) substitutions of eight amino acids at positions 34–41; and (*iii*) a deletion of the entire predicted BH3 domain involving amino acids at positions 31–45 (SI Fig. 14*a*). To verify that nucleotide substitutions of *P2RY5* identified in clinical samples alter its function, we developed additional constructs containing mutant *P2RY5* inserts. These included constructs with: (*i*) a deletion of G at codon 296 causing a frameshift with downstream stop signal at codon 302 identified as a homozygous mutation in one of the cystectomies used for WOHGM (see Fig. 4) and (*ii*) a 1722 T variant allele resulting in the amino acid substitution at position 307 involved in the 1722 G-T polymorphism (see Fig. 5).

Ectopically driven expression of a wild-type *ITM2B*S, as well as of a wild-type *P2RY5*, reduced rates of proliferation in recipient cells (SI Fig. 14 *c* and *d*). These effects were associated with induction of apoptosis as measured by specific proteolytic processing of procaspase-3 and DNA fragmentation (SI Fig. 14 *b*, *e*, and *f*). In contrast, mutant *ITM2B*S constructs lacking a functional BH3 domain had no effect on caspase activation or DNA fragmentation (SI Fig. 14 *b*, *e*, and *f*). Transfections with a wild-type *ITM2B*L showed no evidence of apoptotic activity. Similarly, transfections with constructs containing mutant *P2RY5* inserts showed that nucleotide substitutions identified in cancer samples including a substitution associated with 1722 G-T polymorphism abolished its apoptotic effect. Transfections with a wild-type *CHC1L* construct showed no effect on cell proliferation or survival (data not shown).

Our findings support the existence of distinct genes, referred to by us as FR genes, mapping contiguously to *RB1*. Similar genes may also be present in the remaining five chromosomal regions we have identified. The FR genes are related to tumor suppressors in the sense that they appear to contribute to tumorigenesis by loss of function, but their inactivation precedes the functional loss of tumor suppressors such as *RB1* during tumor development. In fact, we have documented sequential homozygous inactivation of a candidate FR gene *P2RY5* and *RB1* in the development of bladder cancer. Although the mutational inactivation of this candidate FR gene is rare, it provides strong evidence supporting its involvement in early phases of bladder neoplasia. In addition, a 1722 G-T polymorphism in *P2RY5* was associated with an increased risk for bladder cancer development when combined with the exposure to tobacco smoking. Moreover, epigenetic silencing of another candidate FR gene, *ITM2B*, by methylation appears to be a frequent occurrence responsible for silencing of this gene in bladder carcinogenesis. In this scenario, the FR genes provide the initial growth advantage for a neoplastic clone, whereas subsequent inactivation of tumor suppressors is a transforming event associated with the development of a successor clone with features of severe dysplasia/carcinoma *in situ*. Because in some cases allelic loss associated with clonal expansion of *in situ* neoplasia involved not only FR genes but also *RB1*, the possibility that *RB1* haploinsufficiency might play a role in the development of premalignant lesions in these cases cannot be excluded. However, survivors with hereditary retinoblastoma carrying only one wild-type copy of *RB1* in their somatic cells develop normally, and they do not show hyperproliferative changes in the urinary tract or in any other normal tissue including the retina (25). In addition, otherwise normal human cells carrying only one copy of the wild-type *RB1* gene do not show increased growth rates or transformed phenotype *in vitro* compared with the cells carrying two copies of the wild-type *RB1* gene (26). These data indicate that *RB1* haploinsufficiency is unlikely to provide a significant growth advantage either *in vivo* or *in vitro*. It is uncertain at this time whether the FR genes provide growth advantage in the state of haploinsufficiency or they contribute to tumor development after their homozygotic inactivation. The overall pattern of LOH/LOP identified by our mapping studies suggests that multiple FR genes from several chromosomal regions are involved in the development of neoplasia *in vivo*. The FR genes not only provide a new paradigm for cancer development but may also represent early detection and risk markers as well as targets for therapeutic and preventive interventions.

Materials and Methods

Details of *Materials and Methods* are described in *SI Text*.

Mapping Strategy. WOHGM with microsatellites and SNP markers was performed on cystectomies from five patients with high-grade (grade 3) invasive TCC according to previously published protocols (6–8, 13, 14). In brief, testing was performed on 234 DNA samples corresponding to areas with the following: microscopically normal appearing urothelium (NU; $n = 53$), low-grade intraurothelial neoplasia (LGIN; $n = 82$), high-grade intraurothelial neoplasia (HGIN; $n = 50$), and invasive TCC ($n = 49$). Initially, all 787 markers were tested on paired DNA samples from TCC and nontumor DNA. LOH was identified in 196 markers that were subsequently tested on all mucosal samples of five cystectomies. The frequencies of LOH in the six critical chromosomal regions associated with clonal expansion of *in situ* bladder neoplasia identified by WOHGM were additionally tested on paired voided urine and peripheral blood samples of 63 patients with bladder cancer. Then, we focused on one of the regions and performed high-resolution WOHGM by using 661 SNPs mapping within a 27-Mb segment around *RB1*. Initially, all 661 SNPs were tested on normal genomic DNA. Then, those SNPs that exhibited polymorphism were tested on representative DNA samples corresponding to invasive TCC of the same individual. Finally, those SNPs that showed LOP were tested on all mucosal samples of the same cystectomy.

For the 13q14 region, the results of WOHGM were confirmed by allelotyping of 92 SNPs mapped to a 3.16-Mb segment around *RB1*. Testing was performed on 84 paired samples of bladder tumor and peripheral blood DNA using SNP multiplex (SNPlex) technology according to previously published protocol (27).

Expression, Sequencing, and Methylation Assays. We analyzed the expression, methylation, and sequences of FR genes in five cystectomies used for WOHGM, 12 bladder cancer cell lines, and 14 paired samples of adjacent urothelium and TCCs. Because these analyses showed that the two FR genes, *ITM2B* and *P2RY5*, were altered by hypermethylation and mutations, respectively, we analyzed their methylation and sequence in 195 TCCs. Sequencing of *P2RY5* was also performed on peripheral blood DNA from 195 age-, gender-, and race-matched, unaffected

controls. For 131 TCCs, paired nontumor DNA samples were also sequenced.

The epidemiologic analysis of 1722 G-T polymorphism involving *P2RY5* was performed on peripheral blood samples from 790 patients with bladder cancer and 712 age- and sex-matched controls. The allelotyping was performed by using *Taq*Man SNP assay, and the data were analyzed by using STATA software as described previously (28).

Immunohistochemical staining for RB protein was performed according to previously published protocol (29).

Vector-Driven *in Vitro* Transfection Assays. We cloned *ITM2B*L, *ITM2B*S, *P2RY5*, and *CHC1L* cDNA into the mammalian expression vectors: pEGFP-c1 and pflag-c1 (Clontech, San Jose, CA). *ITM2B* and *P2RY5* mutants were generated by site-directed mutagenesis using QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). For transient transfection, 2×10^6 cells were suspended in 100 μl of Nucleofector solution containing 2–5 μg of vector DNA, and electroporated by using Nucleofector apparatus II (Amaxa Biosystems, Gaithersburg, MD). The apoptotic activity was tested by fluorescent flow-cytometric DNA fragmentation and by proteolytic processing of procaspase-3 assays as described previously (30).

This study was supported by the National Cancer Institute Grants U01CA85078 (to B.C.), R01CA066723 (to B.C.), and P50CA91846 (PI Project 1; to B.C.).

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