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Expression of p53 product in Chinese human bladder carcinoma

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Summary. Expression of p53 protein was examined in 67 cases of primary transitional cell carcinoma of the bladder and 6 normal controls using an immunohistochemical method on paraffin sections. Positive nuclear staining for p53 in malignant cells was found in 34 (51%) of the 67 cancer patients; no positive staining for p53 was detected in any of the normal controls or in the benign cells, including stromal and inflammatory cells, within the tumor tissue. There were 8 positive cases (33%) in 24 grade G1 tumors, 12 (48%) in 25 G2 tumors and 14 (78%) in 18 G3 tumors. p53 protein was detected positively in 14 (36%) of 39 superficial tumors (Tis–T1) and in 20 (71%) of 28 invasive tumors (T2–T4). Thus, positive staining for p53 was found more frequently in poorly differentiated tumors (chi-squared test: G3/G1+G2 $P<0.01$) and in invasive tumors (chi-squared test: T2–T4/Tis–T1 $P<0.01$). Expression of p53 was also closely associated with recurrence of tumors. Alterations in p53 expression may be of prognostic value in cases of bladder transitional cell carcinoma.

Key words: Bladder neoplasms – Gene product – Immunochemistry

p53, a 53-kDa nuclear protein, was first described as a cellular protein bound to large T antigen in cells transformed with simian virus 40 (SV40) [14, 16]. The human p53 gene is located on the short arm of chromosome 17. p53 has been found in all mammalian cells so far studied and in the toad *Xenopus*. Its normal function was obscure, but it was implicated in control of the cell cycle by regulating entry into and progression through the normal cycle [19]. Recent transfection studies showed that wild-type p53 has an inhibitory effect on cell proliferation and transformation and that a high incidence of lung, bone and lymphoid tumors was observed in transgenic mice overexpressing mutant alleles of p53 [15]. Current data suggest that wild-type p53 may function as a tumor suppressor gene by inhibiting transformation, while mutant p53 may act as an oncogene, probably by interfering with normal p53 function, acting in a dominant negative fashion [10] and cooperating with mutant *ras* genes in cellular transformation [26].

In normal cells and tissues wild-type p53 has a short intracellular half-life [24] and attains such a low steady-state level that it is not detectable histologically [2, 9]. In contrast, the mutant p53 protein, which forms complexes with heat shock protein 70 in the cytoplasm and also binds wild-type p53 [5], has an extended half-life [6, 29]; this results in an increase in its intracellular concentration and allows its detection by histochemical analysis. It is no means certain that increased stability of p53 is always associated with mutation of the p53 gene. It is possible that elevated levels of wild-type p53 protein accumulate due to another mechanism such as inactivation of an enzymatic pathway responsible for p53 degradation or stabilization by complex formation with a protein from a DNA tumor virus [11]. Another possibility is that significant expression of wild-type protein may occur in the setting of rapid, non-neoplastic cellular proliferation [20].

There have been studies of the molecular alterations in human bladder carcinoma, including allelic loss of chromosome 17 [23] and p53 gene mutation [7]. Recently CM-1, a polyclonal antibody against p53, has proved highly sensitive in staining paraffin-embedded tissue [21]. The aims of the present study were to detect p53 expression in human bladder cancer (paraffin-embedded) tissue by using an immunohistochemical method with CM-1 antibody and to explore any correlation between such expression and the prognosis of the carcinoma.

Materials and methods

Source of tissue samples

Samples were obtained from 67 patients (57 male, 10 female; mean age 59 years) with newly diagnosed primary transitional cell

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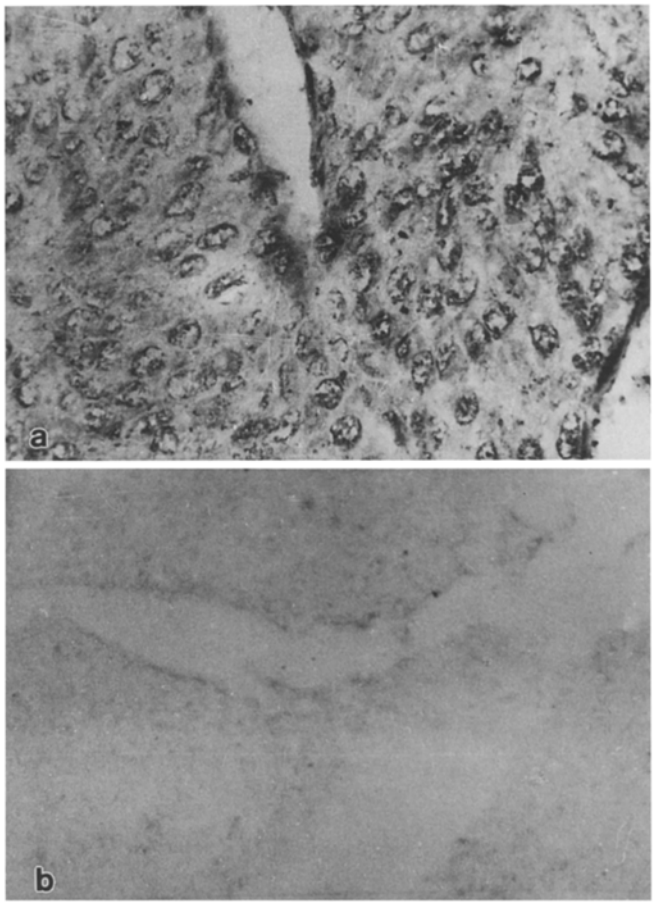

Fig. 1 a, b. Avidin-biotin-peroxidase immunohistochemical analysis of p53 expression in Chinese human bladder transitional cell carcinomas using CM-1 on formalin-fixed, paraffin-embedded tissues. **A** Positive nuclear staining in malignant cells; **B** no positive nuclear staining in malignant cells. (×800, not counterstained)

carcinoma of the bladder at Changhai Hospital in Shanghai between 1980 and 1987. Twenty-three of these cases were recurred within 5 years of resection; the other 44 cases did not recur during the follow-up time or were found to have recurred more than 5 years after resection. Tissue was obtained by means of open or transurethral bladder operations, fixed in 10% buffered formalin and embedded in paraffin. Six subjects with normal bladder tissue were used as controls. Patients were excluded from the study if their clinical records were incomplete or if they had received chemotherapy and radiotherapy before the operation. The tumors were staged at operation according to UICC criteria and were divided into two groups: stages Tis–T1 (39 cases) and stages T2–T4 (28 cases). Embedded sections of tissue were stained with hematoxylin and eosin and the histological grade of each tumor assessed by pathologists according to current WHO criteria. Twenty-four cases were found to be grade 1, 25 cases grade 2 and 18 cases grade 3.

Methods of immunohistochemical staining

p53 in tumor tissue was evaluated by immunohistochemical staining with CM-1 polyclonal antiserum raised against full-length recombinant human p53 protein produced in a bacterial system [21]. Formalin-fixed paraffin-embedded tissue sections were cut at 5 μm, placed on slides coated with poly-L-lysine solution and air-dried at room temperature. Immunohistochemical analysis was performed using the avidin-biotin-peroxidase method as outlined below. After dewaxing, inactivating endogenous peroxidase activity and blocking nonspecific binding with normal goat serum, the sections were incubated with CM-1 diluted in 1/1000 in phosphate-buffered saline (PBS), either overnight at 4°C or for 1 h at room temperature in a humidified chamber. Thereafter the sections were washed in PBS and localization of the primary antibody was achieved by subsequent sequential application of a biotinylated goat anti-rabbit antibody, an avidin-biotin complex conjugated to peroxidase (Vector ABC kit) and diaminobenzidine as chromogen [12]. Substitution of PBS or an irrelevant polyclonal antibody for the CM-1 solution provided negative controls. The control sections were used in each batch of slides to provide consistent immunolabelling results. Counterstaining of nuclei was usually omitted in order to obtain clear results, especially for black and white photography.

The intensity and pattern of p53 immunostaining were evaluated by the authors and scored as follows: negative staining (−), no staining was found; weak staining (+), less than 30% of tumor nuclei were stained; moderate or strong staining (++), more than 30% of tumor nuclei were stained. Statistical analysis of results was performed using the chi-squared test.

Table 1. Correlation between positive staining for p53 and histological grade of tumor

Grade	Staining for p53		Total
	Negative	Positive	
G1	16 (67%)	8 (33%)	24 (100%)
G2	13 (52%)	12 (48%)	25 (100%)
G3	4 (22%)	14 (78%)	18 (100%)

Results

Using the CM-1 polyclonal antiserum, nuclear p53 protein was detected in 34 (51%) of 67 cases of bladder transitional cell carcinoma. In 14 cases staining was either strong or moderate (++), and weak staining (+) was demonstrated in a further 20 cases. Reaction product marking the binding of CM-1 was confined to the nuclei of neoplastic cells (Fig. 1). At high antibody concentrations pale staining was occasionally observed in smooth muscle or blood vessels, but not at the dilution used. Staining within the tumor nuclei had a diffuse, granular or clumped appearance. Pale cytoplasmic staining was seen in some cases where there was nuclear staining. No appreciable staining was detected in tissue from the 6 normal control bladders or in stromal, inflammatory cells or non-cancer cells in the tumor tissue studied. The immunostaining pattern was not uniform in the positive tumors and the cells that stained were distributed without any polarity. No preponderance of staining was observed at either the luminal or the basal side of tumor tissues and the intensity of staining varied among the tumors.

The relationships between p53 expression and tumor clinical stage, histological grade and recurrence are shown in Tables 1, 2 and 3 respectively. Eight of 24 grade 1 tumors, 12 of 25 grade 2 tumors and 14 of 18 grade 3 tumors stained positively for p53. Overexpression of p53 protein was strongly associated with poorly differentiated

Table 2. Correlation between positive staining for p53 and clinical stage of tumor

Stage	Staining for p53		Total
	Negative	Positive	
Tis–T1	25 (64%)	14 (36%)	39 (100%)
T2–T4	8 (29%)	20 (71%)	28 (100%)

Table 3. Correlation between positive staining for p53 and tumor recurrence

	Staining for p53		Total
	Negative	Positive	
Recurrent[a]	7 (30%)	16 (70%)	23 (100%)
Non-recurrent[b]	26 (59%)	18 (41%)	44 (100%)

[a] Recurrent within 5 years of resection
[b] Non-recurrent during follow-up or recurrent more than 5 years after resection

bladder tumors (chi-squared test: G3/G1+G2, $P<0.01$; G3/G1, $P<0.01$; G3/G2, $P>0.05$; G2/G1, $P>0.05$; see Table 1). Positive staining for p53 was observed in 14 of 39 Tis–T1 tumors and in 20 of 28 T2–T4 tumors; the difference was highly significant (chi-squared test: T2–T4/Tis–T1, $P<0.01$; see Table 2). Sixteen of 23 tumors that recurred within 5 years of resection and 18 of 44 in the non-recurrent group stained positively for p53. This difference was also significant (chi-squared test: $P<0.05$; see Table 3).

Discussion

Oncogenes and tumor suppressor genes are believed to play a fundamental role in the development and progression of human neoplasms. Several oncogenes are implicated in human bladder carcinomas: *ras* mutation and altered *ras* expression have been described [8]; superficial or non-recurrent bladder neoplasms had significantly higher c-*myc* oncoprotein levels than invasive or recurrent ones [18]; and expression of c-erbB-2 protein was closely associated with degree of differentiation and clinical stage [32]. Tumor suppressor genes are thought actively to prevent the initiation or progression of numerous types of tumor of diverse origin, the central hypothesis being that the functions of tumor suppressors are abrogated or altered in some way in tumors compared with the normal tissue from which the tumor originates. Though the p53 gene was initially regarded as a classical oncogene, more recent findings have suggested that it may function normally as a tumor suppressor [1, 22].

Allelic deletions on the short arm of chromosome 17 have been observed in a variety of human tumors [17, 31, 33] and are frequently accompanied by mutation of the remaining allele [1, 22]. Mutant p53 expression has been identified as a frequent genetic change in a variety of human tumor types [22]. The molecular basis of overexpression of the p53 protein is becoming clear. Iggo and colleagues [13] found that in a series of primary lung tumors overexpression of the protein was associated with expression of point mis-sense mutant mRNA and loss of expression of the normal p53 mRNA. The studies of Bennett and colleagues [3] suggested that high-level expression of p53 protein correlates with the occurrence of mis-sense point mutations encoding amino acid substitutions in Chinese esophageal cancers. A similar finding came from the analysis of tumors and tumor cell lines from human lung, breast and colon tumors [2, 13, 27]. But this is currently a controversial point in many immunohistological studies dealing with p53 expression. Some researchers found that the percentage of p53 expression in superficial and invasive cancer is much higher when antibody-dependent methods are used than when molecular analysis is performed [30]. Borresen [4] reported that the association between p53 immunocytochemical findings and p53 DNA mutations is not clear-cut.

Investigation of quantitative or qualitative changes in the expression of oncogenes and anti-oncogenes may play a role in classifying tumors into different prognostic categories. Indeed this has been shown to be the case in bladder cancer for the c-*erbB*-2 and the c-*myc* genes [18, 32]. In breast and colorectal tumors the expression of p53 protein might well supplement traditional prognostic factors such as histological grade and clinical stage [25, 28]. In the present study, the staining for p53 protein was detected by using an immunohistochemical method with CM-1 polyclonal antiserum in 67 bladder transitional cell carcinomas. The frequency of positive staining was high (51%). Further molecular biological investigation is necessary to determine the exact incidence of point mutations of the p53 gene in bladder neoplasms. But from the clinical point of view one may speculate that such a high frequency of staining may provide useful prognostic information. The percentage of positive staining is higher in poorly differentiated and muscle-invasive bladder neoplasms than in well or moderately differentiated and superficial bladder neoplasms. These results suggest that tumors containing high levels of altered p53 protein may be at high risk of progression. Tumor markers that would provide the clinician with sufficient information to predict accurately the prognosis of a tumor would be of great interest. In this study it was also found that tumors which recurred within 5 years of resection has stained positively for p53 more frequently than tumors which did not recur during the follow-up time or recurred more than 5 years after resection. Expression of p53 protein may clearly be used as a marker of infiltrative activity or prognosis of bladder cancer. No positive staining for p53 was observed in normal control bladder tissues or in benign cells within tumor tissue. These data are consistent with the results of previous investigations that found that in normal tissues wild-type p53 protein has too short a half-life to accumulate sufficiently to be visualized by histological methods [2, 9].

But Lavigueur [15] found in transgenic animals that even high-level expression of mutant p53 does not produce transformation in some tissues, indicating that changes in p53 alone are not sufficient to cause neoplasms. In human bladder carcinogenesis several other genetic changes occur with high frequency, such as *ras* activation and allelic losses of chromosomes 9, 11 and 17 [8, 31]. The cumulative effect of these events seems more important in determining prognosis.

In conclusion, antibody CM-1 has potential use as a predictive marker for diagnosis of transitional cell carcinoma. Further investigations are needed in larger numbers of patients with complete clinical follow-up data, accompanied by studies of other oncogenes or tumor suppressor genes.

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References

1. Baker SJ, Fearon ER, Nigro JM, Hamilton SR, Preisinger AG, Jessup JM, Tuinen P van, Ledbetter DH, Baker DF, Nakamura Y, White R, Vogelstein B (1989) Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science 244:217
2. Bartek J, Iggo R, Gannon J, Lane DP (1990) Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. Oncogene 5:893
3. Bennett WP, Hollstein MC, He A, Zhu SM, Resau JH, Trump BF, Metcalf RA, Welsh JA, Midgley C, Lane DP, Harris CC (1991) Archival analysis of p53 genetic and protein alterations in Chinese esophageal cancer. Oncogene 6:1779
4. Borresen AL, Hovig E, Smith-Sorensen B, Malkin D, Lystad S, Andersen TI, Nesland JM, Isselbacher KJ, Friend S (1991) Constant denaturant gel electrophoresis (CDGE) as a rapid screening technique for p53 mutations. Proc Natl Acad Sci USA 88:8405
5. Eliyahu D, Goldfinger N, Pinhasi-Kimhi O, Shaulsky G, Skurnik K, Arai N, Rotter N, Oren M (1988) Meth A fibrosarcoma cells express two transforming mutant p53 species. Oncogene 3:313
6. Finlay CA, Hinds PW, Tan T, Eliyahu D, Oren M, Levine AJ (1988) Activating mutations for transformation by p53 produce a gene product that forms an hsc 70–p53 complex with an altered half-life. Mol Cell Biol 8:531
7. Fujimoto K, Yamada Y, Okajima E, Kakizoe T, Sasaki H, Sugimura T, Terada M (1992) Frequent association of p53 gene mutation in invasive bladder cancer. Cancer Res 52:1393
8. Fujita J, Yoshida O, Yuasa Y, Rhim JS, Hatanaka M, Aaronson SA (1984) Ha-*ras* oncogenes are activated by somatic alterations in human urinary tract tumours. Nature 309:464
9. Gannon JV, Greaves R, Iggo R, Lane DP (1990) Activating mutations in p53 produce a common conformational effect: a monocolonal antibody specific for the mutant form. EMBO J 9:1595
10. Herskowitz I (1987) Functional inactivation of genes by dominant negative mutations. Nature 329:219
11. Heuvel SJ van den, Laar T van, Kast WM, Melief CJ, Zantema A, Eb AJ van der (1990) Association between the cellular p53 and the adenovirus 5 E1B-55 kd proteins reduces the oncogenicity of Ad-transformed cells. EMBO J 9:2621
12. Hsu SW, Raine L, Fanger H (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. J Histochem Cytochem 29:577
13. Iggo R, Gatter K, Bartek J, Lane D, Harris AL (1990) Increased expression of mutant forms of p53 oncogene in primary lung cancer. Lancet I:675
14. Lane DP, Crawford LV (1979) T antigen is bound to a host protein in SV-40 transformed cells. Nature 278:261
15. Lavigueur A, Maltby V, Mock D, Rossant J, Pawson T, Bernstein A (1989) High incidence of lung, bone and lymphoid tumors in transgenic mice overexpressing mutant alleles of the p53 oncogene. Mol Cell Biol 9:3982
16. Linzer DIH, Levine AJ (1979) Characterisation of a 54K dalton cellular SV-40 tumor antigen present in SV-40 transformed cells and uninfected embryonal carcinoma cells. Cell 17:43
17. Mackay J, Elder PA, Steel CM, Forrest APM, Evans HJ (1988) Allele loss on short arm of chromosome 17 in breast cancer. Lancet II:1384
18. Masters JRW, Vesey SG, Munn CF, Evan GI, Wason JV (1988) c-*myc* oncoprotein levels in bladder cancer. Urol Res 16:341
19. Mercer WE, Avignolo C, Baserga R (1984) Role of the p53 protein in cell proliferation as studied by microinjection of monoclonal antibodies. Mol Cell Biol 4:276
20. Mercer WE, Beserga R (1985) Expression of the p53 protein during the cell cycle of human peripheral blood lymphocytes. Exp Cell Res 160:31
21. Midgley CA, Fisher CJ, Bartek J, Vojtesek B, Lane D, Barnes DM (1992) Analysis of p53 expression in human tumours: an antibody raised against human p53 expressed in *Escherichia coli.* J Cell Sci 101:183
22. Nigro JM, Baker SJ, Preisinger AG, Jessup JM, Hostetter R, Cleary K, Bigner SH, Davidson N, Baylin S, Devilee P, Glover T, Collins FS, Weston A, Modali R, Harris CC, Vogelstein B (1989) Mutations in the p53 gene occur in diverse tumour type. Nature 324:705
23. Olumi AF, Tsai YC, Nichols PW, Skinner DG, Cain DR, Bender LI, Jones PA (1990) Allelic loss of chromosome 17p distinguishes high grade from low grade transitional cell carcinomas of bladder. Cancer Res 50:7081
24. Oren M, Maltzman W, Levine AJ (1981) Post-translational regulation of the 54K cellular tumor antigen in normal and transformed cells. Mol Cell Biol 1:101
25. Ostrowski JL, Sawan A, Henry L, Wright C, Henry JA, Hennessy C, Lennard TJW, Angus B, Horne CHW (1991) p53 expression in human breast cancer related to survival and prognostic factors: an immunohistochemical study. J Pathol 164:75
26. Parada LF, Land H, Weinberg RA, Wolf D, Rotter V (1984) Cooperation between gene encoding p53 tumor antigen and *ras* in cellular transformation. Nature 312:649
27. Rodrigues N, Rowan A, Smith ME, Kerr IB, Bodmer WF, Gannon JV, Lane DP (1990) p53 mutations in colorectal cancer. Proc Natl Acad Sci USA 87:7555
28. Scott N, Sagar P, Stewart J, Blair GE, Dixon MF, Quirke P (1991) p53 in colorectal cancer: clinicopathological correlation and prognostic significance. Br J Cancer 63:317
29. Sturzbecher HW, Chumakov P, Welch WJ, Jenkins JR (1987) Mutant p53 protein bind hsp 72/73 cellular heat shock-related proteins in SV40-transformed monkey cells. Oncogene 1:201
30. Thompson AM, Anderson TJ, Condie A, Prosser J, Chethy U, Carter DC, Evans HJ, Steel CM (1992) p53 allele losses, mutations and expression in breast cancer and their relationship to clinico-pathological parameters. Int J Cancer 50:528
31. Tsai YC, Nichols PW, Hiti AL, Williams I, Skinner DC, Jones PA (1990) Allelic loss of chromosomes 9, 11 and 17 in human bladder cancer. Cancer Res 50:44
32. Wright C, Mellon K, Neal DE, Johnson P, Corbett IP, Horne CHW (1990) Expression of c-*erbB-2* protein product in bladder cancer. Br J Cancer 62:764
33. Yokota J, Wada M, Shimosato Y, Terada M, Sugimura T (1987) Loss of heterozygosity on chromosomes 3, 13 and 17 in small cell carcinoma and on chromosome 3 in adenocarcinoma of the lung. Proc Natl Acad Sci USA 84:9252