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Original Paper

Expression of *MDR*1 mRNA and Encoding P-glycoprotein in Archival Formalin-fixed Paraffin-embedded Gall Bladder Cancer Tissues

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The aim of this study was to examine the expression of P-glycoprotein (Pgp) and *MDR*1 mRNA, in gall bladder carcinoma, a chemo-resistant tumour. 26 cases of gall bladder cancer and nine samples of normal gall bladder archival paraffin blocks were investigated for the presence of Pgp protein with immunohistochemistry (IHC) and *MDR*1 RNA by reverse transcription–polymerase chain reaction (RT–PCR). Monoclonal antibodies JSB-1 and UIC-2, recognising separate epitopes of Pgp, were used for IHC. For RT–PCR, total RNA was extracted from paraffin-embedded tissue. After RT, the samples were subjected to nested PCR (NPCR) using primers specific for the *MDR*1 gene, and evaluated by electrophoresis. In gall bladder carcinoma, the percentage of positive cases expressing Pgp (77% for JSB-1, 69% for UIC-2) and *MDR*1 mRNA (52%) was significantly higher than those in normal gall bladder. In earlier TNM stages Pgp and *MDR*1 mRNA were more frequently expressed (non-significant) than in advanced stages. The results of this study suggested that overexpression of *MDR1* mRNA and Pgp in gall bladder carcinoma tissue probably is a very important reason why gall bladder cancer is generally not responsive to chemotherapy. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: gall bladder cancer, MDR, P-glycoprotein, paraffin-embedded tissue
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INTRODUCTION

GALL BLADDER cancer is a malignancy with an extraordinarily poor prognosis. Overall, the 5-year survival rate is less than 5% [1, 2] and the correct pre-operative diagnosis is only made in nearly 50% of patients [1]. In 1968, Siewert and Cassau [3] came to the conclusion that there had been hardly any changes in the poor prognosis of carcinoma of the gall bladder during the preceding 50 years. Unfortunately, Cubertafond and colleagues [1] recently came to a similar conclusion, that no progress has been made in the last 10 years in the treatment and prognosis of gall bladder cancer, after they surveyed 724 cases in 73 different institutions and hospitals. However, other surgeons [4, 5] consider that significant improvements have occurred in the last decade and that the overall survival rate has improved after radical surgical procedures. Adjuvant chemotherapy for gall bladder carcinoma has been disappointing, with no significant advantage found even when patients received postoperative chemotherapy [6].

Chemotherapy is one of the main forms of treatment for cancer, but the failure of chemotherapy is predominantly due to drug resistance. Multidrug resistance (MDR) is the phenomenon whereby exposure to one drug induces cross-resistance to a variety of functionally and structurally distinct agents to which the tumour cells have not yet been exposed. This phenomenon is mainly associated with increased expression of the *MDR*1 gene [7], which encodes a plasma membrane glycoprotein of 170 kDa P-glycoprotein (Pgp) [8]. Pgp, in cells with intrinsic or acquired resistance, serves as an ATP-dependent efflux pump [9] for a broad spectrum of anticancer agents. Pgp over-expression often occurs upon relapse from chemotherapy, but may also occur at the time of diagnosis [10]. Studies have demonstrated that over-expression of the *MDR*1 gene and its encoded product Pgp is

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not only an indicator of the poor response to chemotherapy, but also a sign of poor prognosis in some malignant diseases [11, 12].

So far there has not been any detailed report concerning the expression of *MDR*1 and/or Pgp associated with gall bladder cancer. In this study we examined the expression of *MDR*1 mRNA and Pgp in gall bladder cancer and normal gall bladder specimens, and compared the results with different clinicopathological parameters.

MATERIALS AND METHODS

Clinical data

Paraffin-embedded gall bladder cancer tissues from 26 patients (15 females and 11 males), who had undergone surgery in Zhejiang Medical University, China between 1991–1995, were analysed. Their ages ranged from 33 to 71 years (median 59 years). The distribution of the extent of disease according to the TNM classification [13] was as follows: stage I, 4 (15%); stage II, 2 (8%); stage III, 8 (31%); stage IV, 12 (46%). There were nine well differentiated, seven moderately differentiated, seven poorly differentiated, and three undifferentiated tumours. Correct pre-operative diagnosis was made in 62% (16/26).

No patient received chemotherapy and radiotherapy prior to operation.

Nine paraffin-embedded blocks of normal gall bladder tissue were used as the control group. The gall bladders were removed from patients who had had Whipple's operations for chronic pancreatitis in the same period as above (1991–1995). Among them, 6 were women and 3 were men. Their ages ranged from 41 to 73 (median 57 years).

The tissues were fixed in 10% formalin for 2–10 h (according to the size of the tissue) and embedded in paraffin routinely as surgical pathology specimens. All the paraffin blocks were stored at ambient temperature for 2–6 years before analysis.

KB-V1 cell line

The KB-V1 cell line (DSMZ GmbH, Braunschweig, Germany) is a MDR subclone derived from the drug sensitive cell line KB-3-1 which itself is a derivative of HeLa (human cervix carcinoma cell line). KB-V1 cells strongly express *MDR*1 mRNA and Pgp [7, 14]. It is 210 times more resistant to vinblastine than the parental cell line [14] and shows a more than 500-fold increase in *MDR*1 mRNA than KB-3-1 [15]. The KB-V1 cell line was used as a positive control for immunohistochemistry (IHC) and reverse transcription–polymerase chain reaction (RT–PCR).

The cells were cultured in 85% Dulbecco's modified Eagle-medium (Gibco BRL, Eggenstein, Germany) plus 15% fetal bovine serum with antibiotics solution at 37°C in a 10% CO_2 humidified atmosphere in air. Cells were stepwise exposed to increasing concentrations (200, 400, 800 ng/ml) of vinblastine.

IHC

Two monoclonal antibodies (MAb) recognising different epitopes of Pgp were used in this study. MAb JSB-1 (Progen Biotechnik GmbH, Heidelberg, Germany) is a mouse immunoglobulin of the IgG1 subclass. It reacts with a conserved cytoplasmic epitope of the plasma membrane associated human 170 kDa Pgp [16]. Another mouse MAb UIC-2 (Immunotech Co., Marseilles, France), which is of subclass IgG2a, reacts specifically with an extracellular epitope of the human 170 kDa Pgp [17].

Immunostaining was performed with a modified alkaline phosphatase anti-alkaline phosphatase (APAAP) procedure described by Cordell and associates [18]; new fuchsin was used as the chromogen. The KB-V1 cells cultured with 800 ng/ml vinblastine (cytocentrifuged preparation), as well as paraffin sections with strongly expressed Pgp antigen, were used as positive controls. For the negative control, the primary antibodies were substituted by Tris-buffer.

Isolation of total RNA

Total RNA from formalin-fixed paraffin-embedded tissues and KB-V1 cells was recovered by a modification of methods described by Chomczynski and Sacchi [19], and Martin and colleagues [20]. Sections 40–100 μm, according to the area of the tissue embedded in paraffin, were collected in a microfuge tube. After deparaffinisation with 1 ml xylene for 10 min (this step was repeated if there was any visible paraffin left) and extraction with graded ethanol (100, 70 and 50%), the tissue was collected by centrifugation and dried under a flow bench. Then, 0.5 ml digestion buffer [0.1 M Tris–HCl, 25 mM ethylenediamine tetraacetic acid (EDTA), 1% sodium dodecylsulphate (SDS) (pH 7.3)] was added with 500 μg proteinase K (Sigma, Deisenhofen, Germany). This mixture was incubated and agitated at 54°C for 12–30 h or until the tissue was completely solubilised. An additional 250 μg proteinase K was added if the incubation was longer than 24 h. After complete solubilisation, the solubilised tissue was incubated at 95°C for 10 min to inactivate the proteinase K. Then, 1 ml denaturing solution (solution D) [4M guanidinium isothiocyanate (Gibco BRL, Eggenstein, Germany), 25 mM sodium citrate (pH 7.0), 0.5% *N*-lauroyl sarcosine, and 0.1 M β-mercaptoethanol] was added, followed by 100 μl sodium acetate (2 M, pH 4). Water saturated phenol (1.5 ml) was added, followed by 100 μl chloroform:isoamyl alcohol (49:1). The mixture was centrifuged at 15 000 rpm for 20 min at 4°C. The aqueous phase was aspirated to another microfuge tube, and the RNA precipitated with cold absolute ethanol. After centrifugation, the RNA pellet was resuspended in 0.3 ml solution D and again precipitated with absolute ethanol. After a 20 min incubation at −80°C, the RNA was recovered by centrifugation at 15 000 rpm for 20 min at 4°C. The RNA pellet was washed in 70% ethanol, dried under the flow bench, and resuspended in 50 μl water with 1 μl RNasin (40 U/μl) (Boehringer-Mannheim, Mannheim, Germany). RNA preparations were stored at −80°C until required.

KB-V1 cells (1×10^6) cultured with 800 ng/ml vinblastine were mixed with 1 ml solution D, then the lysate was passed through the pipette at least 10 times. Thereafter the procedures were the same as those for isolating total RNA from paraffin-embedded tissue.

The concentration and the purity of the RNA were measured spectrophotometrically by measuring its absorbance at 260 and 280 nm.

RT reaction

A RT reaction was performed with the Ready-To-Go kit according to the manufacturer's recommendations (Pharmacia Biotech, Freiburg, Germany). Total RNA (1 μg) and 1 μg random hexadeoxyribonucleotide primers (Pharmacia Biotech) were used for each reaction. The resultant cDNA was stored at −20°C until required.

Half-nested PCR (NPCR)

To check the integrity of the RNA, the housekeeping gene β2-microglobulin was amplified by RT-NPCR. The sequences of the primers were: first round, sense: 5′-ATCCAGCGTACTCCAAAGATT-3′, antisense: 5′-CATGTCTCGATCCCACTTAACTAT-3′ (expected 296 bp); second round: same antisense primer and another sense primer (5′ -TGAAAAAGTGGAGCATTCAGA-3′), a 160 bp product was desired. The first round of NPCR was carried out in a 50 μl reaction volume containing 5 μl 10×PCR buffer [300 mM Tricine; 20 mM $MgCl_2$; 50 mM β-mercaptoethanol; 0.1% (v/v) gelatin; 1% (w/v) polidocanol; pH 8.4]; 1 μl of 10 mM dNTP (Pharmacia Biotech), 1.25 μl each of sense and antisense primer (20 μM), 0.25 μl *Taq* polymerase (5 U/μl) (Gibco BRL), and finally 1 μl of the cDNA. The reaction mixture was overlaid with mineral oil and subjected to 30 cycles of denaturation at 94°C for 75 sec, annealing at 54°C for 60 sec and extension at 72°C for 90 sec, with an additional 7 min of incubation at 72°C after the final extension. A 0.5 μl aliquot of the first round of NPCR was then used as the template for the second round of NPCR with the same components (except primers) and conditions as the first round. For *MDR*1, the sequences of the primers were: first round, sense: 5′-GTGGGCAGAAGCAGAGGA-3′, antisense: 5′-GGATTCATCAGCTGCATTTT-3′, with an expected 335 bp product. Second round: another antisense primer (5′-GACCTTTTCTGGCCTTATCC-3′) was used with the same sense primer. A 141 bp product was expected. The components were the same as described above. Two rounds of amplification consisted of 35 and 30 cycles, respectively, under the same conditions described above, with the exception that the annealing temperature was set to 57°C.

For all PCR experiments, the cDNA template was replaced by water as a negative control. The cDNA transcribed from the mRNA of the KB-V1 cell line was used a positive control.

The PCR products (10 μl) were electrophoresed through 1.5% agarose gels (prepared with 1×TAE buffer) and visualised by ethidium bromide.

In this study, the antisense primer for β2-microglobulin, which was the same used for both rounds of NPCR, was designed to be interrupted by an intron (1880 bp). At the DNA level, the sequence of the primer is interrupted by the intron. In this way the primer cannot anneal to DNA completely so that the possible contaminating DNA cannot serve as a template. Using this strategy, only cDNA corresponding to spliced mRNA, and not contaminating DNA, can be successfully amplified.

After the first round of NPCR, the electrophoretic results showed no bands of 296 bp of β2-microglobulin, except the samples of the KB-V1 cell line.

21 cases of gall bladder cancer and nine samples of normal gall bladder, positive for the second round of NPCR for β2-microglobulin by agarose gel electrophoresis, were subjected to NPCR for *MDR*1.

There are introns between the two primers of the first round of NPCR for *MDR*1, resulting in a large band (4350 bp) if there was potential contaminating DNA in the RNA preparation. The antisense primer for the second round of NPCR for *MDR*1 was designed to span an intron (3200 bp), just like the antisense primer for the NPCR for β2-microglobulin. There will be no product of DNA if there is any contaminating genomic DNA. It can be concluded that the expected positive bands can only be obtained from cDNA.

Statistical analysis

Fisher's exact test was used to analyse the data. A P value less than 0.05 was considered significant.

RESULTS

Detection of Pgp on gall bladder carcinoma tissues

In the samples of gall bladder cancer, it was noticed that the stained tumour cells were on the glandular epithelium (Figure 1). With MAb JSB-1, the cytoplasm was uniformly stained, a granular staining of cytoplasm was also observed in some positive cells. Significant membrane staining was observed with MAb UIC-2, but in some cases cytoplasmic staining could also be detected. In positive samples of normal gall bladder, the stained cells were also on the glandular epithelium (Figure 1).

In the samples detected with MAb JSB-1, 77% (20/26) had detectable Pgp in the gall bladder cancer group compared with 33% (3/9) in normal gall bladder ($P = 0.026$). With MAb UIC-2, 69% (18/26) and 11% (1/9) ($P < 0.001$) were positive for Pgp in the gall bladder cancer and normal gall bladder group, respectively. This demonstrates that in gall bladder cancer the positive percentage of Pgp expression is significantly higher than that in normal gall bladder tissue.

Twenty-one of the 26 gall bladder cancer samples were positive with JSB-1 or UIC-2 compared with 4 of the 9 normal gall bladder cases (non-significant). Seventeen of the 26 samples of gall bladder cancer were positive for both JSB-1 and UIC-2, while there were no cases in the normal gall bladder group positive for both antibodies ($P < 0.001$).

This demonstrates that Pgp expression is significantly more frequent in gall bladder carcinoma than in normal gall bladder.

All the negative controls of immunostaining were uniformly negative.

Expression of MDR*1 mRNA in gall bladder cancer tissues*

Total RNA was recovered from the paraffin blocks of all 26 cases of gall bladder cancer; nine normal gall bladder paraffin blocks and KB-V1 cells. For the total RNA isolated from paraffin blocks, the average ratio of $A_{260/280}$ was 1.91 and the average yield was 11.4 μg/100 μm tissue. Previous studies have demonstrated that RNA preparations with an absorbance ratio greater than 1.4 are adequate for RT-PCR experiments [21]. All samples evaluated in this study exceeded that level.

In the 21 cases of gall bladder cancer, 52% (11/21) had detectable *MDR*1 mRNA compared with 11% (1/19) in normal gall bladder ($P < 0.05$). This demonstrates that in gall bladder cancer tissue the *MDR*1 mRNA was significantly more often expressed than in normal gall bladder.

Examples of the positive electrophoretic results of the second round of NPCR for *MDR*1 and β2-microglobulin are presented in Figure 2.

Expression of MDR*1 mRNA and Pgp in different TNM stages and histopathological grades of gall bladder cancer*

To estimate the difference between gall bladder cancer TNM stage and the expression of Pgp and *MDR*1 mRNA, stages I and II were merged into 'earlier stage' and stages III and IV into 'advanced stage'. In the earlier stage cases, detected by MAb JSB-1, 100% (6/6) had detectable Pgp

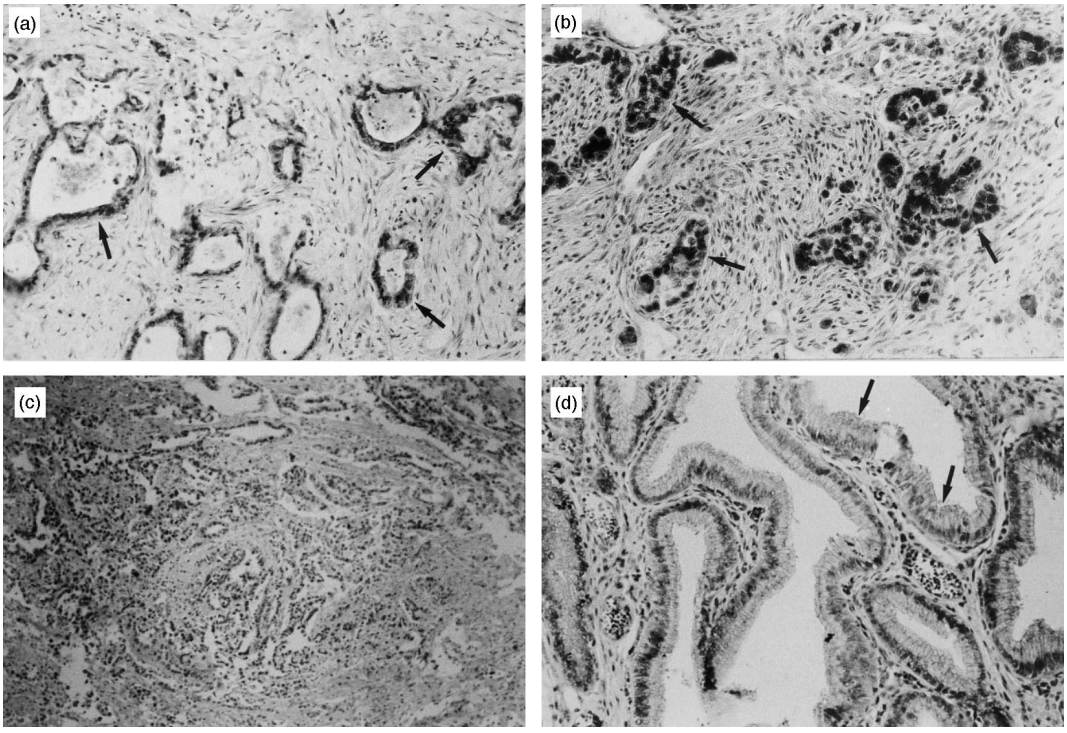

Figure 1. Representative photographs of immunohistochemical staining (a) gall bladder cancer stained with monoclonal antibody (MAb) JSB-1, 200×; (b) gall bladder cancer stained with MAb UIC-2, 200×; (c) unstained gall bladder cancer 100×; (d) normal gall bladder tissue stained with MAb UIC-2, 200× (arrows: stained areas).

compared with 70% (14/20) in advanced stage. With MAb UIC-2, 100% (6/6) were positive for Pgp in earlier stages, while 60% (12/20) were positive in advanced stages. At the mRNA level, 80% (4/5) showed *MDR*1 mRNA expression in earlier stages and 7/16 (44%), in advanced stages. This suggests that Pgp and *MDR*1 RNA expression was more frequent in earlier stages of gall bladder cancer than advanced stages, although this was not significant ($P > 0.05$), since the number of samples in each group was small. There was no significant difference between the expression of Pgp and *MDR*1 mRNA and the histopathological grades of gall bladder cancer ($P > 0.1$).

DISCUSSION

Since the introduction of PCR, the number of reports using formalin-fixed paraffin-embedded tissues for DNA analysis has increased substantially. Although successful amplification of DNA sequences has been carried out with DNA prepared from formalin-fixed paraffin-embedded tissue, there are only a few reports regarding RNA analysis in this kind of material. RNA amplification, through cDNA intermediaries, is of interest because of the relationship between mRNA level and gene expression.

Although RNA is degraded during histological processing, small intact fragments survive and function as templates for RT-PCR. This technique has been successfully used to detect RNA viruses and to examine oncogene expression in paraffin-embedded tissues [22, 23]. Therefore, the demonstration of RNA, which can be amplified by RT-PCR using a housekeeping gene, with no interference by a pseudogene, is a prerequisite for the analysis of fixed or fresh tissue samples. Regarding the use of a housekeeping gene as an internal control, both β2-microglobulin and β-actin represent reliable internal standards to ensure that samples contain high-quality RNA suitable for PCR amplification. β2-microglobulin was chosen in the present study since β-actin can be decreased in drug-resistant cells [24].

The amplification of mRNA isolated from formalin-fixed paraffin-embedded tissue needs more PCR cycles. The degradation of RNA is a likely explanation for this phenomenon. RT-PCR strategies applied to paraffin-embedded tissue should target short sequences to compensate for the inevitable loss of larger RNA species. Mies [25] concluded that 50 amplification cycles and additional *Taq* polymerase were necessary to amplify a 204 bp target, whereas a 150 bp product was clearly visible on a gel after only 40 cycles. In the present study, even after 50 amplification cycles and additional *Taq* polymerase, the 160 bp (β82-microglobulin) and 141 bp (*MDR*1) products were still not detectable. NPCR supplies a promising way to solve this problem. This study has demonstrated that NPCR is an effective method for detecting degraded RNA from paraffin-embedded tissue. Certainly, NPCR is a time-consuming and laborious procedure, and the main problem of NPCR is contamination which results in false positive results. Using fresh autoclaved water, pipette tips with filters, preparing PCR mixtures in the flow bench and the strict separation of the rooms for the isolation of RNA and the analysis of PCR products can reduce contamination efficiently. All the negative controls, consisting of a PCR mix with sense and antisense primers plus water instead of template cDNA, were uniformly negative in the present experiments.

Elevated levels of Pgp are found in some normal tissues, including the adrenal cortex and medulla, the proximal tubules of the kidney, epithelial cells of the small intestine and colon, liver hepatocytes, and ducts of the pancreas [15]

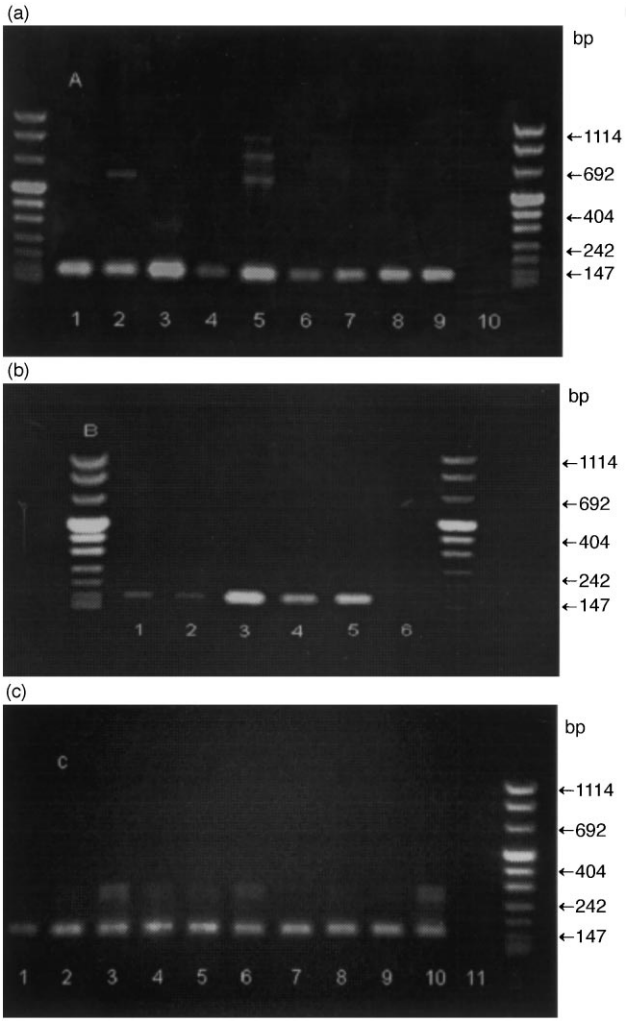

Figure 2. Representative electrophoretic results. (a) Positive samples for second round of nested polymerase chain reaction (NPCR) for *MDR*1, seven gall bladder cancer samples (lanes 1–7), normal gall bladder sample (lane 8), positive and negative controls (lanes 9 and 10). (b) Positive samples for second round of NPCR for *MDR*1, four gall bladder cancer samples (lanes 1–4), KB-V1 cell line (positive control, lane 5), negative control (lane 6). (c) Positive samples for the second round of NPCR for β2-microglobulin, paraffin-embedded samples (lanes 1–9), KB-V1 cells (positive control, lane 10), negative control (lane 11).

[26], and according to the results of the present study, in gall bladder epithelial cells. Its expression in these excretory organs suggests that Pgp plays a physiological role in cell clearance of extrinsic or intrinsic toxic products. Like the gall bladder carcinoma examined in the present study, tumours originating from these tissues that normally express Pgp, usually exhibit high levels of Pgp or its mRNA [15, 27, 28], indicating that the signal for its expression can be maintained during neoplastic transformation [14]. These neoplasms consistently respond poorly to chemotherapeutic agents, including those involved in MDR. Tumours derived from tissues without detectable Pgp (e.g. breast and ovarian carcinomas) show no expression initially, but may have high levels during relapses after exposure to chemotherapy [29].

Pavelic and colleagues [30] detected Pgp with UIC-2 and JSB-1 in normal gall bladder tissue, but did not report how many cases they tested and how many were positive or negative. In the present study, Pgp was detected in the epithelium of normal gall bladder, in agreement with Pavelic and colleagues [30]. The results of IHC in the present study indicate that both MAbs (JSB-1 and UIC-2) can detect Pgp and are likely to have clinical utility. In gall bladder carcinoma taken from patients without previous exposure to chemotherapy, we detected Pgp overexpression probably as a part of the cascade of molecular events associated with tumour progression.

A marked elevation of *MDR*1 mRNA was found for both preneoplastic liver nodules and liver carcinoma, when compared with normal liver tissue in rat liver carcinoma [28] [31]. These studies suggested that overexpression of Pgp may be inherent to carcinogenesis under certain conditions. In the present study, the earlier stage gall bladder cancer compared with advanced stages were more frequently Pgp and *MDR*1 mRNA positive, although there was no statistical significance, since the number of cases in each group was small. These results suggest that Pgp is present at the very beginning of tumour development and not in tumour progression.

Analysis by RT-PCR may be the most specific and most sensitive assay for *MDR*1 mRNA [32], but it does not allow the identification of the actual cells (tumour or normal) that express the gene. For this reason, it is necessary to combine it with IHC so that a more detailed pattern can be seen. Because some commercially available anti-Pgp antibodies recognise epitopes on other molecules, at least two antibodies recognising separate epitopes (one surface, one cytoplasmic) should be used. Only limited confidence can be placed in studies using single antibodies [32].

In conclusion, Pgp is normally present in epithelia of normal gall bladder tissues and it is significantly elevated in gall bladder carcinoma tissue at both the protein and mRNA levels. It may be the main reason for gall bladder tumour's intrinsic resistance to chemotherapeutic drugs.

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