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Inactivation of tumor suppressor genes and deregulation of the c-*myc* gene in urothelial cancer cell lines

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Abstract Recent investigations have demonstrated *p53* and *Rb* alterations in a subset of transitional cell carcinoma (TCC). Further genetic changes during tumor progression include overexpression of the c-*myc* gene in a significant number of mainly invasive bladder tumors. To study the possible interactions between these genes in TCC, urothelial cancer cell lines were chosen as an in vitro model. Expression and mutation of *p53* was studied in 15 bladder cancer cell lines by immunocytochemistry, Western blot, polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) analysis and direct sequencing of double stranded PCR products of exons 4, 5, 7 and 8 of genomic DNA. C-*myc* expression and gene structure were studied using Northern and Southern blot techniques. *Rb* protein expression was analyzed by Western blot. Twelve of 15 cell lines showed either *p53* mutations or abnormal protein expression. Consistent with previous studies, five cell lines did not express *Rb* protein. None of the cell lines studied retained both tumor suppressor genes in a functional form. The c-*myc* gene appeared to be intact in all cell lines and copy numbers were close to normal. Northern analysis demonstrated that all cell lines expressed c-*myc* mRNA but evidence for altered regulation was found in at least two cell lines. Our data suggest that amplification or translocation are not the underlying mechanism for c-*myc* overexpression in urothelial tumors. No correlation between loss of *Rb* protein and c-*myc* expression was observed. The results presented here for the cell lines match well those obtained in vivo. Thus, these cell lines may provide a suitable model for further analysis of molecular alterations in urothelial cancer.

Key words Transitional cell carcinoma · *Rb* · *p53* · c-*myc*

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Earlier reports have demonstrated that mutations and allelic loss of the *p53* locus (17p13) are frequent events in invasive and high-grade bladder cancer [7, 8, 26, 39]. More recent investigations found *p53* alterations even in the early stages of TCC [30, 36]. Different mechanisms of *p53* inactivation have been observed, most frequently point mutations of the gene and loss of the remaining wild-type allele [14, 41]. The majority of point mutations in human cancers occur in four of five highly conserved domains which are relevant for the function of *p53* [11, 34]. A mutation in these domains often results in an altered conformation of the *p53* protein, leading to increased stability and prolonged half-life time [15]. Consequently, whereas wild-type *p53* is usually not expressed at immunocytochemically detectable levels, mutant *p53* protein accumulates in the cell.

The retinoblastoma (*Rb*) tumor suppressor gene was also found to be frequently inactivated in advanced bladder cancer [38]. *Rb* is a negative regulator of the cell cycle, itself regulated by cell-cycle-dependent phosphorylation [4]. The hypophosphorylated form of *Rb* is supposed to mediate growth suppression. Loss of *Rb* function is found in a distinct set of human cancers and is usually due to deletions [12]. Truncated proteins or a total loss of expression are found in these tumors.

Immunohistochemical investigation of bladder cancers has demonstrated frequent overexpression of the c-*myc* gene, especially in invasive tumors [31]. The c-*myc* gene belongs to a group of related genes controlling cell proliferation and differentiation [19]. The c-*myc* gene is expressed in many proliferating tissues

during development [29] and in many adult cells reentering the cell cycle [17]. Activation towards an oncogene found in many human cancers, notably breast, stomach and colon carcinomas and several leukemias, is due to gene amplification, proviral insertion or translocation [35]. Most insertions and translocations occur in the regulatory region comprising sequences around the first exon [2, 35]. These alterations of the c-*myc* gene lead to its deregulated expression, which also frequently takes place without such structural aberrations [17]. Both *p53* and *Rb* tumor suppressor genes have been implicated as potential repressors of c-*myc* [20, 24]. Point mutations are not thought to be important in c-*myc* activation.

Estimation of the impact of specific alterations on carcinogenesis or tumor progression requires functional examinations, which cannot be performed in vivo. Therefore there is a need for the establishment of cell culture models to study the impact of genetic changes in urothelial tumor cells. As a prerequisite, in the present work, 15 urothelial cancer cell lines were characterized for *p53* mutations by detecting altered protein expression and analyzing DNA for mutations. Loss of *Rb* protein expression was examined by Western blot analysis. Deregulation of the c-*myc* gene was analyzed by studying its expression in selected cell lines as well as its gene copy number and integrity.

Materials and methods

Cell lines

Urothelial cancer cell lines were obtained from J. Fogh, Sloan-Kettering Institute, Rye, N.Y., USA, and the melanoma cell line SM-Mel 133 from A. P. Albino, Memorial Sloan-Kettering Cancer Center, New York, N.Y., USA. Cell line SV80 was kindly provided by G. Reifenberger, Department of Neuropathology, Heinrich-Heine University Düsseldorf, and BT-1 by W. Heckl, Julius-Maximilians University, Würzburg, Germany. All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B. All cell culture media and supplements were supplied by Biochrom, Berlin, Germany. The urothelial cell lines used here have been extensively studied with regard to morphology, karyology, tumorigenicity and population doubling time (for review see refs. [18] and [42]). In general they are aneuploid and have been found to be tumorigenic (except for TCCSUP) in at least one animal model.

Immunocytochemistry

Tissue culture cells grown on coverslips were fixed in 9% acetone, 5% formalin in phosphate-buffered saline (PBS) for 5 min. Endogenous peroxidases were blocked using 0.075% HCl in methanol for 15 min at 4 °C and unspecific binding by incubation with 15% dry milk in PBS for 10 min. Cells were incubated overnight with primary antibody PAb 1801 (Dianova, Hamburg, Germany) diluted 1:750 in PBS. PAb 1801 recognizes a denaturation-resistant epitope between amino acids 32 and 79 coded on exon 4 of human *p53* [1]. The primary antibody was visualized using horseradish peroxidase conjugated rabbit anti-mouse immunglobulin (Ig) antiserum (Dako, Hamburg, Germany) diluted 1:200 in PBS and diaminobenzidine.

Western blot analysis

Cell lysates were prepared from subconfluent monolayers (175 cm^2 flask) in 1% NP40, 1% SDS, 0.5% sodium deoxycholate and 100 µg/ml PMSF. Protein concentration was determined as described previously [16]. Samples were diluted in sample buffer containing 50 mmol/l TRIS-acetate pH 7.5 and SDS, dithiothreitol and bromophenol blue were added to obtain final concentrations of at least 10 g/l, 5 mmol/l and 0.1 g/l, respectively. Equal amounts of protein were heated to 95 °C for 3 min and applied to precast ExcelGels SDS with an 8–18% polyacrylamide gradient (Pharmacia, Freiburg, Germany). After blotting using a standard method [13], the nylon membrane was immunostained using monoclonal antibodies against *p53* (PAb1801) or *Rb* (G3-245) as recommended (PharMingen, San Diego, Calif., USA). The primary antibody was visualized using alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin antiserum (Dako) diluted 1:200 in TRIS-buffered saline (TBS) and 5′-bromo-4′-chloro-3′-indolyl phosphate disodium salt (BCIP) as substrate.

Polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP)

Genomic DNA was prepared by the proteinase K/phenol-chloroform extraction method [28]. PCR-SSCP analysis was accomplished using a modification of the previously reported method [23]. Briefly, PCR was performed with 100 ng genomic DNA, 10 pmol each primer, 50 µM dCTP, dGTP, dTTP, 6.25 µM dATP, 0.1 µl α-[^{35}S]dATP (1000 Ci/mmol, 10 mCi/ml; Dupont NEN Research Products, Dreieich, Germany), 10 mM TRIS/HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.5 U *Taq* polymerase (Gibco, Eggenstein, Germany), in a final volume of 10 µl. Thirty cycles of denaturation (94 °C) annealing (55–60 °C; Table 1) and extension (72 °C), 1 min each, were performed in a DNA-thermal cycler (Perkin Elmer/Cetus). To the PCR product one volume of 94% formamide, 20 mM EDTA, 0.05% xylene cyanol and 0.05% bromophenol blue was added. The reaction mixture was heated to 95 °C for 6–8 min, immediately frozen in fluid nitrogen (5–10 s), chilled on ice and loaded (10 µl) onto a 6% nondenaturing polyacrylamide gel (49:1 acrylamide/methylene-bis-acrylamide) in 90 mM TBE (pH 8.3) and 4 mM EDTA. Each exon was analyzed several times in gels with and without glycerol 5–10% at room temperature at 25 W and 15 W for 6–8 h and 12–15 h, respectively. Gels were dried on Whatman 3M paper and exposed to Trimax 3M film for 24–72 h.

Direct sequencing of PCR products

PCR of exons 4–6 and 7–8 was performed with 500 ng genomic DNA, 100 pmol each primer, 200 µM each dATP, dCTP, dTTP and dGTP/^{7}deaza-GTP at a 7:3 ratio, 10 mM TRIS/HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin and 2.5 U *Taq* polymerase (Table 2). Increased yield of the PCR product from exons 4–6 (1261 bp) was obtained by addition of 0.1 µl of *gp32* protein (Pharmacia) [32]. Amplification was performed for 35 cycles: 94 °C, 1 min; 60 °C, 1 min; 72 °C, 2.5 min (final cycle 7 min). PCR products were separated from mineral oil, dried in a vacuum centrifuge, redissolved in 10 µl H_2O and applied to a 2% low-melting agarose gel. Following electrophoresis and ethidium bromide staining, amplified DNA fragments were isolated using Epicentre *Gelase* (Biozym, Hameln, Germany) as recommended. The PCR product was dissolved in 20 µl water. An aliquot of 10 µl, 5 pmol primer, 10% DMSO and annealing buffer was premixed, denatured and frozen in nitrogen. Subsequently, sequencing reactions were performed using

Table 1 PCR-SSCP primers

Exon	Primer	Sequence	Annealing	Fragment
4	P1	TTC ACC CAT CTA CAG TCC CC	58 °C	308 bp
	P2	TCA GGG CAA CTG ACC GTG CA		
5	P3	TTC CTC TTC CTG CAG TAC T	55 °C	209 bp
	P4	AGC TGC TCA CCA TCG CTA T		
7	P5	GTG TTG TCT CCT AGG TTG GC	60 °C	139 bp
	P6	CAA GTG GCT CCT GAC CTG GA		
8	P7	CCT ATC CTG AGT AGT GGT AA	58 °C	165 bp
	P8	TCC TGC TTG CTT ACC TGC CT		
5–6	P3	As above	55 °C	351 bp
	P628	TCT GTC ATC CAA ATA CTC CAC ACGC		

Table 2 Primer pairs for sequencing amplification

Exon	Primer pair	Annealing	Fragment
4–6	P54/P628	60 °C	1261 bp
7–8	P5/P8	60 °C	670 bp

α-[^{35}S]dATP (1000 Ci/mmol, 10 mCi/ml) and the T7-Sequencing Kit (Pharmacia) as recommended. Electrophoresis was performed in a denaturing polyacrylamide gel containing 7 M urea for 2–3 h. Both strands were sequenced for each exon using the primers shown in Table 1.

Analysis of c-*myc* gene status and expression

DNA [28] and RNA [3] were prepared by standard methods. For Southern analysis, 8 μg of DNA from each cell line was digested with *Eco*RI, separated on a 0.7% agarose gel and blotted to Hybond N+ membranes (Amersham, Braunschweig, Germany) in 0.4 N NaOH. For Northern analysis, 25 μg RNA from each sample was run on a 1% agarose gel containing 0.66 M formaldehyde, transferred to Genescreen membranes (Dupont) and UV-fixed. Blots were probed with a 1.3-kb *Cla*I-*Eco*RI fragment from the 3′-end of the *myc* gene, which was labeled by the oligonucleotide priming method using α-[^{32}P]dCTP. Hybridization was performed in 0.25 M sodium phosphate, pH 7.2, 0.25 M NaCl, 1 mM EDTA, 7% (w/v) sodium dodecyl sulfate at 65 °C. Deionized formamide (15%) was added for RNA analysis. Following hybridization overnight, filters were washed under high stringency conditions (15 mM sodium chloride, 1.5 mM sodium citrate, 0.1% sodium dodecyl sulfate, 65 °C) and exposed for autoradiography. Further probes used were a 0.6-kb *Eco*RI-*Dra*I fragment from exon 6 of the human *NQOR* gene [6], a 1.3-kb cDNA for the rat *GAPDH* gene [40], and a 1.3-kb fragment from the human *H3* gene [25]. For quantitation of signals, appropriately exposed autoradiographs were evaluated by laser densitometry.

Results

Status of *p53*

Fifteen bladder carcinoma cell lines were screened for accumulation of *p53* protein by immunocytochemistry and Western blot. By immunocytochemistry, 8 of 15 cell lines consistently showed strong *p53* expression with the *p53* monoclonal antibody PAb 1801. This was confirmed by Western blot analysis (Fig. 1). Cell line 253J showed a particular staining pattern: while most cells were *p53* negative, a small subpopulation of larger cells showed either cytoplasmatic perinuclear staining around a single nucleus or positive double nuclei.

Overexpression of *p53* is known to be frequently associated with point mutations in the highly conserved domains of the gene in exons 4, 5, 7 and 8. Therefore these regions were analyzed by PCR-SSCP and sequencing of PCR products. In addition a fragment including exon 5, intron 5 and a part of exon 6 was examined (using P3 and cDNA-Primer P628, Table 1). Examples for PCR-SSCP and sequence analysis are shown in Fig. 2a–d. Eight of 15 cell lines showed mobility shifts (two only detectable in gels without glycerol) in at least one of the five regions examined and a loss of the wild-type allele. VM Cub 2 showed four bands in exon 5, indicating two different mutations, but no wild-type allele. In three cell lines, EJ, T24 and BT-1, amplification of exon 5 (P3/P4) and exon 5–6 (P3/P628) failed, indicating a mutation within the sequence of primer P3. Only the cell lines TCCSUP and 486P showed neither *p53* overexpression nor mobility shifts and have not been studied by sequence analysis.

Sequencing of SSCP positive exons and of exons 4, 5, 7 and 8 of overexpressing cell lines confirmed and extended these results (Table 3). Mobility shifts and failure of amplification (exon 5 of cell lines EJ, T24 and BT-1) were due to missense point mutations in exons 4 (one case), 5 (five cases), 7 (two cases) and 8 (three cases). Nine of 11 mutations were located within the highly conserved domains (Table 3).

Seven of the eight cell lines showed missense point mutations in *p53* in line with the presence of *p53* overexpression. PCR-SSCP analysis of cell line 647V showed a mobility shift in the fragment including exons 5–6 but not the one containing only exon 5. Since

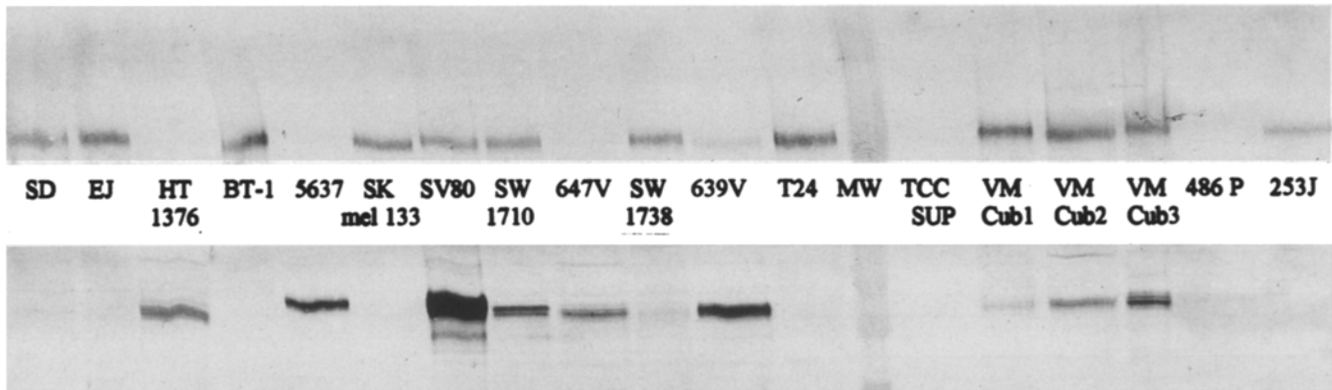

Fig. 1 Western blot of *Rb* and *p53* protein in bladder carcinoma cell lines with monoclonal antibody G3-245 (against *Rb*, *top*) and PAb1801 (against *p53*, bottom), respectively. The protein weight marker is indicated as *MW*. SK mel 133 was used as standard cell line with wild-type *p53* and intact *Rb*. SV80 is a cell line overexpressing *p53* due to transformation with SV40. SW1738 containing normal-sized *Rb* and displaying slight overexpression of *p53* is a bladder carcinoma cell line not further characterized in this study

no mutations were found sequencing exons 4, 5, 7 and 8, *p53* overexpression in this cell line might be caused by a mutation outside the highly conserved domains. Alternatively, this mobility shift could be due to an intron alteration not influencing the *p53* expression. The cell line VM Cub 2, which also overexpresses *p53*, showed two different mutated alleles with point mutations in codons 158 and 163, respectively. These codons do not belong to the highly conserved ones, but are conserved between different species and are functionally and conformationally important [11]. In contrast, cell line SD, with a missense point mutation in codon 116 (Ser- >Cys) directly adjacent to domain II, did not show *p53* overexpression. This amino acid is not conserved and is at the edge of the DNA-binding domain of the *p53* protein [34]. It is questionable whether this mutation affects *p53* function.

Expression of *Rb*

Rb expression was studied by Western blot analysis using monoclonal antibody G3-245. Consistent with previous reports [12], TCCSUP, 5637, HT1376, 486P and 647V did not show detectable *Rb* protein, while all other cell lines showed expression of the protein with the expected molecular weight.

Status of c-*myc*

The c-*myc* gene was investigated by Southern analysis. Digestion with the restriction enzyme *Eco*RI yielded the expected 13-kb fragment [2] in all cell lines (Fig. 3). This fragment comprises the entire coding region and the part of the regulatory region in which most translocations and proviral insertions have been found to occur. The signal strengths obtained were similar to the one in DNA from normal ureter (lane1, Fig. 3), suggesting the presence of only few copies in all cell lines. To further exclude amplifications, the blots were rehybridized with a probe for the single-copy *NQOR* gene residing on chromosome 16p (Fig. 3). The relative signal intensities indicated the presence of one to three copies of the c-*myc* gene per genome. These values exclude amplification, but are compatible with variations in relative chromosome numbers being present in some cell lines, e.g. in T24 (lane 2, Fig. 3).

By Northern analysis, all cell lines showed a signal for c-*myc* mRNA, whereas none was found for N-*myc* (data not shown). Since expression of c-*myc* in cell lines varies with the growth status, it was studied under controlled conditions in selected cell lines. Figure 4 shows the quantitative evaluation of two representative experiments. In both cell lines, T24 and VM Cub 3, c-*myc* expression increased over 48 h when late log-phase cells were treated with fresh medium with fetal calf serum. However, expression did not increase significantly (T24) or even fell (VM Cub 3) after addition of medium without serum. C-*myc* expression did not correlate with the proliferation rate, as indicated by the discrepancy between expression of histone H3 mRNA, which is a marker for S-phase cells, and c-*myc* mRNA at 48 h. A comparison of the signal intensities for T24 and VM Cub 3 showed that the latter cell line expresses c-*myc* approximately 4 times stronger than the former under identical growth conditions (data not shown).

Discussion

In the present study genetic alterations in urothelial cancer cell lines were analyzed. Twelve of 15 cell lines showed either nuclear overexpression or mutations of the tumor suppressor *p53*, suggesting its inactivation. While overexpression of *p53* protein was found in 8 of 15 cell lines only, PCR-SSCP by mobility shifts or loss of amplification suggested DNA mutations in 11 of 15

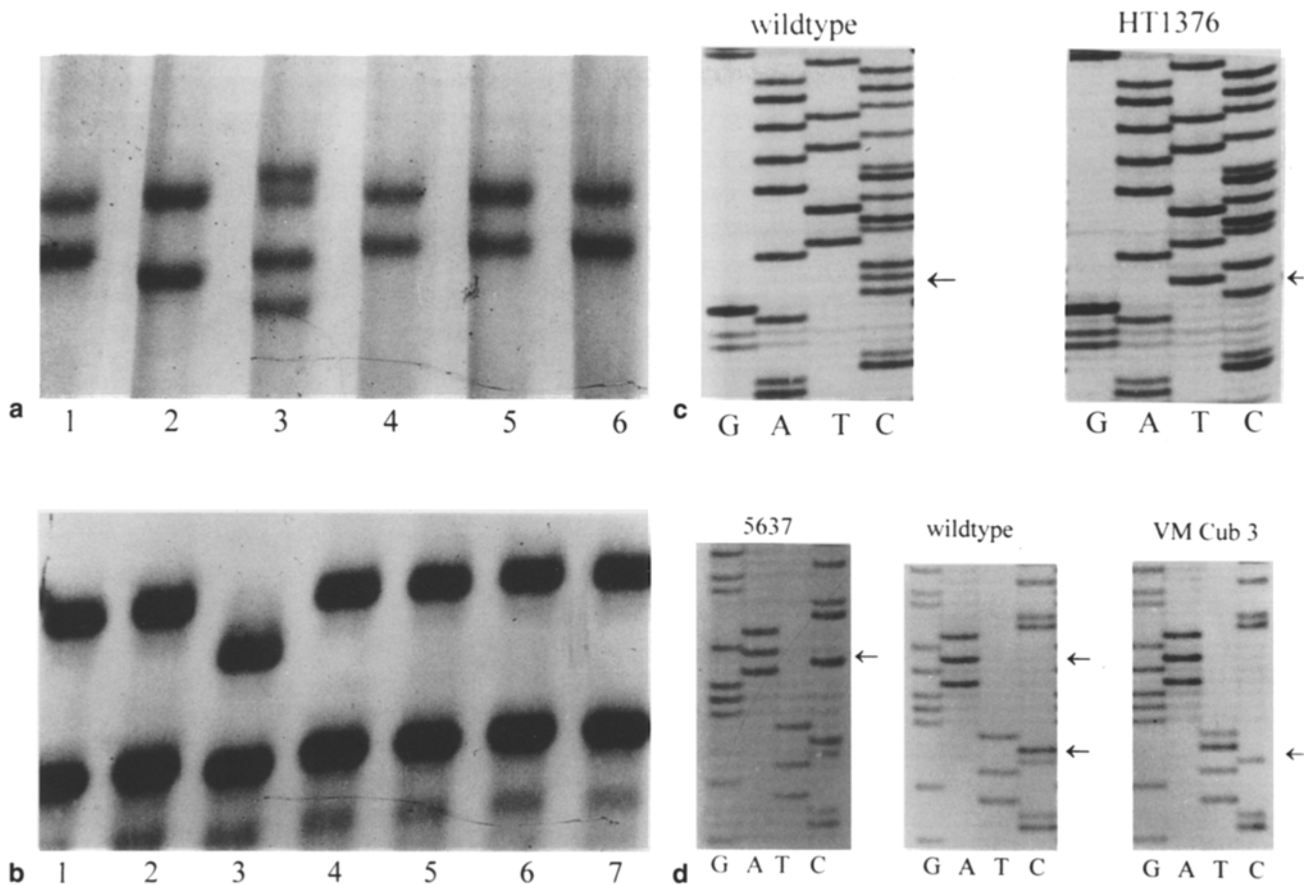

Fig. 2a–d PCR-SSCP and sequencing analysis of *p53* performed as described in "Materials and methods". **a** PCR-SSCP of exon 5 (gel with 10% glycerol). *1* TCCSUP, *2* VM Cub 1, *3* VM Cub 2, *4* VM Cub 3, *5* 486P, *6* 253J. **b** PCR-SSCP of exon 7 (gel with 10% glycerol). *1* 647V, *2* BT-1, *3* HT1376, *4* J82, *5* EJ, *6* SD, *7* SW 1710. **c** Sequencing analysis of exon 7. **d** Sequencing analysis of exon 8

cell lines. Using PCR-SSCP, 1 of 12 mutations identified (2 in VM Cub 2) did not result in a mobility shift, and 2 mutations were only detected in gels without glycerol. Among the immunologically negative cell lines, BT-1, EJ and T24 possessed an identical mutation Tyr → Stop in codon 126, possibly suggesting a "hotspot" for mutation. For EJ and T24, which have been previously reported to have the same c-Ha-*ras* mutation [27, 37], this finding strongly indicates genetic identity or at least cross-contamination between these cell lines. This is supported by isoenzyme data reported by Masters et al. [18] despite different morphological and physiological properties.

Overexpression due to point mutational inactivation occurred in most of the cell lines. However, other mechanisms of *p53* inactivation might be the underlying mechanism of *p53* accumulation in some cell lines, e.g. 647V and 253J. Complex formation with transforming viral proteins (e.g., SV40 large T-antigen, HPV E6 and adenovirus E1B) and oncogene products (e.g. *mdm*-2) is known to abrogate *p53* function and leads to altered expression [21, 22]. A cytoplasmic staining pattern, as observed in cell line 253J, has been described to reflect alterations of the nuclear location signals of *p53* and to abrogate *p53* function [33].The heterogeneous staining pattern of 253J cells could be due to a heterogeneous cell population or could reflect differences in *p53* distribution during the cell cycle.

Regarding the observation that no *p53* gene alteration was seen in cell lines 647V and 253J in spite of abnormal protein expression, one can conclude that the combination of immunological methods and PCR-SSCP is superior to one method alone to evaluate the status of *p53*. Our results are in accord with a recently published study by Cordon-Cardo et al. [5], who evaluated different assays aimed at the detection of *p53* mutations, namely immunohistochemistry, 17p LOH, SSCP and sequencing, ana lyzing archival material of 42 patients with bladder tumors.

Five out of 15 cell lines showed loss of *Rb* expression. In all cell lines either *p53* and/or *Rb* were inactivated. Thus inactivation of these tumor suppressor genes may be an important step in the development of urothelial cancers. However, it cannot be excluded that this observation only reflects a requirement for establishment in vitro.

We have recently reported that the c-*myc* protein is overexpressed in a significant fraction of bladder carcinomas in vivo [31]. Such overexpression may be due to proviral insertions into the *myc* gene or to gene

Table 3 *p53* expression and DNA mutations (*ND* none detected; *MS* mobility shift; E5/E6, PCR fragment including exon 5, intron 5 and part of exon 6)

Cell line	Protein expression ICC/WB	PCR-SSCP	Sequence analysis		
			Exon	Codon	Amino acid change
639V	+/+	MS[a]	7	248	Arg (CGG) → Gln (CAG)
5637	+/+	MS	8	280	Arg (AGA) → Thr (ACA)
647V	+/+	MS (E5/E6)	ND (5)	–	–
T24	–/–	NA	5	126	Tyr (TAC) → Stop (TAG)
EJ	–/–	NA	5	126	Tyr (TAC) → Stop (TAG)
BT-1	–/–	NA	5	126	Tyr (TAC) → Stop (TAG)
HT1376	+/+	MS	7	250	Pro (CCC) → Leu (CTC)
SD	–/–	MS	4	116	Ser (TCT) → Cys (TGT)
SW 1710	+/+	ND	8	273	Arg (CGT) → Cys (TGT)
TCCSUP	–/–	ND	–	–	–
VM Cub 1	+/+	MS	5	175	Arg (CGC) → His (CAC)
VM Cub 2	+/+	MS	5	158	Arg (CGC) → Leu (CTC)
		MS	5	163	Tyr (TAC) → Cys (TGC)
VM Cub 3	+/+	MS[a]	8	278	Pro (CCT) → Leu (CTT)
486P	–/–	ND	–	–	–
253J	–/– [b]	ND	–	–	–

[a] Only detected in gel without glycerol
[b] Characteristic staining pattern, see text

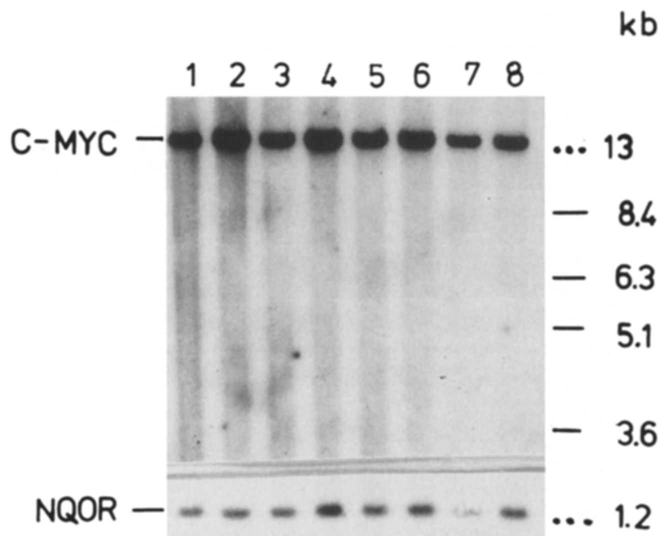

Fig. 3 Southern analysis of the c-*myc* gene in bladder carcinoma cell lines. Eight micrograms of DNA from the indicated cell lines was analyzed by Southern hybridization as described in "Materials and methods", using probes for c-*myc* (*top*) and *NQOR* (*bottom*). The sizes of DNA standard markers and of the observed bands are indicated *on the right*. *1* DNA from normal ureter mucosa, *2* T24, *3* HT1376, *4* 639V, *5* TCCSUP, *6* VM Cub 1, *7* 647V, *8* HepG2 human hepatoma cells

amplification, translocation or deregulation by other means. Alternatively it might reflect the intense proliferation of these tumor cells. The analysis of urothelial carcinoma cell lines presented here did not yield any evidence for gross structural alterations of the c-*myc* gene. Although some cases of translocations and proviral insertions occur far away from the c-*myc* gene [10], most alterations result in a change of the size of the 13-kb *Eco*RI fragment encompassing the c-*myc* gene [2], which was not found in the cell lines studied here. Moreover, amplifications resulting in a significant increase in c-*myc* gene copy number were not observed, although trisomy of the relevant part of chromosome 8 remains a possibility in some cell lines, e.g., cell line T24. Since expression of c-*myc* is expected in proliferating cells, the presence of the c-*myc* mRNA in all cell lines studied does not per se indicate deregulation of the gene. Also, as would be expected in normal cells, c-*myc* expression was increased by addition of fetal calf serum. However, c-*myc* expression did not decrease in confluent cells with diminished proliferation, indicated [25] by a strongly decreased expression of histone H3 mRNA. These observations suggest that deregulation is the underlying mechanism of c-*myc* overexpression in urothelial cancer. The *Rb* protein has been implicated as a negative regulator of c-*myc* gene expression [9] and its loss has been suggested to contribute to c-*myc* gene deregulation [24]. Since both T24 and VM Cub 3 contain normal-sized and therefore presumably functional *Rb* protein, other factors must be involved in the deregulation of the c-*myc* gene in these cell lines.

Our findings in urothelial cancer cell lines are consistent with previous data concerning the frequency of *p53* and *Rb* alterations and the mutational spectra of *p53* in bladder cancer. While the c-*myc* gene is expressed in all cell lines, its regulation was found to be altered in at least some cell lines. Since these observations concur with those made in vivo [31], the cell lines presented here appear to be a promising model for a functional analysis of some aspects of urothelial cancer development and progression.

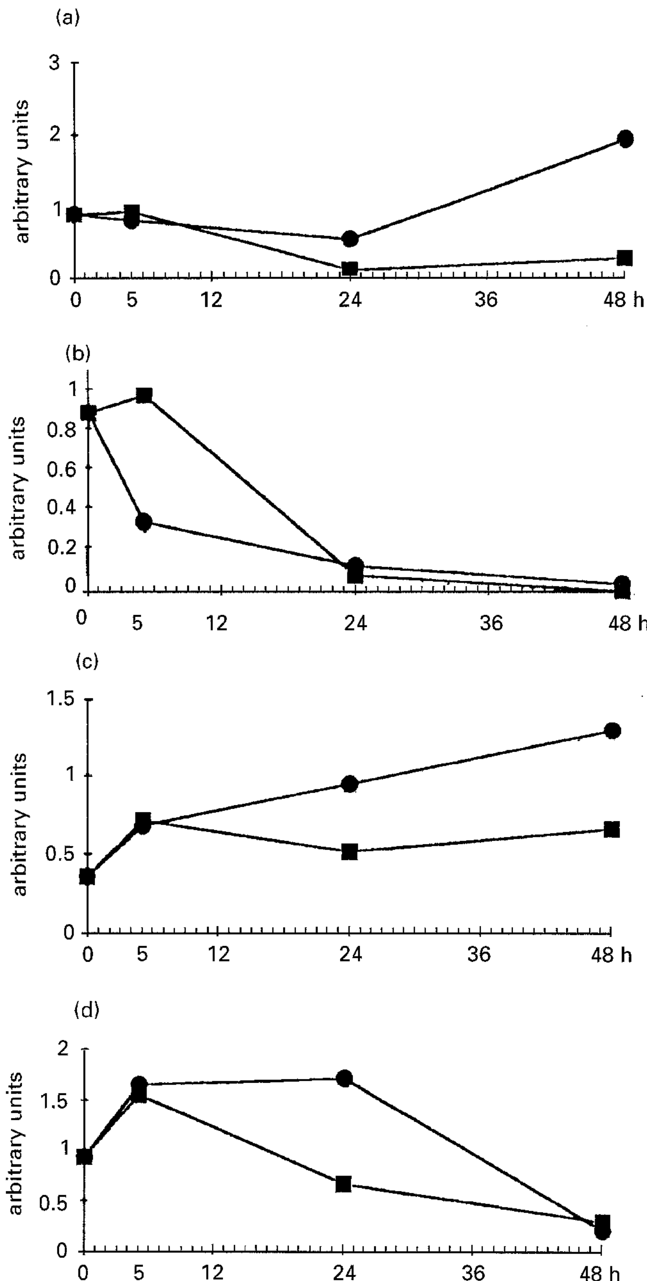

Fig. 4a–d Quantitation of c-*myc* expression in the bladder carcinoma cell lines T24 and VM Cub 3 by Northern blot analysis. Bladder carcinoma cells were seeded at 10^4/cm^2 and 48 h later medium with (*circles*) or without (*squares*) fetal calf serum was added. RNA was isolated at the indicated times and analyzed for c-*myc*, histone H3 and GAPDH mRNAs. Autoradiographs were evaluated by laser densitometry and the c-*myc* and histone signals are presented normalized to GAPDH mRNA. **a** VM Cub 3, c-*myc* expression. **b** VM Cub 3, histone H3 expression. **c** T24, c-*myc* expression. **d** T24, histone H3 expression

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