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Deletions of 9p21 and TP53 in Bladder Cancer

Won-Sang Park, Moon-Ju Oh*, Hun-Kyung Lee, Kyung-Ok Lee*,
Mun-Gan Rhyu#, Seung-Myung Dong, Jung-Young Lee, Sang-Ho Kim

*Departments of Pathology and Microbiology#, Catholic University Medical College, Seoul Clinical Laboratory**

The objective of this study was to characterize the alterations of 9p21 and TP53 in Korean transitional bladder cancer and to assess the relationship between the histopathologic parameter and the alteration of these genes. Allele loss in 29 surgically resected transitional cell carcinoma was examined by using the multiplex PCR with 7 and 1 microsatellite markers for 9p21 and TP53, respectively. Twenty-one(72%) demonstrated allele loss at 9p21 and/or TP53. Deletion at the 9p21 region was detected in 17(61%) of 28 informative cases at one or more loci, and LOH at TP53 was found in 12(55%) of 22 informative cases. Of 7 microsatellite markers for 9p21, allele loss occurred the most frequently at locus D9S162(69%) and D9S104(69%). Additionally, hemizygous deletion was slightly more common than homozygous deletion. Deletion at 9p21 and TP53 was not related with increased grade. These results suggest that the alteration of 9p21 may be an early event in the development of Korean bladder cancer, while p53 gene may be involved in early event of some bladder cancers as well as in their late events.

Key Words: *Deletion, 9p21, TP53, Bladder cancer*

INTRODUCTION

Chromosome region 9p21, which contains a tumor suppressor locus, is involved in chromosomal inversion, translocation, and heterozygous deletion, in a variety of malignant cell lines including those from glioma, non-small cell lung cancer, leukemia, and melanoma (Serrano et al., 1993 ; Kamb et al., 1994a). The region was found to contain a gene, called MTS1, that encodes a previously identified inhibitor (p16) of cyclin-dependent kinase 4(Kamb et al., 1994a ; Kamb et al 1994b). The p16 protein binds to CDK4 and inhibits the ability of CDK4 to interact with cyclin D (Serrano et al., 1993) and arrests normal diploid cells at late G1(Lukas et al., 1995). Surprisingly, the ability of p16 to induce cell-cycle arrest was lost in cells lacking functional retinoblastoma protein. Thus, loss of p16, overexpression of D-cyclins, and loss of retinoblastoma gene have similar effects on G1 progression, and may represent a common pathway to tumorigenesis which results in abnormal cell growth (Lukas et al., 1995).

A high frequency of p16 deletions or mutations was observed in many tumor cell lines (Kamb et al., 1994a ; Kamb et al 1994b ; Nobori et al., 1994), but the study by Cairns et al.(1994), showing lower frequency of mutations in primary tumors, suggests that the p16 mutations seen in cell lines are in vitro artifacts and not evidence of a major role of the gene in the development of a wide variety of malignancies. However, the region 9p21-22, where the p16 gene is located, has been observed to show frequent loss of heterozygosity (LOH) in esophageal

Address for correspondence : *Jung-Young Lee, M.D., Department of Pathology, Catholic University Medical College, 505 Banpo-Dong, Seocho-Gu, Seoul 137-701, Korea. Tel : (02) 590-1194, Fax : (02) 537-3671*

squamous cell carcinoma, pancreatic ductal adenocarcinoma (Liu et al., 1995), bladder transitional cell carcinoma (Williamson et al., 1995), breast and prostatic adenocarcinoma (Cairns et al., 1995), and malignant melanoma (Koh et al., 1995). These results suggest that p16 has a pivotal role in inhibiting the development of human cancer.

The cellular p53 gene was named because of its original identification as an overexpressed 53 kilodalton protein in malignantly transformed cell lines (Crawford et al., 1981). In 11 of 18 invasive bladder cancer, Sidransky et al.(1991) found alteration of p53. In all but 1, the mutations were associated with 17p allelic deletions. This was the first genetic alteration demonstrated in a high proportion of primary invasive bladder cancer.

Transitional cell carcinoma of the bladder is one of the more common malignancies in the world. In Korea, it accounts for 3.2% of total male cancer patients (data reported by Ministry of Health and Social Affairs, Korea, 1994). In this present study, we investigated the frequency of 9p21 and TP53 abnormalities as well as their correlations with histologic grade in transitional cell carcinoma of bladder among Korean.

MATERIALS AND METHODS

Samples and DNA extraction

Sections(5 um thickness) from paraffin-embedded blocks of each tissue sample were stained with hematoxylin-eosin. The samples were histologically subdivided into grade I, II and III ; grade I shows tumor cells having some atypia and rare mitoses; grade II, tumor cells having greater variability in cell size, shape, and loss of polarity; grade III, tumor cells which are barely recognizable as being of transitional origin (Cotran et al., 1994). As the possibility that non-neoplastic transitional cells or inflammatory cells might mask allele losses could not be completely excluded, 29 cases in which more than 80% of bladder mucosa had been replaced by neoplastic transitional cells were included in this study. Ten adjacent sections, of thickness 5 um, were then cut from each block. The areas of transitional cell carcinoma and the surrounding normal transitional cells were scraped from a glass slide by a blade with regard to the microscopic observation of hematoxylin-eosin stained samples. Scraped tissue fragments were incubated overnight at 52℃ in the lytic solution (10 mM Tris-HCl, 1 mM EDTA, 1.25 ug/ul of proteinase K, 1% of sodium dodecyl sulfate) (Goelz et al., 1985). DNAs were successively extracted by phenol- chloroform- isoamyl-alcohol method and ethanol precipitation. DNA concentration was determined by measuring the optical density at 260 nm. Purity was assayed by the ratio of optical density at 260 nm and 280 nm. All patients were Korean and did not have a familial history of bladder cancer.

Analysis of allele loss at 9p21 and TP53

Twenty-nine pairs of samples were examined for LOH at 9p21 loci and TP53. Bladder tumor and corresponding normal DNA from each patient were amplified by PCR (polymerase chain reaction) at the microsatellite markers, D9S104, D9S126, D9S162, D9S163, D9S165, D9S171, and IFNA loci for 9p21, and TP53 for p53. We carried out multiplex PCR (more than one locus amplified simultaneously in one reaction tube) with using ^{32}P-dCTP. For 9p21, PCR conditions consisted of 32 cycles at 95℃ for 50 sec, 55℃ for 90 sec and 72℃ for 90 sec and for TP53, at 95℃ for 50 sec, 60℃ for 90 sec and 72℃ for 90 sec. Reaction products(2 ul) was then denatured and electrophoresed in 6% polyacrylamide gel containing 7 M urea. After electrophoresis, the gel was dried and exposed to X-ray film for 12 hours. Allele loss was determined by absence or a > 50% reduction in the signal of tumor allele compared to that of normal allele. In the cases where the homozygous deletion is suspected, we used D3S647 at 3p23 to confirm the homozygous deletion.

Statistical analysis

A correlation analysis of allele loss and pathological factor was evaluated by Chi Square test.

RESULTS

The 29 sporadic bladder transitional cell carcinomas including 13 grade I, 13 grade II, and 3 grade III were assessed for losses at 9p21 and TP53 using 8 microsatellite instability markers. The male-to-female ratio was approximately 5 : 1. The age distribution of patients were between 50 and 72 years. Of these, deletion of 9p21 was detected in 17(61%) of 28 informative cases at one or more loci, and for

TP53 LOH was detected in 12(55%) of 22 informative cases. Seven cases showed homozygous deletion at one or more loci of 9p21. Nine of 13 (69%) informative cases manifested allele loss at D9S162 and D9S104, 7 of 12 (58%) at D9S126, 5 of 9 (56%) at D9S171, 8 of 15 (53%) at D9S165, 6 of 14 (43%) at IFNA and 3 of 25 (12%) at D9S163.

Loss of 9p21 was found in 8 (67%), 6 (46%) and 3 (100%) of grade I, II and III, respectively (Table 1). LOH at TP53 was found in 4 (44%), 6 (60%) and 2 (67%) of 9, 10 and 3 heterozygous cases, respectively. Moreover, 21(72%) of 29 patients exhibited allele loss at 9p21 and/or TP53, and 8 (28%) demonstrated allele loss at both loci. The latter cases consist of 3, 3 and 2 of grade I, II and III, respectively. Additionally, two cases showed microsatellite instability at D9S165 and D9S104, respectively. Fig. 1 and Table 1 display these results.

DISCUSSION

Cancer is the endpoint of an evolutionary process in which normal cells acquire full malignancy by cumulative genetic or epigenetic alterations, each conferring proliferative, invasive, or metastatic potential to the cells. A critical area of chromosomal loss at region 9p21-22 has been implicated in the genesis of different types of primary tumors. Initial observations defined deletions of this region in leukemia, glioma, and cell lines derived from a wide spectrum of human tumor (Diaz et al., 1988 ; Diaz et al., 1990 ; James et al., 1993). Subsequent analysis in human non-small cell lung cancer demonstrated frequent loss of heterozygosity or homozygous deletion of this region (Merlo et al., 1994). p16, identified as an inhibitor of activated cyclin D-cdk4 complex (Serrano et al., 1993), emerged as a candidate

Table 1. Deletions of 9p21 and TP53 in transitional cell carcinomas of bladder

Sample	Age	Sex	Grade	D9S162 pter-p23-p22	IFNA p22	D9S171 p21	D9S126 p21	D9S104 p21	D9S165 p21-p21	D9S163 p21-p21 - cen	TP53
1	F	54	I	NI	NI	NI	NI	L	-	L	L
2	M	72	I	NI	H	L	NI	H	H	NI	-
3	M	50	II	-	NI	-	-	NI	NI	-	L
4	M	66	II	NI	NI	NI	NI	L	L	NI	NI
5	M	53	I	NI	-	NI	NI	NI	NI	-	NI
6	M	71	III	-	-	L	NI	NI	-	-	L
7	M	73	I	NI	-	NI	NI	L	NI	NI	-
8	M	60	III	H	L	H	H	H	NI	-	L
9	F	56	II	NI	NI	NI	-	-	NI	-	L
10	M	55	II	NI	NI	NI	-	NI	NI	-	L
11	M	54	III	NI	NI,MI	NI	NI	L	L	-	-
12	M	58	II	L	-	NI	L	NI	NI	L	L
13	M	69	I	NI	-	-	H	NI,MI	NI	-	-
14	M	50	I	L	NI	NI	H	L	L	-	L
15	M	65	II	NI	NI	NI	NI	NI	-	-	-
16	M	66	II	NI	L	NI	NI	-	-	-	-
17	F	59	I	NI	NI	NI	NI	NI	NI	-	NI
18	M	53	I	L	L	NI	-	L	L	-	-
19	M	60	I	L	NI	NI	NI	NI	L	-	NI
20	F	67	I	NI	-	-	NI	NI	-	-	-
21	M	54	II	H	NI	NI	NI	NI	L	L	L
22	M	61	II	-	-	NI	-	NI	NI	-	-
23	M	68	I	H	NI	H	H	-	NI	-	L
24	M	59	I	NI	NI	NI	NI	NI	NI	NI	L
25	M	72	II	L	L	L	L	H	NI	-	L
26	M	71	II	NI	-	-	NI	NI	-	-	NI
27	M	71	II	-	NI	NI	NI	-	-	-	NI
28	F	50	II	L	L	NI	L	NI	L	-	-
29	M	68	I	NI	NI	NI	NI	NI	NI	-	NI

NI, non-informative or homozygous ; L, loss of heterozygosity ; H, homozygous deletion ; MI, microsatellite instability ; -, negative for allele loss(retention of both informative alleles) ; pter, 9p terminal ; cen, centromere

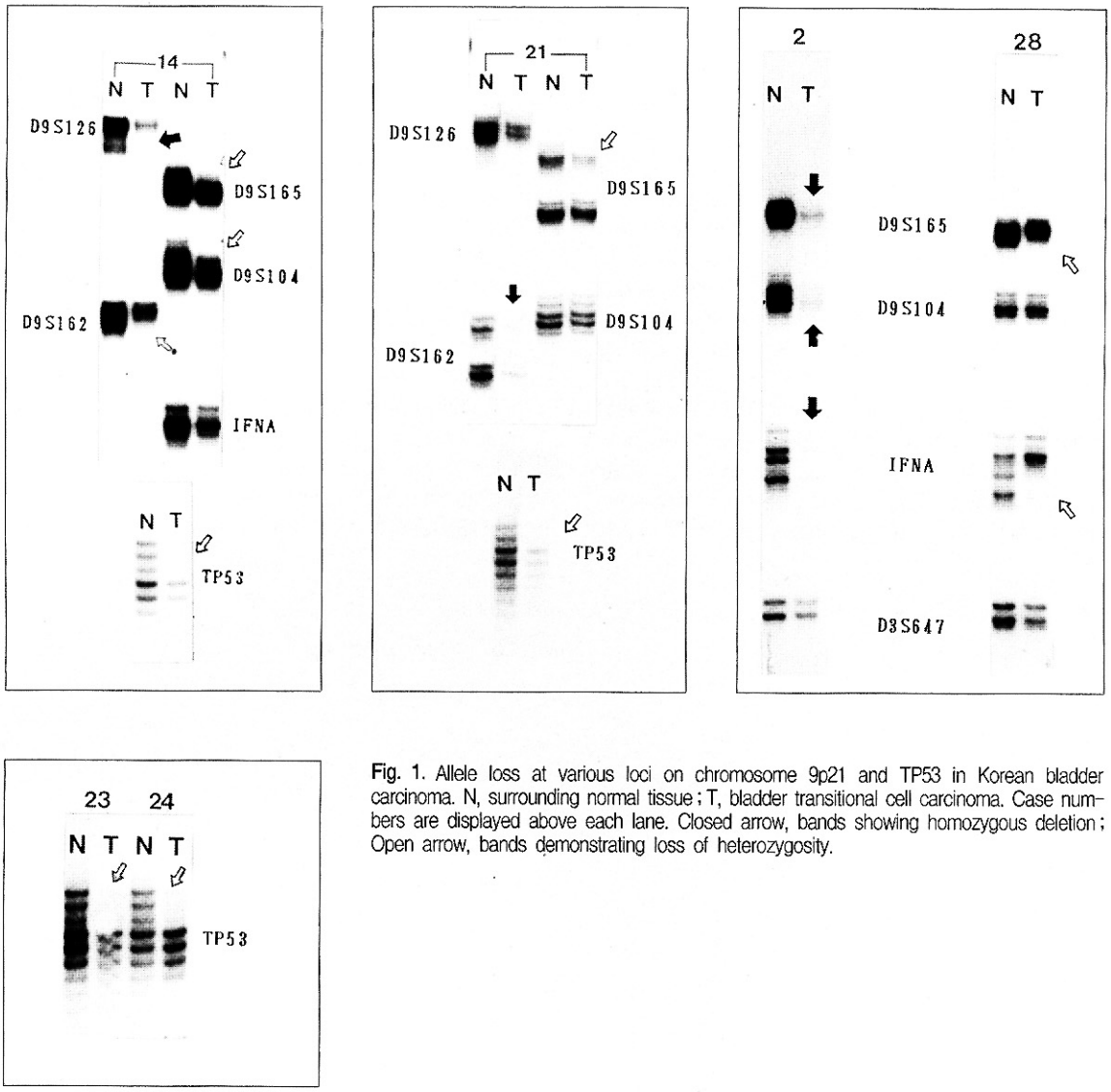

Fig. 1. Allele loss at various loci on chromosome 9p21 and TP53 in Korean bladder carcinoma. N, surrounding normal tissue; T, bladder transitional cell carcinoma. Case numbers are displayed above each lane. Closed arrow, bands showing homozygous deletion; Open arrow, bands demonstrating loss of heterozygosity.

tumor suppressor gene when it was localized to 9p21 and found to be within the critical deleted region (Kamb et al., 1994b; Nobori et al., 1994). More than 60% of bladder tumors have deletions involving chromosome 9p, 9q or both chromosome arms (Tsai et al., 1990; Cairn et al., 1993; Ruppert et al., 1993; Devlin et al., 1994; Keen and Knowles, 1994) and p16 (CDKN2) is a major deletion target at 9p21 in bladder cancer. In order to assess the involvement of the 9p21 region mutation in Korean bladder cancer and the relationship between the 9p21 region mutation and pathological parameters, we analyzed allele losses at 9p21 with 7 microsatellite markers in 29 bladder cancers. We have shown that a significant proportion (61%) of bladder cancers have homozygous or hemizygous deletion of the 9p21 region. However, microsatellite instability was seen in two cases. Cairns et al (1995) found that homozygous or heterozygous deletions at 9p21 represent the predominant mechanism of inactivation of 9p21 in bladder tumors and are present in other tumor types. Also allele loss at 9p21 was not

related with the increased grade (P=0.194). Our results further support that the functional loss at 9p21 may result in abnormal cell growth and that the 9p21 region may be an early event in bladder cancer development.

In our study, allele loss occurred most frequently at locus D9S162(69%) and D9S104(69%), while it was less common at D9S163(12%). These data may suggest that a tumor suppressor gene (or genes) is (or are) located closer to D9S104 and D9S162 than to D9S163. The location of MTS1 relative to these markers is between IFNA and D9S171 ; thus, the frequencies of allele loss at D9S171(57%) and IFNA(43%) is consistent with a role of MTS1 as the target of allele loss, but the high frequency(69%) of allele loss at D9S104 may also support the probability of the existence of an additional tumor suppressor gene or genes between D9S126 and D9S163 on the short arm of chromosome 9.

p53 tumor suppressor gene is considered to play a significant role in carcinogenesis. Mutations in the p53 are the most frequent genetic abnormalities encountered in human malignancy(Hollstein et al., 1991 ; Nigro et al., 1989). We analyzed LOH at 17p with TP53 microsatellite marker. Out of 29, 12(55%) cases showed LOH at p53. And there was no association between loss of TP53 and increased grade (P=0.716). Additionally, allele loss at 9p21 and/or TP53 was found in 21(72%), and TP53 allele loss occurred independently of 9p21 alteration; however, this difference did not achieve statistical significance. These findings suggest that p53 allele loss is a common event in the development of bladder cancer and further support that p53 mutations occur as an early event as well as a late event in the generation of invasive bladder cancer (Miyao et al., 1993; Nakopoulou et al., 1995).

Interestingly, our result indicating that hemizygous deletion was more common(59%) than homozygous deletion in bladder cancer, is similar to that of report of Keen & Knowles(1994), but slightly differs from those reports for bladder tumors (Cairns et al., 1995; Williamson et al., 1995). There are several possibilities in this discrepancy; 1) the use of excess DNA template or high number of PCR cycles may render the detection of such homozygous deletions difficult even in the presence of only small amounts of contaminating normal cells. 2) basically, this difference may depend on genetic, racial or environmental differences between Caucasians and Koreans. 3) technical problem in identifying homozygous deletion may be the explanation for these negative findings

Moreover, the ratio of heterozygosity at microsatellite marker in Korean people was lower than that of Caucasians. For example, heterozygosity ratio at D9S165 was 74% and 48% in Caucasian and Korean, respectively. Although the number of samples analyzed were limited, this difference may depend on genetic or racial differences, which remains to be subjected to further evaluation.

Further study of the genes involved in the development of bladder cancer, and the biochemical and physiological effect of mutations on these genes, should lead to a more detailed understanding of the tumorigenic process, with important implication for bladder cancer prevention, diagnosis, prognostication and treatment.

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