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p53 and c-*erb*B-2 expression in schistosomal urinary bladder carcinomas and schistosomal cystitis with premalignant lesions

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Abstract Immunoreactivity for *p53* and c-*erb*B-2 proteins was studied in 31 schistosomal urinary bladder carcinomas and 21 cases of schistosomal cystitis with hyperplastic, metaplastic and/or dysplastic (premaligant) lesions. The results were compared with 30 carcinomas and 21 premalignant lesions of the urinary bladder without schistosomiasis. Abnormal nuclear *p53* protein accumulation was found in 17/31 schistosomal and in 15/30 non-schistosomal carcinomas and in 8/21 schistosomal cystitis with premalignant lesions of which five showed hyperplasia. No case of non-schistosomal hyperplasia or squamous metaplasia examined showed *p53*-positivity. In non-schistosomal carcinomas *p53* positivity was significantly associated with tumour grade (grade I-II vs grade III tumours: P=0.021) and greater age (P=0.004) while in schistosomal carcinomas no such associations were found. Cytoplasmic membrane-bound positivity for c-*erb*B-2 oncoprotein was found in comparable percentages in schistosomal and non-schistosomal bladder carcinomas (10%), and in both groups was co-expressed with *p53*. *p53* gene alteration is an important event in the development of both schistosomal and non-schistosomal bladder carcinoma.

Key words *p53* · c*erb*B-2 · Bladder carcinoma
Schistosomiasis

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Introduction

The most common genetic change in human cancers is the presence of *p53* gene mutations (Nigro et al. 1989; Hollstein et al. 1991). The *p53* gene encodes a nuclear phosphoprotein which plays an important role in the control of DNA transcription and replication (Steinmeyer et al. 1990; Farmer et al. 1992; Vogelstein and Kinzler 1992). In transfection experiments it has been shown that wild type *p53* can inhibit malignant transformation (Eliyahu et al. 1989; Finlay et al. 1989) and mutations of the *p53* gene can lead to a loss of its tumour suppressor effect (Iggo et al. 1990) and may lead to a loss of production of the *p53* protein or to production of a mutated protein (Iggo et al. 1990; Bartkova et al. 1991; Midgley et al. 1992). Both mutation and binding to viral and cellular proteins may lead to accumulation within the nuclei of the malignant cells to amounts that can be detected by immunohistochemistry and a high correlation between *p53* gene mutations and positive *p53* immunohistochemistry in tumour tissue has been found (Maestro et al. 1992; Vähäkangas et al. 1992).

The c-*erb*B-2 proto-oncogene encodes a membrane-bound glycoprotein with tyrosine kinase activity (Akiyama et al. 1986). It has sequence similarities with the epidermal growth factor receptor (Yamamoto et al. 1986). Amplification and overexpression of c-*erb*B-2 have been found in different types of carcinomas (Slamon et al. 1989; Voravud et al. 1989; Hall et al. 1990; Singleton and Strickler 1992). In breast and ovarian carcinomas, c-*erb*B-2 activation is association with a poor prognosis (Slamon et al. 1989).

Bladder cancer is the twelfth most common cancer globally (Koroltchouk et al. 1987). There are two major types, the first associated with exposure to certain occupational and environmental carcinogens and the second with *Schistosoma haematobium* infection of the urinary bladder (Koroltchouk et al. 1987). The high incidence of carcinoma of the urinary bladder in patients with urinary schistosomiasis is well recognized (McCully et al. 1976; Lucas 1982; Koroltchouk et al. 1987). Approximately

50–80% of malignant urinary bladder tumours associated with schistosomiasis are squamous cell carcinomas, less frequently transitional cell carcinomas or adenocarcinomas are found (McCully et al. 1976; Lucas 1982).

p53 immunohistochemical positivity can be detected in a high percentage of bladder carcinomas (Olumi et al. 1990; Sidransky et al. 1991; Wright et al. 1991; Soini et al. 1993). The association of schistosomiasis with *p53* has not been studied and we thus compared two types of bladder tumours and premalignant lesions, one with a history of schistosomiasis. *p53* immunohistochemistry findings were compared with c-*erb*B-2 cytoplasmic membrane-bound positivity.

Materials and methods

Thirty-one schistosomiasis-associated carcinomas of the urinary bladder and 21 cases of schistosomal cystitis associated with hyperplasia, squamous metamplasia and/or dysplasia, were collected from the files of the Department of Pathology, Zagazig University Central Hospital, Egypt (Tables 1 and 2). There were 20 men (mean age±SD: 50.8±7.8 years) and 11 women (46.9±5.0 years) in our series of urinary bladder carcinomas with a history of schistosomiasis (Table 1). As a control material, 30 cases of non-schistosomal urinary bladder carcinomas and 21 of hyperplasia, squamous metaplasia or dysplasia of the urinary bladder were collected from the files of the Department of Pathology, Oulu University Central Hospital, Finland. There were 16 men (mean age±SD: 67.4±11.0 years) and 14 women (80.6±6.5 years) in the non-schistosomal urinary bladder carcinoma material (Table 3).

All the material included both cystectomy specimens and surgical biopsies. The diagnosis and the grades of the tumours were based on the WHO classification of urinary bladder tumours (Mostofi et al. 1973). The dysplasias were graded into mild, moderate or severe according to Nagy et al. (1982). Hyperplasia was recognized on the basis of the thickening of the epithelium without cellular atypia. Areas of urinary bladder metaplasia were identified as sites with squamous epithelium without cellular atypia. All the tissue materials used in this investigation had been fixed in 10% neutral formalin and embedded in paraffin.

For immunocytochemistry 5 µm sections were cut from the specimens and placed on slides coated with poly-L-lysine solution (Sigma Chemicals, St Louis, Mo.). The specimens were then dewaxed in xylene and rehydrated in graded alcohol. The endogenous peroxidases were blocked by immersing the sections for 20 min in 0.1% hydrogen peroxide in absolute methanol. The nonspecific binding was blocked by incubating the slides in 20% fetal calf serum in phosphate buffered saline (PBS) for 20 min. The avidin-biotin-complex (ABC) method was used (Hsu et al. 1981). With *p53*, the sections were first incubated overnight at 4° C with a primary polyclonal rabbit anti-human *p53* antibody CM-1 at a dilution of 1: 1000 (Midgley et al. 1992) followed by a secondary biotinylated anti-rabbit IgG (dilution 1: 100; Dakopatts, Copenhagen, Denmark) and the ABC (Dakopatts). Careful rinses were

Table 1 *p53* and c-*erb*B-2 protein expression in urinary bladder carcinomas with a history of schistosomiasis (*F* female, *M* male)

Case	Sex	Age (years)	Grade of tumour	History of schistosomiasis (years)	*p53* (number of nuclei/ intensity)	c-*erb*B-2 (number of cells)
Squamous cell carcinomas						
1	M	43	I	12	+(c)/+	–
2	M	39	I	18	–	–
3	F	49	I	12	++++/++	–
4	F	38	I	20	++/+	–
5	M	46	II	20	–	–
6	M	56	II	30	–	–
7	M	47	II	15	–	–
8	F	48	II	20	+(c)/+	–
9	F	47	II	12	–	–
10	M	48	II	30	–	–
11	M	52	III	30	–	–
12	M	45	III	20	–	–
13	F	50	III	20	++++/+++	–
14	M	66	III	50	–	–
15	M	64	III	45	++(c)/++	–
Transitional cell carcinomas						
16	M	48	I	10	+(c)/+	++
17	M	59	II	15	+++/+++	–
18	F	49	II	10	++++/+++	–
19	M	42	III	20	–	–
20	F	41	III	24	+++(c)/++	–
21	M	58	III	40	–	–
22	M	60	III	40	–	–
23	M	46	III	36	+(c)/+	+
24	F	57	III	20	+/+	–
25	F	45	III	20	–	–
26	M	51	III	20	–	–
27	M	56	III	25	++++/+++	–
28	F	44	III	12	+/+	–
29	F	48	III	20	+++/+++	+++
30	M	46	III	6	+(c)/+	–
31	M	43	III	20	++++/+++	–

p53 immunoreactivity; number of nuclei: –=negative, +=1–5%, ++=6–10%, +++=11–40%, ++++=more than 40% of cell nuclei positive, (c)=cytoplasmic positivity. Intensity: +=weak, ++=moderate, +++=strong. c-*erb*B-2 immunoreactivity: –=negative, +=<10% of cells positive, ++=10–50% of cells positive, +++=50–90% of cells positive

Table 2 *p53* protein expression in schistosomal cystitis associated with hyperplasia, squamous metaplasia and/or dysplasia

Case	Sex	Age (years)	Hyper-plasia	Meta-plasia	Dys-plasia	*p53* (number of nuclei/ intensity)
1	M	23	–	–	–	–
2	M	31	–	–	–	–
3	M	21	+	–	–	–
4	F	33	+	–	–	++/++
5	M	22	+	–	–	++(c)/+
6	M	24	–	+	–	–
7	F	16	+	–	–	+(c)/++
8	M	35	+	–	–	+(c)/+
9	M	29	+	–	–	–
10	F	12	+	–	–	–
11	F	26	+	–	–	–
12	M	20	+	–	++	+++/+++
13	M	27	+	–	–	+(c)/+
14	M	30	+	–	++	+/+
15	M	18	–	–	–	–
16	M	22	+	–	+	–
17	F	22	+	–	–	–
18	F	28	–	–	–	–
19	M	24	–	+	–	–
20	M	26	–	+	–	–
21	M	35	–	–	+++	+(c)/+

Hyperplasia: –=absent, +=present; metaplasia: –=absent, +=present; dysplasia: –=absent, +=mild, ++=moderate, +++=severe. *p53* immunoreactivity symbols as per Table 1

done with several changes of PBS between each stage of the procedure. The colour was developed with diaminobenzidine, whereafter the sections were lightly counterstained with haematoxylin and mounted with Eukitt (Kindler, Freiburg, Germany).

A polyclonal sheep antibody to the cytoplasmic domain of human c-*erb*B-2 oncoprotein (Cambridge Research Biochemicals, Cambridge, UK) was used at a dilution of 1: 500. The sections were incubated for 1 h at room temperature with the primary antibody followed by a secondary biotinylated anti-goat IgG (dilution 1: 100) and the ABC (both from Dakopatts).

Negative controls were carried out by replacing the primary antibody with non-immune rabbit serum. As a positive control for the *p53* staining we used a lung carcinoma case previously shown to express abnormal *p53* protein (Soini et al. 1992). As a positive control for the c-*erb*B-2 staining, we used a case of a breast carcinoma that had been shown by in situ hybridization to synthesize high amounts of c-*erb*B-2 mRNA (Pääkkö et al. 1992).

The results of *p53* expression were evaluated quantitatively and divided into five groups (–=negative, +=1–5% of nuclei positive, ++=6–10% of nuclei positive, +++=11–40% of nuclei positive, ++++=>40% of nuclei positive) according to the estimated number of positive nuclei as described in our previous studies (Soini et al. 1992, 1993). The *p53* nuclear positivity was further scored as weak (+), moderate (++) or strong (+++) taking the average of the staining intensity in each case according to Vojtěšek et al. (1993). Also cytoplasmic immunoreactivity for *p53* protein was recorded. With c-*erb*B-2, only cytoplasmic membrane-bound immunoreactivity was interpreted as positive, and results were evaluated quantitatively as described by Soini et al. (1994) as follows: –=no positive cells, +=<10% of cells positive, ++=10–50% of cells positive, +++=50–90% of cells positive, ++++=>90% of cells positive.

The significance of associations was determined using Fisher's exact probability test. Probability values <0.05 were considered significant.

Table 3 *p53* and c-*erb*B-2 protein expression in urinary bladder carcinomas without a history of schistosomiasis

Case	Sex	Age (years)	Grade of tumour	*p53* (number of nuclei/ intensity)	c-*erb*B-2 (number of cells)
Squamous cell carcinomas					
1	F	84	I	+/++	–
2	F	79	I	++++/+++	–
3	F	67	II	–	–
4	F	84	II	++++/++	–
5	M	81	III	++/++	–
6	F	84	III	++++/+++	+
7	F	84	III	++++/++	–
Transitional cell carcinomas					
8	M	53	I	–	–
9	M	42	I	–	–
10	F	86	I	–	–
11	M	70	I	–	–
12	F	78	I	–	–
13	F	69	I	–	–
14	M	69	I	–	–
15	F	79	II	++/+	–
16	M	61	II	–	–
17	M	63	II	+(c)/+	++
18	M	79	II	+(c)/+	–
19	M	81	II	–	–
20	F	75	II	++++/+++	–
21	M	63	II	–	–
22	M	64	II	–	–
23	M	78	II	–	–
24	M	79	II	+/++	+
25	M	60	II	–	–
26	F	88	II	+(c)/+	–
27	F	86	III	++++/+	–
28	M	73	III	++++/++	–
Adenocarcinomas					
29	F	86	I	–	–
30	M	63	II	++/+	–

Symbols as per Table 1

Results

Abnormal nuclear *p53* protein expression was found in 17 out of 31 bladder carcinomas associated with schistosomiasis (55%) (Figs. 1, 2; Table 1). Cytoplasmic immunoreactivity was found in seven cases, always in association with nuclear positivity (Table 1). The high percentage *p53* positive nuclei was often associated with strong *p53* staining (Fig. 1; Table 1). Among the *p53* positive bladder carcinomas there were significantly more women than men (P=0.029). There was no association between the *p53* positivity and the age of patients. There were 68% transitional cell carcinomas with abnormal *p53* protein expression (11/16) while only 40% of squamous cell carcinomas showed *p53* positivity (6/15). There was no association between the *p53* protein expression and the length of the history of schistosomiasis, the clinical stage or the grade of the tumour.

Typical cytoplasmic membrane-bound positivity of varying degree for c-*erb*B-2 oncoprotein was found in 3

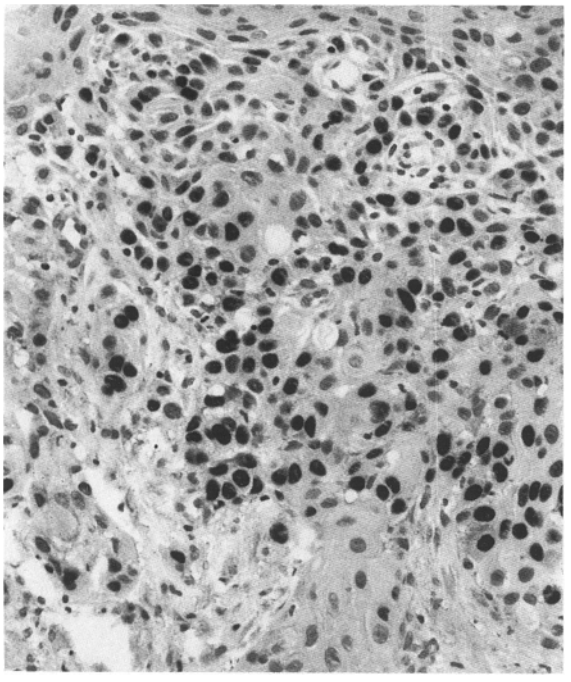

Fig. 1 In this case of a grade III squamous cell carcinoma associated with schistosomiasis (case 13) strong nuclear *p53* immunoreactivity can be seen in most of the tumour cells. Immunoperoxidase stain, × 220

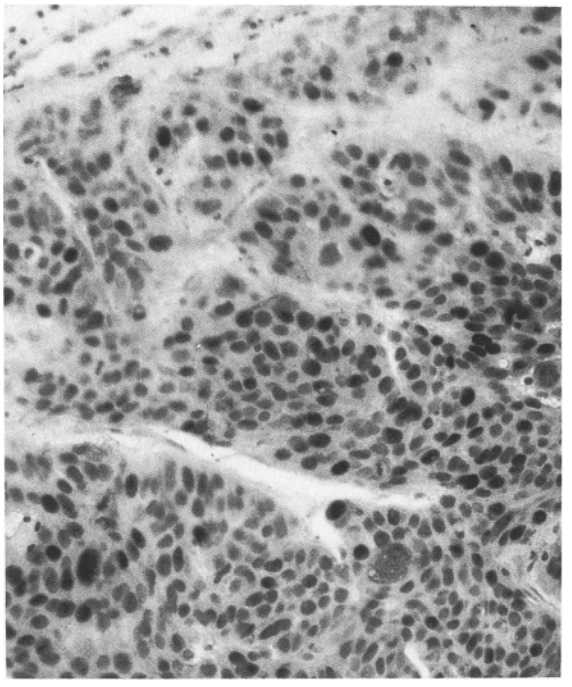

Fig. 2 In a grade III transitional cell carcinoma associated with schistosomiasis (case 27) most of the tumour cell nuclei are *p53* positive. Immunoperoxidase stain, × 220

out of 31 carcinomas (9.7%; Fig. 5; Table 1). All three cases also showed abnormal *p53* protein expression.

Abnormal nuclear *p53* protein expression was found in 8 out of 21 (38%) cases of schistosomal cystitis associated with hyperplasia, squamous metaplasia and/or dysplasia; 5 of these showed epithelial hyperplasia without cellular atypia, 1 dysplasia and 2 both (Figs. 3, 4; Table 2). Cytoplasmic *p53* positivity was found in 5 cases and always co-expressed with nuclear positivity (Table 2). The *p53* staining intensity was weak in 5, moderate in 2, and strong in 1 case (Table 2). None of the cases of schistosomal cystitis associated with hyperplasia, squamous metaplasia and/or dysplasia showed c-*erb*B-2 oncoprotein expression. No *p53* expression was observed in normal epithelia or in the nuclei of adipose, smooth muscle, or inflammatory cells in the bladder wall.

Abnormal nuclear *p53* protein expression was found in 15 of 30 bladder carcinomas without schistosomiasis (50%; Table 3). Cytoplasmic immunoreactivity was found in 3 cases, always in association with nuclear positivity. The strong *p53* staining intensity seemed to associate with the high percentage of *p53* positive nuclei and vice versa (Table 3). There was no significant association between *p53* positivity and the sex of the patients. There were more bladder carcinomas showing abnormal *p53* protein expression in the group of patients with the age of 70 years or more than in the younger patient group (P=0.004). There were significantly more squamous cell carcinomas showing abnormal *p53* protein exprssion (85%) than transitional cell carcinomas or adenocarcinomas with *p53* positivity (40%; P=0.04). There were more carcinomas with abnormal *p53* protein expression among high grade than low grade tumours (grade I vs grade II–III tumours: P=0.025, grade I–II vs grade III tumours: P=0.021).

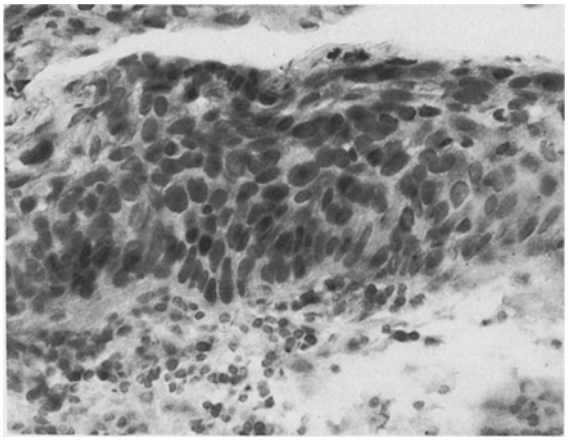

Fig. 3 In schistosomal cystitis with moderate dysplasia of the surface epithelium (case 12) there are several randomly scattered *p53* positive nuclei. Immunoperoxidase stain, × 175

Typical cytoplasmic membrane-bound positivity for c-*erb*B-2 oncoprotein was found in 3 out of 30 bladder carcinomas without schistosomiasis 10% (Table 3). All showed abnormal *p53* protein expression.

Abnormal nuclear *p53* protein accumulation was not

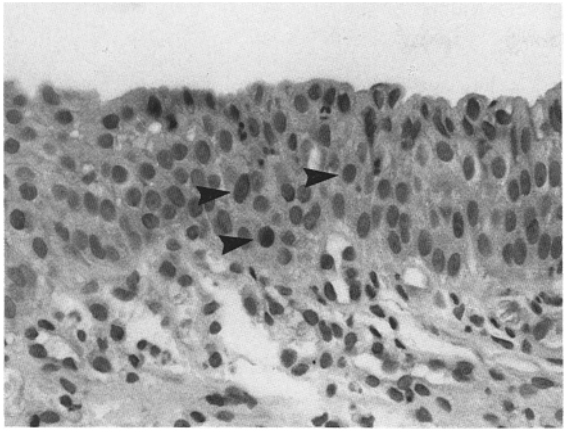

Fig. 4 In schistostomal cystitis with hyperplasia of the surface epithelium (case 8) three randomly scattered *p53* positive nuclei are indicated by *arrowheads*. Immunoperoxidase stain, × 175

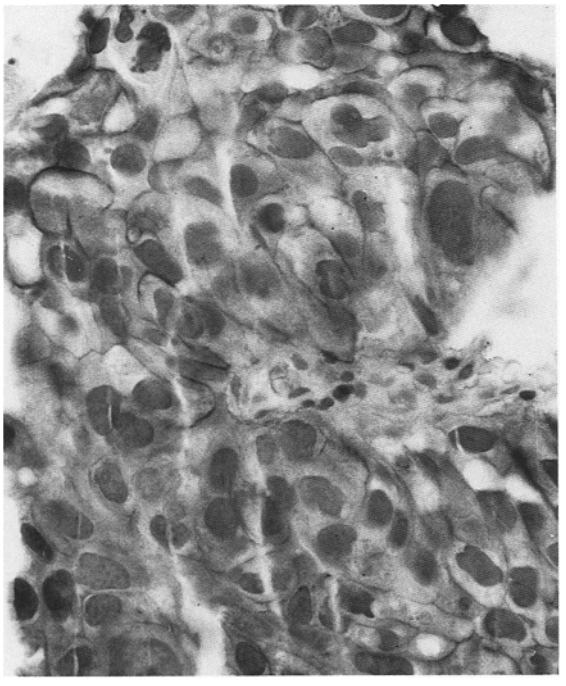

Fig. 5 In a grade III transitional cell carcinoma (case 29) cytoplasmic membrane-bound immunoreactivity for c-*erb*B-2 is visible. Immunoperoxidase stain, × 440

found in any of the nine cases of hyperplasia or in squamous metaplasia of the urinary bladder without schistosomiasis. In contrast, 5 of 12 cases of urinary bladder dysplasias (42%) showed *p53* positive nuclei (4 + cases, and 1 ++ case). The *p53* staining intensity in every case was weak (data not shown). None of the non-schistosomal urinary bladder samples with hyperplasia, squamous metaplasia and dysplasia showed c-*erb*B-2 oncoprotein expression.

Discussion

p53 immunoreactivity has previously been studied in carcinoma and dysplasia of the urinary bladder following or preceding carcinoma (Soini et al. 1993), but there are no reports on abnormal *p53* protein expression in urinary bladder carcinomas or premalignant lesions associated with schistosomiasis. Our results indicate abnormal *p53* protein expression in 55% of bladder carcinomas associated with schistosomiasis and in 50% of those without schistosomiasis, findings in line with previous studies, where mutational analysis of the *p53* gene and immunohistochemical positivity for the *p53* protein has been found in 40–60% or urinary bladder carcinomas (Olumi et al. 1990; Sidransky et al. 1991; Wright et al. 1991; Soini et al. 1993). The results suggest that events leading to the accumulation of *p53* protein play an important role in the evolution of urinary bladder carcinomas both with and without schistosomiasis.

In our urinary bladder carcinomas with a history of schistosomiasis the mean age of the patients was younger than those without schistosomiasis. This is in accordance with the previous knowledge of these carcinomas (McCully et al. 1976; Lucas 1982). There were more *p53* positive cases in the older group of bladder carcinoma patients without a history of schistosomiasis. This suggests that age may favour event(s) leading to accumulation of *p53* protein in bladder carcinomas without schistosomiasis. In those with schistosomiasis there were significantly more *p53* positive bladder carcinomas in women than men. The reason for this association remains obscure.

In the group of carcinomas with schistosomiasis there was no association between abnormal *p53* expression and tumour grade, while we found more *p53* positive cases among high grade than low grade bladder carcinomas in cases without schistosomiasis. This is in agreement with our previous studies which show more *p53* positive cases among high grade lung, breast, gallbladder and urinary bladder tumours (Soini et al. 1992, 1993; Kamel et al. 1993) as well as with other studies from the literature (Olumi et al. 1990; Sidransky et al. 1991). This suggests that alteration in the *p53* gene may play a role in the evolution of high grade tumours in general, and perhaps in the evolution of high grade carcinomas of the urinary bladder without schistosomiasis but not with schistosomiasis.

p53 protein expression has been observed in preneoplastic lesions of oral (Gusterson et al. 1991), laryngeal (Dolcetti et al. 1992), gallbladder (Kamel et al. 1993) and bronchial epithelium (Nuorva et al. 1993), and in metaplastic oesophagal epithelium of a Barrett's lesion (Casson et al. 1991). This suggests that *p53* mutations occur early in the neoplastic process. Our results with a 38% frequency of abnormal *p53* expression in schistosomal cystitis with hyperplasia and/or dysplasia suggest that *p53* gene alterations may also be an early event in the development of invasive bladder carcinoma. Without mutational analysis we cannot exclude the possibility of

mechanisms for *p53* protein accumulation other than mutation, however.

Our results showed a similar (about 10%) frequency of c-*erb*B-2 expression in urinary bladder carcinomas with or without schistosomiasis. Similar, or a somewhat higher frequency or c-*erb*B-2 expression has been found in breast (Singleton and Strickler 1992), lung (Pääkkö et al. 1992; Singleton and Strickler 1992), intestinal (Singleton and Strickler 1992) and gallbladder carcinomas (Kamel et al. 1993). In a larger series of urinary bladder carcinomas c-*erb*B-2 immunoreactivity was found in 26% of the carcinomas and was related to high tumour grade and stage (Sato et al. 1992).

In our material c-*erb*B-2 expression was not found in any of the cases of schistosomal cystitis with or without premalignant lesions. This suggest that c-*erb*B-2 gene alteration is not an early event in urinary bladder tumourigenesis, however, c-*erb*B-2 activation has been found in the dysplasia of the uterine cervix (Brumm et al. 1990).

p53 can act in concert with *ras* in the neoplastic transformation of cells (Finlay et al. 1989). Interestingly, all the urinary bladder carcinomas (with or without schistosomiasis) with c-*erb*B-2 expression were *p53* positive suggesting that the alterations in *p53* gene could co-operate with these in c-*erb*B-2 gene in the neoplastic transformation of the urinary bladder epithelium. In our previous study on gallbladder carcinomas we found an association of the c-*erb*B-2 and *p53* positivity (Kamel et al. 1993). A significant co-expression of c-*erb*B-2 proto-oncogene and *p53* gene inactivation has also been found in breast (Isola et al. 1992) and urinary bladder carcinomas (Wright et al. 1991).

In conclusion, we found abnormal *p53* protein accumulation in 55% of bladder carcinomas associated with schistosomiasis. A high frequency of abnormal *p53* expression was also found in non-neoplastic (hyperplasia) and/or premalignant (dysplasia) urinary bladder lesions with schistosomiasis. These findings were not significantly different from non-schistosomal bladder carcinoma.

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