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Correlation between the expression of oncogenes *ras* and c-*erbB-2* and the biological behavior of bladder tumors

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Summary. Expression of the *ras* and the c-*erbB-2* oncogene products was investigated in 56 cases of human bladder transitional cell carcinoma and 6 samples of human normal bladder tissue using an immunohistochemical method. Thirty of the 56 cases of bladder tumor were found to be immunohistologically positive with the monoclonal anti-*ras* p21 antibody, while 19 of 56 cases were positive with the polyclonal anti-c-*erbB-2* oncoprotein antibody. All 6 controls were negative with both antibodies. The *ras* p21 positive staining was found more frequently in the well or moderately differentiated, superficial and non-recurrent tumors than in the poorly differentiated ($p < 0.01$), muscle invasive ($p < 0.05$) and recurrent tumors ($p < 0.01$), while the c-*erbB-2* gene product was more commonly detected in high-grade ($p < 0.01$), invasive ($p < 0.01$) and recurrent tumors ($p < 0.05$). Thus, the expression of either *ras* or c-*erbB-2* was closely associated with the histological grade, clinical stage and recurrence of bladder transitional cell carcinomas.

Key words: Bladder carcinomas – Gene expression – Oncogene *ras* – Oncogene c-*erbB-2*

It is commonly accepted that the development of cancer is a multistage process requiring the activation of more than one oncogene and/or the inactivation of anti-oncogenes [21]. The activation of particular oncogenes in human tumors, especially when accompanied by aberrant expression of their encoded proteins, may delineate subsets of tumors with specific biological and clinical behavior. Thus, the quantitative and qualitative study of oncogene expression in human cancer may not only provide insight into the mechanisms of cancer development and progression but also give a clinically useful characterization of the tumors.

There is increasing evidence to suggest that members of the *ras* gene family are among the most frequently identified activated oncogenes [3]. The genes of this family (H-*ras*, K-*ras* and N-*ras*) code for structurally and immunologically related proteins with a molecular weight of 21 kDa [14]. The *ras* p21 protein is localized in the internal part of the cell membrane, binds GTP and GDP, and plays a part in growth-signal transduction similar to that of G protein. Increased expression of the *ras* gene can induce neoplastic transformation of cells in vitro. Overexpression of p21 has been found in a variety of recent experiments [7].

The human proto-oncogene c-*erbB-2* or *HER-2/neu* encodes a 185 kDa transmembrane protein homologous to the epidermal growth factor receptor (EGF-R), suggesting a possible role as a membrane receptor [5, 18]. The protein encoded by c-*erbB-2* exhibits tyrosine kinase activity and appears to be a receptor for an as yet unidentified growth factor [1]. The c-*erbB-2* protein, with a molecular weight of 185 kDa, is widely expressed in cells derived from all three germ layers of the human fetus as well as the placenta. In adult tissues expression is localized to the membranes of epithelia, such as those of the gastrointestinal, respiratory, reproductive and urinary tracts, and breast [17]. Overexpression and amplification of c-*erbB-2* were found in a range of tumors [2, 16, 26, 27].

Viola investigated the expression of *ras* p21 in the urinary tract of bladder and found increased expression in all high-grade bladder carcinomas [24]. Gullick studied the expression of c-*erbB-2* in human bladder cancers [10]. The investigation of the expression of both *ras* and c-*erbB-2* products has not been reported in bladder neoplasms. We therefore studied the expression of both these oncogenes in human bladder carcinoma specimens using an immunohistochemical method, and correlated their expression with tumor histological grade, clinical stage and recurrence.

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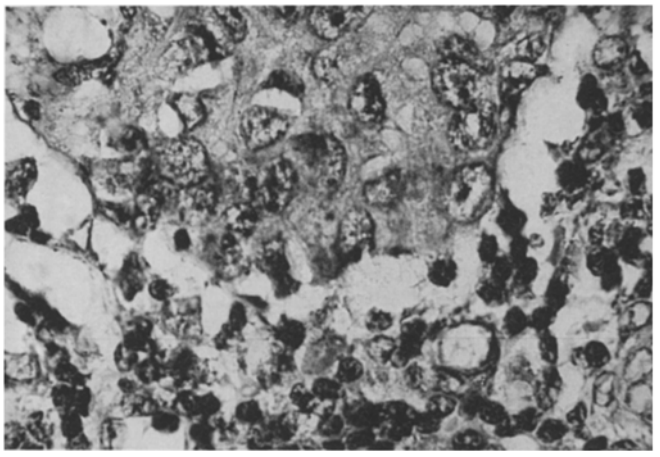

Fig. 1. Avidin-biotin-peroxidase immunohistochemical staining of formalin-fixed paraffin-embedded bladder transitional cell carcinoma using the SCI-oncogema 1 monoclonal antibody, showing cytoplasmic positivity for *ras*. ×330

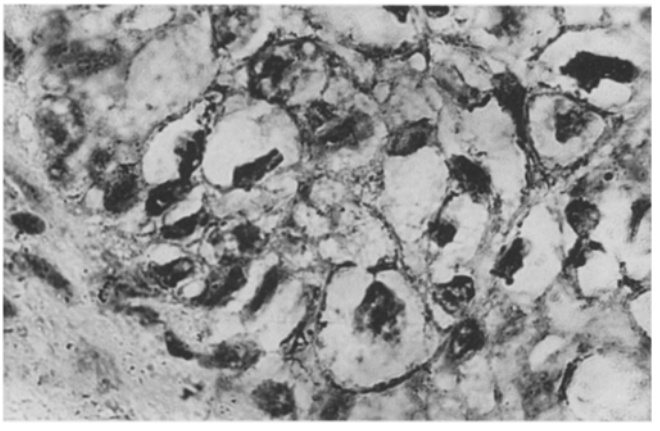

Fig. 2. Avidin-biotin-peroxidase immunohistochemical staining of formalin-fixed paraffin-embedded bladder transitional cell carcinoma using the 21N polyclonal antibody, showing membrane positivity for c-*erbB-2*. ×330

Materials and methods

Source of specimens

Fifty-six patients with primary bladder tumor who underwent an operation (open or transurethral) at Changhai Hospital, Shanghai, between 1980 and 1987 and 6 control cases of normal bladder tissue biopsy (obtained from transurethral or open resections of benign prostate) were included in this investigation. In all cases the resection specimens had been fixed in 10% buffered formalin and embedded in paraffin. Patients without complete clinical records or who had received chemotherapy and radiotherapy before the operation were excluded from the study. The ages of the tumor patients (48 males and 8 females) ranged from 26 to 78 years, with a mean age of 57 years. The patients were divided into two groups according to UICC criteria: 32 cases of Tis–T1 stage disease and 24 cases of T2–T4 stage. All sections of tumor specimens were stained with hematoxylin and eosin and examined and diagnosed by pathologists; all tumors were bladder transitional cell carcinomas. Tumors were classified according to the current World Health Organization standards: 19 as grade 1, 21 as grade 2, and 16 as grade 3.

Immunohistochemical staining

The *ras* p21 protein in human bladder tissue was evaluated by immunohistochemical staining analysis using the SCI-oncogema 1 rat monoclonal antibody (raised by Shanghai Cancer Institute), which reacts with the proteins encoded by the oncogenic members of the *ras* gene family [11, 19]. Oncoprotein encoded by c-*erbB-2* was evaluated by means of a polyclonal antibody raised against synthetic peptide 21N recognizing the C-terminus of the human c-*erbB-2* protein (residue 1243–1255) [9]. Formalin-fixed paraffin-embedded tissue sections were used for all studies. Five-micrometer sections were cut and placed on gelatin-coated slides. Slides were then baked for 60 min at 56°C. The avidin-biotin-peroxidase method was used for the immunohistological study. Endogenous peroxidase activity was eliminated by immersing the sections for 30 min in acidified methanol containing 3% H_2O_2 and then washed in phosphate-buffered saline (PBS). The sections were immunostained with either the SCI-oncogema 1 rat monoclonal antibody (diluted 1:60) or the anti-21N polyclonal antibody (diluted 1:20) and kept in a humidified chamber at 4°C overnight for approximately 16 h. The sections were then washed with PBS and immunostained with anti-rat IgG (for *ras* p21) and anti-rabbit IgG (for c-*erbB-2* oncoprotein), avidin-biotin-peroxidase and *p*-dimethylaminoazobenzene (DAB) sequentially as described previously [12]. Substitution of PBS for the primary antibody solution provided a negative control in each case. Positive control procedures involved immunolabeling with known positive tumor specimens (two colon carcinomas) expressing high levels of *ras* p21 and c-*erbB-2* oncoprotein. Positive control sections were included in each batch of slides to ensure consistent staining results. Immunolabeled sections were evaluated by two independent authors and scored as: (−) negative staining, (+) moderate staining or (++) intense staining, in relation to the amount of the enzyme product. Statistical analysis of the results was carried out using the Chi-square test.

Results

Thirty of 56 bladder tumors were found to be immunohistologically positive with the monoclonal anti-*ras* p21 antibody, while 19 of 56 cases were positive with the polyclonal anti-c-*erbB-2* product antibody. All 6 samples of normal control bladder tissue were negative with both the antibodies. In the group of bladder cancers, the patterns of distribution of *ras* p21 and the c-*erbB-2* oncoprotein were different: the *ras* protein was entirely cytoplasmic, while the c-*erbB-2* protein was found in plasma membrane (Figs. 1, 2). The staining intensities in positive cases varied between tumor tissues: all stained cells were bladder transitional carcinoma cells and were distributed diffusely without any polarity, while all the normal bladder transitional cells and stromal tissues were negative. There was no difference in staining between the luminal and basal sides of the tumor tissues. The stained and unstained cells were diffusely mixed without any regular pattern.

Thirteen of 19 grade G1 tumors, 14 of 21 G2 tumors, and 3 of 16 G3 tumors were positive for staining with SCI-oncogema 1, the anti-*ras* p21 antibody. Twenty-one of 32 stage Tis–T1 tumors and 9 of 24 T2–T4 tumors stained positively for *ras* p21. A correlation was observed between the presence of this antigen and the degree of tumor differentiation and clinical stage. In the well and moderately differentiated tumors cytoplasmic staining was more intense and common than in the poorly differentiated tumors (G1/G2 $p > 0.05$, G1/G3 $p < 0.01$, and G1+G2/

Table 1. Correlation between expression of *ras* p21 staining and histological grade

Grade	Negative	Positive		Total
	(−)	(+)	(++)	
G1	6 (31.6%)	11 (57.9%)	2 (10.5%)	19 (100%)
G2	7 (33.3%)	9 (42.9%)	5 (23.8%)	21 (100%)
G3	13 (81.2%)	3 (18.8%)	0	16 (100%)
Total	26 (46.4%)	23 (41.1%)	7 (12.5%)	56 (100%)

Table 2. Correlation between positivity for *ras* p21 staining and clinical stage

Stage	Negative	Positive		Total
	(−)	(+)	(++)	
Tis–T1	11 (34.4%)	15 (46.9%)	6 (18.7%)	32 (100%)
T2–T4	15 (62.5%)	8 (33.3%)	1 (4.2%)	24 (100%)
Total	26 (46.4%)	23 (41.4%)	7 (12.5%)	56 (100%)

Table 3. Correlation between positivity for the c-*erbB-2* oncoprotein and histological grade

Grade	Negative	Positive		Total
	(−)	(+)	(++)	
G1	15 (79.0%)	3 (15.8%)	1 (5.3%)	19 (100%)
G2	16 (76.2%)	4 (19.0%)	1 (4.8%)	21 (100%)
G3	6 (37.5%)	6 (37.5%)	4 (25.0%)	16 (100%)
Total	37 (66.1%)	13 (23.2%)	6 (10.7%)	56 (100%)

G3 $p < 0.01$: Table 1). In the superficial tumors *ras* p21 was more commonly detected than in muscle invasive tumors (Tis–T1/T2–T4 $p < 0.05$: Table 2). Four of 19 G1 tumors, 5 of 21 G2 tumors and 10 of 16 G3 tumors were positive for c-*erbB-2* . Thus, the c-*erbB-2* gene product was more frequently detected in G3 than in G1 and G2 tumors (G2/G1 $p > 0.05$, G3/G2 $p < 0.05$, G3/G1 $p < 0.05$, and G3/G1+G2 $p < 0.01$: Table 3). The expression of c-*erbB-2* was associated with the clinical stage and was more commonly detected in T2–T4 than in Tis–T1 tumors ($p < 0.01$) (Table 4).

The 56 cancer patient were divided into two groups. The first group (recurrent group) consisted of 15 patients who had recurrent disease within 5 years of surgery and the second group (non-recurrent group) consisted of 41 patients who had recurrent disease after more than 5 years or no recurrence. The *ras* p21 protein was more commonly detected in the non-recurrent group than in the recurrent group ($p < 0.01$), while plasma membrane staining of c-*erbB-2* product was more frequent in the recurrent group than in the non-recurrent group ($p < 0.05$) (Table 5).

Table 4. Correlation between positivity for the c-*erbB-2* oncoprotein and clinical stage

Stage	Negative	Positive		Total
	(−)	(+)	(++)	
Tis–T1	26 (81.3%)	5 (15.6%)	1 (3.1%)	32 (100%)
T2–T4	11 (45.8%)	8 (33.3%)	5 (20.8%)	24 (100%)
Total	37 (66.1%)	13 (23.2%)	6 (10.7%)	56 (100%)

Table 5. Correlation between expression of either *ras* or c-*erbB-2* oncoprotein and the recurrence of tumors

	ras		c-*erbB-2*	
	Negative	Positive	Negative	Positive
Recurrent (15 cases)	12 (80.0%)	3 (20.0%)	6 (40.0%)	9 (60.0%)
Non-recurrent (41 cases)	14 (34.1%)	27 (65.8%)	31 (75.6%)	10 (24.4%)
Total (56 cases)	26 (46.4%)	30 (53.6%)	37 (66.1%)	19 (33.9%)

Discussion

Activation of oncogenes has been implicated in the induction of in vitro malignant transformation of NIH3T3 cells with tumor and may be related to many human tumors. Increased expression of *ras* and c-*erbB-2* has been demonstrated immunohistochemically in a variety of human neoplasms [2, 7, 8, 26, 27]. In this study, the avidin-biotin-peroxidase immunohistochemical technique was used to assess the level of expression of *ras* and c-*erbB-2* oncoproteins in human bladder transitional cell carcinomas. Although this method provides only qualitative and semiquantitative data, it has the advantage of evaluating oncogene expression in each individual cell type and in many formalin-fixed paraffin-embedded sections for retrospective study.

In the present study overexpression of *ras* p21 was commonly found in bladder carcinoma tissues. There is increasing evidence to suggest that overexpression of *ras* oncogene is a common feature in the development of human tumors. Activation of the *ras* gene can be caused by overexpression or point mutation. The latter cannot be identified from immunoperoxidase studies. Although *ras* p21 is localized to the inner surface of the plasma membrane, the avidin-biotin-peroxidase reaction for p21 antigen appears as a generalized cytoplasmic stain [23,

24]. In this study, the more differentiated the bladder cancer the more commonly and intensely *ras* p21 was expressed. There were statistically significant differences in expression between tumors of grade G1, G2, and G3. This is in contrast to Viola's findings [24]. The SCI-oncogema 1 antibody reacts differently from most other antibodies [6, 7, 23], whereas there are some reports indicating that well and moderately differentiated neoplasms show a higher level of *ras* expression than poorly differentiated neoplasms [4, 8, 22]. Shen also found, using an immunohistochemical method with SCI-oncogema 1 antibody, that a high level of *ras* p21 overexpression was associated with well-differentiated but not with poorly differentiated lung carcinomas. They proposed that well-differentiatd tumor cells may have some specific functions and that overexpression of *ras* p21 may be involved in these [19].

In this investigation *ras* p21 was expressed in 21 of 32 (65.6%) superficial cancers (Tis–T1); this percentage was significantly higher than that in invasive cancers (37.5%) (T2–T4). These findings suggest that elevation of *ras* p21 may be a common event in the early stages of bladder tumors. There are reports suggesting that in the early stage of tumors *ras* oncogene activation occurs, and tumor progression may lead to a more autonomous population of cells in which other growth factors supplant the role of this *ras* oncoprotein [8, 13].

The finding of a correlation between *ras* expression and prognosis would be of interest [6, 7]. Our results indicate that there is a higher incidence of *ras* p21 expression in patients with non-recurrent disease than in those with recurrent disease ($p < 0.01$).

Although the c-*erbB-2* product is hardly expressed in normal human adult tissues, various human tumors exhibit overexpression of the c-*erbB-2* oncoprotein as well as amplification of the gene [2, 16, 26, 27]. The expression of c-*erbB-2* in bladder cancers had been reported either to occur infrequently [15] or to be fairly common [10, 25]. In this study, overexpression of c-*erbB-2* was observed in 19 of 56 (33.9%) bladder tumors, which was in agreement with the latter reports. There have been an increasing number of reports suggesting that amplification and/or overexpression of c-*erbB-2* is associated with tumor relapse and survival [20]. In this investigation we found more cases with positive immunostaining for c-*erbB-2* product among the higher grade, muscle-invasive and recurrent tumors. High-grade bladder tumors exhibit a more malignant and invasive biological behavior, and have a poorer prognosis than low-grade bladder neoplasms. This suggests that tumor cells expressing c-*erbB-2* oncoprotein may have a growth advantage over other neoplastic cells and may evolve in more aggressive cancers.

The expression of *ras* p21 was shown to be different from that of the c-*erbB-2* oncoprotein. The percentage of positive-staining cases amongst all the tumors investigated and the distribution of positive cells in various histological grades and stages of tumors were different for the two oncoproteins. This suggests that various oncogenes may be expressed in tumors of different histological grade and may play different roles in the development and progression of the tumors.

In conclusion, the expression of either *ras* or c-*erbB-2* oncoproteins was related to the histological grade, clinical stage and recurrence of bladder transitional cell carcinomas. Further studies are required to investigate the correlation between the expression of *ras* and c-*erbB-2* and tumor metastasis and survival of the bladder carcinoma patients, by increasing the number of samples of cancers for which complete clinical follow-up data are available.

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