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HOMOZYGOUS DELETIONS OF $p16^{INK4}$ OCCUR FREQUENTLY IN BILHARZIASIS-ASSOCIATED BLADDER CANCER

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We have studied $p16^{INK4}$ mutation (by PCR-SSCP) and deletion (by Southern blotting and/or multiplex PCR) in a series of 47 bilharziasis-associated tumors from Egypt and compared the results with those obtained on a series of 17 established bladder cell lines and non-bilharziasis-associated bladder cancers from the Netherlands. In the cell lines we found 9 homozygous deletions and 1 mutation (59% of $p16^{INK4}$ alterations in cell lines), whereas in cases from the Netherlands deletions were found in 4 of 22 samples. No mutations were detected in the 46 samples screened. Interestingly, in bilharziasis-associated bladder cancer, deletions were present in 23 samples and mutations in a further 2 cases (53% of $p16^{INK4}$ alteration in bilharziasis-associated bladder cancer). No correlation was found between $p16^{INK4}$ alteration and histopathological data. Likewise, the same frequency of alteration was found in tumors with different differentiation patterns (squamous, transitional or adenocarcinoma). Three conclusions can be drawn from our findings: (i) $p16^{INK4}$ alterations are more frequent in cell lines than in primary tumors; (ii) in primary bladder tumors (bilharziasis-associated or not), $p16^{INK4}$ deletions are much more frequent than $p16^{INK4}$ mutations; (iii) $p16^{INK4}$ alterations are more frequent in bilharziasis-associated bladder tumors than in other bladder tumors. This high frequency of deletion is not related to a specific histological type but to the specific etiology of these tumors.

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Changes in cell-cycle control are thought to be critically associated with cancer development. Progression from the G₁ to the M phase is controlled by a family of enzymes called the cyclin-dependent kinases (CDKs) (for review see Kamb, 1995 and references there in). The phosphorylated down-stream effectors transduce the signal and lead ultimately to DNA synthesis and mitosis. CDK activity is dependent on positive regulators called cyclins and inhibited by a set of proteins termed CDK inhibitors. In addition, other kinases and phosphatases participate in the regulation of CDKs. Cyclins are low m.w. proteins whose function is markedly modulated during different phases of the cell cycle. The role played by these proteins in human cancer has long remained elusive. However, the fact that in some parathyroid adenomas, the promoter region of the parathyroid hormone gene is fused to the gene encoding cyclin D1, provided evidence that cyclins can be directly involved in cancer development. Subsequent analysis showed that cyclin D1 can substitute totally or partially for certain oncogenes in cellular transformation assays in various cancers, and cyclin D1 has been found to be over-expressed in several cancers (Kamb, 1995), including bladder (Bringuiet *et al.*, 1996).

Seven CDK inhibitors have been characterized so far: p57, p21, p27, p19, p18, $p16^{INK4}$ (also called CDKN2 and MTS1) and p15. $p16^{INK4}$ binds specifically to CDK4 and CDK6 and inhibits these 2 kinases (Serrano *et al.*, 1993). Interestingly, cyclin D1 activates CDK4 and CDK6. Thus, $p16^{INK4}$ is a specific regulator of cyclin D1-dependent kinases. It is thus likely that $p16^{INK4}$ alterations can also be involved in cancer development. Mutations in the $p16^{INK4}$ gene were revealed in cell lines. In 46% of 290 studied cell lines, the $p16^{INK4}$ gene was found to be homozygously deleted (Kamb *et al.*, 1994a; Nobori *et al.*, 1994). Moreover, $p16^{INK4}$ has been mapped to 9p21, a region prone to loss of heterozygosity in bladder cancer. It has thus been hypothesized that $p16^{INK4}$ is the target gene in this region (Cairns *et al.*, 1994; Williamson *et al.*, 1995).

Bilharziasis is one of the most widespread diseases related to parasitic infections and is particularly endemic in tropical and subtropical countries; according to statistical data from the World Health Organisation (WHO), 200 million infected people were recorded in 74 different countries (WHO, 1985). Egypt is considered to be a hyperendemic area, with an overall prevalence rate of 50% and an early age of onset (WHO, 1985). Bilharziasis is often associated with bladder cancer, which occurs with a frequency of 31% of total cancer incidence in Egypt (Rosin *et al.*, 1994; Ramchurren *et al.*, 1995). These tumors have a different etiology and histology than transitional-cell carcinoma (TCC) as found in Western countries. It has been suggested, however, that bladder tumors of both origins share some molecular alterations (*e.g.*, over-expression of EGFR and *c-erbB-2*) but also that some alterations differ in respect to etiology [*e.g.*, frequent loss of retinoblastoma expression in bladder tumors found in Western countries but not in bilharziasis-associated tumors (Ramchurren *et al.*, 1995)]. To determine whether $p16^{INK4}$ is differentially involved in these 2 types of bladder cancer, we studied $p16^{INK4}$ mutations and deletions in bilharziasis-associated tumors from Egypt in comparison with established cell lines and bladder tumors from the Netherlands.

MATERIAL AND METHODS

In this report, we limited our analysis to exon 2 alterations in the $p16^{INK4}$ gene. Exons 1 and 3 were omitted since they cover only a minor part of the $p16^{INK4}$ gene (32%) and most mutations/deletions reported so far are clustered in exon 2 (Kamb *et al.*, 1994b; Nobori *et al.*, 1994).

Cell lines and patient samples

The following bladder cancer cell lines, obtained from the Sloan-Kettering Cancer Center (New York, NY), were used in our study: SCaBER, J82, VMcub1, VMcub2, VMcub3, SW-800, SW-780, SW-1710, T24, RT4, RT112, 575A, 647V, 253 J, SD, Jon and 5637. Cells were grown in RPMI medium containing 10% FCS until they became subconfluent.

Forty-six frozen tumor samples from the Netherlands were analyzed by SSCP. In none of these was evidence for schistosomiasis infection found. Two were squamous-cell carcinoma (SCC), both grade 2, stage 3. The other tumors were TCC: 22 were superficial and 22 invasive, 11 grade 1, 16 grade 2 and 17 grade 3. The 2 SCC and 20 TCC (10 superficial and 10 invasive; 4 grade 1, 7 grade 2 and 9 grade 3) were also analyzed by Southern blot and multiplex PCR. DNA was extracted as previously described (Bringuiet *et al.*, 1996). Only samples with more than 75% tumor material were included.

Forty-seven formalin fixed, paraffin-embedded bilharziasis-associated tumors were analyzed by both SSCP and multiplex PCR. Twenty were SCC, 20 were TCC and 5 were adenocarci-

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nomas. In 2 cases, pathological data were not available. Six tumors were superficial and 39 invasive. Twelve were grade 1, 25 grade 2 and 8 grade 3. After microdissection, DNA was extracted according to Wright and Manos (1990).

Polymerase chain reaction

We used 2 sets of primers covering exon 2: 5'-GCAGCAC-CACCAGCGTGTCC-3' and 5'-GGAAATTGGAACTG-GAAGC-3'; 5'-TCTGTTCTCTCTGGCAGGTC-3' and 5'-TCTGAGCTTTGGAAGCTCT-3'. Fifty nanograms of purified DNA were amplified in a total volume of 50 μ l using 50 pmol of sense and anti-sense primers, 200 μ M of each dNTP, 1 \times amplification buffer (50 mM KCl, 10 mM TRIS, pH 8.3, 1.5 mM MgCl₂), 0.5 μ l (2.5 units) of *Taq* polymerase (Perkin Elmer, Branchburg, NJ) and 5% of DMSO. Forty cycles of 50 sec at 94°C, 40 sec at 61°C and 40 sec at 72°C were carried out. PCR products were analyzed on 2% agarose gels.

Single-strand conformation polymorphism (SSCP) analysis

PCR was carried out as described above except that 0.3 μ l [α -³²P]dATP (3,000 Ci/mmol; Amersham, Aylesbury, UK) were added to the reaction. Three microliters of the reaction product were then mixed with 10 μ l of loading buffer containing 96% formamide. Samples were denatured at 94°C for 3 min and chilled on ice for 5 min, and 2 μ l were loaded onto a 6% nondenaturing polyacrylamide gel with and without 10% glycerol. Gels were electrophoresed at 5 W (with glycerol) and 3 W (without glycerol) for 16 hr at room temperature, using 0.5 \times TRIS-borate-EDTA buffer (66 mM TRIS-HCl, 22 mM borate, 1 mM EDTA). Gels were dried and films exposed at -80°C for 3 days.

Sequence analysis

PCR products displaying a shift on the SSCP gel were sequenced both directly, using the Ampli Cycle sequencing kit (Perkin Elmer), and after being cloned into a TA cloning vector (pCR II; Invitrogen, San Diego, CA). Sequence comparison was based on the EMBL/GenBank data base (access number L27211).

Southern blotting

Ten micrograms of DNA were digested by EcoRI (for cell lines) or HindIII (for primary tumors) restriction enzymes, separated on a 0.8% agarose gel, transferred onto Hybond N⁺ (Amersham) according to the manufacturer's protocol and hybridized as previously described (Bringuier *et al.*, 1996) with a probe of 500 bp corresponding to the entire exon 2 PCR product. For loading control, bladder cancer blots were hybridized with the pHE 5.4 probe corresponding to the *ETS* oncogene, which is located at 11q23-24 (de Taisne *et al.*, 1984). For the cell lines, the HPG Ca 2.1 probe (Franke *et al.*, 1989), corresponding to human plakoglobin, localized on 17q21 (Aberle *et al.*, 1995), was used.

Comparative multiplex PCR

Comparative multiplex PCR was performed essentially as described by Walker *et al.* (1995). The primer pair for exon 2 and one control primer pair for a gene located on chromosome 9q were mixed in a PCR reaction and amplified for 30 cycles (1 min 94°C, 1 min 56°C, 1 min 72°C). PCR products were electrophoresed through a 2% agarose gel. After ethidium bromide staining, signals from *p16*^{INK4} and the internal control were compared and homozygous deletions scored if the *p16*^{INK4} signal was missing or highly reduced.

RESULTS

p16^{INK4} deletions and mutations in cell lines

In 9 of 17 (53%) cell lines, no amplification of exon 2 of the *p16*^{INK4} gene was possible, suggesting homozygous deletions. To confirm whether in samples with no PCR product the gene

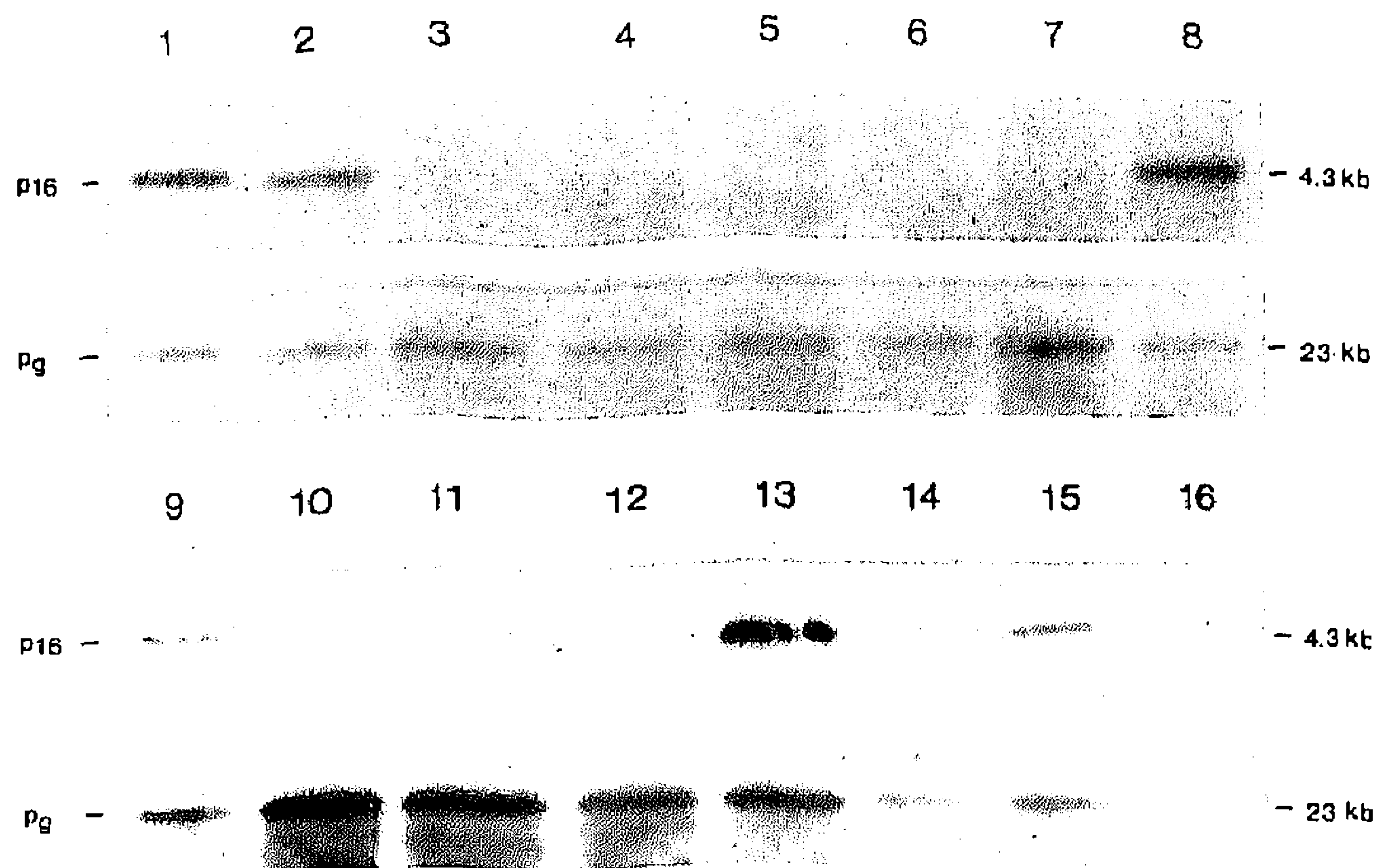


FIGURE 1 – Homozygous deletions of *p16*^{INK4} (exon 2) by Southern blotting. Numbers 1, 2, 3, 4, 5, 6, 7 and 8, correspond to cell lines 647V, TSU (prostatic cell line), VMcub2, 5637, VMcub3, SW-780, RT4 and SCaBER, respectively. Samples 3, 4, 5, 6 and 7 show homozygous deletions. Numbers 9, 10, 11, 12, 13, 14, 15 and 16 correspond to T24, 253J, SD, SW-800, SW1710, RT42, J82 and 575A bladder cell lines, respectively. Note the homozygous deletions displayed by samples 10, 11, 12 and 14.

was indeed homozygously deleted, Southern blot analysis was performed. No signal was obtained in 9 of the 17 cell lines subjected to this analysis, confirming homozygous deletion (Fig. 1).

We subsequently investigated the presence of point mutations using SSCP analysis, which revealed 2 shifts. One of them (SCaBER) has already been reported (Spruck *et al.*, 1994). In the other (Jon), the sequence alteration at position 454 (codon 138) did not lead to any amino acid change (AGA \rightarrow AGG, Arg \rightarrow Arg).

p16^{INK4} deletions and mutations in bladder tumors from the Netherlands

In all of these tumors, bands of the predicted size (250 and 340 bp) could be amplified by PCR. However, the presence of a low percentage (10–25%) of normal tissue prevents the identification of homozygous deletions. Therefore, DNA of 24 frozen samples were tested for homozygous deletions by Southern blot analysis. In 4 of the 22 TCC samples (18%), *p16*^{INK4} appeared to be homozygously deleted. Multiplex PCR on the same samples confirmed deletion in 3 of these samples. Therefore, PCR is an appropriate method to measure *p16*^{INK4} deletions, albeit one that tends to underestimate the frequency. In the 2 SCC samples, no deletions were found.

Band migration shifts, as revealed by SSCP analysis, were not found in bladder cancer not associated to bilharziasis, no evidence for *p16*^{INK4} mutation in such tumors being detected.

p16^{INK4} deletions and mutations in bilharziasis-associated tumors

As shown in Figure 2, after comparative multiplex PCR, the band specific for *p16*^{INK4} was often reduced in intensity when compared with the control. Indeed, homozygous deletion of the *p16*^{INK4} gene was found in 23 of 47 (49%) bilharziasis-associated bladder carcinomas.

Using SSCP analysis, 3 band migration shifts in the coding region of *p16*^{INK4} (exon 2) were found. In one case, the sequence alteration at position 395 of codon 126 did not lead to any amino acid change (GCG \rightarrow GCA, Ala \rightarrow Ala). For the other 2 tumors (Fig. 3), the sequence alterations could not be determined.

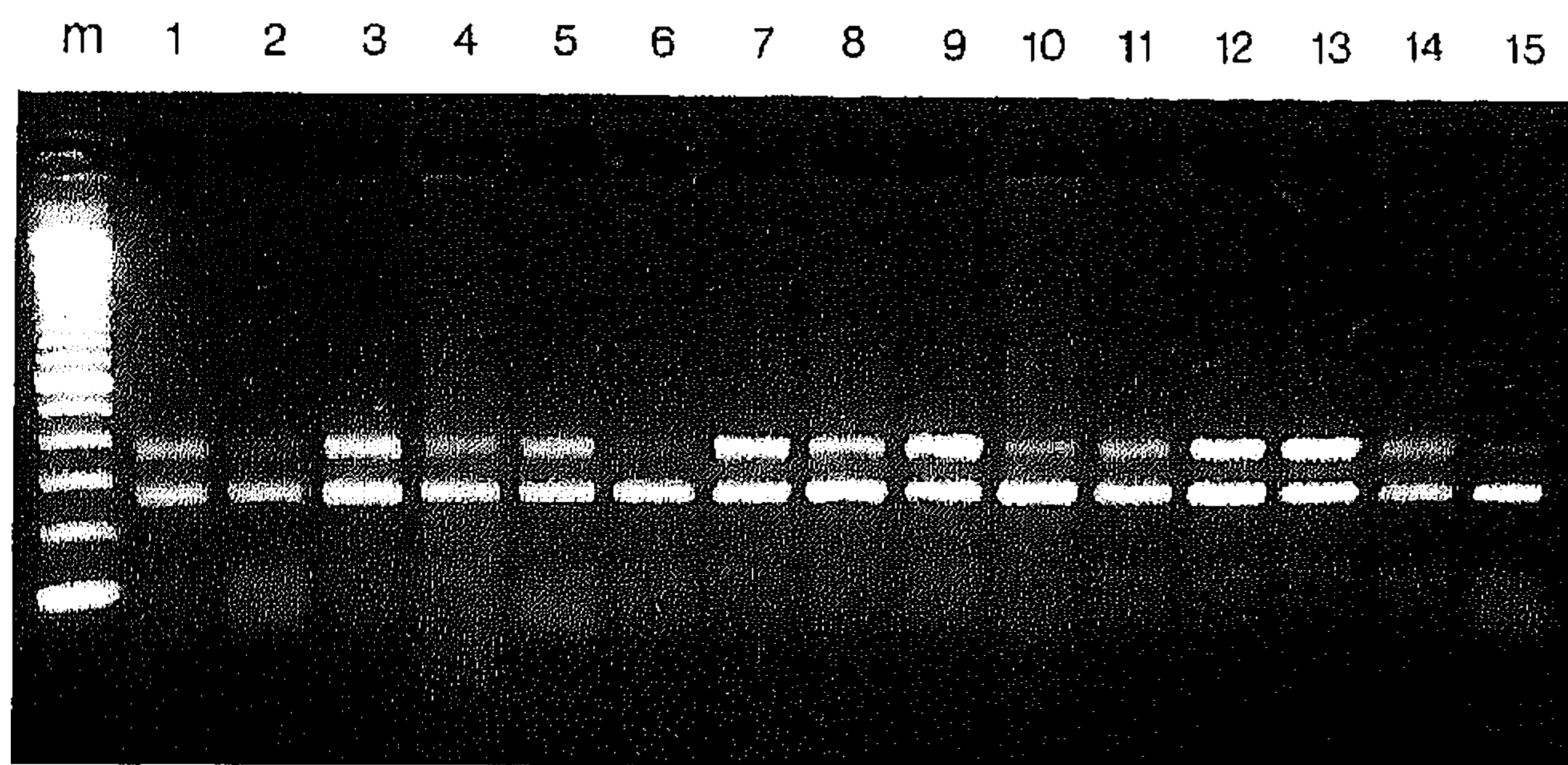


FIGURE 2 – Multiplex PCR analysis of bilharziasis-associated bladder tumors: samples with reduced signal for the *p16*^{INK4}-specific primer set (upper band) have been considered as deleted (lanes 2, 4, 6, 8, 10, 11, 14 and 15). The lower band is our internal control.

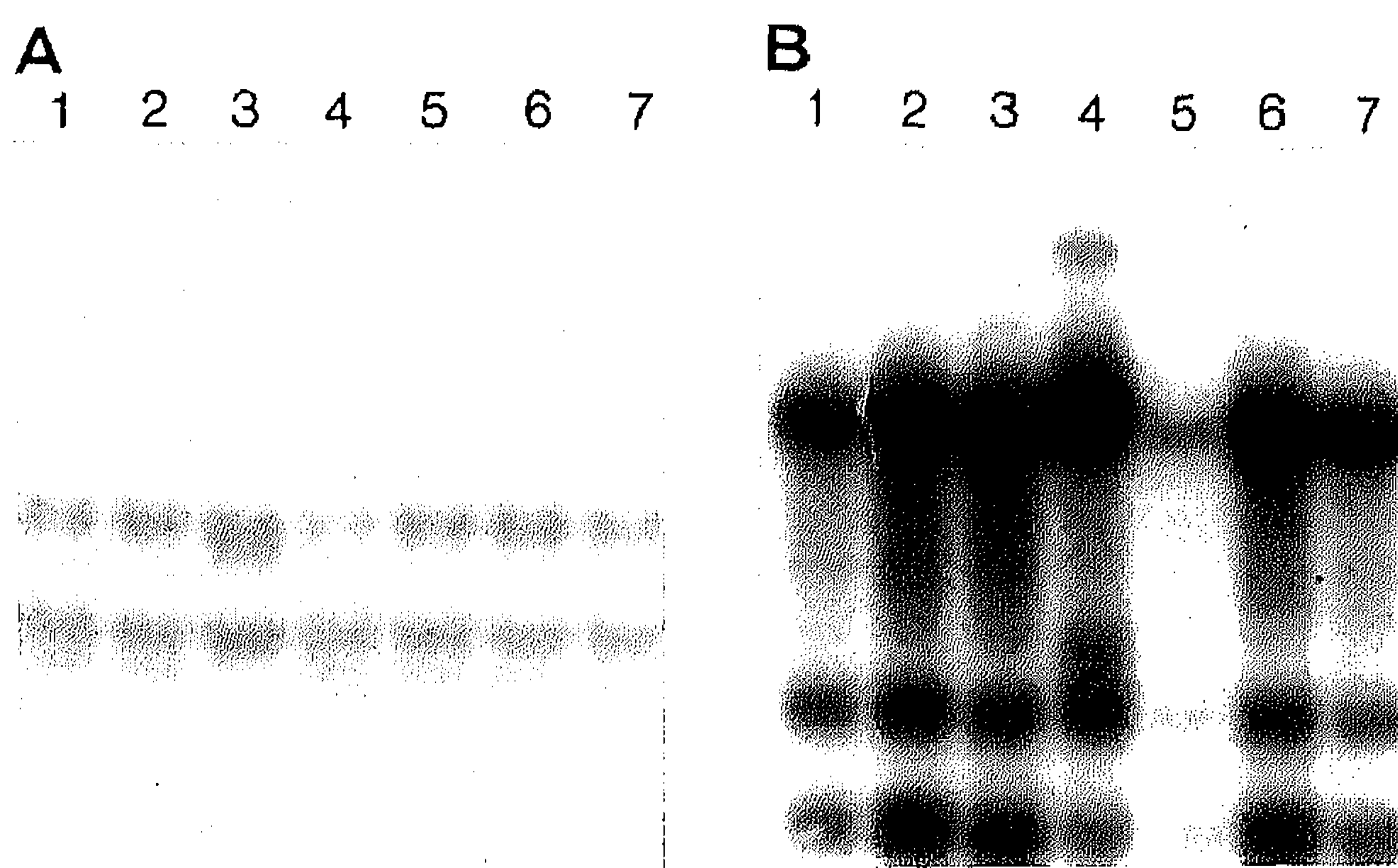


FIGURE 3 – SSCP analysis of exon 2 of the *p16*^{INK4} gene. The 2 migration shifts found in bilharziasis-associated tumors are shown. (a) SSCP for the 5' part of *p16*^{INK4} exon 2 with a shift in lane 3; (b) SSCP for the 3' part of *p16*^{INK4} exon 2 with a shift in lane 4.

Altogether, 25 of 47 (53%) tumors exhibited a *p16*^{INK4} alteration. The distribution of such alterations among the different histological types of tumor is shown in Table I. No correlation was found between *p16*^{INK4} alteration and either grade or stage.

DISCUSSION

In this work, we have studied *p16*^{INK4} gene alteration in a series of 47 bilharziasis-associated bladder tumors from Egypt and compared the results with those obtained for a series of bladder tumors from the Netherlands. As samples from Egypt were paraffin-embedded, deletions were detected by multiplex PCR. We first compared this technique with Southern blot and found no false-positive cases but one false-negative: only 3 of the 4 deletions found by Southern blot could be detected by multiplex PCR. Thus, the frequency of deletion we report here is an under-estimate rather than an over-estimate. This has also been noticed by Williamson *et al.* (1995). The false-negative case was probably due to amplification of the DNA from normal tissue, which was more abundant for the DNA extracted for Southern blot (up to 25% normal tissue as estimated by step section) than in DNA from paraffin-embedded material, which was obtained after microdissection. Indeed, the results of multiplex PCR were much more clear-cut for the bilharziasis-associated bladder tumors than for the tumors not associated with bilharziasis. Another explanation might be related to the fact that in order to be certain that partial deletion and not simple losses of chromosome 9 were

detected, we choose our control PCR set on chromosome 9q, a region prone to loss of heterozygosity in TCCs found in Western countries but not in bilharziasis-associated bladder tumors (Gonzalez-Zulueta *et al.*, 1995).

We believe that the 2 tumors with a shift should be considered as mutated since such a shift has been repeatedly observed and since direct sequencing is less sensitive than SSCP (Knowles and Williamson, 1993). If these shifts were caused by polymorphism, the variant sequences would have represented at least 50% of the DNA, which is well above the detection limit of direct sequencing. Conversely, a mutation can be present in only one part of the sample analyzed, which makes it undetectable by direct sequencing.

Whatever the real status of these 2 tumors, 3 conclusions can be drawn from our work: (i) *p16*^{INK4} alterations are more frequent in cell lines than in primary tumors as described and discussed in detail by Spruck *et al.* (1994); (ii) in primary bladder tumors (bilharziasis-associated or not), *p16*^{INK4} deletions are much more frequent than *p16*^{INK4} mutations; (iii) *p16*^{INK4} alterations are more frequent in bilharziasis-associated bladder tumors than in other bladder tumor types.

Numerous mechanisms have been proposed to explain the association of bilharziasis and bladder cancer (WHO, 1983). One theory on the possible role of carcinogens is that cancer is most likely to occur along with bilharziasis infection. The *N*-nitroso compounds, a potential group of carcinogens widely present in the environment and appearing in the urine of patients with bilharziasis infection, may be implicated in the initiation of bladder cancer (Gentile *et al.*, 1985). Methylation in DNA of bilharziasis-infected human bladder tissue has also been observed (Badawi *et al.*, 1992).

The fact that deletions occur more frequently than single mutations has been noticed in other tumor types, such as brain tumors (Jen *et al.*, 1994; Schmidt *et al.*, 1994; Giani and Finocchiaro, 1994; Walker *et al.*, 1995), lung cancers (Xiao *et al.*, 1995) and mesothelioma (Cheng *et al.*, 1994). In bladder, the rate of homozygous deletions has been found to be higher than the rate of mutations. Our data on bladder cancer from the Netherlands are thus in agreement with other results (Cairns *et al.*, 1994; Williamson *et al.*, 1995), though the rate of homozygous deletion varies from one series to another: 3 of 22 in our study and 5 of 25 in the study of Cairns *et al.* (1994). However, this frequency was estimated at 38% by Williamson *et al.* (1995), who studied a large series of tumors (140 samples). The lower frequency we found cannot be explained by the low sensitivity of the PCR technique, as proposed by Williamson *et al.* (1995), since we have also used Southern blot analysis.

To explain the higher frequency of deletion, it has been proposed that inactivation of *p16*^{INK4} alone does not provide a strong selective advantage and that deletion could lead to the inactivation of several genes (Jen *et al.*, 1994); *p15* is, of course, an appealing candidate for co-inactivation together with *p16*^{INK4} since the gene lies immediately beside *p16*^{INK4} and since both proteins exhibit similar biochemical properties. However, in bladder tumors, a number of deletions have been found which do not involve the *p15* gene, leading to the hypothesis that another gene, telomeric to *p16*^{INK4}, is co-deleted (Williamson *et al.*, 1995). However, in some tumor types, *e.g.*, esophageal SCC (Mori *et al.*, 1994; Zhou *et al.*, 1994; Igaki *et al.*, 1995), *p16*^{INK4} is frequently inactivated by point mutation. It thus appears that the selective advantage provided by *p16*^{INK4} inactivation varies according to tissue types.

Experimental evidence that *p16*^{INK4} may play a role in tumor progression has emerged from studying both sporadic and familial malignancies. In familial melanoma, a locus responsible for the disease has been mapped by linkage analysis to 9p21, a region that harbors the *p16*^{INK4} gene as well (Cannon-Albright *et al.*, 1992, 1994). Furthermore, an association

TABLE I - $p16^{INK4}$ STATUS ACCORDING TO HISTOLOGICAL TYPE IN BILHARZIASIS-ASSOCIATED AND NON-ASSOCIATED TUMORS AND BLADDER CANCER CELL LINES

Tumor type	Histological type	Homozygous deletions	Mutations	% Alterations	Total % alterations
Bilharziasis-associated tumors	SCC	11/20	0/20	55%	53%
	TCC	9/20	2/20	53%	
	Adenocarcinoma	3/5	0/5	60%	
	Unknown	0/2	0/2	0%	
Tumors not associated with bilharziasis	TCC	4/22	0/22	18%	18%
	SCC	0/2	0/2	0%	
Cell lines		9/17	1/17	59%	59%

between germ-line $p16^{INK4}$ mutation and familial melanoma has been observed in some kindreds, suggesting that the $p16^{INK4}$ gene is an inherited melanoma susceptibility gene (Ohta *et al.*, 1994; Kamb *et al.*, 1994b). Hussussian *et al.* (1994) have indeed shown that in 9/15 families with inherited melanoma linked to 9p21, 6 $p16^{INK4}$ mutations co-segregate with the occurrence of melanoma (Hussussian *et al.*, 1994). A somatic mutation (Pro-81-Leu) was also detected in a tumor from an individual with the (Arg-87-Pro) germ-line mutation (Hussussian *et al.*, 1994). A similar study on 15 Dutch families revealed a 19 bp exon 2 deletion in 13 kindreds (Gruis *et al.*, 1995). Surprisingly, all of the kindreds carried the same deletion, suggesting a common ancestry of the affected population. Alternatively, none of the previously described melanoma-associated mutations was found in a study carried out on 17 Australian melanoma kindreds, with one family exhibiting an exon 1 mutation not described so far. This was shown to co-segregate with melanoma and to cause an amino acid substitution (Arg-24-Pro) (Holland *et al.*, 1995). Sporadic cutaneous melanoma accounts for approximately 90% of all cases of melanoma and probably arises as an cumulative result of genetic defects (Herlyn, 1993). In a melanoma cell line established from an individual patient, it has been shown that deletion of 9p21 can occur before metastasis in sporadic melanoma (Glendening *et al.*, 1995).

The most interesting finding of our study is the high deletion rate in tumors from Egypt. In non-small cell lung cancer, it has been suggested that $p16^{INK4}$ alteration is a late event during the carcinogenic process (Okamoto *et al.*, 1995). If this is also true for bladder, this could contribute to the high frequency of alteration found in bilharziasis-associated bladder tumors as these are generally detected at an advanced stage. However, we do not favor this explanation since we did not observe any

increased frequency of alteration along with either grade or stage in such samples. The increased frequency of $p16^{INK4}$ alteration thus appears to be truly associated with schistosome infestation. Actually and while our work was in progress, a study of genetic alterations in SCCs has been published (Gonzalez-Zulueta *et al.*, 1995), in which $p16^{INK4}$ alterations were found in 6 of 9 SCCs from Egypt. The results we have obtained on a larger series confirm the high prevalence of $p16^{INK4}$ alterations in bilharziasis-associated bladder tumors. Gonzalez-Zulueta *et al.* (1995) suggest that $p16^{INK4}$ deletions might be a part of a pathway specific for SCC development. Indeed, they have also analyzed 3 SCCs from Sweden and found $p16^{INK4}$ alteration in 2. Our data do not support this hypothesis since: (i) the 2 SCCs from the Netherlands we have analyzed showed no $p16^{INK4}$ alteration; (ii) in bilharziasis-associated bladder tumors, $p16^{INK4}$ alterations were not restricted to SCC but were found with a similar frequency in any type of tumor. According to our results, $p16^{INK4}$ alteration is rather associated with a specific etiology (the presence of schistosome eggs in the bladder wall) than with a specific histological type.

Since D-type cyclin, CDK4, $p16^{INK4}$ and the retinoblastoma (RB) protein act *via* the same pathway, it has been proposed that inactivation or activation of one of these elements would deregulate the G_1 transition. Indeed, aberrations of $p16^{INK4}$ and RB occur in distinct subsets of cell lines (Aagaard *et al.*, 1995). The fact that in bilharziasis-associated bladder tumors RB expression is often conserved, whereas $p16^{INK4}$ is often inactivated, supports this hypothesis. The study of bladder cancer samples from the Netherlands, which we analyzed for activation of cyclin D1 and inactivation of $p16^{INK4}$ and RB, provides indications in favor of this hypothesis (Bringuier *et al.*, 1996).

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