

Analysis of *p53*, *p16^{MTS1}*, *p21^{WAF1}* and *H-ras* in archived bladder tumours from workers exposed to aromatic amines

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Summary Exposure to aromatic amines is considered a major risk factor for the development of bladder cancer. In this study, we have analysed the pattern of point mutations in several tumour genes in 21 cases of bladder cancer arising among western European workers exposed to aromatic amines in an attempt to determine whether this exposure may be associated with a unique spectrum of mutations. Of the four genes analysed (*p53*, *p16^{MTS1}*, *p21^{WAF1}* and *H-ras*), only *p53* showed a high frequency of mutations (in 8 out of 21 cases, 38%). Two mutations were found in *p16*, one in *H-ras* and none in *p21* exon 3. All mutations were at G:C base pairs, mostly at non-CpG residues. This spectrum of mutations, which is highly suggestive of an involvement of exogenous carcinogens, is however identical to the spectrum of *p53* mutations detected in bladder cancers of the general population. In exposed workers, *p53* mutations were associated with tumour grade and with high occupational and tobacco exposure. Taken together, our data suggest that the same carcinogens may be responsible for the development of bladder cancers in workers exposed to aromatic amines and in the general population.

Keywords: bladder cancer; aromatic amines; *p21^{WAF1}*; *p53*; *p16^{MTS1}*; *H-ras* mutations

Bladder cancer is the eighth most common cancer worldwide in men and is associated with several risk factors, the contributions of which vary greatly in different countries and populations (Parkin et al, 1993). Risk factors for bladder cancer include tobacco smoking, exposure to certain therapeutic drugs, such as phenacetine, chronic infection by the parasite *Schistosoma haematobium* and exposure to aromatic amines (IARC, 1987, 1994).

Epidemiological studies have clearly shown that an increase in incidence of bladder cancer is observed in humans occupationally exposed to β -naphthylamine, benzidine and 4-aminobiphenyl. Experimental studies in rodents and dogs have also shown that these agents are carcinogenic. Thus, there is clear evidence that these aromatic amines are potent bladder carcinogens (IARC, 1972).

Aromatic amines are involved in many industrial processes, such as chemical dye production, plastic and rubber manufacturing and textile and leather industries. Bladder cancer was recognized as early as 1895 among workers of the dye manufacturing industry. Although the recognition of this association has led to dramatic changes in working practices and industrial processes over the past 40 years, recent studies indicate that workers in the dyestuff production still experience a 2.6–4.6 relative risk of bladder cancer, even after adjustment for age and smoking (Boyko et al, 1985; La Vecchia, 1990).

Another potentially important source of exposure to aromatic amines is tobacco smoking. In addition to a number of well-characterized human carcinogens, tobacco smoke contains 4-aminobiphenyl (4-ABP) and 2-naphthylamine (β -naphthylamine, 2-NA). In developed countries, between 40% and 70% of bladder cancers occurring in men are attributable to tobacco exposure and it has been proposed that aromatic amines may be responsible for the excess of bladder cancers observed in smokers (Pisani et al, 1993). Moreover, the analysis of DNA adducts in urothelial cells of smokers indicates that DNA damage may be due to aromatic amines rather than to other tobacco carcinogens, such as benzo(a)pyrene (Vineis et al, 1996).

In 1995, we published a preliminary report indicating that the *p53* gene was mutated with a frequency of 30% in a group of 12 bladder cancers occurring in workers who had been occupationally exposed to aromatic amines in western Europe (Esteve et al, 1995). The results reported here are an extension of the preliminary study and include 21 tumours from patients for whom data on occupational exposure and cigarette smoking were available. In addition to *p53*, we have analysed other genes that may be the targets of chemical carcinogens, such as *H-ras* and the cyclin kinase inhibitor *p16*.

Point mutation in the *p53* tumour-suppressor gene is a common genetic alteration in human cancer. Mutations usually occur at more than 150 distinct codons within the central portion of the gene, and the nature and location of these mutations can be informative in assessing the role of putative carcinogenic agents (Hainaut et al, 1997). In bladder cancers, the *p53* gene is mutated with a frequency that varies from 18% to 50% (Williamson et al, 1994; Vet et al, 1994; Taylor et al, 1996), with one report describing a frequency of

Received 5 August 1997

Revised 27 October 1997

Accepted 29 October 1997

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62% in the endemic area of 'black foot disease' in Taiwan, a region at high risk for bladder cancer (Shibata et al, 1994).

Deletions at 9p21-22 are frequent in bladder cancers and are often detectable in low-grade tumours, suggesting that one (or several) tumour-suppressor genes located in this region may play an important role in the genesis of bladder cancer (Southgate et al, 1995; Williamson et al, 1995). One candidate gene at this locus is *p16*, which encodes an inhibitor of cyclin-dependent kinases 4 and 6, active at the G₁/S transition of the cell cycle. In mouse and rat, the *Ha-ras* gene is often activated by mutation in liver tumours induced by exposure to benzidine, with the most frequently detected mutation being a C:G to A:T transversion at the first nucleotide of codon 61. The cyclin kinase inhibitor *p21^{WAF1}* is rarely mutated in human cancers but is transcriptionally activated by p53 and is responsible for cell cycle arrest at the G₁/S border (Levine, 1997).

Our results indicate that only *p53* is frequently mutated in workers exposed to aromatic amines. The frequency of mutations increases with tumour grade, but the spectrum of *p53* mutations in exposed workers is similar to the one in the general population. We also show that the frequency of *p53* mutations increases with high occupational exposure and tobacco consumption. These results are compatible with those reported by Taylor et al (1996) in a cohort of exposed workers in North America.

MATERIAL AND METHODS

Tumour samples

Twenty-one bladder cancer cases were identified from a cohort of dyestuff manufacturing workers employed at Bayer A-G,

Leverkusen, Germany. All tumours were archived at the Institute of Biology Monitoring, Ärztliche Abteilung, Bayer AG. Tissues were fixed in formalin and embedded in paraffin. Data on exposure to aromatic amines are based on the occupational history of each individual. Data on smoking history are from the company medical records and are based on self-reported information via questionnaires (Table 1).

Immunohistochemical analysis of p53 and p21^{WAF1} proteins

Immunohistochemical detection of p53 protein was performed using the avidin-biotin-peroxidase complex (ABC) method (Hsu et al, 1981). Sections were deparaffinized, inactivated for endogenous peroxidase activity and incubated with normal serum to block cross-reactivity before incubating overnight at 4°C with the p53 polyclonal antibody CM-1 (1:100). Immunostaining with the p21 monoclonal antibody Ab-1 (1:300, Oncogene Science) was performed using the same procedure, except that sections were microwaved in citrate buffer to unmask the epitopes of the protein before incubation with normal serum. The immunoreactivity was classified into five categories by estimating the percentage of positive nuclei in the tumour tissue: -, no immunoreactivity; +, <5%; ++, 5-20%; +++ 20-50%; +++++, >50%.

Detection of mutations in p53, H-ras, p16 and p21

Haematoxylin- and eosin-stained sections (5 µm) were used to identify neoplastic regions to be analysed for gene alterations, and tumour areas were microdissected from 10-µm matched, unstained

Table 1 Distribution of bladder cancer cases according to occupational exposure, smoking history and tumour grade

ID	Occupational exposure					Smoking			
	Benzidine	2-NA	4APB	Azobenzol	Age at first exposure (years)	Duration (years)	Pack/year	Duration (years)	Years quit
<i>Grade 1</i>									
6/1	+				45	14	27.3	22	
12/1	+		+		43	1	182.5	35	2
14/1		+			24	32	NS		
24/1	+	+			19	31	91.25	55	
27/1	+				43	11	228.72	28	11
<i>Grade 2</i>									
8/1	+			+	44	20	NS		
11/1	+		+		24	25	NS		
17/1		+			37	4	136.87	38	
19/1		+			31	28	319.37	60	
21/1	+	+			14	49	273.75	33	11
23/1		+			NA		182.5	41	
28/1	+				18	2	NS		
30/1	+				41	16	182.5	40	
31/1	+				33	27	182.5	9	10
<i>Grade 3</i>									
1/1-2	+				37	13	146	30	
16/1	+	+			24	18	200.75	67	
20/1		+	+		32	5	182.5	31	
22/1		+			32	24	365	40	
25/1	+				56	7	NS		
26/1	+				25	?	NS		
29/1	+	+			31	29	NS		

NS, non-smoker; NA, not applicable.

Table 2 Distribution of *p53* and *p16^{MTS1}* mutations and of *p21^{WAF1}* expression by tumour grade

Case no.	<i>p53</i> Analysis				<i>p16</i> Mutation			<i>p21^{WAF1}</i> Expression
	IHC ^a	Codon	Mutation	AA change	Codon	Mutation	AA change	IHC ^a
<i>Grade 1</i>								
6/1	-							-
12/1	-							++++
14/1	-				28	GTG>ATG	Val>Met	+
24/1	-							NA
27/1	-							+
<i>Grade 2</i>								
8/1	++							+++
11/1	-							+
17/1	-							++
19/1	++							++
21/1	-	231	ACC>ACT	Silent				NA
23/1	-							++
28/1 ^b	-							-
30/1	+	276	GCC>ACC	Ala>Thr				+++
31/1	+++	192	CAG>AAG	Gln>Lys	148 ^d	GCG>ACG	Ala>Thr	-
		214	CAT>TAT	His>Tyr				
<i>Grade 3</i>								
1/1-2	++++	273	CGT>CTT	Arg>Leu				-
16/1	+ ^c	117	GGG>GAG	Gly>Glu				++
20/1	++++	273	CGT>CTT	Arg>Leu				++
22/1	++++	242	TGT>TAC	Cys>Tyr				++
25/1	+++							NA
26/1					110	TGG>TGA	Trp>stop	+
29/1	-	282	CGG>TGG	Arg>Trp				+

^aIHC, immunohistochemistry scoring is defined in the text. ^bThe patient has a homozygous polymorphism at codon 213 (CGA>CGG). ^cThe tumour shows staining only in the cytoplasm. ^dPolymorphism. NA, tumour tissue not available.

tissue sections. Extraction of DNA from microdissected regions was performed as described previously (Esteve et al, 1993, 1995). Exons 5-8 (in some cases also 4 and 9) of the *p53* gene were amplified using a nested polymerase chain reaction (PCR) strategy with the primers described by Lehman et al (1991, 1993). PCR products were subjected to direct sequencing after conversion to single-stranded DNA by (a) asymmetric PCR or (b) using biotinylated primers and streptavidin-coated magnetic beads. For *H-ras*, exons 1 and 2, covering codons 12, 13 and 61, were amplified by PCR (Lehman et al, 1991) and the PCR products were analysed by sequencing. For *p16*, exons 1 and 2 were analysed using the asymmetric approach and direct sequencing as described in Esteve et al (1996). For *p21*, exon 3 (not exons 2 and 3 because of methodological difficulties) was amplified with GC-clamped primers and the products were analysed by constant denaturing gradient gel electrophoresis (CDGE) (Børresen et al, 1996). All mutations were confirmed by repeating all steps in the procedure.

RESULTS

Study subjects

The tumours analysed here were all transitional cell carcinomas, grade 1-3, occurring in 21 male workers exposed to aromatic amines. During their employment, most patients were exposed to multiple aromatic amine derivatives, including benzidine (15 patients), 4-amino-biphenyl (4-ABP, three patients), 2-naphthylamine (2-NA, ten patients) and azobenzol (one patient). Age at first exposure varied from 14 to 56 years, and time of exposure

Table 3 Distribution of *p53* mutations by exposure and tumour grade^a

Grade	Cases with <i>p53</i> mutations (total number of cases)			
	Occupational exposure		Exposure to tobacco	
	Low	High	Non-smoker	Heavy smoker
Total	2 (7)	5 (9)	1 (7)	5 (9)
1	0 (3)	0 (2)	0 (1)	0 (2)
2	1 (2)	2 (4)	0 (3)	2 (4)
3	1 (2)	3 (3)	1 (3)	3 (3)

^aPatients were classified in two groups corresponding to low and high exposure to either aromatic amines (occupational exposure) or smoking (exposure to tobacco). Criteria for distinction between low and high exposure are defined in text.

ranged from 1 year (patient 12/1) to 49 years (patient 21/1). The period of exposure covered years 1927-81. Of these 21 patients, ten had a continuous cigarette-smoking history at the time of the diagnosis, four were ex-smokers and seven reported themselves as non-smokers (Table 1).

Analysis of *p53* mutations

p53 exons 4-9 (including flanking intronic splicing sites) were first analysed for the presence of mutations using PCR and direct

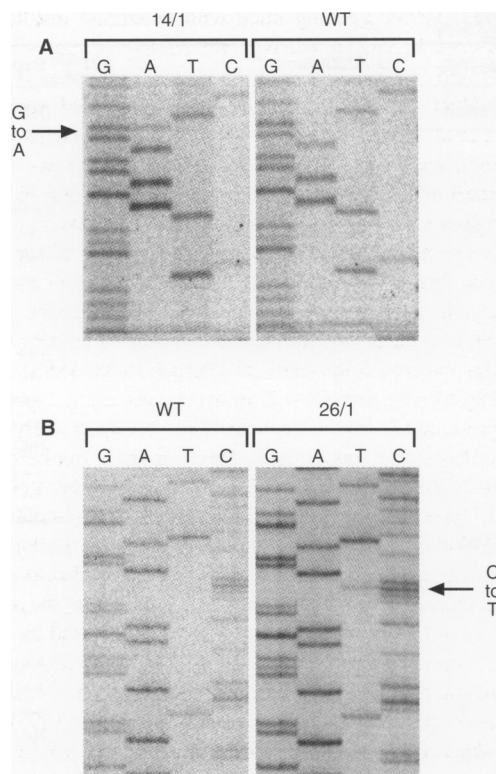


Figure 1 Results of sequence analyses of the *p16^{MTS1}* gene in two of the bladder carcinomas from workers exposed to aromatic amines. (A) Case 14/1 showing a mutation at codon 28 (GTG→ATG, valine to methionine). (B) Case 26/1 showing a mutation at codon 110 (TGG→TGA, tryptophane to Stop). The sequence of the non-transcribed strand is shown with a C→T mutation

sequencing. In parallel, p53 protein expression was analysed by immunohistochemistry using the rabbit antibody CM-1. The data on p53 analysis are summarized in Table 2. p53 mutations were found in eight patients (38%). One patient (31/1) had two independent mutations in the p53 coding sequence (codons 192 and 214), and another (21/1) had a silent mutation at codon 231 that does not correspond to a known polymorphism. This mutant allele coexisted with the wild-type allele in the tumour tissue, suggesting that the mutation is heterozygous. Non-neoplastic tissue from this patient was not available. In addition, one patient (28/1) was homozygous for a well-described polymorphism at codon 213 (CGA to CGG, silent). Four of the nine mutations were in exon 8, at codons 276, 282 and 273 (two mutations). Other mutations were in exon 7 (codons 231 and 242), exon 6 (codon 214), exon 5 (codon 192) and exon 4 (codon 117).

All of these mutations occurred at G:C base pairs. Six of them were C to T transitions, including one at a CpG dinucleotide (codon 282, CGG to TGG, patient 29/1). The other three mutations were G to T transversions. Interestingly, the two mutations at codon 273 were G to T transversions (CGT to CTT, Arg to Leu), which represent only 9% of all the mutations at codon 273 described in the p53 mutation database (Hainaut et al, 1997).

Table 2 also shows that the frequency of p53 mutations increases with tumour grade and that the presence of a mutation is generally associated with nuclear accumulation of the p53 protein. However, nuclear p53 accumulation was also found in three tumours without detectable mutations in exons 5–8 (patient 19/1,

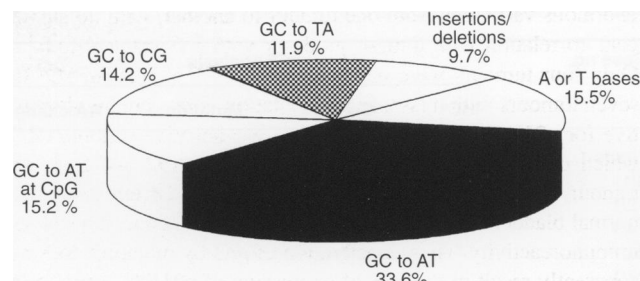


Figure 2 Mutation spectrum of p53 in bladder cancers from the general population (data from Hainaut et al, 1997)

grade 1; patient 8/1, grade 2; patient 25/1, grade 3) and, in one case (patient 16/1), a mutation in exon 4 (codon 117, Gly to Glu) was associated with cytoplasmic staining and nuclear exclusion of the p53 protein (data not shown). MDM2 overexpression or amplification has been reported in bladder cancers (Lianes et al, 1994), and it is possible that inactivation and stabilization of the p53 protein derive from interaction with this cellular oncoprotein in these tumours.

Associations between p53 mutation and exposure to aromatic amines and tobacco

The subjects analysed were separated into several groups according to their level of occupational or tobacco exposure. For occupational exposure, we defined as 'high exposure' the group of subjects who have been employed in the dye manufacturing industry for more than 18 years, starting before 35 years of age (nine cases). The 'low exposure' group comprised individuals who have been employed for less than 17 years, starting after 36 years of age (seven cases). For exposure to tobacco, we compared the subjects reported as 'non-smokers' with the group of 'heavy smokers', defined as the consumption of at least 180 cigarette packs per year for at least 10 years, without cessation before diagnosis.

Table 3 shows that the frequency of p53 mutations is higher in both of the high-exposure groups than in the low-exposure counterparts. Moreover, in each group, the frequency of mutation increased with tumour grade. Of the four tumours from patients who were highly exposed to both occupational aromatic amines and tobacco, only one (patient 19/1) had no detectable p53 mutation, but expressed high levels of histochemically detectable p53 protein. On the other hand, the two patients with grade 3 tumours and no p53 mutations were non-smokers and of the low-occupational-exposure group. Although the numbers in these groups are small, these results suggest that the frequency of p53 mutations increases with occupational and/or tobacco exposure.

Analysis of p16, H-ras and p21

Sequencing of exons 1 and 2 of *p16* (plus flanking intronic splicing sites) revealed three point mutations (Table 4), one of them being a common polymorphism at codon 148 (Kelley et al, 1995; Smith-Sørensen and Hovig, 1996). The two other mutations were in exon 1 (GTG to ATG, valine to methionine at codon 28) and exon 2 (TGG to TGA, tryptophane to stop at codon 110) (Figure 1).

Analysis by constant denaturing gel electrophoresis (CDGE) revealed no mutation in exon 3 of *p21^{WAF1}*. However, immunohistochemical analysis of the expression of *p21^{WAF1}* showed

enormous variations from one tumour to another, with no significant correlation with tumour grade or with p53 status (Table 2). Only four tumours were negative for p21^{WAF1} expression. Of the seven tumours with missense p53 mutation, five were highly positive for p21^{WAF1} expression and two were negative (tumour 31/1, which contains two p53 mutations at codon 192 and 214, and tumour 1/1–2, with a mutation at codon 273). Of ten cases with normal bladder epithelium analysed as controls, none showed any immunoreactivity. Thus, inactivation of p53 by mutation does not apparently result in the loss of expression of p21^{WAF1}, a transcriptional target of p53.

In this series of bladder cancers, only one mutation was detected in the H-ras gene at the first base of codon 61 in patient 6/1 (CAG to AAG). A low prevalence of Ha-ras mutations (6%) was also reported in bladder cancers from non-occupationally exposed patients (Knowles et al, 1993).

DISCUSSION

Despite substantial epidemiological and experimental evidence for the role of aromatic amines and tobacco carcinogens in the aetiopathogenesis of bladder cancers, the exact nature of the carcinogens involved in human bladder carcinogenesis is still unclear. In this study, we have analysed the distribution of point mutations in several cancer-related genes in a cohort of 21 bladder cancer patients with well-defined occupational exposure to aromatic amines and for which information on tobacco consumption was available.

Point mutations were found in p53 (nine mutations in eight patients), p16^{MTS1} (two somatic mutations) and H-ras (one mutation), but not in p21^{WAF1}. All these mutations occurred at G:C base pairs, and eight of them were C to T transitions at non-CpG dinucleotides; one was a C to T transition at a CpG dinucleotide. The other three mutations were all G to T transversions in p53 (two at codon 273 and one at codon 192). The very low prevalence of transitions at CpG dinucleotides, a type of mutation thought to occur spontaneously by methylation and deamination of cytosines, suggests that endogenous mutagenic events do not play a major role in the natural history of these cancers (Jones et al, 1991; Greenblatt et al, 1994). Thus, the profile of point mutations in the bladder cancers analysed here is indicative of the involvement of carcinogens of exogenous origin and is consistent with previous observations in bladder cancers from occupationally exposed patients (Taylor et al, 1996) or from patients exposed to tobacco smoke (Spruck et al, 1993).

Mutations of p53 at G:C base pairs are also frequent in bladder cancers of the general population (Figure 2). Of the 310 bladder cancers mutations listed in the January 1997 update of the p53 mutation database (Hainaut et al, 1997), 75% occur at G or C bases, and about half of these are GC to AT transitions at non-CpG sites. Transitions at CpG dinucleotides are infrequent (except in squamous cell carcinomas of the bladder associated with schistosomiasis (Habuchi et al, 1993)). Although G to T transversions are relatively rare (12%), 4 of the 12 mutations at codon 273, a frequent site for CpG transitions in breast and colon cancers, are G to T transversions (compared with 9% in all other cancers).

Several experimental studies have shown that, after activation to electrophilic metabolites, aromatic amines could bind covalently to DNA. Aromatic amines bind preferentially at the C8 position of guanine, although significant mutagenic activity was also observed at certain A-T base pairs (Lasco et al, 1988; Essigmann

and Wood, 1993). Feeding mice with benzidine resulted in the formation of hepatic *N*-(deoxyguanosin-8-yl)-*N*-acetylbenzidine adducts, and *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl was the major adduct formed in the bladder of dogs fed with 4-ABP (Talaska et al, 1990). This binding specificity is consistent with a mutation mechanism affecting primarily G:C base pairs.

In experimental systems, the principal mutations induced by aromatic amines are G to C and G to T transversions. G to T transversions are also a typical molecular signature of the tobacco carcinogen benzo(a)pyrene, and these mutations occur at high frequency in lung cancers associated with tobacco smoking (Denissenko et al, 1996). G to C transversions have been observed in bladder cancers from smokers (Spruck et al, 1993), but it is interesting to note that G to T transversions are not particularly well represented in bladder cancers from occupationally exposed patients (this study; Taylor et al, 1996) or from smokers (Spruck et al, 1993). Instead, G:C to A:T transitions are the predominant mutation type in both occupationally exposed patients and in smokers. Although this observation suggests that similar carcinogens are responsible for bladder cancers in exposed workers and in smokers, there is no direct evidence at this point that the p53 mutations observed in bladder cancers are directly caused by aromatic amines. Indeed, G to A transitions at non-CpG sites may result from mutagenesis by other carcinogens, such as alkylating nitrosamines or nitric oxide (Essigmann and Wood, 1993).

The absence of a specific p53 mutation pattern in exposed workers raises the question of whether occupational exposure, rather than environmental exposure and tobacco consumption, has a significant role in the genesis of bladder cancer. In this respect, it is interesting to note that the frequency of p53 mutation is higher in the group of workers who have been exposed to aromatic amines for a long time and at an early age (see Table 2). Although a similar relationship also exists for tobacco consumption, it is tempting to speculate that prolonged, occupational exposure may result in an increased risk to acquire p53 mutations leading to bladder cancer. As the number of cases involved in the present study is small, this issue warrants further investigation to evaluate with precision the adequacy of the protection measures presently in force in the dye manufacturing industry.

Mutations of the other genes analysed in this study are apparently infrequent in bladder cancer. In the case of p16, the occurrence of deletions of large segments of chromosome 9p is a well-described genetic alteration, even in superficial bladder cancer. Homozygous or heterozygous deletions of 9p21 are sometimes detected in the early stages of bladder carcinogenesis. In most cases, the deleted area encompasses the p16 locus (Williamson et al, 1995). However, in cases with heterozygous deletions, the remaining p16 allele is rarely inactivated by mutation. Infrequent p16 mutations have been reported in bladder cancer from non-occupationally exposed patients (Kai et al, 1995; Pakenham et al, 1995; Okajima et al, 1996). Furthermore, the deleted area on chromosome 9 is usually large and also involves several other well-characterized loci, including p15, interferon alpha 1 (IFNA1), interferon beta 1 (IFNB1), methylthioadenosine phosphorylase (MTAP) (Stadler et al, 1996; Zhang et al, 1996; Balacz et al, 1997). Taken together, these data suggest that other suppressor gene(s), in addition to p16, may be the target of deletions on 9p in bladder cancers. In the case of p21^{WAF1}, it is remarkable to note that the levels of protein expression were neither correlated with those of p53 nor with p53 mutational status. This discrepancy is however consistent with the notion that the p21^{WAF1}

promoter can be transactivated by several distinct signalling pathways in addition to p53 (Sheikh et al, 1994; Zhang et al, 1995). In addition, that most tumours show elevated levels of p21^{WAF1} suggests that in these cells p21^{WAF1} is not capable of exerting its suppressor effects and that the functioning of the cell cycle-regulatory mechanisms may be altered through other pathways not involving p21^{WAF1}.

Several additional genetic alterations have been observed in bladder cancers, involving allelic losses of various chromosomal loci or chromosomal instability (Gonzalez-Zulueta et al, 1993; Reznikoff et al, 1993; Knowles et al, 1994), as well as mutations or altered expression of different genes, namely the retinoblastoma gene (Miyamoto et al, 1995), *MDM2* (Lianes et al, 1994), *erbB-2* (Sauter et al, 1993), connexin (Grossman et al, 1994) and angiogenic peptide basic fibroblast growth factor (Nguyen et al, 1993). The temporal occurrence of these alterations is still not well understood and the order in which these changes occur in the natural history of bladder cancer may depend on the aetiology of this cancer (Reznikoff et al, 1993; Lianes et al, 1994). Our results also show that the frequency of p53 mutation is closely associated with tumour grade. This observation may be interpreted as being indicative of a late involvement of p53 in bladder carcinogenesis (Williamson et al, 1994; Vet et al, 1994). This is apparently in contradiction with the interpretation of the p53 mutation spectrum as indicative of the involvement of exogenous carcinogens. One explanation might be that these mutations may have been acquired very early during carcinogenesis, but confer a significant selective advantage in later stages on tumour progression. It would therefore be of great interest to use very sensitive detection methods to investigate the presence of p53 mutations in low-grade tumours, as well as in the normal mucosa of exposed subjects and to follow prospectively the biological behaviour of this genetic lesion.

ACKNOWLEDGEMENTS

The support of EC grant 'Environmental and Climate' EV5V-CT92-0199 to RM and the Norwegian Cancer Society to A-L B-D is gratefully acknowledged. TS is a research fellow of the Norwegian Cancer Society.

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