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Expression of the Multidrug Resistance-associated Protein (MRP) Gene in Human Lung Tumours and Normal Tissue as Determined by *In situ* Hybridisation

G.A. Thomas, M.A. Barrand, S. Stewart, P.H. Rabbitts, E.D. Williams and P.R. Twentyman

In a number of cell lines with a multidrug resistant phenotype, there is no overexpression of the putative efflux pump, P-glycoprotein. Some such lines do, however, overexpress the MRP gene which encodes a protein bearing considerable amino acid homology to P-glycoprotein. We have used *in situ* hybridisation to study expression of the MRP gene in human cell lines, lung tumours (representing all the major histologies) and normal lung tissue. Considerable heterogeneity of expression was seen in parental cell line COR-L23/P whereas relatively uniform high-level expression was seen in the resistant line COR-L23/R. Normal bronchial epithelium was strongly positive, but the major epithelial component of all eight lung tumours studied showed only a negative to weak signal. However, the leading edge of the tumours consistently produced a more intense signal similar to that in normal epithelium. Areas of lymphocytic infiltrate were more strongly positive than the tumour epithelium. These results suggest that expression of the MRP gene may be a significant factor determining response of lung tumours to chemotherapy, but that considerable caution is needed in the interpretation of expression studies carried out on homogenised tissue biopsies.

Key words: multidrug resistance, MRP, lung cancer, *in situ* hybridisation
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INTRODUCTION

CELLS EXPOSED to a single cytotoxic drug frequently assume resistance not only to the inducing agent but also to a range of other agents. One such pattern of cross-resistance, usually termed classical multidrug resistance (MDR), involves over-expression of the 170–k P-glycoprotein (Pgp) believed to act as a drug efflux pump located in the plasma membrane [1]. Cells possessing this phenotype show cross-resistance to, and defective accumulation of, a wide range of drugs including doxorubicin, vincristine, etoposide and paclitaxel but can be, at least partially, resensitised by the use of resistance modifiers such as verapamil or cyclosporin A [2]. Pgp is the product of the human MDR1 gene located on chromosome 7 and, in resistant cells, the gene is amplified and/or overexpressed at the mRNA level [1]. Use of antibodies to Pgp has revealed that, in humans, Pgp is expressed constitutively at relatively high levels in a number of normal tissues including adrenals, kidney, liver and gastro-intestinal epithelium [3]. Various types of tumour, including those of the breast, colon, and kidney, also express high levels of Pgp; in contrast, expression in lung tumours is generally low [4, 5].

A number of cell lines have, however, been reported which, whilst having an MDR phenotype, do not overexpress the MDR1 gene at either the mRNA or protein levels [6–9]. Some of these cell lines, at least, show defective accumulation of the relevant cytotoxic drugs and it has, therefore, been speculated that an alternative transporter molecule must be involved. Recently, Cole and colleagues [10] reported the cloning of a gene (MRP, multidrug resistance-associated protein) which apparently codes for such an alternative transporter. The gene was initially cloned from an MDR small cell lung cancer cell line, and has subsequently been found to be overexpressed in three additional lung cancer MDR cell lines which lack Pgp [11, 12]. In the initial report, Cole and colleagues used northern blotting to show MRP mRNA to be present at high levels in normal lung and testis and also in peripheral blood mononuclear cells [10]. Levels were much lower in several other normal human tissues. Use of RT-PCR enabled Zamon and associates [11] to demonstrate more widespread expression in many human normal tissues.

Assays of mRNA expression at the tissue level are difficult to interpret as they do not reveal the identity of the cells within the tissue which express a given gene, nor do they allow assessment of heterogeneity of expression. In tumours, it may be that

Correspondence to P.R. Twentyman.
G.A. Thomas, S. Stewart and E.D. Williams are at the University of Cambridge, Department of Histopathology, Addenbrooke's Hospital; and M.A. Barrand, P.H. Rabbitts and P.R. Twentyman are at the MRC Clinical Oncology and Radiotherapeutics Unit, Hills Road, Cambridge CB2 2QH, U.K.
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expression is due to normal cells present within the tumour stroma rather than to malignant cells. Such information has, therefore, to be obtained either by the use of antibodies to the relevant protein or by *in situ* hybridisation to detect mRNA at the cellular level. As appropriate antibodies to MRP are not available, we have used *in situ* hybridisation to study expression of MRP in human lung tumours and normal tissues in order to understand the possible significance of such expression for the chemotherapy of human lung cancer.

MATERIALS AND METHODS

Cells

Verification of MRP probe specificity was carried out using the human large cell lung cancer cell line COR–L23/P, and its MDR subline COR–L23/R [9]. Subline COR–L23/R, derived by exposure to doxorubicin, does not overexpress Pgp [9] but shows considerable overexpression of the MRP gene and its 190-k protein product [12]. Detectable levels of MRP in mRNA are seen in the parental line COR–L23/P and high levels in the resistant subline COR–L23/R [12].

Probes

Two antisense oligoprobes, each 30 nucleotides in length, were synthesised and labelled at the 5′ end with digoxigenin (R and D Systems (Europe) Ltd, Abingdon, Oxon, U.K.). Probe A (CAAGCTGGCGCTGCCCGACACTGAGGTTCT) was complementary to the sequence of bases (4508–4479) corresponding to amino acids situated close to the nucleotide binding site in the C-terminal half of MRP [10]. Probe B (CAGACAGGTTCACGCCCTTCTCGCCAATCT) was complementary to the sequence of bases (2504–2475) corresponding to amino acids situated close to the nucleotide binding site in the N-terminal half of MRP. The specificities of these probes for MRP mRNA were determined by hybridising them to northern blots of RNA prepared from COR–L23/P parent cells and from COR–L23/R resistant cells. The probes were labelled at the 3′ end with [α-^{32}P]CTP using terminal transferase. Following separation from unincorporated [α-^{32}P]CTP by centrifugation through Sephadex G50, the labelled probes were hybridised to the RNA filters at 50°C for 18–24 h in 5 ml of hybridisation buffer containing 5% dextran sulphate, 1 M NaCl, 1% SDS and 0.005% salmon sperm DNA. The filters were washed at 50°C, for 2–5 min each wash, twice in 2 x SSC/0.1% SDS and twice in 1 x SSC/0.1% SDS, and then autoradiographed. Adjacent strips from the same RNA filters were also probed with [α-^{32}P]CTP-labelled cDNA fragments amplified by PCR from RNA prepared from COR–L23/R using primers corresponding to bases 1999–2595 and 4005–4619 in the MRP sequence [12].

In situ *hybridisation*

Serial section (4 μm) were taken from formalin-fixed, paraffin-embedded blocks containing cells from parental cell line COR–L23/P and its MDR subline COR–L23/R. In addition, serial sections were taken from routine formalin-fixed, paraffin-embedded blocks from an unselected series of lung tumours—two small cell carcinomas, two squamous cell carcinomas, three adenocarcinomas and one carcinoid. All sections were mounted on slides coated with 3-aminopropylethoxysilane.

The *in situ* hybridisation technique used has been described fully elsewhere [13]. Briefly, sections were pre-incubated in proteinase K (1 μg/ml) for 30 min at 37°C, followed by hybridisation buffer for 1 h at 37°C and hybridised overnight in 45 μl of hybridisation buffer containing either 1 ng/μl probe A or probe B. After washing in graded concentrations of SSC to remove unbound probe, bound probe was detected by alkaline phosphatase linked anti-digoxigenin antibody (1:500) and standard histochemistry using nitro blue tetrazolium (NBT) as the reporter molecule. Control sections were either hybridised with a 5′ labelled control probe of the same length (30 mer) complementary to EBER 1 mRNA [14] or in the absence of labelled probe. To ensure that mRNA had not been degraded by previous handling and fixation of the surgical tissue, sections were also hybridised with 0.5 ng/μl oligo dT probe [R and D Systems (Europe) Ltd].

RESULTS

Northern blotting

Following hybridisation, both probe A and probe B were able to detect an RNA species on northern blots of RNA prepared from cell lines COR–L23/P and COR–L23/R [12] (Figure 1). No signal was detectable with the control oligoprobe to EBER 1 mRNA which was labelled and used in the same manner as the probes A and B. The signal obtained from probe A was consistently weaker than that from probe B. The RNA species detected by the digoxigenin probes A and B were identical in size to those recognised by the larger PCR-amplified MRP-specific cDNA fragments and consistent with RNA encoding a protein containing 1522 amino acids [10].

In situ *hybridisation*

Results from the two probes showed the same qualitative cellular localisation and, as had been observed on the northern blots, probe A was consistently weaker than probe B.

COR–L23/R showed an homogeneously strong staining pattern, whereas cell line COR–L23/P showed a weaker and more heterogeneous staining pattern on *in situ* hybridisation (Figure 2). Normal bronchiolar epithelium from areas adjacent to the tumour was strongly positive (Figure 3), but in all cases, the

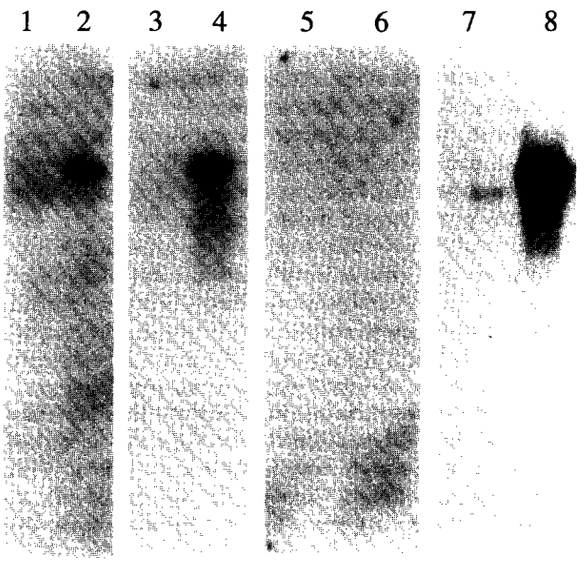


Figure 1. mRNA from COR–L23/P (tracks 1, 3, 5 and 7) and COR–L23/R (tracks 2, 4, 6 and 8) resolved on the same northern blot, hybridised with end-labelled 30-mer oligoprobes A (tracks 1, 2), B (tracks 3, 4) or EBER I (tracks 5, 6) under conditions described in Materials and Methods or with oligolabelled PCR-generated 615-mer MRP probe (tracks 7, 8) under conditions described previously [12].

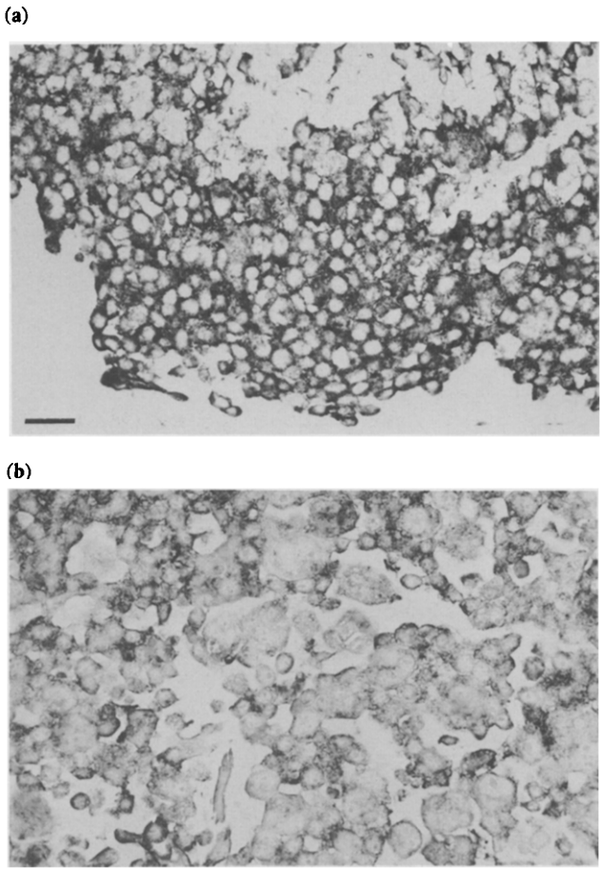


Figure 2. (a) Section from a formalin-fixed, paraffin-embedded cell block of cell line COR–L23/R hybridised with 1 ng/μl probe B. All cells show high expression of MRP mRNA, with little heterogeneity of expression. (b) Section from a formalin-fixed, paraffin-embedded cell block of cell line COR–L23/P hybridised with 1 ng/μl probe B. In contrast to the results observed with cell line COR–L23/R, this cell line expresses a lower and more heterogeneous content of MRP mRNA. The scale bar represents 50 μm. These results are consistent with the relative contents of MRP mRNA observed on northern blots performed on resistant and parent cell lines.

major epithelial component of the tumours showed a weak to negative hybridisation signal. However, the leading edge of the tumour was consistently stronger and was particularly strong in areas of tumour either within lymphatics (Figure 4) or close to vessels (Figure 5). Endothelial cells were consistently stained positive and, in addition, where there was a considerable lymphocytic infiltrate, populations of lymphocytes at various sites within the tumour were much more strongly positive than the tumour epithelium (Figure 5). The control oligo dT probe showed homogeneous strong staining over the entire tumour, showing that the focality of MRP mRNA expression was not due to fixation artefact or poor tissue preservation.

Control sections were consistently negative when hybridised with probe to EBER 1 mRNA or in the absence of labelled probe.

DISCUSSION

We have demonstrated the localisation of MRP mRNA using two separate digoxigenin-labelled oligoprobes, in two cell lines (COR–L23/P and COR–L23/R) and on tissue sections taken from a series of lung tumour samples. The results for the cell lines confirm our previous data [12], obtained using northern blotting, that overall expression of MRP is much greater in the resistant line. We now see, however, that there is considerable heterogeneity of expression in the parental line, with some cells producing a strong hybridisation signal. This observation would suggest that a selection process for such MRP-high cells may have been involved in the derivation of the resistant subline.

We have also demonstrated the presence of MRP mRNA in a series of lung tumours and shown there to be considerable variation between regions of the same section and different cell types within the tumour. Such variation within the malignant population could be due to a number of reasons including clonal

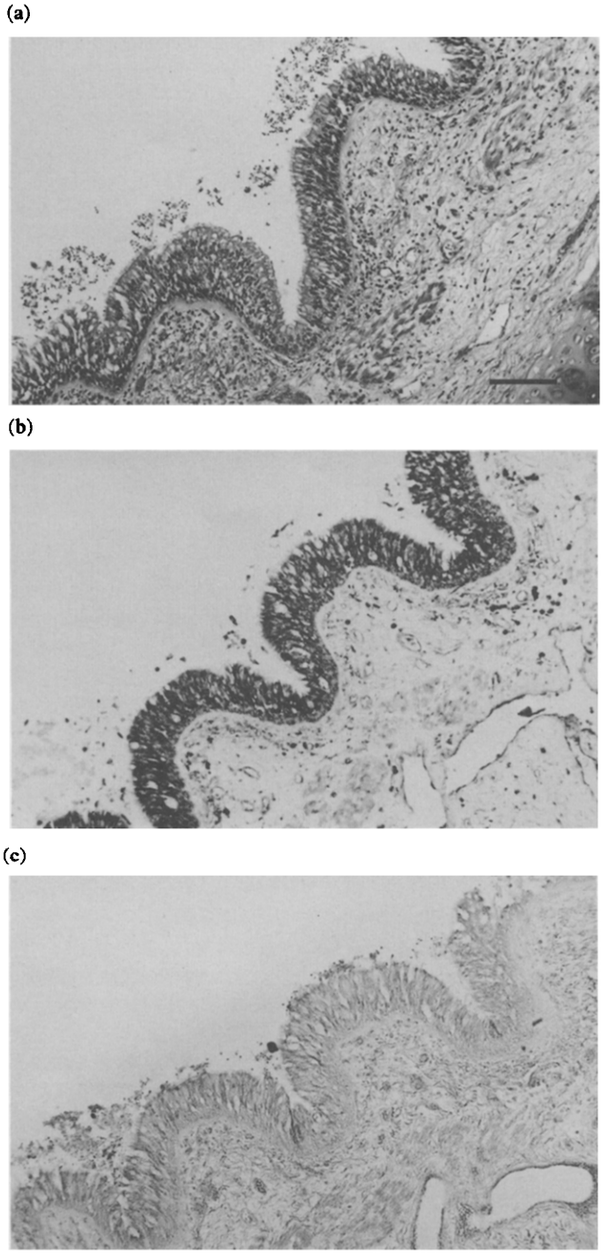
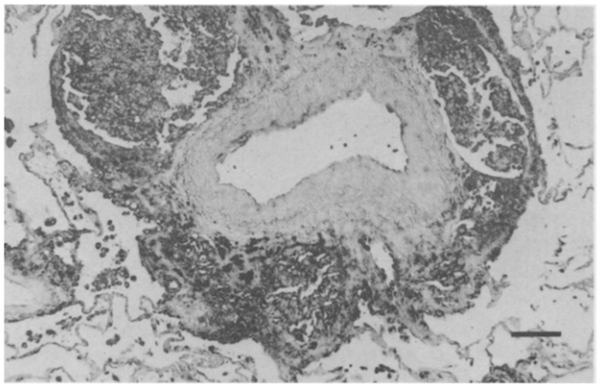


Figure 3. (a) Haematoxylin and eosin-stained section of normal bronchus. (b) Serial section of 3a hybridised with 1 ng/μl probe B. There is strong staining of the bronchial epithelium indicating a high MRP mRNA content. The surrounding stromal tissue is negative. (c) Serial section of 3b, hybridised with 1 ng/μl control probe complementary to EBER 1 mRNA, showing negativity in both bronchiolar epithelium and stroma. The scale bar represents 100 μm.

variation, micro environmental factors or cell cycle dependency. High expression of MRP in lymphocytes is in accordance with a previously reported observation on blood mononuclear cells [12]. Normal bronchiolar epithelium expresses relatively high levels of MRP, but is weaker than the lymphocytic infiltrate observed in some tumours. MRP mRNA expression is limited to tumour epithelial cells, and is more prominent in cells at the leading edge of the tumour and in tumour deposits within lymphatics close to vessels, suggesting an involvement either in invasion or related to mitotic activity. This observation is very similar to that previously reported for Pgp in human colon carcinoma [15]. Due to the small sample size in this preliminary study, it would be inappropriate to draw any conclusions regarding expression of MRP and the histological type of tumour. Further, more comprehensive, studies will also be required to correlate tumour architecture or patient treatment and prognosis with expression of MRP mRNA. However, this study has demonstrated the value of using *in situ* hybridisation to study cellular localisation of MRP mRNA in pathological archival material, and indicates that qualitative results from simple northern blotting could be misleading, particularly in a series of normal tissues or tumours with variable lymphocytic infiltration.

(a)



(b)

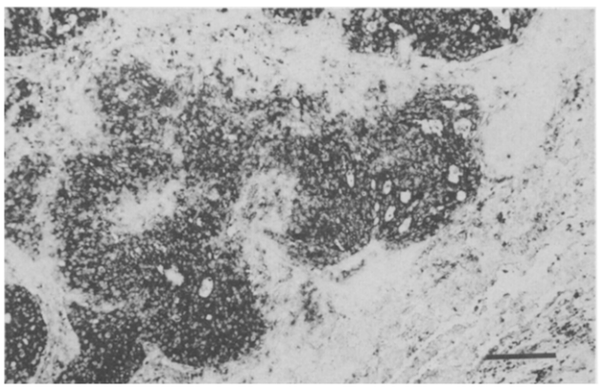


Figure 4. (a) Section hybridised with probe B (1 ng/μl) showing a tumour deposit from an adenocarcinoma within lymphatics surrounding a blood vessel. The tumour epithelium is strongly positive for MRP mRNA. The scale bar represents 100 μm. (b) Different region from the same section showing the leading edge of the tumour which expresses relatively high levels of MRP mRNA in the epithelial component of the tumour. The scale bar represents 100 μm.

(a)

(b)

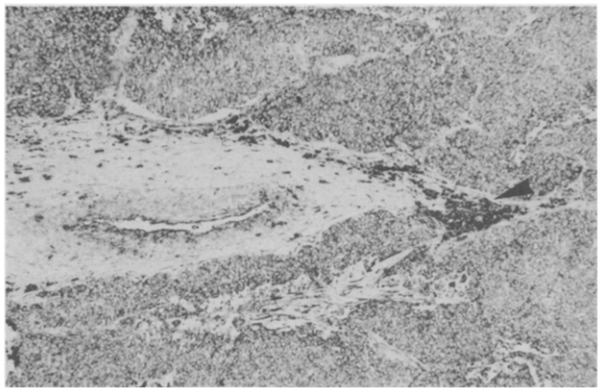


Figure 5. (a) Section stained with haematoxylin and eosin showing a region from a lung adenocarcinoma surrounding a vessel. A lymphocytic infiltrate can be observed to the right side of the vessel (large arrow), and small deposits of carbon are also seen (small arrow). (b) Serial section to 5a hybridised with probe B (1 ng/μl), showing moderate expression of MRP mRNA in the epithelial tissue of the tumour. The lymphocytic infiltrate (arrowed), expresses much higher levels of MRP mRNA. The scale bars represent 100 μm.

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Mutational Analysis of the First 14 Exons of the Adenomatous Polyposis Coli (APC) Gene

Z. Dobbie, M. Spycher, R. Hürliman, R. Ammann, T. Ammann, J. Roth, A. Müller, Hj. Müller and R.J. Scott

In the present study, the polymerase chain reaction single strand conformation polymorphism (PCR-SSCP) technique has been applied to the mutation analysis of the adenomatous polyposis coli (APC) gene. We examined the first 14 exons of the APC gene in 46 polyposis coli patients. Five germline mutations were observed, including a single-nucleotide substitution and small (1–4 bp) deletions leading, in 4 cases, to a stop codon. A missense mutation in exon 3 and a 1 bp deletion in exon 4 of the APC gene were observed in patients presenting with the attenuated form of FAP.

Key words: APC gene, familial polyposis coli, genetics, mutation
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INTRODUCTION

FAMILIAL ADENOMATOUS polyposis (FAP) is a rare autosomal dominant inherited disorder predisposing to colorectal cancer. Patients with FAP develop hundreds to thousands of adenomatous polyps in the colon and rectum during their second or third decades of life which, if left untreated, will almost certainly develop into colorectal cancer. Recently, several reports have indicated the evidence of a phenotypically different form of polyposis coli [1–4] known as the attenuated or variant form of APC (AAPC). It is characterised by a low and variable number of colonic polyps (from only a few to over a hundred), a more advanced age of onset and/or slower evolution of the disease.

Correspondence to Z. Dobbie.
Z. Dobbie, M. Spycher, Hj. Müller and R.J. Scott are at the Division of Human Genetics, Department of Research of the University Clinics, Hebelstrasse 20, CH-4031 Basel, Switzerland; R. Hürliman, R. Ammann, T. Ammann and A. Müller are at the Department of Gastroenterology and J. Roth is at the Department of Pathology, University Hospital, Zürich, Switzerland.
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Germline mutations in the APC gene are believed to be responsible for FAP. This gene was first localised to 5q21-22 and subsequently cloned [5–9]. The full length APC transcript consists of 15 exons, and encodes a protein of 2843 amino acids [9]. The coding region of the APC gene is preceded by several alternatively spliced 5′ non-coding exons [10].

Mutations in the APC gene have also been detected in sporadic carcinomas and adenomas [11–13]. The simultaneous existence of somatic and germline alterations of the APC gene have been observed in a high portion of desmoid tumours [14]. These results strongly suggest an important role for the APC gene product in epithelial proliferation and/or differentiation. Somatic mutations in APC have been suggested to initiate colorectal tumour development in the general population, whereas germline mutations result in FAP.

We have used the technique of single strand conformation polymorphism (SSCP) to determine the germline mutations in the first 14 exons of the APC gene in 46 unrelated Swiss polyposis coli patients. Identification of five mutations occurring in this region is reported here and discussed in the light of the patients' phenotypes.