Pergamon

European Journal of Cancer, Vol. 34, No. 1, pp. 81–86, 1998

Printed in Great Britain
0959-8049/98 $19.00+0.00

PII: S0959-8049(97)00356-0

Original Paper

Expression of the Multidrug Resistance Protein (MRP) in Squamous Cell Carcinoma of the Oesophagus and Response to Pre-operative Chemotherapy

K. Nooter,[1] T. Kok,[1] F.T. Bosman,[2]* K.E. van Wingerden[1] and G. Stoter[1]

[1]Department of Medical Oncology, University Hospital Rotterdam, 3015 GD Rotterdam and Rotterdam Cancer Institute (Daniel den Hoed Kliniek); [2]Department of Pathology, Erasmus University, Rotterdam, The Netherlands

One of the major problems in the treatment of squamous cell carcinoma of the oesophagus (ESCC) is the unresponsiveness to cytotoxic drugs. So far, the mechanisms underlying the intrinsic drug resistance of ESCC remain unclear. The aim of this study was to determine the role of the newly recognised drug resistance protein, the multidrug resistance protein (MRP), in ESCC drug resistance. Tumour biopsies from ESCCs were analysed by RNase protection assay (RPA) as well as by immunohistochemistry (IHC) for the presence of MRP mRNA or protein, respectively. The ESCC samples were obtained from patients participating in a prospective randomised clinical phase III trial, evaluating pre-operative chemotherapy (cisplatin and etoposide) followed by surgery versus surgery alone in patients with operable ESCC. For most patients, tumour biopsies taken at diagnosis by endoscopy as well as surgically resected primary tumours were available. Of 58 ESCC patients enrolled, 28 received chemotherapy before surgical resection of their tumours, and 30 were treated with surgery alone. 12 patients (3 complete and 9 partial responses; 43%) showed a major response after chemotherapy, 10 patients (36%) had stable disease (SD), and 6 (21%) progressive disease (PD). On 14 surgically resected, untreated, primary ESCCs, the IHC scores correlated with the MRP mRNA levels, quantitated by RPA (multiple testing, $P < 0.01$). MRP expression was detected by IHC in the vast majority (52/58; 90%) of the diagnostic biopsies. MRP expression did not differ significantly between CR+PR, and patients with SD or PD. In addition, multivariate analysis by logistic regression did not show any effect of tumour cell differentiation or UICC tumour stage on the outcome of pre-operative chemotherapy in relation to MRP expression. However, a difference became apparent (Sign-test, $P < 0.05$) for higher MRP expression in tumours from patients with PR or SD, when comparing MRP levels in paired tumour samples before and after chemotherapy, suggesting that chemotherapy selected for drug-resistant cell clones. © **1998 Elsevier Science Ltd.**

Key words: MRP, squamous cell carcinoma of the oesophagus, clinical response, pre-operative chemotherapy, immunohistochemistry
Eur J Cancer, Vol. 34, No. 1, pp. 81–86, 1998

INTRODUCTION

STANDARD TREATMENT for squamous cell carcinoma of the oesophagus (ESCC) consists of surgical resection. Cure is rare, with 5-year survival of less than 10%. Response rates to chemotherapeutic agents, given singly or in combination, are low and range from 15 to 40% [1]. However, a promising development in the treatment of ESCC is the use of pre-operative chemotherapy with the primary goal of debulking the tumour load, followed by surgical resection of the tumour. In 1989, a multicentre prospective randomised phase III trial was initiated in the University Hospital Rotterdam (The Netherlands), in which pre-operative chemotherapy (cisplatin and etoposide) followed by surgery was

Correspondence to K. Nooter.
*Present address: Department of Pathology, University of Lausanne, Switzerland.
Received 24 Mar. 1997; revised 2 Jul. 1997; accepted 9 Jul. 1997.

compared with surgery alone in patients with operable ESCC. Clinical response to chemotherapy was evaluated after the second cycle, and patients with a major response were given two additional cycles of chemotherapy followed by surgery, whereas non-responding patients were operated upon at once. Interim analysis showed very favourable results in terms of survival for those who responded well to preoperative chemotherapy [2]. The median survival after randomisation was 18.5 months for the 'chemotherapy plus surgery' arm, versus 11 months for the 'surgery alone' arm ($P = 0.0017$). Among the chemotherapy-treated patients, approximately 40% had a major response (with approximately 10% complete responders).

In a previous study [3] we have shown that a variety of human cancers, including ESCC, overexpress the newly recognised drug resistance gene, the multidrug resistance protein (MRP) gene [4]. *MRP* encodes a 190 kDa membrane-bound glycoprotein of 1531 amino acids and is a member of the ATP-binding cassette superfamily of transport proteins [4, 5]. Transfection experiments with different eukaryotic expression vectors containing full-length complementary DNAs of the *MRP* gene have shown that MRP confers multidrug resistance (MDR) to a broad range of natural product drugs, among which are anthracyclines, vinca alkaloids and epipodophyllotoxins [6–9]. As yet, the mode of action by which MRP makes cells multidrug resistant is not known. However, the available data suggest that MRP acts both as a plasma membrane outward drug pump and as a pump for drug accumulation in intracytoplasmic vesicles [6, 7, 10, 11]. By both mechanisms cytoplasmic concentrations of free drug may be reduced to sublethal levels, and in that way MRP would promote cell survival. Although expression of MRP has been demonstrated in a variety of solid tumours [3, 12–20] and leukaemias [21–23], the question of whether elevated levels of MRP are associated with clinical drug resistance has not yet been fully answered. In the present study, we examined the hypothesis that the unresponsiveness of ESCC to chemotherapy might be related to the presence of MRP.

MATERIALS AND METHODS

Tumour samples

Tissue samples were subjected to this research with informed consent of the patients. In total, 58 primary ESCCs, resected at the University Hospital Rotterdam between 1989 and 1993, were analysed for MRP expression. The ESCC samples were obtained consecutively in a prospective randomised clinical phase III trial, evaluating preoperative chemotherapy (cisplatin and etoposide) followed by surgery versus surgery alone in patients with operable ESCC. All chemotherapy-treated patients received two cycles of chemotherapy. Patients with a major response, as defined by a tumour reduction of $>50\%$, were given two additional cycles of chemotherapy, followed by surgery, whereas patients with stable (SD) or progressive disease (PD) were subjected to surgery at once. Cisplatin (80 mg/m^2) was given on day 1 as a 4-h infusion preceded by etoposide (100 mg) as a 2-h infusion. On day 2 only etoposide (100 mg) was given as a 2-h infusion, and on days 3 and 5 etoposide (200 mg/m^2) was administered orally. The specimens included in the study were 30 tumours resected without prior chemotherapy, and 28 tumours for which patients received chemotherapy before surgical resection of their tumours. Clinical response to preoperative chemotherapy was categorised as complete (CR), indicating the pathological absence of tumour in the resection; or partial (PR), indicating at least a 50% reduction in the size of the tumour. SD was defined as no change in tumour volume, and in the case of PD tumour volume increased during treatment. The reduction in tumour size was estimated by comparing tumour dimensions obtained before and after chemotherapy by endoscopic and radiological examination. Endoscopic biopsies and resected tumours were routinely processed at the pathology department of the hospital for diagnostic purposes. Patient and tumour characteristics are summarised in Table 1.

RNase protection assay

Total RNA was isolated from tissue biopsies by the lithium chloride–urea method [25], and quantitated spectrophotometrically at A260. Expression of MRP mRNA was quantitated by RNase protection assay (RPA) as described previously [21, 26]. Briefly, 10 μg of total RNA were hybridised under standard conditions with α^{-32}P labelled RNA transcripts complementary to sequences (nucleotides 239–503) at the 5′ end of the MRP mRNA [4, 26]. This probe does not cross-react with the human MDR1 or MDR3 mRNAs [21]. Radiolabelled protected probes were visualised by electrophoresis through a denaturing 6% acrylamide gel, followed by autoradiography. In all assays a human γ-actin probe was included as control for RNA integrity and recovery. All individual experiments included tRNA, as well as RNA isolated from the drug-resistant cell line GLC4/ADR and its parental cell line GLC4 (kindly provided by Dr E.G.E. de Vries, University of Groningen, Groningen, The Netherlands), as positive and negative controls [26]. Expression levels were quantitated by densitometric scanning of the autoradiographs.

Immunohistochemistry

MRP expression was estimated by immunohistochemistry (IHC) on paraffin-embedded tissue sections (5 μm) prepared from tissue blocks used for routine diagnostic purposes, as well as on cryostat sections (5 μm) prepared from frozen biopsies. Sections of paraffin-embedded tissue were placed on slides coated with 3-aminopropyl-triethoxysilane (Sigma, St Louis, Missouri, U.S.A.) in acetone (1:50), dried overnight at 37°C, deparaffinised, rehydrated and washed in phosphate-buffered saline (PBS: 0.13 mol/l NaCl, 7 mmol/l

Table 1. *Patient and tumour characteristics of* 58 *patients with squamous cell carcinoma of the oesophagus*

Median age		60.4 years (range 36–77)
Gender	male	43
	female	15
Race	Caucasian	58
Grade of tumour differentiation		
	well	$n = 4$ (7%)
	moderately	$n = 34$ (59%)
	poorly	$n = 19$ (33%)
	undifferentiated	$n = 1$ (2%)
Tumour stage (UICC)*		
	I	$n = 7$ (12%)
	II	$n = 22$ (38%)
	III	$n = 6$ (10%)
	IV	$n = 23$ (40%)

*Ref. [24].

Na_2HPO_4, 3 mmol/l NaH_2PO_4, pH 7.6). Cryostat sections were fixed in cold acetone (10 min, 0°C) and air-dried. Endogenous peroxidase activity was blocked by immersing the slides for 30 min in 0.3% (v/v) H_2O_2 in methanol at room temperature. Endogenous avidin and biotin activity was blocked by the avidin and biotin blocking kit (Vector Lab, Burlingame, U.S.A.). The slides were pre-incubated with normal goat serum (NGS) (Gibco-Life Technologies, Paisley, U.K.) (1:50) diluted in 1% (v/v) bovine serum albumin (BSA) in PBS for 20 min at room temperature. Incubation with the MRP-specific monoclonal antibodies (MAb) was performed overnight at 4°C. Two MAbs specific for MRP were used: the rat MAb MRPr1 and the mouse MAb MRPm6 [27]. Prior to use, MRPr1 and MRPm6 were diluted 1:1500 and 1:25, respectively, in PBS containing normal rabbit serum (Gibco) (10%, w/v), NGS (1%, w/v) and normal human AB serum (NHS) (Sigma) (1%, w/v). Subsequently, the slides were incubated for 60 min with biotinylated goat anti-rat MAb or goat anti-mouse MAb (Sigma) diluted 1:50 in PBS supplemented with 1% BSA, 2% NHS and 2% NGS. Antibody binding was detected using streptavidin-conjugated horseradish peroxidase (Zymed, San Francisco, California, U.S.A.) (1:200 in PBS with 1% BSA) in combination with 3,3′-diaminobenzidine tertrahydrochloride (Sigma). The slides were counterstained with haematoxylin and mounted. The specificity of MRPr1 and MRPm6 have been documented in detail elsewhere [3, 13, 27, 28] and are suitable for protein blot analysis, flow cytometry and immunohistochemical studies. MRPr1 and MRPm6 do not cross-react with the human MDR1 and MDR3 glycoproteins [27]. Paraffin-embedded or frozen, MRP-overexpressing, drug-resistant human lung cancer cell line GLC4/ADR and its drug-sensitive parental line GLC4 were used in each assay as positive and negative controls, respectively. Each assay also included the use of isotype-matched irrelevant MAbs (rat IgG2a; mouse IgG1). Staining of the tumour cells was scored on the following semi-quantitative scale: negative with only weak staining of the stromal tissues (−); weak cytoplasmic staining of the tumour cells (±); clear cytoplasmic staining of the tumour cells (+); strong cytoplasmic and weak membranous staining of the tumour cells (++) and strong cytoplasmic and strong membranous staining of the tumour cells (+++). The MRP staining was scored by two independent observers (F.T.B., K.v.W.), one of which is a board-certified pathologist (F.T.B.), who had no further clinical information of the patients whose tumours were analysed.

RESULTS

Expression of MRP protein and mRNA

MRP expression was determined by IHC in 14 surgically resected, primary untreated ESCCs. MRPr1 was used on paraffin-embedded sections, and MRPm6 on cryostat sections. The vast majority of the tumour samples stained with MRPr1 as well as with MRPm6, while incubation with the control, irrelevant rat and mouse MAbs was always negative. The intensity and cellular localisation of the staining varied among the different ESCC samples, and based on these parameters the tumour samples were qualitatively divided into five groups (−, ±, +, ++, +++). As the intensity of the MRP staining increased, the percentage of stained tumour cells increased also. The IHC staining group ±, and + mostly had 30–50% of the tumour cells stained, while for the stronger stained tumours (IHC score: ++, +++), this figure generally was more than 50%. Since MRPr1 has a higher affinity than MRPm6 [26], it was used at a higher dilution than MRPm6 (1:1500 versus 1:25). The IHC scores obtained with the two different MAbs were very similar (Spearman rank correlation coefficient, $r_s = 0.8$; $P < 0.01$) (Table 2).

To correlate MRP protein expression, as estimated by IHC, with mRNA expression, MRP mRNA levels were determined with a sensitive and quantitative RPA in RNA samples isolated from freshly obtained ESCC biopsies, as described previously [3, 21, 22, 26]. The MRP-overexpressing drug-resistant lung cancer cell line GLC4/ADR and its drug-sensitive parental cell line GLC4 were used in each experiment as positive and negative controls, and to compare MRP expression levels in different experiments. Expression levels were quantitated by densitometric scanning of the autoradiographs and the signal obtained with 10 μg of total RNA, isolated from GLC4/ADR cells, was assigned an arbitrary expression level of 100 U. In all 14 ESCC samples we could detect MRP mRNA (Table 2). The expression levels among the various tumours ranged from 2 to 33 U. These MRP mRNA levels of the ESCC samples, quantitated by RPA, strongly correlated with the MRP protein levels, estimated by IHC (Spearman rank correlation, multiple testing $P < 0.01$).

MRP expression in endoscopic biopsies

MRP expression was determined by IHC with MRPr1 on paraffin-embedded sections of endoscopic biopsies of 58 pri-

Table 2. MRP expression determined by immunohistochemistry and RNase protection assay in surgically resected, primary untreated ESCC

	Immunohistochemistry*		
Tumour	MRPr1	MRPm6	RNase protection assay†
1	++	++	33
2	+++	+	31
3	++	++	27
4	+	+	17
5	++	++	14
6	++	ND‡	13
7	++	++	11
8	+	±	9
9	++	+	8
10	+	+	8
11	±	±	7
12	+	ND‡	5
13	−	−	3
14	±	−	2

*Staining of the tumours was scored according to the following categories: negative (−); weak cytoplasmic staining of the tumour cells (±); clear cytoplasmic staining of the tumour cells (+); strong cytoplasmic and weak membranous staining (++); and strong cytoplasmic and strong membranous staining of the tumour cells (+++). MRP-specific MAb MRPr1 was applied on paraffin-embedded tissue sections, while MRPm6 was used on cryostat sections. †MRP mRNA levels were determined with an RNase protection assay [3, 21, 26], and expressed in arbitrary units (U). Each RNA isolate obtained from each individual sample was determined twice, i.e., in two separate assays, and the average of each sample was used to calculate the expression relative to the expression of MRP in the human non-Pgp MDR cell line, GLC4/ADR, which was set arbitrarily at 100 U. ‡ND, not done, because of lack of frozen material.

Table 3. MRP expression in primary ESCC (n = 28) *at time of diagnosis versus clinical response to chemotherapy*

	Immunohistochemical score*				
Clinical response	–	±	+	++	+++
CR ($n = 3$)		1	1	1	
PR ($n = 9$)	2	2	4	1	
SD ($n = 10$)	2	4	3	1	
PD ($n = 6$)		2	1	2	1

*Staining of the tumours was scored as indicated in the legend of Table 2. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

Table 4. MRP expression of surgically resected squamous cell carcinoma of the oesophagus estimated by immunohistochemistry with the MRP-specific MAb MRPr1

	Immunohistochemical score*				
Patient treatment	–	±	+	++	+++
Untreated ($n = 30$)	2 (7%)	3 (10%)	11 (37%)	11 (37%)	3 (10%)
Treated ($n = 21$)	0 (0%)	4 (19%)	9 (43%)	5 (24%)	3 (14%)
All tumours ($n = 51$)	2 (4%)	7 (14%)	20 (39%)	16 (31%)	6 (12%)

*Staining of the tumours was scored as indicated in the legend of Table 2.

mary, untreated ESCCs. 52 of 58 (90%) of the tumour samples stained with MRPr1, while incubation with the control, irrelevant rat MAb was always negative. The intensity and cellular localisation of the staining varied among the different ESCC samples from –, ±, +, ++, to +++ with regard to MRPr1 staining. 6 of 58 (10%) ESCC specimens were scored as negative, 19/58 (33%) showed weak cytoplasmic staining of the tumour cells (±), 19/58 (33%) had a clear cytoplasmic staining (+), in 12/58 (21%) the staining was scored as ++, and in only 2/58 (3%) as +++. The intensity of the staining in the highest MRP staining group (IHC score: +++) equalled the intensity observed in the MRP-positive, drug-resistant GLC4/ADR cells.

28 of 58 patients received pre-operative chemotherapy with cisplatin and etoposide, and the clinical response of the primary tumour is shown in Table 3. 12 patients (43%) had a major response (3 CRs (11%) or 9 PRs (32%)), 10 patients (36%) were classified as SD and 6 patients (21%) had PD. The IHC MRP score of the diagnostic biopsies of the tumours before chemotherapy are also shown in Table 3. There was no obvious correlation between the IHC score and response to chemotherapy. Figure 1 shows the percentage of major responses (CR plus PR) of each IHC staining group. Again, no correlation was evident between IHC staining and response to chemotherapy (χ^2-test, n.s.).

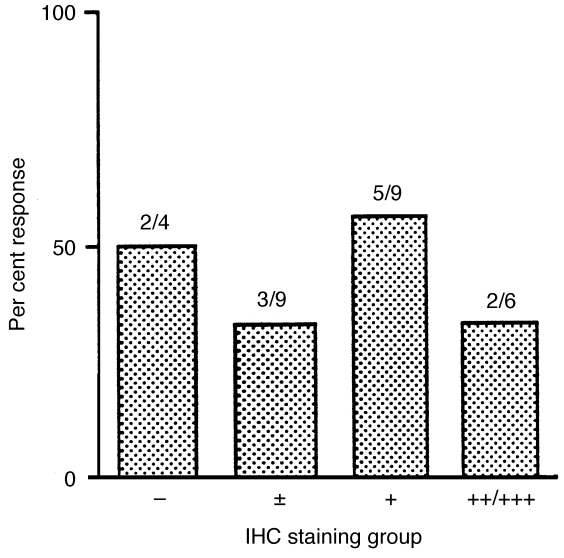

Figure 1. Major clinical response, as defined by reduction in tumour volume of > 50%, on pre-operative chemotherapy in relation to MRP expression. The number of responses/total number of patients in the respective IHC staining groups is shown above the bars.

The patients were stratified the basis of tumour cell differentiation and tumour stage, and MRP expression analysed in relation to clinical outcome. Tumour cell differentiation was dichotomised into differentiated, well and moderately differentiated tumours (combined), and undifferentiated, representing poorly and undifferentiated tumours. In the stratification on tumour stage, the tumours were divided into two categories: locoregional disease (UICC stage I, II, and III) versus stage IV (metastasised tumour). The response to chemotherapy was divided into major responders (CR and PR) and non-responders (SD and PD). Multivariate analysis by logistic regression did not show any effect of tumour cell differentiation and UICC tumour stage on the outcome of pre-operative chemotherapy in relation to MRP expression.

MRP expression before and after chemotherapy

The resected tumours available for MRP IHC included 30 tumours resected without prior chemotherapy, and 21 tumours for which patients had received chemotherapy before surgical resection. No differences were found in MRP expression between the treated and untreated tumours (Table 4). However, when MRP expression was compared in paired samples of diagnostic biopsies and resected tumours obtained after chemotherapy, a statistically significant difference was seen, with higher MRP expression upon drug treatment in SD and PR (Sign-test, $P < 0.05$). This phenomenon was not observed in PD nor in the control 'surgery alone' group. In SD and PR, respectively, 7/9 (78%) and 5/7 (71%) had an increased MRP expression after chemotherapy (Figure 2). For the patients with PD and the 'surgery alone' group, these figures were 2/5 (40%) and 4/13 (31%), respectively.

DISCUSSION

One of the major problems in the treatment of ESCC is the unresponsiveness to cytotoxic drugs. So far, the mechanisms underlying the intrinsic drug resistance of ESCC are unclear. The well-known drug resistance mechanisms that have been elucidated in the laboratory, using cell lines made drug-resistant by *in vitro* challenging, are probably not involved in ESCC. Enhanced cellular drug efflux by the P-glycoprotein drug pump [reviewed in 29], decreased or altered topoisomerase IIα [30] and changes in cellular detoxification systems [31], have not been shown to play a role in the unresponsiveness of ESCC to chemotherapy. In the present study, we analysed the expression of the newly recognised drug resistance *MRP* gene in relation to response to chemotherapy in ESCCs obtained in a pre-operative chemotherapy phase III study. MRP expression was determined

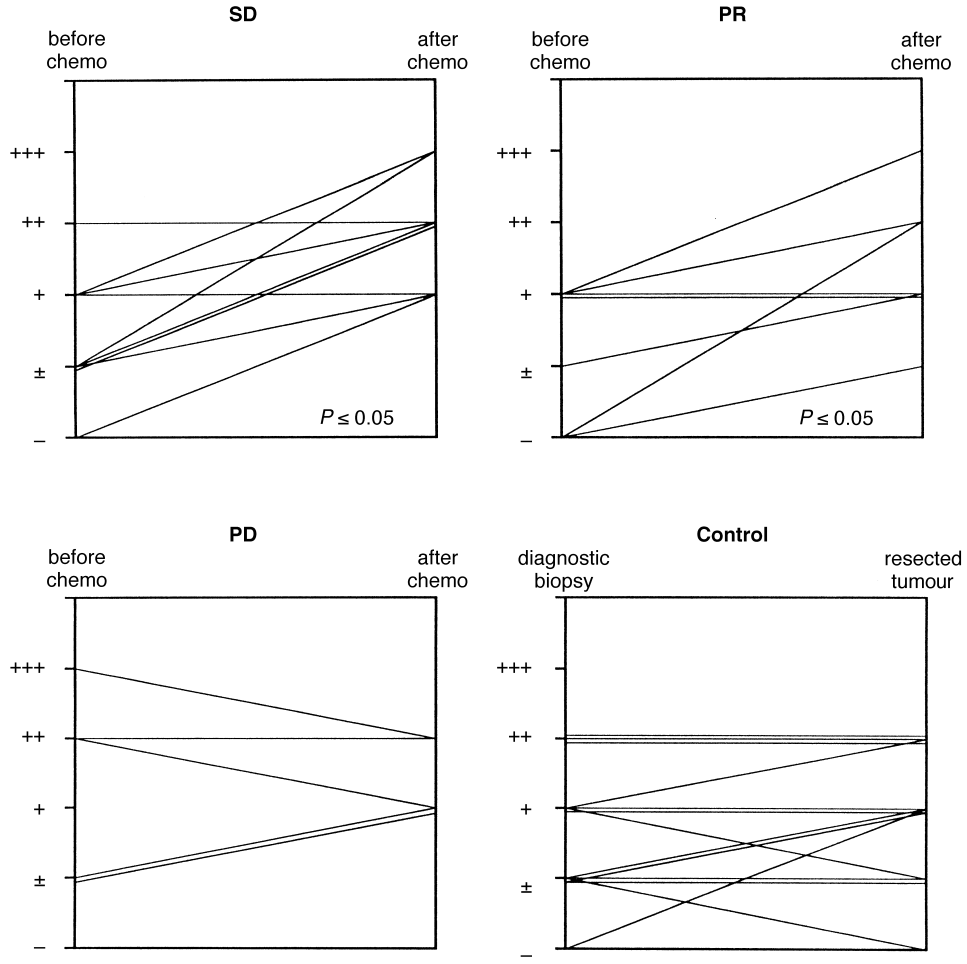

Figure 2. MRP expression in paired tumour samples, before and after chemotherapy, from patients with stable disease (SD), partial response (PR) or progressive disease (PD). In PR and SD, expression of MRP was increased significantly (Sign-test, $P < 0.05$) after chemotherapy. Each line represents 1 patient.

by IHC with two different MRP-specific MAbs, MRPr1 and MRPm6, directed against two different epitopes of the MRP protein [27]. Similar results were obtained with the two MAbs. In addition, the protein levels, estimated by IHC, correlated with MRP mRNA levels, as quantitated by RPA. Subsequently, we used the MRPr1 antibody for detection of MRP in paraffin-embedded sections of diagnostic biopsies. The vast majority of the diagnostic biopsies were positive for MRP, and the protein level, as estimated by IHC, varied between low to very high.

Our study aimed to elucidate a possible role for MRP in clinical drug resistance in ESCC. In classical adjuvant strategies, the efficacy of chemotherapy cannot be assessed directly, because drug treatment is given after surgery when measurable tumour is no longer present. However, in the pre-operative chemotherapy setting, when cytoreductive drugs are administered before surgery, the effects of therapy can be measured more accurately. Indeed, the present phase III study, in which pre-operative chemotherapy (cisplatin and etoposide) followed by surgery was compared with surgery alone in patients with operable ESCC, allowed us to do so. Moreover, the response to pre-operative chemotherapy in our study cohort varied dramatically from CR (11%) to PD (21%). No correlation was found between MRP-staining of the tumours at diagnosis, tumour stage and differentiation, and response to subsequent chemotherapy. The intensity of MRP staining was more or less equally distributed over the clinical response groups being CR, PR, SD and PD, and all IHC staining groups (–, ±, +, ++, +++) had approximately an equal response rate.

Expression of MRP is widely distributed among a large variety of normal and malignant tissues [3, 12–23, 26–28]. However, evidence suggesting a role of MRP in clinical drug resistance is scarce [18–20]. A significant association was found between high levels of MRP expression and poor treatment outcome in neuroblastoma [18], independent of *N-myc* amplification. In two recent studies [19, 20], we showed that in breast cancer MRP might be of prognostic significance. Our data suggest that MRP is related to resistance to chemotherapy in an adjuvant setting [19] as well as in advanced disease [20]. In our present study, a tendency became apparent towards higher MRP expression in tumours from patients with PR or SD, when comparing MRP levels in paired tumour samples before and after chemotherapy. This observation suggests that chemotherapy either directly enhanced the expression of MRP in the tumour cells by, for example, transcriptional activation, or that chemotherapy selected for cell clones with higher MRP expression levels already present in the tumour before treatment. Indeed, for the MDR1/P-glycoprotein drug pump, stress-induced activa-

tion of the MDR1 promoter has been reported [32]. In our study selection of drug-resistant cell clones seems to be likely, since the phenomenon of enhanced MRP expression levels after chemotherapy was more prominent in responsive tumours, including SD. Etoposide belongs to the spectrum of drugs involved in the multidrug resistance phenotype of MRP, and could account for such an *in vivo* selection. MRP has not been shown to confer resistance to cisplatin *in vitro*. However, MRP might function as an efflux pump for glutathione-*S*-conjugates [33–35], and cisplatin can be a substrate for glutathione conjugation [36]. Therefore, theoretically cisplatin might also contribute to such an *in vivo* selection. In conclusion, MRP expression is widely found among ESCCs and does not seem to predict unresponsiveness to chemotherapy. These results indicate that drug resistance in ESCC is due to yet unknown mechanisms, and further studies are needed to elucidate these mechanisms of clinical drug resistance in ESCC.

1. Kok TC. Chemotherapy in esophageal cancer. *Cancer Treat Rev* 1997, **23**, 65–85.
2. Kok TC, van Lanschot J, Siersema PD, van Overhagen H, Tilanus HW. Neoadjuvant chemotherapy in operable esophageal squamous cell cancer; final report of a phase III multicenter randomized controlled trial. *Proc Am Assoc Clin Oncol* 1997, **16**, 277.
3. Nooter K, Westerman AM, Flens MJ, *et al.* Expression of the multidrug resistance-associated protein (MRP) gene in human cancers. *Clin Cancer Res* 1995, **1**, 1301–1310.
4. Cole SPC, Bhardwaj G, Gerlach JH, *et al.*, Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* 1992, **258**, 1650–1654.
5. Hipfner DR, Gauldie SD, Deeley RG, Cole SPC. Detection of the M_r 190,000 multidrug resistance protein, MRP, with monoclonal antibodies. *Cancer Res* 1994, **54**, 5788–5792.
6. Cole SPC, Sparks KE, Fraser K, Loe DW, Grant CE, Wilson GM, Deeley RG. Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. *Cancer Res* 1994, **54**, 5902–5910.
7. Grant CE, Valdimarsson G, Hipfner DR, Almquist KC, Cole SPC, Deeley RG. Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. *Cancer Res* 1994, **54**, 357–361.
8. Kruh GD, Chan A, Myers K, Gaughan K, Miki T, Aaronson SA. Expression complementary DNA library transfer establishes MRP as a multidrug resistance gene. *Cancer Res* 1994, **54**, 1649–1652.
9. Zaman GJR, Flens MJ, van Leusden MR, *et al.* The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. *Proc Natl Acad Sci USA* 1994, **91**, 8822–8826.
10. Breuninger LM, Paul S, Gaughan K, *et al.* Expression of multidrug resistance-associated protein in NIH/3T3 cells confers multidrug resistance associated with increased drug efflux and altered intracellular drug distribution. *Cancer Res* 1995, **55**, 5342–5347.
11. Paul S, Breuninger LM, Tew KD, Shen H, Kruh GD. ATP-dependent uptake of natural product cytotoxic drugs by membrane vesicles establishes MRP as a broad specificity transporter. *Proc Natl Acad Sci USA* 1996, **93**, 6929–6934.
12. Ota E, Abe Y, Oshika Y, *et al.* Expression of the multidrug resistance-associated protein (MRP) gene in non-small-cell lung cancer. *Br J Cancer* 1995, **72**, 550–554.
13. Nooter K, Bosman FT, Burger H, *et al.*, Expression of the multidrug resistance-associated protein (MRP) gene in primary non-small-cell lung cancer. *Ann Oncol* 1996, 7, 75–81.
14. Endo K, Maehara Y, Ichiyoshi Y, *et al.* Multidrug resistance-associated protein expression in clinical gastric carcinoma. *Cancer*, 1996, 77 1681–1687.
15. Kavallaris M, Leary JA, Barrett JA, Friedlander ML. MDR1 and multidrug resistance-associated protein (MRP) gene expression in epithelial ovarian tumors. *Cancer Lett* 1996, **102**, 7–16.
16. Filipits M, Suchomel RW, Dekan G, *et al.* MRP and MDR1 gene expression in primary breast carcinomas. *Clin Cancer Res* 1996, **2**, 1231–1237.
17. Clifford SC, Neal DE, Lunec J. Alterations in expression of the multidrug resistance-associated protein (MRP) gene in high-grade transitional cell carcinoma of the bladder. *Br J Cancer* 1996, **73**, 659–666.
18. Norris MD, Bordow SB, Marshall GM, Haber PS, Cohn SL, Haber M. Expression of the gene for multidrug-resistance-associated protein and outcome in patients with neuroblastoma. *N Engl J Med* 1996, **334**, 231–238.
19. Nooter K, Brutel de la Riviere G, Look MP, *et al.* The prognostic significance of expression of the multidrug resistance-associated protein (MRP) in primary breast cancer. *Br J Cancer* 1997, **76**, 486–493.
20. Nooter, K, Brutel de la Riviere G, Klijn JGM, Stoter G, Foekens JA. Multidrug resistance protein (MRP) in recurrent breast cancer. *The Lancet*, 1997, **349**, 1885–1886.
21. Burger H, Nooter K, Zaman GJR, *et al.* Expression of the multidrug resistance-associated protein (MRP) in acute and chronic leukemias. *Leukemia* 1994, **8**, 990–997.
22. Burger H, Nooter K, Sonneveld P, van Wingerden KE, Zaman GJR, Stoter G. High expression of the multidrug resistance-associated protein (MRP) in chronic and prolymphocytic leukemia. *Br J Haematol* 1994, **88**, 348–356.
23. Schneider E, Cowan KH, Bader H, *et al.* Increased expression of the multidrug resistance-associated protein gene in relapsed acute leukemia. *Blood* 1995, **85**, 186–193.
24. Hermanek P, Sobin LH. *TNM classification of Malignant Tumours*. Berlin, Springer, 1992.
25. Fellous M, Nir U, Wallach D, Merlin G, Rubinstein M, Revel M. Interferon-dependent induction of mRNA for the major histocompatibility antigens in human fibroblasts and lymphoblastoid cells. *Proc Natl Acad Sci USA* 1982, **79**, 3082–3086.
26. Zaman GJR, Versantvoort CHM, Smit JJM, *et al.* Analysis of the expression of MRP, the gene for a new putative transmembrane drug transporter, in human multidrug resistant lung cancer cell lines. *Cancer Res*, 1993, **53**, 1747–1750.
27. Flens MJ, Izquierdo MA, Scheffer GL, *et al.* Immunochemical detection of multidrug resistance-associated protein MRP in human multidrug-resistant tumor cells by monoclonal antibodies. *Cancer Res* 1994, **54**, 4557–4563.
28. Flens MJ, Zaman GJR, van der Valk P, *et al.* Tissue distribution of the multidrug resistance protein. *Am J Pathol* 1996, **148**, 1237–1247.
29. Clynes M. *Multiple Drug Resistance in Cancer. Cellular, Molecular and Clinical Approaches*. Dordrecht, Kluwer, 1993.
30. Hofmann GA, Mattern MR. Topoisomerase II in multiple drug resistance. *Cytotechnology* 1993, **12**, 137–154.
31. Moscow JA, Dixon KH. Glutathione-related enzymes, glutathione and multidrug resistance. *Cytotechnology* 1993, **12**, 155–170.
32. Chaudhary PM, Roninson IB. Induction of multidrug resistance in human cells by transient exposure to different chemotherapeutic drugs. *J Natl Cancer Inst* 1993, **85**, 632–639.
33. Müller M, Meijer C, Zaman GJR, *et al.*Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione *S*-conjugate transport. *Proc Natl Acad Sci USA* 1994, **91**, 13033–13037.
34. Jedlitschky G, Leier I, Buchholz U, Center M, Keppler D. ATP-dependent transport of glutathione-*S*-conjugates by the multidrug resistance-associated protein. *Cancer Res* 1994, **54**, 4833–4836.
35. Leier I, Jedlitschky G, Buchholz U, Cole SPC, Deeley RG, Keppler D. The *MRP* gene encodes an ATP-dependent export pump for leukotriene C_4 and structurally related conjugates. *J Biol Chem* 1994, **26**, 27807–27810.
36. Ishikawa T, Ali-Osman F. Glutathione-associated *cis*-diammine-dichloroplatinum(II) metabolism and ATP-dependent efflux from leukemia cells: molecular characterization of glutathione–platinum complex and its biological significance. *J Biol Chem* 1993, **268**, 20116–20125.

Acknowledgements—We thank Dr E.G.E. de Vries (University of Groningen, Groningen, The Netherlands) for providing cell lines. Miss M. Look is also thanked for statistical analysis. This work was