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Genetic Alterations as Clonal Markers for Bladder Cancer Detection in Urine

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Abstract Bladder cancer is the result of a clonal expansion of cancer cells in which multiple genetic alterations have accumulated. Point mutations of the *p53* gene are frequently observed in bladder cancer. Loss of a retinoblastoma (*Rb*) allele is also common in bladder cancer. Recent data have shown frequent loss of heterozygosity (LOH) and homozygous deletion of 9p21, including the region of $p16^{INK4A}$, a putative tumor suppressor gene, in bladder cancer. LOH is also observed frequently at several other chromosome regions in bladder cancer. These genetic changes have proved useful as clonal markers in the detection of cancer cells in urine. Because of their complexity, most molecular diagnostic approaches are not considered promising cancer screening tools in patients or high-risk populations. However, a new molecular approach, the examination of microsatellite alterations in bladder cancer and urine specimens, is a promising screening tool for the disease. The common genetic alterations in bladder cancer and their use as clonal markers in screening or diagnosis strategies will be discussed. J. Cell. Biochem. 25S: 191–196. © 1997 Wiley-Liss, Inc.

Key words: bladder tumor; cancer screening; molecular markers

Bladder cancer accounts for about 50,000 new cases of cancer each year in the United States and is the fourth most common cancer in men and seventh most common one in women [1]. Bladder cancer may exist several years before the clinical diagnosis because of a lack of apparent symptoms or definite clinical evidence of cancer. The recurrence rate of bladder cancer, after surgical resection or chemotherapy is very high, accounting for about 70% of superficial bladder tumors [2]. This may reflect the presence of residual lesions in the bladder or continuing exposure of urothelium to carcinogens. Therefore, it is important to have efficient tools that can detect these cancers early and monitor their recurrence.

Cystoscopy is a powerful tool in the diagnosis of bladder cancer by the precise biopsy and the following histological evaluation. However, it is not useful for screening because the procedure is invasive and expensive. Also, some flat lesions, especially carcinomas in situ, may evade correct localization for biopsy and be indistinguishable from chronic inflammation. Urine cytology is a widely used noninvasive procedure for the diagnosis or screening of the disease. Although very useful in clinical evaluation, urine cytology may miss grade 1 papillary tumors and 30–52% of grade 2 tumors [3, 4]. To promote earlier diagnosis and improve the overall survival rate of the disease, more sensitive noninvasive screening strategies need to be developed. With our expanding understanding of bladder tumorigenesis, our efforts toward the development of a tumor progression model of bladder cancer, and the translational studies that have been done in the past several years, it has become clear that molecular approaches can augment traditional methods for bladder cancer diagnosis and screening.

GENETIC ALTERATIONS IN BLADDER CANCER

Bladder cancer arises and progresses through the accumulation of serial genetic alterations, including successive mutations which activate proto-oncogenes or inactivate tumor suppressor genes. This leads to the uncontrolled proliferation of progeny cells. In the genetic progression model of colorectal tumors developed by Fearon and Vogelstein, multiple genetic alterations involving functional changes in the gene

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products have been documented [5, 6]. Although no genetic progression model has been defined for bladder cancers, multiple genetic alterations have been identified in primary bladder tumors even at their very early stages.

A point mutation of the H-*ras* proto-oncogene was initially discovered in a bladder carcinoma cell line [7]. In human cancers, the majority of point mutations in H-*ras* and K-*ras* have been observed in codons 12, 13, and 61. These hot spots are potentially useful as targets for cancer detection. However, only 10% of primary bladder carcinomas contain H-*ras* gene mutations and no consistent correlation between the mutation and stage has been shown [8, 9], making H-*ras* an unlikely screening target for bladder tumors.

Amplification of the c-*erb*B-2/*neu*, an oncoprotein which is encoded by the HER-2/*neu* gene, has been suggested to play a role in human bladder carcinogenesis [10]. Although overexpression of c-*erb*B-2/*neu* oncoprotein has been observed in up to 40% of bladder tumors [11], the ligand with the genetic alterations in their encoding gene is still unknown. High expression of the *myc* protein and loss of normal *myc* regulation are observed in most proliferating cells in many different tumor cell lines and tumors. Also, rearrangements of the *myc* cellular oncogene are found in different types of tumors [12]. Although overexpression of c-*myc* has been observed in bladder tumors, alteration of the c-*myc* gene has not yet been identified [13].

Loss of heterozygosity (LOH) of 17p13, including the region of *p53* gene, is a frequent event in high-grade bladder tumors, and the gene in the remaining allele is usually inactivated by point mutations and is associated with tumor invasion [14–16]. Overexpression of mutant *p53* protein is usually observed in the late stages of bladder cancer and correlates with mutations in the conserved regions of the *p53* gene [17]. It has been reported that 61% of tested bladder tumors contain *p53* gene mutations [18], and point mutations are the most common alterations in the gene. Point mutations may lead to structural alterations of the *p53* protein that inactivate its wild type function. The mutant protein may bind to wild type *p53* protein and inactivate it by eliminating its binding activity. LOH at the *Rb* locus is frequent and associated with tumor progression in bladder cancer [19]. Immunostaining assay has shown altered pRB expression in 37% of the bladder tumor specimens. Most of the tumors which contained altered pRB expression were at late stages and were associated with a poor prognosis [20, 21].

Recently, $p16^{INK4A}$, located at chromosome 9p21 was found to be altered in many human cancers, including bladder cancer [22–24]. As an inhibitor of cyclin D/cyclin-dependent kinases which promote cells from G1 to S phase, $p16^{INK4A}$ is an ideal candidate tumor suppressor gene [25, 26]. In our recent study of 285 primary bladder tumors, more than 60% showed LOH at 9p21 centering around interferon-α and 71% of those tumors had a homozygous deleted region including $p16^{INK4A}$ [27], supporting the concept that $p16^{INK4A}$ is a target of inactivation in primary bladder cancer. In another recent study, 31 patients with carcinoma in situ of the bladder were examined for LOH at 13 chromosome arms. Loss of chromosome 9p21 was found in 61% of lesions [28] suggesting that this genetic alteration occurs relatively early in bladder cancer progression. Our recent study demonstrated that methylation of $p16^{INK4A}$ exon 1 may inactivate the transcription of the gene. This has been shown to be the case in 20–30% of various primary tumors including bladder tumors [27, 29]. Thus, loss of 9p21 or $p16^{INK4A}$ may be the most common genetic alteration so far identified in bladder cancer.

Apart from chromosomes 13q (*Rb*), 17p (*p53*), and 9p ($p16^{INK4A}$), LOH also occurs frequently at several other chromosomes in bladder cancer, including 3p, 4q, 5q, 6q, 8p, 9q 11p, 14q, and 18q; this suggests that other tumor suppressor genes may hide in these regions and play roles in tumor initiation or progression [28, 30]. Among these alterations, losses of 4q, 8p, 9q, 9p, 17p, 13q, and 11p have been found frequently at the carcinoma in situ stage, according to a recent report [28]. Microsatellite instability has often been found in bladder cancer when selected markers, especially the tri- and tetra-nucleotide repeat markers, were utilized [31]. Unlike microsatellite instability in hereditary nonpolyposis colorectal carcinomas, that in sporadic bladder tumors usually is identified in certain markers but rarely in dinucleotide markers [31]. Although the mechanisms are unclear, microsatellite instability is considered a potential clonal marker for detection of cancer cells.

GENE MUTATIONS AS CLONAL MARKERS

Early diagnosis of bladder cancer and monitoring its recurrence is critical for improving the overall survival rate. Moreover, identification of high-risk populations especially groups exposed to high doses of carcinogens such as benzidine, β-naphthylamine, and tobacco, is necessary, since identifying these groups would allow intensive follow-up or intervention with chemoprevention strategies, with the potential for eventually decreasing the incidence of the disease or increasing the survival rate. While the traditional approaches may not be sufficient to accomplish the goal, effort has been made to develop novel approaches that utilize emerging biological and molecular technologies.

The theory of clonal expansion of tumor cells through the accumulation of genetic alterations and the establishment of the tumor genetic progression models have made investigators consider the possibility that clonal cancer cells may be detectable by using genetic clonal markers. The development of polymerase chain reaction (PCR) technology has made it possible to examine genetic alterations such as mutations in a very small clinical specimen. Sidransky et al. [18] first successfully demonstrated that *p53* gene mutations can be detected in urine specimens of patients with bladder cancer. They amplified DNA isolated from the cytologically negative urine specimens of patients with bladder cancer by PCR to get the *p53* gene fragment and subcloned PCR products into bacteriophages, transferred them to nylon membranes, and hybridized them with specific radioisotope-labeled oligonucleotide probes according to the mutations identified in the patients' primary tumors. Of the cells in the urine sediments, 1–7% contained the same *p53* mutations as the corresponding tumors.

The same approach was utilized in a study of the urine sediment of Hubert H. Humphrey, a former vice president of the United States, which was collected several years prior to the clinical diagnosis of bladder cancer [32]. A *p53* gene mutation which was identified in his primary bladder cancer was also found in 9% percent of cells in his cytologically negative urine sediment obtained 9 years prior to the cystectomy, 6 years before receiving any therapy for the disease, and 2 years before the clinical diagnosis of carcinoma in situ. These studies prove that genetic clonal markers such as specific gene mutations involved in tumorigenesis and progression may identify tumor cells prior to the routine clinical means and therefore might be useful in the early diagnosis of bladder cancer. Moreover, the sensitivity of this approach is very high. It can identify one cancer cell among 10,000 normal background cells. According to one of our studies, a pathologist cannot make a diagnosis from surgical margins of head and neck tumors or lymph nodes if only 5% or fewer clonal tumor cells are present in the background [33]. However, several factors limit the use of this approach as a screening tool. Although *p53* gene mutations have been found in more than 50% of bladder tumors, the mutations are widely varied, and a large number of probes have to be used to cover the different tumors, which is difficult practically because of the complexity and cost. The H-*ras* gene mutations yield some relative hot spots, but only 10% of bladder tumors contain these alterations. Moreover, both *p53* and H-*ras* mutations may occur relatively late in tumorigenesis, making it possible that only a part of the tumor (or one of the tumors in cases of multiple bladder tumors) has these mutations, which may decrease the sensitivity of the assay. The current finding of frequent homozygous deletion in 9p21 in bladder tumors, especially this alteration, may occur early in the tumor progression. Strategies such as in situ PCR can be developed to examine the genetic alterations in urine sediments.

MICROSATELLITE ALTERATIONS AS CLONAL MARKERS

Microsatellite markers have been widely used as markers for the detection of LOH in mapping suppressor genes in human tumors and instability in neurodegenerative diseases such as Huntington's disease, myotonic dystrophy, and fragile X syndrome [34, 35]. Microsatellite instability is frequently observed in hereditary nonpolyposis colorectal tumors (HNPCC); it is termed replication error (RER+) because it involves the inactivation of one of the DNA mismatch repair genes [36].

In general, LOH is the most common genetic alteration in human cancers, including bladder cancer. In a genotype study, 95% of transitional cell carcinomas of the bladder had LOH in at

least one locus tested [37]. Because only a few markers have been utilized for each chromosome arm in the study, it is expected that the percentage of LOH might reach 100% in bladder transitional cell carcinoma if more markers were examined. Microsatellite instability is also detected frequently in tumor types other than HNPCC, including bladder cancer, if selected markers are used [31, 38]. In one study, we observed microsatellite instability in 34% of bladder tumors by using nine tri- or tetranucleotide repeat markers [31]. Recently, we further examined 60 more trinucleotide or tetranucleotide markers in 50 bladder tumor/normal tissue pairs. By using 10 selected markers, we identified microsatellite instability in 26 of 50 or 52% of the bladder tumors (data not shown). The shifted bands can be clearly seen in panels comparing tumor to normal tissues (Fig. 1). This clonal alteration is identical to the one seen in the patients' urine sediments [31].

To demonstrate whether this approach can be used to screen for bladder tumors in urine sediments, a panel of urine specimens of patients with and without bladder cancer were collected prior to the clinical procedures. By utilizing a panel of selected markers, we were able to identify microsatellite alterations in urine specimens of patients with bladder cancer at a rate of 95%, including 45% whose urine cytologic analysis reported negative or atypia [39]. These alterations were then identified in the resected or biopsied primary tumors. Because this assay requires only PCR amplification and gel separation steps, it is cost effective and can be done in any laboratory. Because microsatellite alterations are so common in bladder cancer and some of the changes occur early in tumor progression, microsatellite alteration analysis is very promising for the early detection of bladder tumors or screening of high-risk populations.

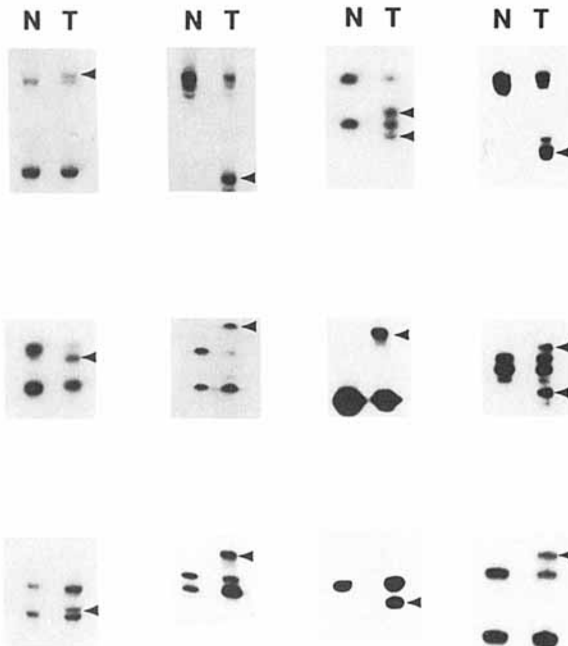

Fig. 1. Microsatellite instability observed in primary bladder tumors. N indicates DNA from normal white blood cells and T from primary bladder tumors.

FUTURE DIRECTIONS

A large clinical trial of screening microsatellite alterations in urine sediments needs to be organized to validate these findings. One proposal is to examine urine sediments of patients who may have bladder tumors, patients who have been treated for primary bladder cancer, heavy smokers, and workers with occupational exposure to carcinogens.

For early detection and screening, it is important to use markers that are altered during the very early stages of tumorigenesis. Therefore, continuing the search for genetic alterations which occur at initiation or early progression stages of bladder tumors and genetic markers which relate with high risk populations is critical. When a more complete and precise genetic model of bladder cancer is developed, it may guide us to appropriate markers that can be used for either the detection or screening of specific genetic alterations in patients.

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