Pergamon

European Journal of Cancer, Vol. 35, No. 6, pp. 935–938, 1999

Printed in Great Britain
0959-8049/99/$ - see front matter

PII: S0959-8049(99)00035-0

Original Paper

Multidrug Resistance-associated Protein (MRP) Expression is Correlated with Expression of Aberrant p53 Protein in Colorectal Cancer

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Multidrug resistance-associated protein (MRP) is one of the major factors responsible for non-P-glycoprotein (Pgp)-mediated multidrug resistance of human tumour cells. In this study, we examined MRP and aberrant p53 expression in 54 colorectal cancers (CRC), 35 carcinoma in adenomas (CIA) and 40 adenomatous polyps by immunohistochemical procedures. 38 of 54 (70%) CRCs, 16 of 35 (46%) CIAs and 3 of 40 (8%) adenomatous polyps were MRP positive (χ^2 test, $P < 0.0001$). 36/54 (67%) CRCs, 10/35 (29%) CIAs and 0/40 adenomatous polyps were p53 positive. 30 of the 36 p53-positive CRCs were also MRP positive and 8/10 CIAs were both p53 and MRP positive. MRP overexpression correlated with aberrant p53 accumulation in CRCs and CIAs (χ^2 test, $P \leq 0.01$). Coexpression of MRP and p53 in the same cells was confirmed in the CRCs and CIAs by double staining procedures. These results suggested that MRP overexpression is related to aberrant p53 expression in CRC. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: multidrug resistance-associated protein (MRP), p53, coexpression, colorectal cancer (CRC), carcinoma in adenoma (CIA)
Eur J Cancer, Vol. 35, No. 6, pp. 935–938, 1999

INTRODUCTION

MULTIDRUG RESISTANCE to anticancer agents is a problem in patients with cancer and a major obstacle preventing effective treatment of disseminated neoplasms. Several types of drug resistance to anticancer agents have been characterised in human carcinomas [1–3]. Multidrug resistance-associated protein (MRP) was isolated as a 190-kDa membrane transporter protein from a doxorubicin-selected multidrug-resistant lung cancer cell line by Cole and colleagues, and seems to be the major molecule responsible for non P-glycoprotein (Pgp)-mediated multidrug resistance. MRP is a member of the ATP-binding cassette superfamily of membrane transporter proteins and has minor sequence homology to Pgp [4, 5].

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Received 23 Oct. 1998; accepted 12 Jan. 1999.

MRP expression has been examined in several malignant human tumours [6, 7] including non-small cell lung cancer (NSCLC), [8, 9], thyroid carcinoma [10], gliomas [11] and gastrointestinal tract carcinomas [12, 13]. Whilst we reported that MRP mRNA expression was correlated with the histopathology and prognosis of NSCLC [8], the clinicopathological relevance of MRP remains unknown. Some reports have suggested that Pgp/multidrug resistance gene (*MDR*) 1 expression is regulated by aberrations in *TP53* [14]. Missense mutation of the *TP53* gene showed good correlation with positive immunohistochemical staining for p53 in cancer cells [15]. Recently, we reported that MRP expression was significantly correlated with aberrant p53 accumulation in NSCLC [16]. In this study, we immunohistochemically evaluated expression and localisation of MRP in 54 colorectal cancers (CRC), 35 carcinoma in adenomas (CIA) and 40 adenomatous polyps. We analysed expression of MRP and

p53 in the same cells by double staining procedures to investigate the relationships between MRP overexpression and p53 aberration, and to discuss its clinical significance in CRC and polyps.

MATERIALS AND METHODS

Patients and tumours

54 CRC specimens (25 from females and 29 from males, mean age 60.7 years) were obtained by surgical resection (October 1989 to February 1992, at Tokai University Hospital). 75 colon polyps (35 CIAs, 40 adenomatous) were obtained from 75 patients by endoscopic polypectomy. Specimens were processed for routine histopathological analysis (fixation in 10% formalin followed by embedding in paraffin). Of the 54 CRC specimens, 50 were colonic and 4 rectal adenocarcinomas (well differentiated, 26; moderately differentiated, 24; poorly differentiated, 1; mucinous adenocarcinoma, 3). Tumours were histopathologically classified according to standard criteria [17, 18], and pathological stage was also estimated by TNM score [19].

For preparation of total cellular RNA, the specimens were rapidly frozen and stored at − 80°C until analysis. Total cellular RNA was prepared from the frozen specimens by standard procedures.

Immunohistochemical detection of MRP and p53

Immunohistochemical detection of MRP was performed using a rabbit antihuman MRP polyclonal antibody established in our laboratory, as described previously [16]. Deparaffinised sections were incubated with antihuman MRP antibody (1:200) at 4°C for 16 h after antigen retrieval, and blockage of endogenous peroxidase activity (methylalcohol, 3% hydroxygen) and non-specific binding (normal goat serum). Sections were serially incubated with peroxidase-labelled polymer-conjugated goat antirabbit antibody (DAKO, Carpinteria, California, U.S.A.). Immunoreaction products were visualised with 3,3′-diaminobenzidine tetrahydrochloride (DAB). The drug-resistant cell line KB8-5 was used as a positive control for MRP expression. The drug-sensitive cell line KB3-1 lacking MRP expression was used as a negative control.

p53 was detected with monoclonal antibody DO-7 (Novocastra, Newcastle, U.K.). Sections prepared as described above were incubated with the primary antibody for 60 min at room temperature and serially incubated with peroxidase-labelled polymer-conjugated goat antirabbit antibody. Immunoreaction products were visualised with DAB.

We also performed double staining with anti-MRP and anti-p53 antibodies to confirm both MRP and p53 expression in the same cells. Immunohistochemical detection of MRP was performed as described above, then p53 was detected using alkaline phosphatase-conjugated rabbit antimouse immunoglobulin as the secondary antibody and fast blue BB salt (Sigma, St Louis, Missouri, U.S.A.) instead of DAB. We judged the specimens to be MRP- or p53-positive when over 10% of cancer cells showed immunoreactivity.

MRP gene expression

MRP gene expression was examined by RT-PCR with the following primers: sense primer (MRP-S6); 5′-TCTGGGACTGGAATGTCACG-3′, antisense primer (MRP-A6); 5′-CAGGAATATGCCCCGACTTC-3′. PCR consisted of 30 rounds of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min (Gene Amp PCR System 9600, Perkin Elmer, Foster City, California, U.S.A.). Probes were prepared by PCR amplification with the above primers and total RNA of KB8-5, a drug-resistant epidermoid carcinoma cell line [20], as the template. The sequences of the probes used were confirmed by subcloning into the pT7 vector (Novagen, Madison, Wisconsin, U.S.A.) and sequencing with universal primers (Genetic Analyzer 310, Perkin-Elmer). Blots of RT-PCR products (Zeta-Probe, BIO-RAD, Herucles, California, U.S.A.) were hybridised with photochemically labelled probes (ECL; enhanced chemiluminescence, Amersham, Buckinghamshire, U.K.), and exposed to Kodak AR film. RT-PCR was performed with primers for the housekeeping gene β_2m as a control.

Statistical analysis

The differences between the two groups of specimens were compared by χ^2 test using the StatView statistical software package (Abacus, California, U.S.A.).

RESULTS

Expression of MRP

Expression of MRP was observed in 38 of 54 (70%) cases of CRC, 16 of 35 (46%) CIA and 3 of 40 (8%) adenomatous polyps. The positive rate of MRP was significantly increased in the hypothetical manner of carcinoma generation; adenomas, CIA and colorectal cancer (χ^2 test, $P < 0.0001$). MRP was mainly positive in the cytoplasm of the cancer cells, and was not apparent in the stroma. MRP gene expression was examined in 24 of 38 MRP-positive colorectal cancer specimens by RT-PCR, and confirmed in 20 of 24 specimens (83%, Figure 1). The relationship between MRP expression and clinicopathological features including TNM score, pathological stage and prognosis were not significant in the colorectal cancers (Table 1).

Co-expression of MRP and p53

Accumulation of p53 was detected in 36 of 54 (67%) CRCs. 30 (83%) of the 36 CRCs with p53 accumulation were positive for MRP, whilst only 8 of 18 (44%) CRCs without p53 accumulation were positive for MRP. MRP was more frequently expressed in p53-positive than p53-negative

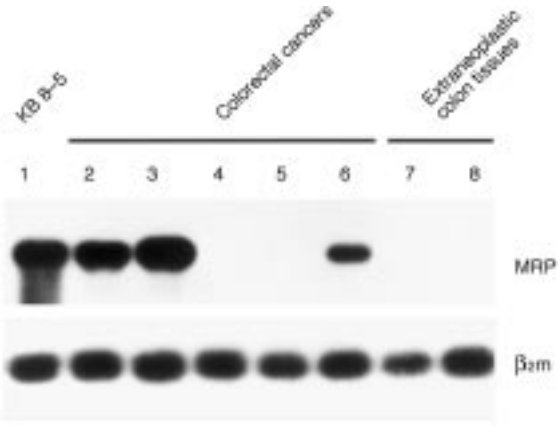

Figure 1. MRP gene expression in colorectal cancers (lanes 2–6) and in the extraneoplastic colon tissues (lanes 7,8). β_2m gene expression was examined as a control for the quality of RNA specimens used as templates. KB8-5 was used as a positive control for MRP expression (lane 1).

Table 1. *MRP expression and clinicopathological features in colorectal cancer*

	MRP-negative (%)	MRP-positive (%)	
Colorectal cancer	16 (30)	38 (70)	$P = 0.041$*
Sex			NS
Male ($n = 29$)	8 (28)	21 (72)	
Female ($n = 25$)	8 (32)	17 (68)	
Degree of differentiation			NS
Well† ($n = 26$)	8 (31)	18 (69)	
Moderately‡ ($n = 24$)	6 (25)	18 (75)	
Poorly§ ($n = 1$)	0 (0)	1 (100)	
Mucinous‖ ($n = 3$)	2 (67)	1 (33)	
Primary tumour			NS
T0 ($n = 2$)	2 (100)	0 (0)	
T1 ($n = 3$)	0 (0)	3 (100)	
T2 ($n = 4$)	1 (25)	3 (75)	
T3 ($n = 37$)	9 (24)	28 (76)	
T4 ($n = 8$)	4 (50)	4 (50)	
Regional lymph nodes			NS
N0 ($n = 29$)	9 (31)	20 (69)	
N1 ($n = 13$)	2 (15)	11 (85)	
N2 ($n = 12$)	5 (42)	7 (58)	
Distant metastasis			NS
M0 ($n = 41$)	12 (29)	29 (71)	
M1($n = 13$)	4 (31)	9 (69)	
Stage grouping			NS
Stage 0 ($n = 2$)	2 (100)	0 (0)	
Stage 1 ($n = 6$)	1 (17)	5 (83)	
Stage 2 ($n = 18$)	5 (28)	13 (72)	
Stage 3 ($n = 15$)	4 (27)	11 (73)	
Stage 4 ($n = 13$)	4 (31)	9 (69)	
p53 accumulation			$P = 0.0032$*
Negative ($n = 18$)	10 (56)	8 (44)	
Positive ($n = 36$)	6 (17)	30 (83)	

*χ^2 test. †well differentiated adenocarcinoma. ‡moderately differentiated adenocarcinoma. §poorly differentiated adenocarcinoma. ‖mucinous adenocarcinoma. NS, not significant.

colorectal cancers (χ^2 test, $P = 0.0032$). Accumulation of p53 was detected in 10 of 35 (29%) CIA specimens, eight (80%) of which were also positive for MRP. Of the 25 CIAs without p53 accumulation, eight (32%) were positive for MRP (χ^2 test, $P = 0.01$). Positivity for aberrant p53 had a significant correlation with MRP overexpression in CRCs and CIA. Double staining with anti-MRP and anti-p53 antibodies showed co-expression of MRP (cytoplasm) and p53 (nucleus) in the same cancer cells, whilst cancer cells without p53 showed little expression of MRP (Figure 2). The carcinoma cells also demonstrated coexpression of MRP and p53 in the CIA (data not shown).

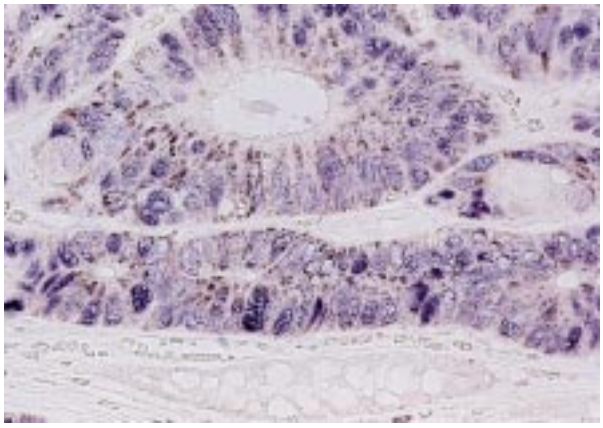

Figure 2. Immunohistochemical double staining showed MRP (brown in the cytoplasm) and p53 (blue in the nuclei) in the same cancer cells in colorectal cancer.

DISCUSSION

Previously, we reported that MRP gene expression was correlated with multidrug resistance of NSCLC [8], and directly evaluated MRP production in NSCLC by immunohistochemical analysis with anti-MRP polyclonal antibody [16]. CRC is one of the most multidrug-resistant neoplasms, and MRP has been suggested to be responsible for multidrug resistance of colon cancer [20, 21].

The classical multidrug-resistance gene *MDR1* is activated by aberrant p53 protein through stimulation of the MDR1 promoter [14]. However, it is unclear whether the expression of MRP is regulated by aberrant p53. In this study, we demonstrated that p53-positive neoplastic epithelial cells coexpressed MRP more frequently than p53-negative cells; 83% of colorectal cancers and 80% of CIA with p53 accumulation were also positive for MRP, whilst 44% of cancers and 32% of CIA without p53 accumulation were MRP-positive. Double staining confirmed coexpression of both molecules (MRP and p53) in the same cancer cells. Accumulation of *p53* in cancer cells reflects aberration or missense mutation of the *TP53* gene in various neoplasms [15]. These results suggest that MRP expression may be affected by aberrant p53 protein in CRC. We recently reported that there was a significant co-relation between MRP expression and accumulation of p53 in NSCLC [16], which is consistent with the results of this study.

Aberration of *TP53* is considered to have an important role in carcinogenesis of CRC [23], and aberration of *TP53* has been detected in 8% of adenomas [24], 40–50% of CIA [24, 25] and around 60–70% of colon cancers [26, 27]. Our results indicated that expression of MRP protein was correlated with p53 accumulation and with histological grade, increasing from adenoma to carcinoma (8% of adenomatous polyps, 46% of CIA and 70% of colorectal cancers were MRP positive). These observations suggest that aberration of *TP53* increases during the development of colon cancer and may induce MRP overexpression in cancer cells.

We also analysed clinocopathological features including TNM score, pathological stage and prognosis. Fillpits and colleagues reported that there was no association between MRP expression and clinicopathological variables in colorectal carcinoma [28]. Takebayashi and colleagues reported that there was a correlation between MRP expression and degree of differention in colorectal carcinomas [12]. We observed no significant relationship between MRP overexpression and clinocopathological features. Although MRP was suggested to be responsible for multidrug resistance of CRC cells *in vitro* [21, 22], it is unclear whether MRP plays a role in the multidrug resistance of colorectal cancer patients *in vivo*.

This study showed that MRP overexpression affected by aberrant p53 occurred more frequently in CRC/CIA than in adenomas. Our observation also suggested that MRP expression may be regulated by aberrant p53 molecules in CRC cells.

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Acknowledgements—We thank Mr Yuichi Tada, Mr Johbu Itoh and Ms Kyoko Murata for their technical assistance. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture numbers 09680835 (Y. Ueyama), 09670239 (H. Kijima) and 09670205 (M. Nakamura).