p15INK4b in bladder carcinomas: decreased expression in superficial tumours

MA Le Frère-Belda1,2, D Cappellen3, A Daher2, S Gil-Diez-de-Medina1,3, F Besse3, CC Abbou2, JP Thiery3, ES Zafrani1, DK Chopin2,4 and F Radvanyi3

Summary

The p15 gene which encodes a cyclin-dependent kinase inhibitor, is located in the 9p21 chromosomal region that is frequently deleted in human bladder transitional cell carcinomas (TCCs). The aim of the present paper is to study the potential involvement of the p15 gene in the evolution of TCCs. p15 mRNA expression was investigated by semi-quantitative RT-PCR in a series of 75 TCCs, 13 bladder cell lines and 6 normal bladder urothelia. p15 mRNA levels were significantly decreased in 66% of the superficial (Ta-T1) TCCs (P = 0.0015). In contrast, in muscle-invasive (T2-T4) TCCs, p15 expression differed widely between samples. p16 mRNA levels were also studied and there was no correlation between p15 and p16 mRNA levels, thus indicating that the two genes were regulated independently. Lower p15 expression in superficial tumours did not reflect a switch from quiescence to proliferative activity as normal proliferative urothelial controls did not present decreased p15 mRNA levels relative to quiescent normal urothelia. We further investigated the mechanisms underlying p15 down regulation. Homozygous deletions of the p15 gene, also involving the contiguous p16 gene, were observed in 42% of the TCCs with decreased p15 expression. No hypermethylation at multiple methylation-sensitive restriction sites in the 5'-CpG island of p15 was encountered in the remaining tumours. Our data suggest that decreased expression of p15 may be an important step in early neoplastic transformation of the urothelium and that a mechanism other than homozygous deletions or hypermethylation, may be involved in p15 down regulation. © 2001 Cancer Research Campaign

Keywords: bladder; human transitional cell carcinoma; cyclin-dependent kinase inhibitor; p15

In eukaryotes, cell cycle progression is particularly controlled at two steps, before the transitions from G1 to S and from G2 to M (Hall and Peters, 1996). Progression through both checkpoints is controlled by cyclin-dependent protein kinases (CDKs) sequentially regulated by cyclins D, E and A (Sherr, 1996). P15 (also called p15INK4a, MTS2, INK4b, CDKN2B) and p16 (also known as p16INK4a, p16INK4, MTS1, CDK4, CDKN2A) prevent CDK activation, specifically that of CDK4 and CDK6 associated with D-type cyclins, by blocking the binding to the cyclin regulatory subunits, inducing G1 phase arrest (Hannon and Beach, 1994; Serrano et al., 1993). However, the activities of p15 and p16 are regulated differently. P15 is an effector of transforming growth factor-β (TGF-β)-induced cell cycle arrest whereas p16 is not involved in TGF-β-induced growth inhibition (Hannon and Beach, 1994). P15 is located on chromosome band 9p21 adjacent to the INK4a/ARF locus, which encodes two unrelated proteins, p16INK4a and p14ARF, through the use of shared coding regions and alternative reading frames. P14ARF is a potent negative regulator of the cell cycle that functions in a manner different from that of CDK inhibitors, via a p53-dependent pathway (Sherr, 1998; Sharpless and DePinho, 1999). The INK4a/ARF locus is a frequent site of chromosomal deletion in human tumours (Hannon and Beach, 1994; Jen et al., 1994). Numerous studies have identified p16 as the principal target of these deletions (Kamb, 1995). Mutational analysis has shown that p16 is commonly mutated or homozygously deleted in human cancer. In particular, germline mutations specifically affecting p16 have been identified in familial melanoma (Hall and Peters, 1996; Sherr, 1996). The methylation of the 5'-CpG island of p16 has been proposed as another mechanism for the inactivation of this gene (Merlo et al., 1995; Herman et al., 1995). The neighbouring p15 gene has been considered as a putative tumour suppressor gene due to the high level of sequence identity and functional similarity between p15 and p16. The analysis of cell lines and primary tumours of various origins has not resulted in the identification of any p15 gene point mutations (Stone et al., 1995; Hall and Peters, 1996; Sherr, 1996). Homozygous deletions of the p15 gene in primary tumours and tumour cell lines almost invariably involves the nearby p16 gene as well. aberrant methylation of p15 is associated with the loss of transcription of this gene in leukemias and gliomas (Herman et al., 1996). This mechanism, which seems to involve the p15 gene selectively, provides the sole evidence so far of a tumour suppressor role for this gene in human neoplasia.

Urinary bladder transitional cell carcinomas (TCCs) are the fourth most common cancer in men and the ninth most common cancer in women in Western countries. TCCs are either superficial [Ta-T1 tumours including TCCs confined to the urothelium (Ta) and those invading only the lamina propria (T1)] or muscle-invasive. The 9p21 region, surrounding the INK4a/ARF locus and the p15 gene, has been found to be lost in about 50% of bladder tumours (Reznikoff et al., 1996). Several analyses of bladder tumours have failed to identify p15 gene point mutations (Orlow et al., 1995; Packenham et al., 1995; Miyamoto et al., 1995). Although the homozygous deletions found in bladder tumours generally
include both p15 and p16, rare examples of selective p15 deletion have been described (Orlow et al., 1995; Packenham et al., 1995; Williamson et al., 1995). No study has reported the inactivation of p15 through a loss of expression. Basing our study on the clear involvement of the loss of the 9p21 region in bladder cancer, we evaluated the importance of the cell cycle inhibitor p15 in bladder carcinogenesis, by investigating changes in p15 expression at the mRNA level in a series of 75 primary TCCs of various stages and grades and 13 bladder cancer cell lines. As we found a decreased expression of p15 in a significant number of bladder tumours, we investigated several potential mechanisms for this down regulation. Considering the known involvement of p16 in some tumours, we have also analysed p15 expression in conjunction with p16 expression.

MATERIALS AND METHODS

Cell lines

Human bladder cell lines 647V, EJ138, J82, JON53, RT112, T24, TCCSUP were obtained and cultured as previously described (Gil Diez de Medina et al., 1999). Lysates in 4M guanidinium thiocyanate of the bladder cell lines HCV29, HT1376, RT4, UM-UC-3, VM-CUB-1, VM-CUB-3, were kindly provided by Dr J Southgate (Leeds, U.K.).

Tissue samples

Tumour tissues were obtained from transurethral resections or radical cystectomy samples from 75 patients with transitional cell carcinomas of the urinary bladder. Tumours were classified by stage according to the TNM classification (UICC, 1992) and by grade according to criteria recommended by the World Health Organisation (WHO, 1973). The tumours studied were: 9 Ta (papillary superficial non-invasive tumours), 14 T1a (lesions invading the superficial lamina propria), 15 T1b (lesions invading the deep lamina propria), 10 T2 (lesions invading the inner layer of vesical muscle), 17 T3 (outer layer of the muscle invaded with or without adipous perivesical tissue tumour invasion), and 10 T4 (tumour extension beyond the bladder). Sixteen were grade G1 (low grade), 26 grade G2 (intermediate grade), and 33 grade G3 (high grade). A representative sample was taken from each tumour for histological assessment, and an adjacent fragment (high grade). A representative sample was taken from each tumour for histological assessment, and an adjacent fragment (high grade). A representative sample was taken from each tumour for histological assessment, and an adjacent fragment (high grade). A representative sample was taken from each tumour for histological assessment, and an adjacent fragment (high grade). A representative sample was taken from each tumour for histological assessment, and an adjacent fragment (high grade).

Primary cultures of human urothelia and 5-bromo-2′-deoxyuridine (Brdu) incorporation

An organo-typic culture model (de Boer et al., 1996) was used for three normal urothelia. In order to compare p15 mRNA expression to proliferation data in these primary cultures of human normal urothelia, BrdU incorporation was studied at different times of the culture (de Boer et al., 1994). Briefly, upon termination of the culture, cells were incubated with 40 μg/ml of BrdU in serum-free medium for 2 h. Cultures were then rinsed with PBS, PH 7.2, and fixed with 96% ethanol for at least 1 h for immunocytochemistry. Before the primary anti-BrdU antibody incubation, cultures were treated with HCl and Borax buffer. Chain-specific cyto keratin and BrdU expressions were visualized using appropriate dilutions of the primary mouse monoclonal antibodies in a conjugated immunoenzyme assay. The anti-cytokeratin and anti-BrdU antibodies were kindly donated by Professor FCS Ramaekers (Maastricht, Netherlands). Secondary rabbit anti-mouse (Dako, Glostrup, Denmark) antibodies were either peroxidase-conjugated (used for BrdU staining) or alkaline phosphatase-conjugated (used for cytokeratin staining). 3,3′-diaminobenzidine tetrahydrochloride (DAB) or the diazonium salt served as chromogens, and fast red violet LB with naphtol AS-MX phosphate served as coupling reagent (Sigma, St Louis, USA). The number of cells immunostained with the anti-BrdU antibody was then compared to the total cell number assessed with the anti-cytokeratin antibody in 1 mm² surface.

RNA and DNA extraction

RNA and DNA were extracted simultaneously using the caesium chloride cushion method essentially as described elsewhere (Coombs et al., 1990), but with slight modifications (Cappellen et al., 1997; Gil Diez de Medina et al., 1997).

RT-PCR

p15 and p16 messenger RNA levels were determined by radioactive semi-quantitative RT-PCR using TBP (TATA-binding protein) or GAPDH as internal controls. cDNA synthesis and PCR analysis were performed as previously described (Radvanyi et al., 1993; Gil Diez de Medina et al., 1997). Briefly, the number of cycles was chosen to be in the exponential part of the PCR (23 cycles for the co-amplification of p15 and TBP, 24 for the co-amplification of p16 and TBP and 21 for the co-amplification of TBP and GAPDH). The primer sequences used for p15 and p16, located in exons 1 and 2 of these genes, were: 5′-CGCTGCCCATCATGAC-3′ (sense) and 5′-CTAGTGAGAAGGTGCGACA-3′ (antisense) for p15 and 5′-CACCGCACCAGAATTAC-3′ (sense) and 5′-CACCGGTCGGGATG-3′ (antisense) for p16. Primer sequences for TB and GAPDH were as described elsewhere (Gil Diez de Medina et al., 1997). The PCR-amplified products were subjected to electrophoresis in 8% polyacrylamide gels. Signals were quantified with a Molecular Dynamics 300 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). There was no amplification if reverse transcriptase was omitted from the reverse transcription reaction.

Homozgous deletions analysis for the p15 and p16 genes

Homozogous deletions were detected by a PCR based assay. A fragment located in exon 1 of p15 (or p16) was co-amplified with a genomic fragment of either GAPDH or PSA as a control. GAPDH and PSA are located in chromosomal regions that are infrequently the target of allelic loss in bladder carcinomas (less than 3% and 7%, respectively). The primer sequences for p15, p16, GAPDH and PSA are listed below.

5′-GGGCCAGGCGTTTGAG-3′ (p15 sense) and 5′-CTGG GCTAGCCTCATTACC-3′ (p15 antisense), 5′-TCGTTGTA GGAGGTGGCGG-3′ (p16 sense) and 5′-GATCGGCCCTC- GACCCTAACT-3′ (p16 antisense), 5′-TGGCGTGCTGAATA
CC ATGT-3′ (GAPDH sense) and 5′-AAGGCATGCTGCAACTGAA-3′ (GAPDH antisense), 5′-AGGCTGGCGGACGAT-3′ (PSA sense) and 5′-CACCTCGAGAGTGACATTG-3′ (PSA antisense). PCR were performed with 50 ng of genomic DNA and the number of cycles was selected so as to be in the exponential part of the two amplification reactions (i.e. 23 cycles). PCR products were analysed as described in the reverse transcription-PCR section. The relative intensity of the products obtained for the test (p15 or p16) and control (GAPDH or PSA) sequences in normal and tumour DNA samples was compared and the relative representation of p15 and p16 calculated as follows:

| Intensity of control in tumour DNA | Intensity of test sequence in tumour DNA | Intensity of control in normal DNA | Intensity of test sequence in normal DNA |
|-----------------------------------|----------------------------------------|----------------------------------|----------------------------------------|

Given the potential for tumour heterogeneity and contamination with non-neoplastic cells, tumours with ratios below 0.3 were considered to have homozygous deletions.

**Methylation analysis**

A quantitative PCR assay based on the inability of some restriction enzymes to cut methylated sequences (Singer-Sam et al, 1990) was used to analyse the methylation status of the first exon of the p15 gene. Three sets of primers flanking three different regions of exon 1 of p15 gene were designed (Figure 3). The sites examined were: one HpaII site in fragment 1, one EagI, two HpaII and five CfoI sites in fragment 2 and ten HpaII, one SacII and ten CfoI sites in fragment 3. DNA was digested according to the manufacturer’s instructions (New England Biolabs). DNA (1 µg) was digested overnight at 37°C, with 10 units of enzyme/µg of DNA. The primer sets used for methylation analysis of p15 exon 1 were 5′-CTTGGCCACGT-GAAACG-3′ (sense) and 5′-ACGCAGCGAGCTCAG-3′ (antisense) for fragment 1 and, 5′-CGGGAACCGGTGAT-TATCC-3′ (sense) and 5′-CACACCTCGGCAACGACG-3′ (antisense) for fragment 3. The primer set for fragment 2 and the amplification reactions were as described in the homoyzogous deletions analysis section except that 25 and 26 cycles were performed to amplify fragments 1 and 3 of exon 1 of the p15 gene respectively, so as to be in the linear range of the assay. The PCR-amplified products were subjected to electrophoresis in 8% polyacrylamide gels and an autoradiograph was produced. AflII restriction enzyme was used as a positive control (restriction site outside the amplified fragments) and MspI restriction enzyme as a negative control (methylation-insensitive enzyme) for each template.

**Statistical analysis**

mRNA levels were analysed according to stage and frequencies were analysed with the Mann-Whitney test. Correlation was estimated between p15 and p16 mRNA expression.

**RESULTS**

**Decreased expression of p15 mRNA in superficial TCCs**

p15 mRNA levels were determined in normal bladder tissues (urothelium, lamina propria, muscle) and in a series of 75 TCCs (38 superficial and 37 muscle-invasive tumours) by semi-quantitative RT-PCR, using two different internal controls, TBP and GAPDH (Figures 1, 2A and data not shown). The six normal human urothelia studied all expressed p15 mRNA, and the levels in the various urothelia were similar (mean value = 0.86). p15 mRNA was also detected in lamina propria (n = 3) and muscle (n = 5) (mean value 0.51 and 0.53 respectively). The superficial TCCs (Ta, T1a, T1b) had significantly lower levels of p15 mRNA than normal urothelium (P = 0.0015). Twenty-five of the 38 superficial TCCs (66%) contained low levels of p15 mRNA (less than 30% of the mean value for normal urothelium) and in 19 of these 25 TCCs, p15 mRNA levels were close to zero. Such a decrease in p15 mRNA levels was more frequent in the Ta-T1a tumours. In this group, 18 out of 23 tumours (78%) presented low levels of expression, versus 7 out of 15 T1b tumours (47%). In the superficial tumours that expressed p15, p15 mRNA levels were similar those for normal urothelium. In contrast, in the 37 invasive TCCs (T2–T4), p15 mRNA levels varied widely from non-detectable to more than 5 times higher than the level found in normal urothelium and no significant difference in p15 mRNA level was found between normal urothelium and invasive TCCs (P = 0.55). We compared

![Figure 1](image.png)
mRNA expression with tumour grade and found that 12 out of 16 mRNA expression in the TCCs (correlation coefficient R² = 0.26) (Figure 2B). p16 mRNA levels were non-detectable in any of the normal bladder tissues whether they were urothelium, lamina propria or muscle samples. In the same way, p16 mRNA levels were non-detectable in most of the superficial TCCs; only 6 superficial tumours (1 Ta, 3 T1a and 2 T1b) out of the 37 (16%) expressed p16. On the other hand, p16 positive tumours were more frequent in the invasive TCCs group and concerned 13 (2 T2, 8 T3, 3 T4) of the 37 tumours (35%). No correlation was found between p15 and p16 mRNA expression in the TCCs (correlation coefficient R² = 0.26). p16 mRNA expression was also studied according to tumour grade. p16 mRNA levels were non-detectable in all the 15 G1 tumours as well as in 20 of the 26 G2 (77%) and 20 of the 33 G3 (61%) tumours.

**Homozygous deletions of p15 in bladder tumours**

The results are summarized in Table 2. Twenty-eight TCCs for which DNA was available were tested for deletions of exon 1 of p15 using a PCR-based assay. They comprised 12 TCCs (1 Ta, 5 T1a, 2 T1b, 1 T2, 1 T3 and 2 T4) with p15 mRNA levels less than 30% of the mean for normal urothelium and 16 TCCs (1 Ta, 2 T1a, 2 T2, 4 T3 and 7 T4) with higher levels of p15 expression. Homozygous deletions of the p15 gene were observed in 5 of the 12 tumours lacking p15 mRNA. No homozygous deletions were detected in the 16 TCCs that expressed p15. The frequency of p15 homozygous deletions was 18% if the entire cohort of TCCs was considered (5 out of 28) and 42% if the cohort was restricted to the 12 TCCs that did not express p15 (5 out of 12). Homozygous deletions of p15 were found in two (1 T1a, 1 T1b) of the eight superficial TCCs and in three of the four invasive tumours. The 12 TCCs lacking p15 expression comprised 3 G1, 6 G2 and 3 G3 tumours and the 5 homozygous deletions of p15 involved 3 of the 6 G2 and 2 of the 3 G3 tumours.

**Homozygous deletion of p16 in bladder tumours**

To determine whether p15 deletions were selective or also involved the p16 gene, the 5 TCCs with homozygous p15 deletions were tested

### Table 1

Quantitation of parameters for proliferation and p15 mRNA expression in six normal quiescent urothelia and in three primary cultures of human urothelia. p15 mRNA expression/TBP mRNA is the mean of the ratio of p15 mRNA to TBP mRNA in semi-quantitative RT-PCR ± the standard deviation. The proliferation was determined as described in Materials and Methods and is given as the mean of the percentage of BrdU positive nuclei relative to the total number of nuclei ± the standard deviation.

| Day of culture | p15 mRNA/TBP mRNA | BrdU incorporation |
|---------------|--------------------|-------------------|
| D0            | 0.9 ± 0.2          | 3 ± 0.5           |
| D10           | 4.9 ± 2.7          | 19.4 ± 3.4        |

**Table 2**

| Tumour stage | p15 mRNA expression |
|--------------|----------------------|
|              | Superficial | Invasive | Superficial | Invasive |
|              | (n = 8)     | (n = 4)   | (n = 3)     | (n = 13)  |
| Homozygous deletion | 2/8         | 3/4       | 0/3         | 0/13      |

p15 mRNA expression was not expressed in any of the normal bladder tissues whether they were urothelium, lamina propria or muscle samples. In the same way, p16 mRNA levels were non-detectable in most of the superficial TCCs; only 6 superficial tumours (1 Ta, 3 T1a and 2 T1b) out of the 37 (16%) expressed p16. On the other hand, p16 positive tumours were more frequent in the invasive TCCs group and concerned 13 (2 T2, 8 T3, 3 T4) of the 37 tumours (35%). No correlation was found between p15 and p16 mRNA expression in the TCCs (correlation coefficient R² = 0.26). p16 mRNA expression was also studied according to tumour grade. p16 mRNA levels were non-detectable in all the 15 G1 tumours as well as in 20 of the 26 G2 (77%) and 20 of the 33 G3 (61%) tumours.

**p15 mRNA expression in proliferative normal human urothelia in an organo-typic culture model**

Cell proliferation on the cultures of normal urothelia was assessed by BrdU incorporation. The highest level of proliferation was obtained after 5 days’ culture (19.4% of cells immunostained with the anti-BrdU antibody) and then it decreased slowly after 10 days’ culture. The analysis of p15 mRNA expression in this model showed that p15 mRNA expression, present in quiescent urothelia, did not decrease in proliferative urothelia (Table 1).

**Absence of p16 mRNA expression in normal bladder tissues, in most superficial TCCs and in some invasive TCCs**

p16 mRNA expression was studied in all the samples of normal bladder tissues and in 74 (37 superficial and 37 muscle invasive tumours) out of the 75 TCCs previously studied for p15 mRNA levels with tumour grade and found that 12 out of 16 p15 mRNA levels with tumour grade and found that 12 out of 16 mRNA expression in the TCCs (correlation coefficient R² = 0.26) (Figure 2B). p16 mRNA levels were non-detectable in any of the normal bladder tissues whether they were urothelium, lamina propria or muscle samples. In the same way, p16 mRNA levels were non-detectable in most of the superficial TCCs; only 6 superficial tumours (1 Ta, 3 T1a and 2 T1b) out of the 37 (16%) expressed p16. On the other hand, p16 positive tumours were more frequent in the invasive TCCs group and concerned 13 (2 T2, 8 T3, 3 T4) of the 37 tumours (35%). No correlation was found between p15 and p16 mRNA expression in the TCCs (correlation coefficient R² = 0.26). p16 mRNA expression was also studied according to tumour grade. p16 mRNA levels were non-detectable in all the 15 G1 tumours as well as in 20 of the 26 G2 (77%) and 20 of the 33 G3 (61%) tumours.

**Homozygous deletions of p15 in bladder tumours**

The results are summarized in Table 2. Twenty-eight TCCs for which DNA was available were tested for deletions of exon 1 of p15 using a PCR-based assay. They comprised 12 TCCs (1 Ta, 5 T1a, 2 T1b, 1 T2, 1 T3 and 2 T4) with p15 mRNA levels less than 30% of the mean for normal urothelium and 16 TCCs (1 Ta, 2 T1a, 2 T2, 4 T3 and 7 T4) with higher levels of p15 expression. Homozygous deletions of the p15 gene were observed in 5 of the 12 tumours lacking p15 mRNA. No homozygous deletions were detected in the 16 TCCs that expressed p15. The frequency of p15 homozygous deletions was 18% if the entire cohort of TCCs was considered (5 out of 28) and 42% if the cohort was restricted to the 12 TCCs that did not express p15 (5 out of 12). Homozygous deletions of p15 were found in two (1 T1a, 1 T1b) of the eight superficial TCCs and in three of the four invasive tumours. The 12 TCCs lacking p15 expression comprised 3 G1, 6 G2 and 3 G3 tumours and the 5 homozygous deletions of p15 involved 3 of the 6 G2 and 2 of the 3 G3 tumours.

**Homozygous deletion of p16 in bladder tumours**

To determine whether p15 deletions were selective or also involved the p16 gene, the 5 TCCs with homozygous p15 deletions were tested

### Table 2

| Tumour stage | p15 mRNA expression |
|--------------|----------------------|
|              | Superficial | Invasive | Superficial | Invasive |
|              | (n = 8)     | (n = 4)   | (n = 3)     | (n = 13)  |
| Homozygous deletion | 2/8         | 3/4       | 0/3         | 0/13      |
for deletions of exon 1 of p16. All 5 tumours with homozygous p15 deletions also had homozygous deletions of p16 (data not shown).

**Lack of methylation of the CpG island in exon 1 of the p15 gene in bladder tumours**

The 5’ region of the p15 gene contains a CpG island located around the transcription start site. It is therefore a good candidate for hypermethylation-associated inactivation (Herman et al, 1996). A methylation-sensitive restriction map of approximately 600-bp extending from the promoter region through exon 1 of the p15 gene is shown in Figure 3. A PCR-based assay was used to analyse the methylation status of this CpG island. Double digestion with a restriction enzyme cutting the flanking regions (AflII) and methylation-sensitive enzymes (CfoI, HpaII, EagI, SacII) was followed by amplification of regions 1, 2 and 3 (Figure 3). Normal urothelium showed no methylation in any of the three regions tested (Figure 4 and data not shown). Eight of the nine primary TCCs expressing p15 that were tested were also unmethylated. Only one p15-expressing tumour was methylated in region 3 of exon 1, heavily at a SacII and at a lower level at HpaII restriction sites (Figure 4). After excluding the possibility of homozygous deletions of p15, the methylation status of five of the seven tumours that did not express p15 was assessed. The five available samples showed no detectable methylation in any of the three regions tested. Restriction of genomic DNA from normal urothelium and 10 tumours with the flanking enzyme HindIII, plus the methylation-sensitive enzyme EagI, and Southern blotting with a p15 exon 1 probe, confirmed the results obtained by the PCR-based assay (data not shown). The p15 gene was unmethylated at this EagI site in normal urothelium. All tumour tissues, both expressing and not expressing p15, were unmethylated at this site.

**mRNA levels and homozygous deletions of p15 in bladder tumour cell lines**

Of the 13 human bladder cell lines for which mRNA levels were analysed, 6 cell lines had no detectable p15 mRNA and one cell line (EJ138) had low levels of p15 mRNA (Table 3). In contrast with the tumours, all 6 bladder cell lines with no p15 expression presented homozygous deletions of p15, always deleted with p16 (data not shown). As expected, in the seven remaining tumour cell lines, which expressed p15, no homozygous deletions were detected. In the EJ138 cell line, which had very weak p15 expression, the methylation status of p15 exon 1 was studied by PCR and Southern blotting, and no abnormal DNA methylation was detected (data not shown).

**DISCUSSION**

A critical area of chromosomal deletion at region 9p21–22 has been implicated in the genesis of various types of primary tumours, including bladder carcinomas. Three negative cell cycle regulators, p15, p16 and p14ARF, encoded by two genes located in tandem in this region, have been identified as potential tumour suppressors. The role of p15 in carcinomas is unclear. Indeed, no intragenic mutation in p15 has been reported and homozygous deletions of p15 have not been observed in any of the tumours examined. It should be noted that the p15 gene is present in chromosome region 9p21–22 and that homozygous deletions of the p14ARF gene have been observed in the tumour cell lines used in this study (12). As the p14ARF gene is located in chromosome region 9p21–22, the lack of homozygous deletions of the p15 gene in these cell lines is not surprising. These findings suggest that the p15 gene is not a frequent target of homozygous deletions in bladder carcinomas.
deletions of p15 almost always include the neighbouring INK4a/ARF locus. We investigated the possible involvement of p15 in bladder carcinomas, by comparing p15 mRNA levels in normal urothelium and a series of derived carcinomas.

We found that p15 was expressed in the normal urothelium and that p15 mRNA levels were significantly decreased in most superficial TCCs (66%). This decreased expression was not due to contamination of the tumour samples by normal tissue and that for several reasons: all the TCC samples used in this analysis were primarily composed of tumour cells, as assessed by histological examination; contamination of the tumour by the underlying compartments, the lamina propria and the smooth muscle, which express p15 at a level similar to that in urothelium, would have masked a decrease in p15 mRNA level. Furthermore, the analysis of p15 mRNA expression according to proliferation in normal urothelia showed that p15 mRNA levels did not decrease in proliferative urothelia thus demonstrating that lower p15 mRNA levels encountered in Ta-T1 tumours were in fact a tumour specific alteration and did not reflect a proliferative state.

Considering the known involvement of p16 in the genesis of various tumours and because p16 was located nearby p15, we have also investigated p16 mRNA expression in those tumours. We found that p16 mRNA levels were non-detectable in any of the normal bladder samples and increased with stage and grade of the TCCs. The absence of p16 expression in normal bladder urothelium was in agreement with several other studies that did not find any p16 expression in normal urothelium cultured or uncultured neither by RT-PCR nor Western blotting nor immuno-histochemistry (Yeager et al, 1995; Stadler et al, 1996; Benedict et al, 1999). In TCCs, we have found that p16 mRNA expression was not correlated to p15 mRNA levels (correlation coefficient R² = 0.26) thus indicating that the expression of the two neighbouring p15 and p16 genes encoding related CDK inhibitors was differently regulated in normal and tumour tissues.

We investigated several possible explanations for p15 mRNA down-regulation. Although loss of heterozygosity on chromosome band 9p21 is a common event, p15 mRNA down-regulation in this case cannot be due to the loss of one copy of the p15 gene, as in 26 of the 34 TCCs with decreased p15 mRNA levels there was almost no p15 mRNA. As the p15 and p16 loci are targets for homozygous deletions in bladder carcinomas, the loss of two copies of the p15 gene may account for the down-regulation of p15 reported herein. To avoid underestimation of homozygous deletions due to the presence of even small amounts of contaminating normal tissue, a semi-quantitative PCR assay was performed with a limited number of PCR cycles (see Materials and Methods). The frequency of homozygous deletions at the p15 locus in primary TCCs in this study (18%) was similar to that in several other studies (Spruck et al, 1994; Orlow et al, 1995; Packenham et al, 1995; Williamson et al, 1995). The p15 homozygous deletions that we observed were always associated with p16 deletions and were present in less than half the primary bladder tumours with little or no p15 expression. In primary tumours, homozygous deletions were not the sole mechanism of p15 down-regulation. In contrast, the frequency of homozygous p15 deletions was much higher in the bladder cell lines tested (about 50%) than in primary tumours. These deletions, in all but one case, account for the lack of expression in cell lines. Differences in the frequency of homozygous deletions in primary tumours and cell lines for certain tumour types including bladder carcinomas have been reported independently by many studies (Spruck et al, 1994; Southgate et al, 1995; Williamson et al, 1995). Homozygous deletions in cell lines may confer a long-term growth advantage in vitro (Spruck et al, 1994). Transcriptional repression by DNA methylation of the Cpg island in the 5’ region of p15 may be an additional mechanism of inactivation of this gene in primary bladder carcinomas. This mechanism, specifically involving the p15 gene, has already been reported by Herman et al (1996) in leukaemias and some gliomas. In bladder tumours, and similarly to Gonzalez-Zulueta et al (1995), we observed no hypermethylation-associated inactivation of p15 suggesting that this epigenetic mechanism is not involved in bladder carcinomas.

Figure 4  Methylation status of the first exon of the p15 gene. A representative methylation-sensitive PCR analysis (region 3 of exon 1) of normal urothelium and neoplastic cells from 3 TCCs is shown. p15+ and p15− are samples with or without p15 expression respectively. The lanes are: PCR products obtained from DNA samples digested with AflII (A : cutting outside the region analysed) as positive control; AflII plus MspI (A+M : cutting within the region analysed and methylation-sensitive) as negative control; AflII plus CfoI (A+C) or SacII (A+S) or HpaII (A+H) (all 3 cutting within the region analysed and methylation-sensitive)
The decreased p15 expression in tumours without homozygous deletions, may be due to a pathway involved in p15 regulation. As the p15 protein is a major mediator of the antiproliferative effects of TGF-β (Hannon and Beach, 1994), an abnormality in the TGF-β signalling pathway associated with the resistance of cancer cells to TGF-β-induced growth inhibition (Markowitz et al, 1996) could result in p15 mRNA down-regulation.

It is interesting to note that p15 mRNA levels were significantly decreased in most superficial TCCs, whereas 76% of invasive TCCs had p15 mRNA levels similar to or higher than those found in normal urothelium. P15 and p16 are cell cycle regulatory proteins that prevent CDK4 activation. The cyclinD-CDK4 complex catalyses the phosphorylation of pRb, which releases E2F, resulting in G1 to S cell cycle progression. It has been reported that tumour cells with mutations in Rb express very high levels of wild-type p16 whereas pRb-positive tumour cells frequently show little or no p16 (Yeager et al, 1995; Hall and Peters, 1996). Similarly, we can suppose that a target molecule downstream from p15 may be inactivated in some invasive tumours, thereby inducing p15 up-regulation.

In conclusion, our results suggest that p15 mRNA down-regulation is a frequent event in early neoplastic transformation of the urothelium and provide the first evidence for the possible involvement of this gene in carcinomas.

ACKNOWLEDGEMENTS

We would like to thank Dr Christian Larsen for the critical review of the manuscript and Dr Jennifer Southgate for kindly providing cell lysates. This work was supported by the Association Claude Bernard, Université Paris XII, CNRS, Ligue Contre le Cancer (Comité de Paris and Comité du Val de Marne), Délégation à la Recherche Clinique (PHRC, AOA94015), the GEFLUC. D. Cappellen was awarded a fellowship from ARC and S. Gil Diez de Medina from the Ligue Contre le Cancer-Comité du Val de Marne.

REFERENCES

Benedict WF, Lerner SP, Zhou J, Shen X, Tokunaga H and Czerniak B (1999) Level of retinoblastoma protein expression correlates with p16 (MTS-1/INK4A/CDKN2) status in bladder cancer. Oncogene 18: 1197–1203

Cappellen D, Gil Diez de Medina S, Chopin D, Thiery JP and Radvanyi F (1997) Frequent loss of heterozygosity on chromosome 10q in muscle-invasive transitional cell carcinomas of the bladder. Oncogene 14: 3059–3066

Coombs LM, Pigott D, Proctor A, Eydmann M, Denner J and Knowles MA (1990) Simultaneous isolation of DNA, RNA and antigenic protein exhibiting kinase activity from small tumor samples using guanidine isothiocyanate. Anal Biochem 188: 338–343

de Boer WJ, Rebel JM, Vermeys M, de Jong AAW and van der Kwast TH (1994) Characterization of distinct functions for growth factors in murine transitional epithelial cells primary organotypic culture. Exp Cell Res 214: 510–518

de Boer WJ, Vermeys M, Gil Diez de Medina S, Bindels E, Radvanyi F, van der Kwast T and Chopin D (1996) Functions of fibroblast and transforming growth factors in primary organoid-like cultures of normal human urothelium. Lab Invest 75: 147–156

Gil Diez de Medina S, Chopin D, El Marjou A, Delouvee A, Larochelle WJ, Hoznek A, Abbou C, Aaronson SA, Thiery JP and Radvanyi F (1997) Decreased expression of keratinocyte growth factor receptor in a subset of human transitional cell bladder carcinomas. Oncogene 14: 323–330

Gil Diez de Medina S, Popov Z, Chopin DK, Southgate J, Tucker GC, Delouvee A, Thiery JP and Radvanyi F (1999) Relationship between E-cadherin and fibroblast growth factor receptor 2b expression in bladder carcinomas. Oncogene 18: 5722–5726

Gonzalez-Zulueta M, Bender CM, Yang AS, Nguyen T, Beart RW, Van Tournout JM and Jones PA (1995) Methylation of the 5′CpG island of the p16CDKN2 tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. Cancer Res 55: 4531–4535

Hall M and Peters G (1996) Genetic alterations of cyclins, cyclin-dependent kinases, and cdk inhibitors in human cancer. Adv Cancer Res 66: 67–108

Hosaka M (1995) Infrequent somatic mutations of the p16 and p15 genes in human bladder cancer: p16 mutations occur only in low-grade and superficial bladder cancers. Oncol Res 7: 327–330

Hosaka M, Lacombe L, Hannon GJ, Serrano M, Pellicer I, Dalbagni G, Deaver TR, Williams MP, Elder PA, Shaw ME, Devlin J and Knowles MA (1995) P16 homozygous deletions but no sequence mutations in coding regions of p15 or p16 in human primary bladder tumors. Cancer Res 55: 722–727

Hosaka M, Lacombe L, Hannon GJ, Serrano M, Pellicer I, Dalbagni G, Reuter VE, Zhang, ZF, Beach D and Cordon-Cardo C (1995) Deletion of the p16 and p15 genes in human bladder tumors. J Natl Cancer Inst 87: 1524–1529

Kamb A (1995) Cell-cycle regulators and cancer. TIG 11: 136–140

Markowitz SD and Roberts AB (1996) Tumor suppressor activity of the TGF-β pathway in human cancers. Cytokine & Growth Factor Reviews 7: 93–102

Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, Baylin SB and Sidransky D (1995) ’5′CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nature Medicine 1: 686–692

Miyamoto H, Kubota Y, Fujimani K, Dobashi Y, Kondo K, Yao M, Shinn T and Hosaka M (1995) Infrequent somatic mutations of the p16 and p15 genes in human bladder cancer: p16 mutations occur only in low-grade and superficial bladder cancers. Oncol Res 7: 327–330

Packenham JP, Taylor JA, Anna CH, White CM and Devereux TR (1995) Homozygous deletions but no sequence mutations in coding regions of p15 or p16 in human primary bladder tumors. Mol Carcinogenesis 14: 147–151

Radvanyi F, Christgau S, Baekkeskov S, Jolicoeur C and Hanahan D (1993) Pancreatic beta cells cultured from individual preneoplastic foci in a multistage tumorgenesis pathway: a potentially general technique for isolating physiologically representative cell lines. Mol Cell Biol 13: 4223–4232

Reznikoff CA, Belair CD, Yeager TR, Savelieva E, Blelloch RH, Puthenveettil JA and Cuthill S (1996) A molecular genetic model of human bladder cancer pathogenesis. Semin Oncol 23: 571–584

Serrano M, Hannon GJ and Beach D (1999) A new regulatory motif in cell-cycle dependent kinase inhibitor genes and chromosome 9 karyotypic abnormalities in human bladder cancer. Oncogene 29: 1577–1584

Sherr CJ (1998) Tumor surveillance via the ARF-p53 pathway. Genes & Dev 12: 2984–2991

Singer-Sam J, Yang TP, Mori N, Tanguay RL, Le Bon JM, Flores JC and Riggs AD (1990) In: Nuclear Acid Methylation, Clawson GA, Willis DB, Weissbach A and Jones PA (eds) pp 285–289. A R Liss: New York

Southgate J, Profitt J, Roberts P, Smith B and Selby P (1995) Loss of cyclin-dependent kinase inhibitor genes and chromosome 9 karyotypic abnormalities in human bladder cancer cell lines. Br J Cancer 72: 1214–1218

Spruck CH, Gonzalez-Zulueta M, Shibata A, Simoneau AR, Lin MF, Gonzales F, Tsai LS, Baker K and Vande Woude F (1996) Loss of p15 and p16 genes in primary organotypic culture. Nature 382: 704–707

Stadler WM and Olopaide OI (1996) The 9p21 region in bladder cancer cell lines: large homozygous deletions inactivate the CDKN2, CDKN2B and MTAP genes. Urol Res 24: 239–244

Stone S, Dayanath P, Jiang P, Weaver-Feldhaus, JM, Tavigian SV, Cannon-Albright L and Kamb A (1995) Genomic structure, expression and mutational analysis of the p15 (MTS2) gene. Oncogene 11: 987–991

UCCC-American Joint Committee on Cancer (1992) Manual for staging of cancer. 4th edition. Lippincott: Philadelphia

WHO (1973) Histological typing of urinary bladder tumours. World Health Organization: Geneva

Williamson MP, Elder PA, Shaw ME, Devlin J and Knowles MA (1995) P16 (CDKN2) is a major deletion target at 9p21 in bladder cancer. Hum Mol Genet 4: 1569–1577

Yeager T, Stadler W, Beliar C, Puthenveetil J, Olopaide O and Reznikoff C (1995) Increased p16 levels correlate with pRb alterations in human urothelial cells. Cancer Res 55: 493–497

© 2001 Cancer Research Campaign

British Journal of Cancer (2001) 85(10), 1515–1521