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Co-amplification and over-expression of two *mdr* genes in a multidrug-resistant human colon carcinoma cell line

Chuck C.-K Chao[1], Chang M. Ma[1] and Sue Lin-Chao[2]

[1]*Tumor Biology Laboratory, Departments of Biochemistry and Medicine, Chang Gung Medical College, Taoyuan, Taiwan 33332* and [2]*Institute of Molecular Biology, Academia Sinica, Taipei 11529, Taiwan, Republic of China*

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The human P-glycoprotein gene family contains the *mdr*1 and the *mdr*3 gene The *mdr*1 P-glycoprotein is over-expressed in multidrug resistant (MDR) tumor cells and is believed to play a role in the elimination of certain cytotoxic drugs used in the chemotherapy of cancer The *mdr*3 gene has not been found to be amplified or over-expressed in MDR cells In this study, gene-specific *mdr* gene probes were developed for the detection of the gene and the total mRNA level Southern and Northern hybridization analyses showed that the *mdr* genes and the mRNA levels were increased 30–40-fold in a MDR human colon cancer cell line In addition, this MDR cell line had an altered growth rate and morphology and detectable double minute chromosomes

Co-amplification, *mdr* Genes, Multidrug resistance

1 INTRODUCTION

Drug resistance in cancer cells is a major limitation to the effective treatment of human cancers Multidrug resistant (MDR) cells are typically resistant to a wide variety of structurally and functionally unrelated hydrophobic drugs to which they have not been previously exposed [1] This phenomenon, based on in vitro studies of mammalian cell lines, is most often associated with an increased ATP-dependent drug efflux, a decreased intracellular drug accumulation, gene amplification and the over-expression of a group of plasma membrane glycoproteins (~170 kDa) designated P-glycoproteins [2] P-glycoproteins are encoded by a family of genes, termed *mdr* or *pgp* which comprises 3 members in rodents and 2 members in humans [3–9] Overexpression of an *mdr* gene can also be found in tumor samples obtained from patients after relapse from chemotherapy [10–12]. In mouse cells, it has been independently found that *mdr*1 and *mdr*3 (human *mdr*1 counterparts), but not *mdr*2 are over-expressed in MDR cells [13–15] In human cells, *mdr*1 is over-expressed in most MDR cell lines [16–18] Transfection and over-expression experiments indicate that the human *mdr*1 counterpart of the mouse genes (i.e. *mdr*1 and *mdr*3) and human *mdr*1 cDNA are sufficient to confer the complete MDR phenotype to otherwise drug-sensitive cells [4,8,19] However, low levels of human *mdr* mRNA or P-glycoprotein are also detectable in many normal tissues in a tissue-specific manner [20–21] In addition, the *mdr* gene is induced in hepatocarcinogenesis and regenerating rat liver [22] and in the endometrial lining of the human uterus during pregnancy [23], suggesting a normal functional role of P-glycoprotein in general This is supported by the substantial lines of evidence indicating that P-glycoprotein-like molecules are present in the lower organisms, including bacteria [24] and the malarial parasite *Plasmodium falciparum* [25–26] However, the reason as to how a normally regulated *mdr* gene becomes over-expressed is not clear

A second class of human *mdr* gene, termed *mdr*3 which has recently been cloned, yields a transcript of 4100 bases with a deduced protein size of 140 kDa [5]. *mdr*3 is genetically linked to *mdr*1 within 230 kb as estimated by pulse field electrophoresis [27] Substantial amounts of *mdr*3 mRNA have only been found in liver [28] and in prolymphocytic leukemia cells of the B-cell lineage [29] Although its function is not clear, it is interesting to note that alternative splicing of *mdr*3 transcript was detected in human liver [28], implying a putative function of this new *mdr* gene However, transcripts of *mdr*3 are marginally detectable in the MDR cell lines examined thus far [17,30] and it does not show any activity in transfection experiments [5] These data provide useful information, however they also open a great deal of questions in terms of the normal functions and the role of *mdr*3 in MDR formation To delve further into the possible role of the *mdr*3 gene in MDR cells, we have analyzed a human MDR colon cancer cell line [31]. We are particularly interested in the MDR mechanism of human colon cancer because it has only a 15–20% response rate, even when the highly effective

Correspondence address: C C -K Chao, Tumor Biology Laboratory, Departments of Biochemistry and Medicine, Chang Gung Medical College, Taoyuan, Taiwan 33332 Fax (886) (3) 3283031.

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drug 5-FU and its analogues are used [32] In this paper, we are reporting that this human MDR cell line has a coordinated over-expression of mRNA and amplification of *mdr*1 and *mdr*3 genes The significance of the regulation of the *mdr* genes will be discussed.

2 MATERIALS AND METHODS

2 1 *Cell lines and culture conditions*

The established human colonic cancer cell line SW620 (ATCC CCL221), and its adriamycin-resistant SW620-ADR [31] were used as study materials Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (Gibco), penicillin (100 u/ml) and streptomycin (100 μg/ml) and maintained according to the supplier's protocol SW620-ADR cells were grown in culture medium containing 1000 ng/ml of adriamycin to maintain drug resistance

2 2 *Chemicals and cytotoxicity*

Stock solutions of 500 μg/ml of adriamycin (Sigma) were prepared in sterile distilled water, dispensed in aliquots and kept frozen at −20°C in the dark Stocks were quickly thawed before use Appropriate numbers of exponentially growing cells were harvested and washed before seeding into 96-well microtiter plates containing medium with increasing concentrations of cytotoxic drugs All the platings of cells were in 5 duplicates The treated cells were allowed to grow for 5 days and processed for toxicity analysis by colorimetric MTT assays [33] Fold resistance was determined by the ratio of the ID_{50} (the drug concentration causing 50% cell killing) of SW620 to the ID_{50} of SW620-ADR cells

2 3 *Morphology and chromosome analysis*

For morphological analysis, cell preparations were stained with May-Grunwald Giemsa, according to routine procedures For chromosome analysis, the cells were prepared as follows Three hours before harvesting, 0 05 μg/ml colcemid was added, after which the cells were centrifuged for 5 min at 250 × *g* The pellets were resuspended in 0 06 M KCl, incubated for 20 min at 37°C, centrifuged, resuspended in a mixture of ice-cold ethanol/glacial acetic acid (3 1), centrifuged, resuspended again, and left in the tube for 20 min After a final centrifugation, the suspension was pipetted onto slides and air dried After staining with Giemsa for 30 min, 10 metaphases were analyzed and 25 were chromosomally counted

2 4 *MDR hybridization probe*

The probe used in Northern and Southern analysis was a DNA fragment cut out from human *mdr*1 and *mdr*3 cDNA clones in the 5′ structural region Human *mdr*1 and *mdr*3 cDNA sequences were cloned in pGEM3Zf(−)-mdr1 and pFRCMVmdr3, respectively (a kind gift from Dr Piet Borst, The Netherlands Cancer Institute) *mdr*1 was subcloned into pBS(+) (Stratagene) through *Bam*HI and *Sal*I restriction digestion resulting in pBSmdr1 For labeling, the cDNA fragments were purified by electrophoresis on a NA45 DEAE membrane (Schleicher and Schuell) and radiolabeled by the random priming method with [α-^{32}P]dCTP [34] to a specific activity of ~10^8 cpm/μg DNA

2 5 *Northern, Southern, and slot blot hybridizations*

All solutions and procedures involving electrophoresis, blot transfers, and hybridizations were carried out by standard methods [35] For Northern blots, 10 μg of total RNA from the cell lines were fractionated by electrophoresis on 1% (w/v) agarose containing 6 7% (v/v) formaldehyde. For Southern blots, 10 μg of DNA was digested with restriction enzymes *Hind* III or *Eco*RI (New England Biolabs) under the conditions specified by the supplier and separated in a 1% agarose gel. After electrophoresis, gels were processed and RNA or DNA was transferred to Hybond-N filters (Amersham), UV-cross-linked by Stratalinker (Stratagene), and hybridized at 42°C for 16 h in hybridization buffer (6× SSC, 50% (v/v) deionized formamide, 10× Denhardt's solution, 10 mM EDTA and 0 1% (w/v) SDS) containing 5× 10^5 cpm of probe per ml The filter was then washed at 65°C in 2× SSC and 0 1% SDS, followed by exposure on X-ray film with an intensifying screen at −80°C for 2–3 days The X-ray film was scanned in a densitometer to estimate the density of the hybridization bands For slot blot hybridization, heat denatured RNA or DNA was directly loaded onto a filter using a slot blotter (Schleicher and Schuell), and processed for hybridization as above For some experiments, blots were retrieved and rehybridized using different probes by standard methods [35]

3 RESULTS

3 1. *Drug resistance accompanies changes in morphology*

The data for ID_{50}, the drug concentration that causes 50% cell killing in parental and MDR cells, are shown in Table I There is a 100-fold resistance to adriamycin in MDR cells. Characteristic changes in cellular morphology and double-minute chromosomes were observed in MDR cells. The MDR cells are rounded-up, contrasted to the flat-shaped parental cells. Growth is slower in MDR cells; the population doubling time is 30 h whereas it is only 18 h for parental cells However, there is no significant difference in the chromosome number, ranging from 45 to 53 with an average of 49 or 50 chromosomes per cell.

3.2 *Establishment of* mdr *gene specific probe*

In order to analyze the expression of *mdr* genes, we sought to establish specific DNA probes for *mdr*1 and *mdr*3 genes By comparing *mdr*1 [3] with *mdr*3 [5] sequences using the Pustell's matrix method and the Myers and Miller's method (PC/Gene, Intelligenetics), a 368 bp *Sac*I–*Eco*RV cDNA segment (termed f368) at the 5′-end of the gene starting from the transcriptional initiation site of the *mdr*1, and a *Eco*RV–*Kpn*I cDNA segment (termed f212) containing the first 212 bp of the *mdr*3 gene were used as a gene- specific probe A typical DNA hybridization pattern was shown in Fig. 1 One μg of plasmid DNA containing *mdr*1 cDNA (pBSmdr1), *mdr*3 cDNA (pFRCMVmdr3) or an unrelated control vector DNA pBS(+) (Stratagene) was hybri-

Table I

Cytotoxicity parameters, cellular and cytogenetic characteristics of MDR and parental cells

	Parental	MDR
ID_{50} (μg/ml)[a]	0 5	50
Cell morphology	flat	rounded-up
Doubling time (h)	18	30
Chromosome modal value (range)	50 (45–54)	49 (46–53)
Double minutes[b]	−	+

[a]ID_{50} is the concentration of the drug effective in inhibiting 50% of cell growth measured after 4 days of continuous exposure to adriamycin
[b]Arbitrary value −, not detectable, +, detectable.

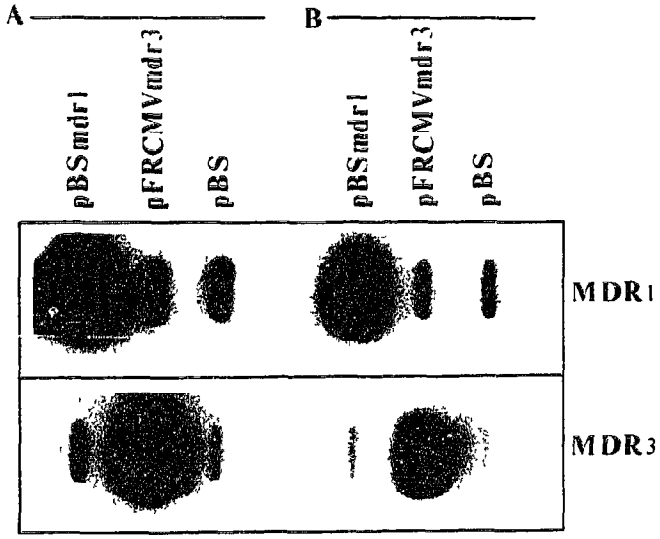

Fig 1 Gene specific probes assayed by slot blot hybridization f368 (MDR1) and f212 (MDR3) (see section 3 for details) were used to probe plasmids carrying full-length *mdr*1 cDNA (pBSmdr1), *mdr*3 cDNA (pFRCMVmdr3), or negative control vectors (pBS) Panel A, 1 μg, panel B, 0 1 μg

dized to an *mdr* 1 f368 or *mdr* 3 f212 probe A dramatic hybridization signal from pBSmdr1 was observed with the f368 *mdr*1 probe, whereas a greater hybridization signal from pFRCMVmdr3 was detected using the f212 *mdr*3 probe (Fig 1, panel A) A hybridization pattern using a10-fold dilution (i e, 0 1 μg) of the tested DNA was shown in panel B Continuous dilutions or shorter X-ray film exposure eliminated non-specific hybridization, whereas significant gene-specific signals remained (data not shown) These data indicate that f368 and f212 are appropriate probes for the *mdr*1 and *mdr*3 genes, respectively, and they were used for the entire studies

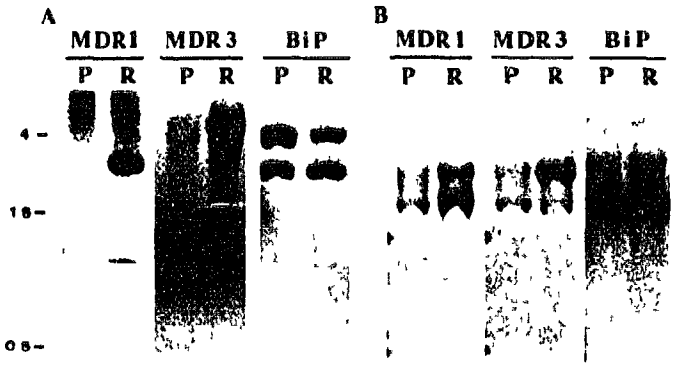

Fig 2 Southern blot hybridization of the genomic DNA of parental and MDR cancer cells Ten μg of *Hin*dIII (panel A) or *Eco*RI digested (panel B) genomic DNA of cells was loaded on each lane Probes used are indicated on top The same blot was hybridized to gene-specific probes in the following order MDR1, MDR3, and BiP (see section 2 for details) P, parental cells, R, MDR cells Size markers in kb are indicated on the left side of each panel

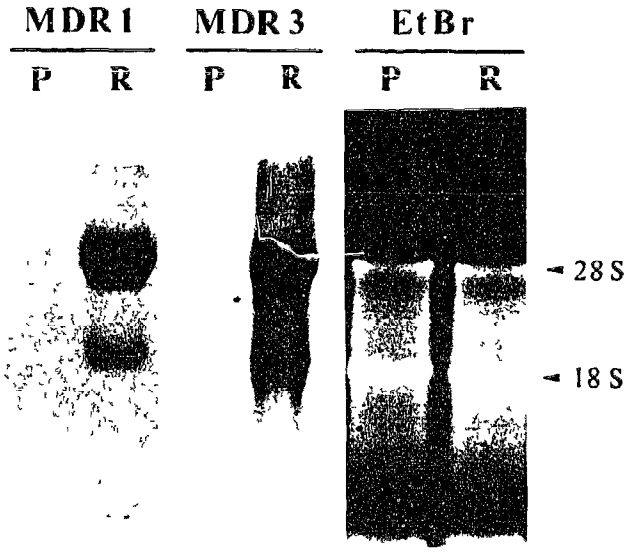

Fig 3 Northern blot hybridization of the steady-state RNA of parental and MDR cancer cells Ten μg of total cellular RNA was loaded in each lane The same blot was hybridized to the MDR1 probe, retrieved and rehybridized with the MDR3 probe The RNA gel which was stained with ethidium bromide (EtBr) before blotting is also shown The gel and the hybridization blots were adjusted to the same size 28S and 18S rRNA are indicated as reference size markers

3.3. *Co-amplification of* mdr1 *and* mdr3 *genes in MDR cells*

Total genomic DNA was prepared from parental or MDR cells and the *mdr* genomic organization was investigated. Southern blotting of cellular DNA digested with either *Hin*dIII (panel A in Fig 2) or *Eco*RI (panel B) was hybridized with the *mdr*1 probe (indicated on top) The hybridization pattern for *mdr*1 from *Hin*dIII-digested DNA showed two bands (1 1 kb and 2.6 kb), and from *Eco*RI-digested DNA, 2 bands (2 8 kb and 4 8 kb) were also seen By contrast, the same blot of *Hin*dIII or *Eco*RI digests rehybridized with the *mdr*3 probe showed unique 5 kb bands The *mdr*3 hybridization pattern is consistent with findings in human COLO 320 DM cells from the other investigators [28]. Both restriction enzyme digested DNA indicates that the amounts of *mdr*1 and *mdr* 3 are dramatically amplified ~30-fold in MDR cells (R) compared to parental cells (P). As control, the same DNA blot was reprobed with BiP (immunoglobulin heavy chain-binding protein) cDNA, an actively expressed gene in most of the mammalian cells [31]. A comparable level of BiP gene was detected in parental and MDR cells It also indicates that the amplified *mdr* 1 and *mdr* 3 genes are not the result of sample loading

3.4 *Coordinated over-expression of* mdr1 *and* mdr3 *genes in MDR cells*

Expression of *mdr*1 and *mdr* 3 in parental and MDR cells was analyzed by Northern blot hybridization. Typical results are shown in Fig 3 The level of *mdr*1 and

*mdr*3 mRNA for parental cells is too low to be seen in this hybridization autoradiogram Overexposure of the X-ray film showed a ~40-fold increase in *mdr*1 and *mdr*3 mRNA (both around 28S) in MDR cells (panel MDR1 and MDR3). This is not the result of sample loading because an equal RNA staining by ethidium bromide was detected (panel EtBr) A hybridization band around 18S was also seen for both *mdr* genes We do not know presently whether it is a non-specific hybridization or an alternative splicing transcript

4 DISCUSSION

In this report, we have established gene-specific probes for the *mdr*1 and *mdr*3 genes Using these probes, we have shown that the MDR cells co-amplified both *mdr*1 and *mdr*3 genes ~30-fold A ~40-fold over-expression of the steady-state RNA of both genes was detected in MDR cells, suggesting that the over-expression of *mdr* genes was controlled at the copy number of the *mdr* genes. Co-amplification of *mdr*3 has also been reported in a human MDR cell line [36], and both amplification and over-expression were found in some MDR derivatives of the human KB cell line [30] However, other investigators failed to detect amplification and/or over-expression of the mouse *mdr*2 gene [14] Borst and colleagues [28] have reported an alternative splicing of *mdr*3 in human liver, and speculated that these alternatives, when superimposed on differential expression of P-glycoprotein homologues, could provide an explanation for the large variation in cross-resistance patterns observed in cell lines that were selected for MDR with different drugs [28] The genetic linkage of *mdr*1 and *mdr*3 genes (within 500–1000 kb) was detected using pulse-field gradient electrophoresis in a human cell line COLO 320 DM [28]. Further studies showed that the human *mdr* locus covers about 230 kb [27] This may explain, at least in part, the coordinated increase of both *mdr* genes in our colon cancer cells. In addition, it has recently been shown that both human *mdr* genes are transcribed in the same orientation [27] It is possible that the expression of *mdr* genes shares common regulatory transcription factors Chromosome structure may also participate alternatively and/or coordinately in regulating the activity of the 2 closely linked *mdr* genes We have consistently detected the ~170 kDa (*mdr*1 gene product) but not the ~140 kDa (*mdr*3 gene product) protein by silver staining of cell membrane extracts (data not shown) This suggests that posttranscriptional regulation may play a role in the surface expression of P-glycoproteins. The results suggest that the acquired MDR phenotype does not result from the over-production of *mdr*3 P-glycoprotein Alternatively, absence of a ~140 kDa protein does not necessarily mean that the *mdr*3 P- glycoprotein is absent from SW620-ADR cells since it has recently been shown that the human *mdr*3 P-glycoprotein co-migrates with the *mdr*1 P- glycoprotein in SDS–protein gels [37] Thus, one can not entirely eliminate the possibility that *mdr*3 products may have a role in human MDR or in normal physiological function where changes, as an example, may affect cell morphology. Co-amplification and over-expression of *mdr*1 and *mdr*3 genes may result in an increase of *mdr*3 P-glycoprotein in human MDR colon cancer cells. We believe that when an antibody against the *mdr*3 product is available, the function of the *mdr*3 product will be determined in detail

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