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Absence of structural alterations of the multidrug resistance genes in transitional cell carcinoma*

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Summary. Tumor DNA from 27 patients with treated or untreated transitional cell carcinomas of the urinary tract was screened for genomic alterations of the multidrug resistance genes in order to determine whether structural changes of these genes are important in primary urothelial tumors. None of the tumors showed evidence of amplification or rearrangements of either mdr1 or mdr2. The lack of amplification or rearrangements observed in these tumors suggests that structural alterations of the mdr1 and mdr2 genes are not important mediators of drug resistance in TCC.

Key words: Multidrug resistance genes – Transitional cell carcinoma – Gene amplification

The ineffectiveness of chemotherapeutic regimens in many solid tumors represents a major challenge in clinical oncology. In some instances drug refractoriness is evident at the start of chemotherapy; in others resistance emerges during the administration of chemotherapy or subsequently after a period of clinical remission. Recent evidence suggests that the establishment and maintenance of intrinsic or acquired drug resistance is caused by mutations that result in the altered expression of certain genes. These mutations are postulated to occur in the stem cell compartment of the tumor, resulting in a subset of cells which can survive drug exposure, become the dominant cell population within the tumor, and result in treatment failure [17, 21]. Drug resistance may result from a mutation which confers resistance to a specific agent or to an entire class of drugs [11, 17].

Recent studies have demonstrated that the amplification and/or overexpression of one or more "multidrug resistance genes" in response to sequential exposure to increasing concentrations of cytotoxic agents is an important mechanism of cellular drug resistance. This multidrug resistance phenotype is characterized by overexpression and/or amplification of a gene (mdr1) coding for the plasma membrane-associated P-glycoprotein that mediates intracellular drug accumulation [11]. Overproduction of this protein results in cellular resistance to a variety of naturally occurring cytotoxic agents [9] including two (vinblastine and doxorubicin) which are used in the treatment of invasive and metastatic transitional cell carcinoma (TCC). Multidrug resistance for vinblastine and doxorubicin mediated by amplification of the mdr1 gene has been demonstrated in human [14, 19] and mammalian [7, 12, 13, 18] cell lines. Other studies have demonstrated increased levels of mdr1 and mRNA in a variety of treated and untreated human tumors [5, 6] and increased levels of P-glycoprotein in response to therapy in two cases of drug resistant ovarian adenocarcinoma [1]. The role of a second gene of this family (mdr2) in multidrug resistance is unknown. No published studies have yet identified amplification of the mdr genes in human tumors.

In order to determine whether structural alterations of the multidrug resistance genes are important in primary urothelial tumors we screened 27 treated and untreated TCC of the urinary tract for amplification and rearrangements of mdr1 and mdr2. The lack of amplification or rearrangements observed in these tumors suggests that structural alterations of the mdr1 and mdr2 genes are not important mediators of drug resistance in TCC.

Materials and methods

Specimens

Tumor and normal tissue was obtained from an unselected series of 27 patients undergoing surgery for transitional cell carcinoma of the renal pelvis, ureter, or bladder. Twenty of the tumors were never exposed to chemotherapeutic agents recognized as important in the

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Table 1. Pathologic stage of untreated tumors

Specimen number[a]	Pathologic stage[b]	Specimen number[a]	Pathologic stage[b]	Specimen number[a]	Pathologic stage[b]
Bladder		Renal pelvis		Ureter	
U1	PIS, P4aNoMo	U2	PISNoMo	U15	PIS, P3N2Mo
U6	PIS, P3bNoMo	U5	PIS, P3N2Mo	U4	PaNoMo
U8	P3aN1Mo	U9	PaNoMo		
U11	PISNoMo	U10	PIS, P1NoMo		
U14	P3bNoMo	U12	PISNoMo		
U7	PIS, P3bNoMo	U3	P1NoMo		
U13	PIS, P3bNoMo				
U17	PIS, P3bNoMo				
U18	P3bN2Mo				
U19	PIS, P3bNoMo				
U20	P3bN2Mo				
U21	PIS, P3bNoMo				

[a] U denotes untreated tumor
[b] Staging according to the TNM system for genitourinary sites of the American Joint Committee on Cancer

Table 2. Treated tumors

Specimen number[a]	Treatment (# courses)	Pathologic stage[b]	Response
R4	MVAC (4)	PISN1Mo	CR
R3	CMV (1)	P1NoMo	NR
R5	MVAC (4)	P1NoMo	NR
R7	MVAC (1)	PIS, P3bNoMo	NR
R1	MVAC (6)	PoN3Mo	NR
R2	MVAC (3)	PIS, P3bNoMo	NR
R6	Adriamycin[c]	PIS, P4a, N2Mo	NR

MVAC = methotrexate, vinblastine, doxorubicin, and cis-platinum; CMV = cis-platinum, methotrexate, and vinblastine; CR = complete response; NR = no response

[a] R denotes treated tumor
[b] Staging according to the TNM system for genitourinary sites of the American Joint Committee on Cancer
[c] given intravesically 68 times

multidrug resistance phenotype and are referred to as "untreated" tumors (Table 1). The remaining 7 tumors were obtained from excised bladders at the time of cystectomy. These were all previously exposed to at least one dose of doxorubicin and/or vinblastine, usually as part of systemically administered combination chemotherapy, and are referred to as "treated" tumors (Table 2). One treated tumor was exposed only to doxorubicin administered intravesically. Pretreatment tumor samples were not available in these patients. In most cases grossly normal-appearing transitional cell mucosa was obtained from the surgical specimens for use as germline control. In two cases, normal tissue from the kidney and prostate was used. In cases where normal tissue was not available, placental DNA was used as control.

The tumors studied spanned the pathologic spectrum from papillary noninvasive TCC to deeply invasive and metastatic tumors (Tables 1 and 2).

DNA analysis

High molecular weight DNA was extracted from frozen tissue as described [23]. Ten micrograms of DNA per sample were digested with an excess of restriction endonuclease (BglII and/or HindIII) in the appropriate buffer, and transferred to nitrocellulose following electrophoresis on a 0.7% agarose gel [22]. Digests were then hybridized to human- or hamster-derived mdr probes labeled with 32P by the random oligonucleotide primer technique [4]. Hybridization was performed at 65°C in a mix of 0.05 M $NaPO_4$, 0.01 M EDTA, 5% Dextran Sulfate, 4 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 4 × Denhardts, 0.3% sodium dodecylsulfate (SDS), and 5 μg/ml single stranded salmon sperm DNA. Following hybridization, filters were washed in 0.1 × SSC and 0.1% SDS and exposed to Kodak XAR film for 1–3 days at -70°C. Following exposure, autoradiographs were inspected for rearrangements and amplification visually and by laser densitometry. Hybridization with a probe for the human bcl-1 gene was used as a control for variations in loading DNA in those tumors suspected to have amplification by densitometry.

Tissues were analyzed using four probes derived from the mdr1 and mdr2 genes (Fig. 1): pHDRVI 5′, a 1.2 kb cDNA from the 5′ end of the human mdr1 gene [3] pHDRVI 3′, a 3 kb cDNA from the 3′ end of human mdr1 [3] pcDR1.3, a 1.2 kb probe from the central portion of the hamster mdr1 gene [7]; and pMDR2, a genomic probe from the human mdr2 gene [14]. The human mdr probes were provided by I. Roninson of the University of Illinois and the hamster probe by D. Housman of the Massachusetts Institute of Technology.

Results

Of the 20 untreated tumors, none showed structural rearrangements or amplification of either mdr1 (Fig. 2) or mdr2 (data not shown).

Of the 7 treated tumors, only one (R4) had a complete response to systemic chemotherapy (Table 2). This patient had complete disappearance of a muscle-invasive tumor

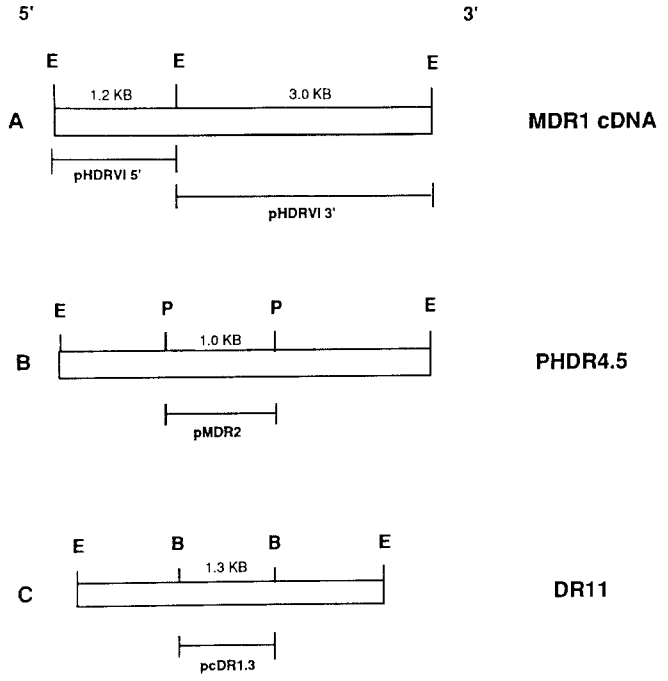

Fig. 1 A–C. Simplified diagram of oligonucleotide probes for the mdr1 and mdr2 genes used in Southern hybridizations. **A** pHDRVI 5′ and pHDRVI 3′ derived from mdr1 cDNA as described in [3]; **B** pMDR2 derived from clone pHDR4.5 as described in [14]; and **C** pcDR1.3 derived from clone lambda DR11 as described in [14]. E = EcoRI; P = PstI; B = BglII; KB = kilobases

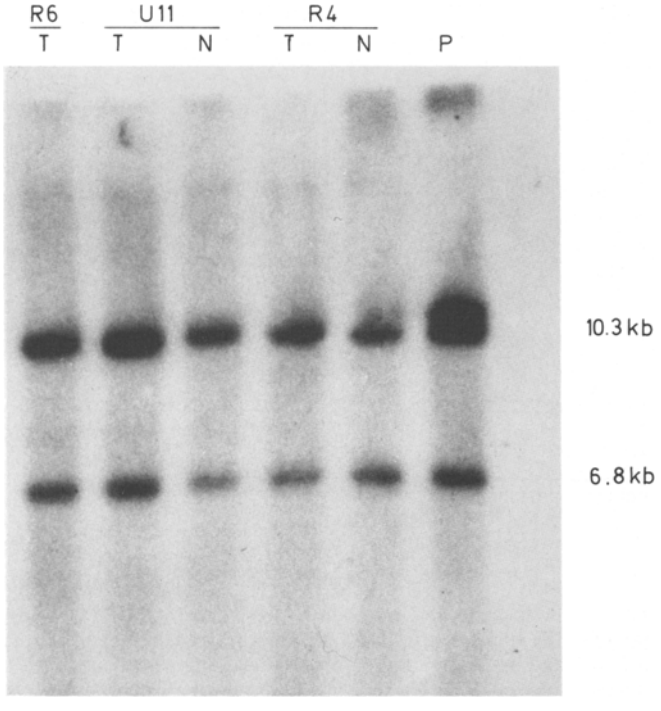

Fig. 2. Germline configuration of mdr1 in two treated (R4, R6) and one untreated (U11) patient. Identical bands of 10.3 and 6.8 kb are present in each tumor and control DNA sample. Samples were digested with BglII and hybridized with probe pHDRVI 5′. *T* = tumor; *N* = normal tissue; *P* = placenta; *kb* = kilobases

after two cycles of chemotherapy including methorexate, vinblastine, doxorubicin, and cis-platinum (MVAC), followed in six months by a relapse of papillary and in situ tumor confined to the mucosa (at which time cystectomy was performed). No alterations of mdr1 nor mdr2 were detected in this tumor. The remaining treated tumors were unresponsive to systemic or intravesical chemotherapy (Table 2). All of these tumors had germline configurations of both mdr1 (Fig. 2) and mdr2 (data not shown).

Quantitative Southern blotting using a human bcl-1 probe as a control suggested one extra copy of mdr1 in one treated tumor (R3) (Fig. 3). This finding might be accounted for by the presence of an extra copy of chromosome 7 (upon which mdr1 resides [2]) rather than by true gene amplification, although cytogenetic study was not performed on this tumor.

Discussion

Until recently, standard treatment modalities for muscle-invasive transitional cell carcinoma of the bladder were limited to radical cystectomy or external irradiation. Although treatment by these techniques alone or in combination produce local control of tumor in up to 90% of patients, 5-year survival rates rarely exceed 50% [10, 20, 25]. Because most of the nonsurvivors succumb to metastatic disease, it was recognized that improved survival would require systemic treatment. These observations spurred the development and use of platinum-based combination chemotherapy for initial treatment of TCC in patients with metastatic or locally unresectable disease and preoperatively as "neoadjuvant" therapy in patients with localized but invasive tumor in the bladder [24].

Two trials at our institution have assessed the efficacy of a four drug combination (methotrexate, vinblastine, doxorubicin, and cis-platinum: MVAC) in the treatment of TCC. In 92 patients with metastatic or locally unresectable TCC of the renal pelvis, ureter, or bladder, complete response (CR) was observed in 39%, partial response (PR) in 28%, and incomplete response (IR) in 33% [16]. In the trial evaluating MVAC as neoadjuvant therapy prior to cystectomy in patients with invasive but resectable disease, the corresponding CR rate was 24%, PR rate 39%, and IR rate 37% [16]. Analysis of factors predicting response in these trials has identified only one criterion with predictive value: the presence of non-transitional cell elements, such as adeno- or squamous cell carcinoma, which predict incomplete response to therapy [16]. Because treatment with any chemotherapeutic regimen is associated with significant morbidity, and because current regimens fail to eradicate disease in a significant proportion of patients, the ability to predict response to chemotherapy would be a valuable asset in patient management. This ability would have the obvious advantages of avoiding unnecessary toxicity and the capacity to choose an alternative form of therapy. The demonstration of altered structure or overexpression of mdr1 or mdr2 in pretreatment samples of tumors which ultimately fail

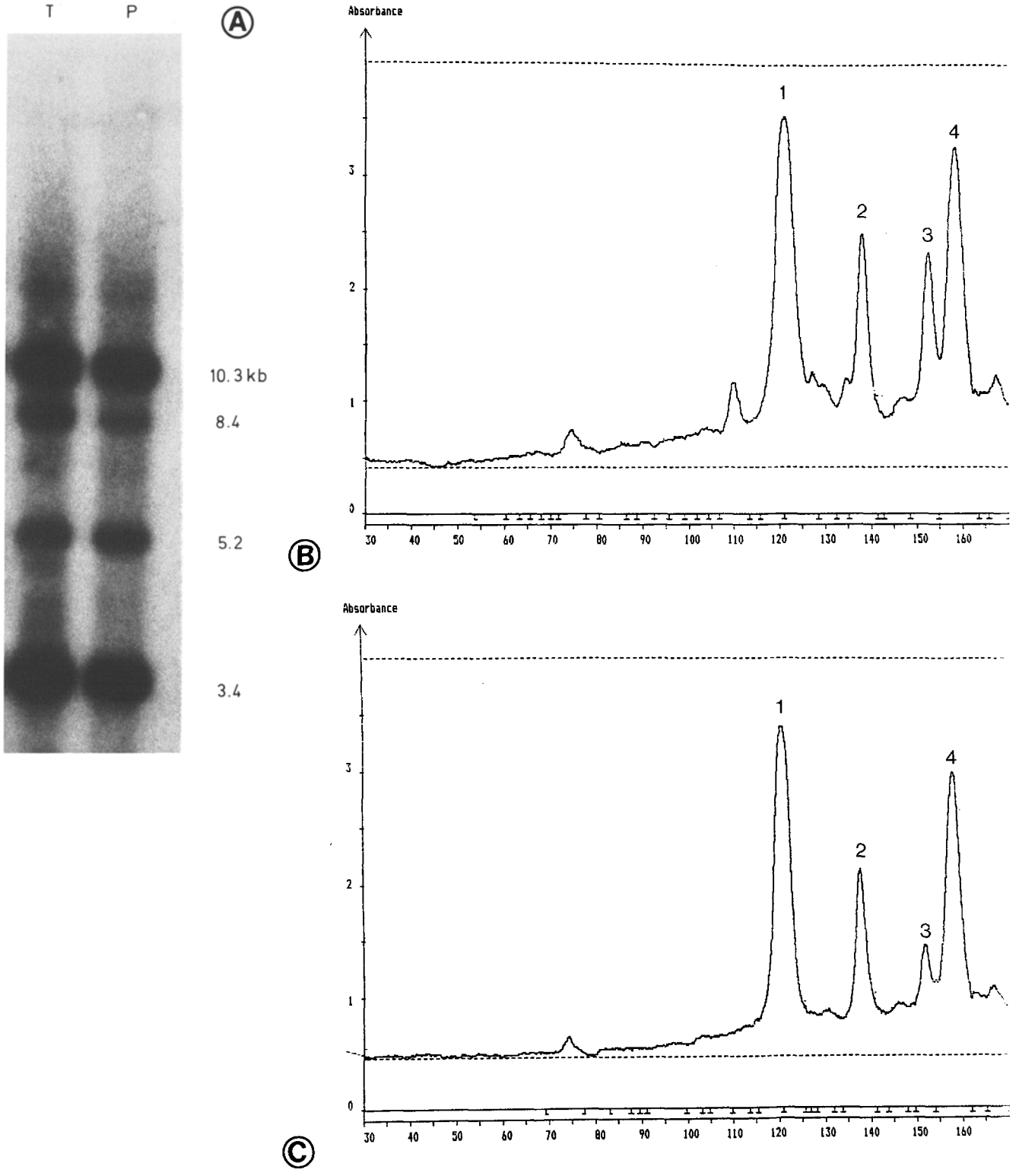

Fig. 3 A. Quantitative Southern blot of tumor DNA from patient R3 using probe for bcl-1 as control. Densitometric scan of initial Southern blot (not shown) suggested amplification of mdr1 in this tumor. Ten micrograms of tumor (*T*) and placental (*P*) DNA were digested with BglII and hybridized with pcDR1.3 and bcl-1 (a human cDNA probe for the bcl-1 gene). Bands of 10.1, 8.4, and 5.2 kb represent germline configuration of mdr1 with pcDR1.3; 3.4 kb band corresponds to germline bcl-1. **B and C** Densitometric scans of blots from specimen R3 and control respectively. Peak 1 corresponds to the 3.4 kb band of bcl-1. Peaks 2, 3, and 4 correspond to the 5.2, 8.4 and 10.1 kb bands of MDR-1. Using the hybridization intensity of peak 1 (bcl-1) as control and normalizing the ratio of peak 1 between tumor and normal to a value of 1, the resulting ratios of peaks 2, 3, and 4 are 1.5, 2.7, and 1.6 suggesting the presence of an extra copy of MDR1 in the tumor DNA

chemotherapy may be one way to develop predictive criteria for tumor response.

The role of multidrug resistance and the molecular events associated with its occurrence in human tumors is incompletely defined. In mammalian cell lines multidrug resistance appears to arise by two mechanisms. In cells selected for low level resistance, transcriptional deregulation of mdr1 results in overexpression of mdr1 mRNA [11, 19]. In cells which are selected for higher levels of resistance by stepped exposure to increasing drug concentrations, resistance is invariably associated with amplification of the mdr1 gene [11, 19]. The full multidrug resistance phenotype can result from either mdr1 amplification or overexpression in vitro. Which of these mechanisms are operative in primary human tumors is unknown. Bell et al. detected increased levels of the mdr1 protein product P-glycoprotein in two cases of drug resistant ovarian adenocarcinoma, but whether this resulted from mdr1 amplification or overexpression is unknown [1]. Other studies have suggested that overexpression without amplification of mdr1 is important in at least some malignancies. Fojo et al. demonstrated increased levels of mdr1 mRNA in a variety of treated and untreated human tumors [5]. In one drug refractory pheochromocytoma an increase in mdr1 mRNA expression between samples assayed before and after drug exposure was demonstrated. Increased levels of mdr1 mRNA in primary human renal adenocarcinomas have also been reported [6]. Although coamplfication and rearrangements of a second gene of the multidrug resistance family (mdr2) is often seen in vitro in cells which have amplified mdr [22] the role of mdr2 in drug resistance is unknown. No studies have addressed whether drug resistance in TCC is mediated by amplification or overexpression of mdr1 or mdr2.

Although we have studied only a relatively limited number of tumors, the lack of observed structural alterations or amplification of the mdr genes suggests that such changes are not important in mediating drug resistance in TCC in vivo. The significance of the single extra copy of mdr1 observed in one drug-unresponsive tumor (R3) is unknown because neither a pretreatment sample nor mdr1 mRNA expression were assayed in this tumor. The presence of this extra gene could be explained by partial or complete duplication of chromosome 7 in this tumor, a hypothesis consistent with recent observations of trisomy 7 as a characteristic cytogenetic change in TCC [15]. If expression of the mdr genes is important clinically, the most likely alternative mechanism of action is by transcriptional deregulation, as observed for mdr1 in tumor cell lines and some primary tumors. Although we did not assay for the expression of mdr1 mRNA in this study, the limited data available from other sources suggest that mdr1 mRNA expression is not important in TCC, with only low level expression observed in the study of 7 tumors [8]. These results suggest that neither amplification nor overexpression of the mdr genes are important in mediating drug resistance in TCC.

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