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p53 and *erb*B-2 protein overexpression are associated with early invasion and metastasis in bladder cancer

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Abstract. Overexpression of p53 and *erb*B-2 was studied by immunohistochemistry in formalin-fixed tissue samples of 179 patients with transitional cell carcinoma of the urinary bladder. p53 immunostaining was strongly correlated with tumour stage ($P<0.0001$). This was driven by a marked difference in p53 expression between pTa (37% positive) and pT1 (71%) tumours, while there was no difference between pT1 and pT2–4 tumours. Similarly, a strong overall association between p53 expression and grade ($P<0.0001$) was driven by a marked difference between grade 1 (28%) and grade 2 tumours (71%), and there was no significant difference between grade 2 and grade 3 tumours. Surprisingly, the frequency of *erb*B-2 overexpression was higher in pT1 tumours (74%) than in either pTa (49%; $P=0.0265$) or pT2–T4 (56%; $P=0.0645$) tumours. Both p53 and *erb*B-2 expression was also associated with metastasis. Metastases were found in 77% of patients with p53 positive primary tumours, but in only 50% of the patients with p53 negative primary tumours ($P=0.022$). Metastases were found in 66% of patients with *erb*B-2 positive primaries, but in only 37% of the *erb*B-2 negative primaries ($P=0.020$). Of 32 patients with positivity for both p53 and *erb*B-2, 84% developed metastases, as compared to 49% of patients with positivity for either one or neither positive ($P=0.002$). We conclude that both p53 and *erb*B-2 overexpression are associated with early invasion in bladder cancer. Furthermore, p53 and *erb*B-2 may be important predictors for metastasis.

Key words: Bladder neoplasm – p53 – *erb*B-2 – Metastasis

Introduction

Transitional cell carcinomas (TCC) of the bladder vary greatly in their biological behaviour. Between 50 and 70% of superficial TCC (pTa/T1) will eventually recur and 10–20% will progress to muscle invasion (pT2 or greater). About 50% of patients with muscle invasive tumours subsequently develop metastases. Tumour recurrence or progression cannot be predicted by histological criteria alone. Since better estimates of eventual clinical outcomes for individual bladder cancers might significantly modify further treatment, there is a considerable need for better prognostic evaluations.

p53 is a cell-cycle-related DNA binding protein, thought to be involved in transcription regulation (Steinmeyer and Deppert 1988). It is usually classified as a tumour suppressor gene, since in many tumours one p53 allele is mutated and the other allele deleted (Weinberg 1991). Mutation of the p53 gene is, in most cases, detectable by immunohistochemistry (Bartek et al. 1990; Iggo et al. 1990; Marks et al. 1991). This is because most p53 mutations lead to a significantly longer half-life of the protein, causing an apparent overexpression (Levine 1989). p53 protein overexpression correlates with clinicopathological as well as prognostic parameters in several malignant tumours (Davidoff et al. 1991; Morkve and Laerum 1991; Scott et al. 1991).

The c-*erb*B-2 proto-oncogene codes for a 185000-kDa transmembrane phosphoglycoprotein (*erb*B-2 protein), which is closely related to the epidermal growth factor receptor (Hsuan et al. 1989). Overexpression of *erb*B-2 protein is a frequent and prognostically relevant event in a variety of human cancers (McCann et al. 1991; Stenman et al. 1991; Ueda 1992; Yonemura et al. 1991).

Several reports have described overexpression of p53 and *erb*B-2 in bladder cancer (Asamoto et al. 1990; Coombs et al. 1991; McCann et al. 1990; Moriyama et al. 1991; Serio 1991; Wood et al. 1991; Wright et al. 1991; Zhau et al. 1990). Immunohistochemical studies in bladder cancer have all shown p53 overexpression in more than 50% of cases (Sarkis et al. 1993; Wright et al. 1991) and p53 protein expression has most recently been suggested as a prognostic marker in pT1 TCC, indicating a higher risk for tumour progression (Sarkis

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et al. 1993). However, previous reports of *erb*B-2 overexpression are conflicting in bladder cancer. The prevalence of *erb*B-2 overexpression has been reported between 2% and 70% (Asamoto et al. 1990; Coombs et al. 1991; McCann et al. 1990; Moriyama et al. 1991; Serio 1991; Wood et al. 1991; Wright et al. 1991; Zhau et al. 1990). Both p53 and *erb*B-2 have recently been implicated in tumour metastasis, including studies demonstrating a higher prevalence of metastasis in p53 positive breast cancers (Davidoff et al. 1991) and *erb*B-2 positive colon cancers (Omori 1991).

The purposes of this study were: (1) to determine the prevalence of p53 and *erb*B-2 overexpression in TCC; (2) to determine the correlation of overexpression of both markers with grade, stage, and with each other; and (3) to determine whether p53 and/or *erb*B-2 expression is predictive for the development of metastases in muscle invasive TCC.

Materials and methods

Formalin-fixed, paraffin-embedded, tissue samples of 178 patients with TCC were collected from the files of the Department of Pathology, University of Basel, Switzerland. The collection consisted of all specimens of pT2–T4 invasive tumours examined between 1985 and 1991 ($n=63$), all biopsies of primary tumours of pT2–4 TCC prior to 1985 from patients with subsequent autopsy performed between 1980 and 1991 ($n=37$), 47 randomly selected transurethral biopsies of non-invasive TCC (pTa), 31 randomly selected transurethral biopsies of minimally invasive TCC (pT1) and normal bladder urothelium from autopsies of 10 patients without a history of bladder tumour.

All immunohistochemical examinations were performed on 5-μm sections using the avidin-biotin-enhanced immunoperoxidase technique (Hsu et al. 1981). For detection of p53 protein a rabbit polyclonal antibody CM1 (1:4000; Medac, Hamburg, Germany) was used. CM1 recognizes wild-type and mutant p53 protein (Visakorpi et al. 1992). For detection of *erb*B-2 overexpression a polyclonal rabbit antibody (1:1000; Dako, Glostrup, Denmark) was used. For both antibodies the optimal titre was interpreted as the dilution that gave clearly identifiable membrane (*erb*B-2) or nuclear (p53) staining, minimal cytoplasmic staining and negligible background on positive breast cancer samples. Sections were incubated with antibodies at 4° C overnight. Sections were then incubated for 30 min with normal goat serum. Subsequently a biotinylated goat anti-rabbit antibody (1:2000; Vector, Burlingame, Calif.) was used, followed by staining with streptavidin-biotin-peroxidase complex (ABC-Elite, Vector). The peroxidase reaction was developed with 3'3-diaminobenzidine (Jansen, Belgium) as chromogen. To enhance the reaction, an additional incubation in osmium tetroxide was performed for 2 min and the sections were counterstained with haematoxylin. To assess the specificity of the reaction in all cases, positive (immunoreactive breast cancer tissue) and negative (primary antibody substituted by phosphate-buffered saline) controls were used.

Tumour staging was performed according to the criteria of the International Union Against Cancer (UICC 1978). Because of the limitations of transurethral biopsies in determining the degree of invasion in higher stage TCC accurately, all tumours showing muscle invasion were included in one group (T2–T4). All slides were reviewed by one investigator (H.M.). Grading was performed according to the World Health Organization (Mostofi 1973). The degree of p53 and *erb*B-2 immunoreactivity in each tumour was classified into one of five categories by estimating the percentage of tumour cells staining: 0, no staining; 1, 1–10% of tumour cells staining; 2, 10–50% of tumour cells staining; 3, 51–80% of tumour cells staining; 4, >80% of tumour cells staining. For *erb*B-2 only membrane staining, and for p53 only nuclear staining, was considered.

Contingency table analysis was performed for comparisons between p53 staining, *erb*B-2 staining, grade, stage and metastasis.

Results

Of the 178 tumours examined, 47 were pTa, 31 pT1 and 100 pT2–4. Forty-six tumours were grade 1, 72 grade 2 and 60 grade 3. Data about metastases were available in 72 pT2–4 tumours. In 48 pT2–4 tumours metastasis to distant organ sites or lymph nodes was found in subsequent autopsies or biopsies [follow up 25 (mean) ± 30 (SD) months]. The absence of metastases was shown by autopsy in another 24 cases (follow up 18 ± 12 months).

Immunostaining was interpretable for p53 in 178 and for *erb*B-2 in 177 biopsies. Histologically normal urothelium adjacent to tumours was also examined in 37 cases.

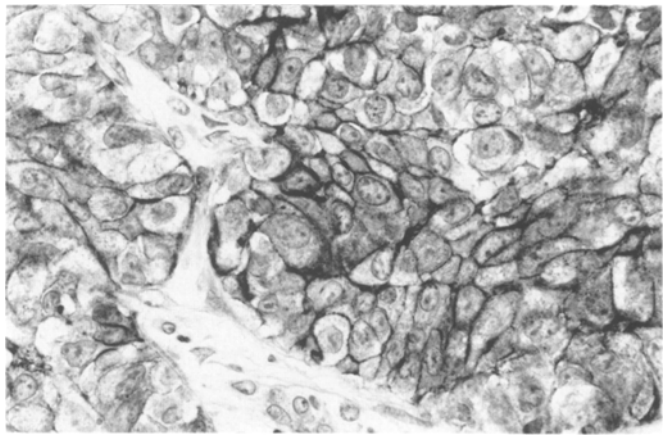

Fig. 1. *erb*B-2 immunostaining. Grade 3 bladder cancer with distinct membraneous staining for *erb*B-2. Haematoxylin counterstaining, ×400

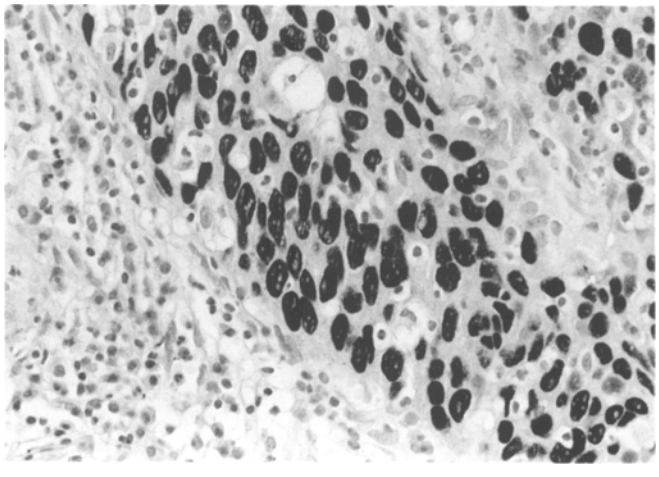

Fig. 2. p53 immunostaining. Grade 2 bladder cancer with strong positivity for p53 staining in >80% of tumour cells. Haematoxylin counterstaining, ×400

Table 1. Expression of p53 and *erb*B-2 in bladder cancer

Fraction of positive cells	Fraction of tumours (%)	
	p53 ($n=178$)	*erb*B-2 ($n=177$)
0	38	33
1–10%	16	10
11–50%	17	23
51–80%	9	17
>80%	20	17

p53 positivity was not seen in histologically normal urothelium. Slight but distinct *erb*B-2 positivity was seen in less than 10% of cells in normal-appearing urothelium in 6 of 37 patients with bladder cancer. No *erb*B-2 staining was seen in the remaining 31 cases or in 10 samples of normal urothelium from autopsies of patients without bladder cancer. Figures 1 and 2 show representative examples of tumours with *erb*B-2 and p53 positivity. The extent of immunostaining observed for both antibodies in tumours is summarized in Table 1. For statistical analysis, a cutoff of 1% positive cells was used for p53 because background nuclear staining was almost nonexistent, and low p53 expression levels have been reported in solid tumours having p53 mutations (Bodner et al. 1992; Cunningham et al. 1992). A higher cutoff value of 10% was used for *erb*B-2 staining to minimize the risk of false positivity.

The relationship of immunostaining with tumour stage and grade is shown in Table 2. A strong association between p53 and tumour stage was present ($P<0.0001$). This association was caused by the difference between stage pTa (37% positive) and pT1 tumours (71%, $P=0.0026$), while there was no difference between pT1 and pT2–T4 (both 71%). p53 immunostaining was also strongly correlated with tumour grade ($P<0.0001$). This was driven by a strong difference in positive p53 staining between grade 1 (28%) and grade 2 tumours (71%, $P<0.0001$), whereas there was no significant difference between grade 2 (71%) and grade 3 tumours (77%, $P=0.38$).

Overall the association between *erb*B-2 staining and tumour stage was not significant ($P=0.08$). Interestingly stage pT1 tumours showed higher *erb*B-2 expression (74%) than either pTa tumours (49%, $P=0.0265$) or pT2–T4 tumours (56%, $P=0.0645$). No significant difference in *erb*B-2 expression was found between pTa and pT2–T4 tumours. The frequency of *erb*B-2 positivity was highest for grade 2 tumours (66%), while a smaller fraction of positive tumours was found for grade 1 (48%, $P=0.053$) or grade 3 (54%, $P=0.178$) tumours. There was a significant association between *erb*B-2 and p53 immunostaining. *erb*B-2 was positive in 66% of the p53 positive tumours, but in only 43% of the p53 negative tumours ($P=0.003$). Finally, there was a strong correlation, as expected, between tumour grade and stage ($P=0.0001$).

The relationship between p53 and *erb*B-2 staining to the subsequent development of metastases is shown in

Table 2. Immunostaining and histological phenotype

Immunostaining		Number of tumours (%)					
		Stage			Grade		
		pTa	pT1	pT2–4	1	2	3
p53	negative	30 (64%)	9 (29%)	29 (29%)	33 (72%)	21 (29%)	14 (23%)
	positive	17 (36%)	22 (71%)	71 (71%)*	13 (28%)	51 (71%)	46 (77%)*
*erb*B-2	negative	24 (51%)	8 (26%)	44 (44%)	24 (52%)	25 (35%)	27 (46%)
	positive	23 (49%)	23 (74%)	55 (56%)**	22 (48%)	47 (65%)	32 (54%)**

* $P<0.0001$ (three group contingency table analysis); ** $P=0.08$ (three group contingency table analysis); *** $P=0.15$ (three group contingency table analysis)

Table 3. Immunostaining and subsequent metastasis

Immunostaining		Number of tumours (%)		P (χ^2 test)
		No metastasis	Metastasis	
p53	negative	12 (50%)	12 (50%)	
	positive	11 (23%)	37 (77%)	0.02
*erb*B-2	negative	15 (63%)	9 (37%)	
	positive	16 (34%)	31 (66%)	0.02
p53/*erb*B-2	both negative	6 (55%)	5 (45%)	
	*erb*B-2 or p53 positive	13 (46%)	15 (54%)	
	*erb*B-2 and p53 positive	5 (16%)	27 (84%)	0.002[a]

[a] Tumours positive for both *erb*B-2 and p53 vs tumours positive for either one or neither

Table 3. The primary tumours in these patients were all stage pT2–T4. Subsequent metastases were defined by biopsy or autopsy. Both *erb*B-2 and p53 staining were associated with metastases. p53 positivity was seen in 77% of tumours with metastases as compared to 50% in the non-metastatic group ($P=0.0222$). *erb*B-2 immunostaining was found in 68% of metastatic tumours, while only 35% of the non-metastatic tumours were *erb*B-2 positive ($P=0.0202$). Interestingly metastases were seen in 84% of tumours positive for both p53 and *erb*B-2, but only in 54% of those staining positively for either p53 or *erb*B-2 and in 45% not staining for any antibody ($P=0.0034$). No association was found between tumour grade and subsequent metastases (data not shown).

Discussion

Strong associations were found between p53 expression, *erb*B-2 expression, tumour stage, tumour grade and metastases in transitional cell carcinoma of the urinary bladder. Analysis of a large number of pTa and pT1 tumours revealed a major difference in p53 expression between non-invasive pTa tumours (36%) and minimally invasive pT1 tumours (71%), whereas there was no difference in p53 expression between pT1 and muscle invasive tumours (71%). These data suggest that p53 is associated with early invasion in bladder cancer. Our findings are consistent with previous reports showing a high prevalence of p53 alterations in pT2–4 tumours and pT1 tumours. For example, Dalbagni et al. (1992) found p53 protein expression in 6 of 11 pT1 TCC and 26 of 39 pT2–T4 TCC, Sidransky et al. (1991) reported p53 gene mutations by DNA-sequencing in 11 out of 18 advanced TCC, and Sarkis et al. (1993) found 81% p53 positive cases among 43 pT1 TCC. Other studies have concluded that p53 expression or mutation is a characteristic feature of muscle invasive tumours (Fujimoto et al. 1992; Wright et al. 1991). However, this may be due to the fact that in these studies pT2–T4 TCC were compared with superficial TCC, consisting of both pTa and pT1 lesions.

It has recently been suggested that p53 may play a cell cycle check-point role, leading to an S-phase delay in cells with genetic damage in order to provide extra time for DNA repair (Lane 1992). Thus, tumours with mutant p53, having positive immunostaining, may be expected to accumulate increased genetic aberrations. Since *erb*B-2 overexpression may be the result of genomic alterations an association between p53 and *erb*B-2 expression, as observed by Wright et al. (1991), was expected and was confirmed in this study. A key role for the p53 gene in tumour development is also consistent with the observed strong association of p53 immunopositivity with grade and stage in this study, given the known high prevalence of DNA aneuploidy (Tribukait 1987) and specific genomic lesions in bladder tumours of advanced grade and stage (Olumi et al. 1990; Presti et al. 1991; Tsai et al. 1990).

There is little agreement on the significance of *erb*B-2 overexpression in bladder cancers. Previous reports have compared *erb*B-2 overexpression in muscle invasive (pT2–T4) and superficial (pTa-T1) TCC and found (Moriyama et al. 1991), or did not find (Serio 1991; Wright et al. 1991), a significantly higher frequency of *erb*B-2 expression in pT2–T4 tumours. The reported prevalence ranges from 2% to 74% (Asamoto et al. 1990; Coombs et al. 1991; McCann et al. 1990; Moriyama et al. 1991; Serio 1991; Wood et al. 1991; Wright et al. 1991; Zhau et al. 1990). This might be due in part to different tissue preparations as well as to the use of different antibodies [pAb1 (Zhau et al. 1990), mAb3 (Coombs et al. 1991; Zhau et al. 1990), NCL-CB11 (Wright et al. 1991), 3B5 (Serio 1991), SV2-61 (Asamoto et al. 1990; Moriyama et al. 1991), 21N (Coombs et al. 1991; McCann et al. 1990). Different definitions of *erb*B-2 positivity, variously described as "any positivity" (Coombs et al. 1991; Serio 1991; Wright et al. 1991; Zhau et al. 1990), "distinct positivity" (Asamoto et al. 1990; Moriyama et al. 1991), or "membranous positivity" (Wood et al. 1991; Wright et al. 1991) might also contribute to these controversial results.

In this study a cutoff level of 10% positive tumour cells was used to define *erb*B-2 positivity. This was to avoid false positivity as a result of staining artefacts due to crush or edge effects. The selected cutoff level was also supported by the fact that none of the 47 histological normal urothelia examined showed more than 10% *erb*B-2 positive cells. However, we cannot rule out true *erb*B-2 positivity in 6 of these normal appearing urothelia, especially since genetic damage may be present in apparently normal cells of bladder cancer patients as a result of a "field defect". Surprisingly, the highest frequency of *erb*B-2 overexpression was seen in pT1 tumours (74%). This was significantly higher than in pTa tumours (49%), whereas only borderline significance was found if compared to pT2–4 tumours (56%). This finding is comparable to the situation in ductal breast cancer, where *erb*B-2 overexpression is more frequent in early stage neoplasia (ductal carcinoma in situ), whereas higher stage tumours have less frequent *erb*B-2 overexpression (Somerville et al. 1992). Two different mechanisms might explain the higher frequency in pT1 tumours. First, it is possible that pT2–T4 tumours are a heterogeneous group; some may develop from pT1 lesions (mostly *erb*B-2 positive), and others may originate de novo or evolve from other lesions, such as carcinoma in situ. *erb*B-2 positivity may be less frequent in invasive tumours arising de novo or evolving from carcinoma in situ. A second explanation for high *erb*B-2 positivity in pT1 neoplasms is that *erb*B-2 overexpression is important in a distinctive early step of tumour development situated at the transition from non-invasive to the invasive phenotype. After this stage, *erb*B-2 overexpression might no longer be advantageous for the tumour and some TCC might therefore revert to *erb*B-2 negativity.

Several previous studies have proposed that *erb*B-2 (Kallioniemi et al. 1991; Omori 1991) and/or p53 (Davidoff et al. 1991; Kim et al. 1991) may play a role in tumour metastasis. In this study a higher rate of eventual

metastasis was seen for both p53 and *erb*B-2 positive tumours. Moreover, tumours expressing both proteins were especially likely to develop metastases (27 of 32 lesions, 85%). The highly malignant phenotype of tumours expressing both *erb*B-2 and p53 is consistent with a previous report of Isola et al. (1992), showing particularly high tumour proliferation in breast cancers positive for both p53 and *erb*B-2.

Despite reports of prognostic significance of minimal invasion in bladder cancer (Abel et al. 1988), pTa and T1 tumours are still frequently grouped together as "superficial" bladder cancers, and thought to have similar prognosis (Flamm and Havelec 1990; Raghavan et al. 1990). Whereas there was no difference in p53 expression between pT1 and pT2–4 tumours, major differences were found in expression between pTa and pT1 tumours in this study, suggesting relevant biological differences between pTa and pT1 lesions. Given their frequent overexpression in early stage bladder cancer p53 and *erb*B-2 protein expression are promising candidates as prognostic markers for tumour progression. The combination of both markers may identify a subgroup of muscle invasive tumours with high risk for metastasis.

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Note added in proof

In the light of recent reports showing *erb* B-2 expression in normal urothelium and p53 expression in normal squamous epithelium of the uterine cervix all cases with normal urothelium adjacent to tumours were carefully reviewed after finishing the study. It showed that focal *erb* B-2 expression not limited to the upper cell layer was seen in a few urothelial fragments which must be considered histologically normal. There were also cases ($<20\%$) showing some p53 positive cells ($<10\%$) in normal urothelium.