MDR1/P-glycoprotein and MRP-1 mRNA and Protein Expression in Non-small Cell Lung Cancer

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Abstract. *Background: Multiple drug resistance (MDR), both inherent and acquired, is a serious problem in non-small cell lung carcinomas (NSCLC). The purpose of this study was to investigate the prevalence of expression of genes encoding drug efflux pumps, MDR1 and MRP-1, at both the mRNA and protein levels, in this type of cancer. Materials and Methods: Tumour specimens (38 cases) were analysed using immunohistochemistry and, where possible (30 cases), also using reverse-transcriptase polymerase chain reaction. Results: The results from this analysis indicated that either, or both, drug efflux pumps were frequently expressed in NSCLC. Expression of mrp1 was found to be predominant over mdr1 at the mRNA level, while MDR1 P-gp was more frequently detected than MRP-1 protein. In some cases, proteins encoding pumps were detected without corresponding mRNAs – possibly due to differing sensitivities of the analysis techniques. Conclusion: Future studies of mdr1 and mrp1 using increased-sensitivity qPCR techniques, in parallel with protein analysis, in larger cohorts of cases may help to elucidate the role of drug efflux pumps in NSCLC multiple drug resistance.*

Lung cancer is the most common cancer worldwide, with approximately 1.2 million new cases diagnosed annually (1). It is estimated that approximately 80-85% of lung cancers are non-small cell carcinomas (NSCLC) and approximately 65% of these are advanced stage disease at the time of diagnosis (2). The management of NSCLC is generally dependent on tumour stage: early stage (I and II) are often treated by surgical resection with or without tumours adjuvant chemotherapy, while multi-modality (chemotherapy, radiotherapy and surgery) is commonly used for more advanced cancers. Unfortunately, despite the use of best available treatments, the overall survival rate for NSCLC patients is poor, ranging from ~70% in early stage disease to 25%, or less, with advanced disease for those tumours that are operable (3). Approximately 20-25% of NSCLC patients have tumours at diagnosis that are not surgically resectable (4).

Chemotherapy plays a role in increasing the median survival time in advanced NSCLC and inhibiting recurrence in resected early stage tumours. While platinum-based doublets are currently considered to be the standard treatment for advanced stage NSCLC (5), chemotherapy, with or without radiotherapy, provides only a modest survival benefit, with median survival measured in months (6). A contributing factor to NSCLC currently being a leading cause of cancer deaths worldwide is its intrinsic drug resistance (*i.e.* multiple drug resistance/MDR) which affects response to a broad range of structurally and mechanistically unrelated drugs (7).

The ATP-dependent membrane-bound drug efflux pumps, including MDR1 P-glycoprotein (MDR1 P-gp) and MRP-1, may be mediators of clinically relevant MDR, and, as summarised in Table I, a number of studies on their expression in NSCLC have been reported. However, many of these studies have been restricted to analysis of *mdr1* and/or *mrp1* mRNA or MDR1 P-gp and/or MRP-1 protein(s). To determine the potential involvement of these drug efflux pumps in the clinical setting, here we report an analysis of MDR1 P-gp and MRP-1 proteins and *mdr1* and *mrp1* mRNA expression in NSCLC.

Patients and Methods

Patients. The patient group studied comprised 38 individuals with NSCLC. All patients were treated at St. Vincent's University Hospital (SVUH), Dublin, between 2002-2003 and approval to conduct this study was granted by SVUH Ethics Committee. Pathologic material was examined in each case by SK. Formalin-fixed paraffin-embedded material was available for all patients. Representative 4 μm sections of tissue blocks were cut using a

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Key Words: Non-small cell lung carcinoma, drug efflux pump, MDR1 P-gp, MRP-1, mRNA, protein, immunohistochemistry, reverse-transcriptase polymerase chain reaction.

Table I. *Summary of previously reported MDR-1 and MRP-1 studies in human NSCLC specimens.*

No. of NSCLC specimens	MDR-1 (method)	MRP-1 (method)	Results	Reference
6 (+ 7 normal)	*mdr1* mRNA (Slot Blot)	Not studied	low levels *mdr1* mRNA in NSCLC and normal lung tissue	(8)
107 (+ 20 normal)	*mdr1* mRNA (RT-PCR)	Not studied	57% (61/107) tumours, 90% (18/20) normal tissue positive; 1 tumour only > level than normal	(9)
104	*mdr1* mRNA (RT-PCR)	*mrp1* mRNA (Northern blot)	*mrp1*: 31.7% (33/104) with high level expression; some α worse prognosis	(10)
29	MDR1 P-gp (IHC)	Not studied	45% MDR1 P-gp-positive, with no with disease stage	(11)
11	*mdr1* mRNA (RT-PCR)	*mrp1* mRNA (RT-PCR)	most co-express *mdr1* and *mrp1* mRNA; generally low level *mdr1* mRNA	(12)
84	*mdr1* (Northern Blot)	Not studied	15% *mdr1* positive; generally low level; no α drug resistance and tumour progression	(13)
27	MDR1 P-gp (IHC)	MRP-1 (IHC)	MDR1 P-gp protein: 11% (3/27) had >5% positive cells; MRP-1 protein: 18.5% (5/27) had >5% positive cells	(14)
50 (pre-paclitaxel)	MDR1 P-gp (IHC)	Not studied	100% (28/28) of "good responders" were MDR1 P-gp positive; 68% (15/22) "poor responders" were MDR1 P-gp positive	(15)
72	MDR1 P-gp (IHC)	MRP-1 (IHC)	35% (25/72) MDR1 P-gp-positive; 78% (56/72) MRP-1-positive No significant α with chemo-response or survival	(16)
116	MDR1 P-gp (IHC)	MRP-1 (IHC)	MDR1 P-gp expression levels similar to surrounding normal tissue; MRP-1 levels significantly increased in NSCLC. High levels of expression with longer overall survival.	(7)
4	4 cases: *mdr1* (RT-PCR)	4 cases: *mrp1* (RT-PCR)		

IHC: immunohistochemistry; α: associated with; RT-PCR: reverse transcriptase-polymerase chain reaction.

microtome, mounted onto poly-l-lysine coated slides and dried overnight at 37˚C. Slides were stored at room temperature until required. Snap-frozen tissue from 30 of these tumours was also available for mRNA analysis.

Immunohistochemical analysis of MDR-1/Pgp and MRP-1 protein expression. All immunohistochemical studies on formalin-fixed paraffin wax-embedded tissue sections were performed following the method of Hsu *et al.* (17), using an avidin-biotin horseradish peroxidase (HRP) conjugated kit (avidin-biotin complex; ABC) plus an appropriate secondary antibody. In brief, sections were dewaxed in xylene (2x5 min), rehydrated in grading alcohols 100%, 90% and 70% (2x3 min), and placed in tris-buffered saline (TBS/0.1% Tween-20). Endogenous peroxidase activity was quenched by placing tissue sections in 3% (v/v) H_2O_2/distilled water for 5-7 min at room temperature. All slides were blocked for non-specific staining with 20% normal rabbit serum (Dako, High Wycombe, UK: X-902)/TBS for 20 min at room temperature. Primary antibodies were applied to each sample optimally diluted in TBS/0.1% Tween-20 (anti-MDR-1, clone 6/1C (National Institute for Cellular Biotechnology)) (18): ascites diluted 1:40; anti-MRP-1 monoclonal antibody (diluted 1:15; ALX-801-007-C125: Alexis, Nottingham, UK). Primary antibodies were incubated overnight at 4˚C. Specimens were then washed (3x5 min) with TBS/0.1% Tween-20, followed by a 30 min incubation with biotinylated secondary antibody (rabbit anti-mouse IgG; 1/300 dilution in TBS/0.1% Tween-20) (Dako, E345)) or rabbit anti-rat (1/500 dilution in TBS/0.1% Tween-20). Finally, following another 3x5 min wash step, Vectastain Elite ABC reagent (HRP-conjugated; Vector Laboratories, UK) was applied for 25 min at room temperature. The peroxidase substrate, 3',3-daminobenzidine tetrahydrochloride (DAB) containing 0.02% H_2O_2 (Vector Laboratories) was added for 10 min at room temperature. Tissue sections were then lightly counter stained with Crazzi's haematoxylin (Vector Laboratories). Following this, slides were dehydrated in grading alcohols: 70%, 90% and 100% (2x3 min). Specimens were then cleared in xylene and mounted in DPX (BDH, UK). Negative control specimens, in which primary antibodies were replaced by 1x TBS/0.1% Tween-20, and positive controls (normal kidney and lung tissue) using the same experimental conditions, were included in all experiments.

Table II. *Primers used to amplify cDNA formed by reverse transcription of mRNA templates.*

cDNA	Primer sequences	Annealing temp. (˚C)	Amplified product (bp)
mrp1	(F): 5' GTACATTAACATGATC TGGTC 3'	54	203
	(R): 5' CGTTCATCAGCTTGAT CCGAT 3'		
mdr1	(F): 5' GTTCAAACTTCTGCTC CTGA 3'	55	157
	(R): 5' CCCATCATTGCAATAG CAGG 3'		
β-actin	(F): 5' GAAATCGTGCGTGACA TTAAGGAGAAGCT 3'	AR	383
	(R): 5' TCAGGAGGAGCAATGA TCTTGA 3'		

Note AR: As *β-actin* was co-amplified with *mrp1* and *mdr1* (respectively) cDNAs as endogenous control, annealing temperature (temp.) used was that of relevant cDNA of interest. (F): Forward; (R): Reverse. (All primers used were selected in our laboratory (19)).

Immunohistochemical scoring. MRP-1 and MDR1 P-gp immunohistochemical staining was evaluated semi-quantitatively, according to the percentage of cells showing specific immunoreactivity.

Reverse transcriptase-polymerase chain reaction. For RNA analyses, dissected tumours were homogenised, on ice, in 2 ml TriReagent (Sigma; Poole, England) and total RNA was subsequently isolated according to the manufacturer's instructions. First-strand cDNA was synthesised from 1 μg total RNA using oligo (dT) primer (Oswel; Southampton, England). cDNA (5 μl) was then amplified in a 50 μl PCR reaction solution containing 1.5 mmol/l $MgCl_2$, 0.2 mmol/l deoxynucleotide triphosphates, 20 μmol/l oligonucleotide primers and 2.5 U *Taq* polymerase enzyme (Sigma). Forward and reverse primers for amplification of cDNAs of interest and the amplified product sizes are detailed in Table II. β-actin was co-amplified in all cases as endogenous control. Following optimisation of conditions, the PCR cycle used was as follows: 94˚C for 2 min; 30 cycles of 94˚C for 30 sec, relevant annealing temperature (Table II) for 30 sec, 72˚C for 30 sec; completion step of 72˚C for 5 min.

Results

Patient characteristics. This study involved analysis of tumours from 38 NSCLC cancer patients. At the time of diagnosis, their ages ranged between 41 years and 81 years (median age=65 years). Twenty-one of the patients were male; 17 were female. Nineteen of the tumours were squamous, 15 were adenocarcinomas and 4 were large cell carcinomas. Two tumours were classified as grade 1, 17 as grade 2, 17 as grade 3 and 2 as grade 4. Twelve tumours had metastasised at the time of diagnosis, while the remaining 26 apparently had not. Thirty-seven of the patients were smokers.

Table III. *(A) Comparison of MDR1 P-gp and MRP-1 protein co-expression in all 38 NSCLC cases.*

Drug Efflux Pump Protein	% Cases
$MRP1^+/MDR1\ P\text{-}gp^+$	18.4
$MRP1^-/MDR1\ P\text{-}gp^+$	52.6
$MRP1^+/MDR1\ P\text{-}gp^-$	2.6
$MRP1^-/MDR1\ P\text{-}gp^-$	26.4

(B) Comparison of mdr1 and mrp1 mRNA co-expression in all 30 NSCLC cases.

Drug Efflux Pump mRNA	% Cases
$mrp1^+/mdr1^+$	13.3
$mrp1^-/mdr1^+$	3.3
$mrp1^+/mdr1^-$	73.4
$mrp1^-/mdr1^-$	10

MDR1 P-gp and MRP-1 protein expression. MDR1 P-gp specific staining was observed in 71% of NSCLC specimens analysed, with staining localised to cell membrane/cytoplasm. Approximately 29% percent (11/38) of tumours did not express MDR1 P-gp protein. Twenty-one percent (8/38) of tumours analysed were found to express MRP-1 protein, while 79% (30/38) of tumours had no detectable MRP-1 protein expression.

As summarised in Table III (A), the majority (73.6%) of NSCLCs express drug efflux pumps analysed in this study while only 26.4% expressed neither MDR1 P-gp nor MRP-1 proteins. No significant association was found between expression of MDR1 P-gp or MRP-1 proteins and clinical and pathological information available on these NSCLC patients.

mdr1 and mrp1 mRNA expression. RT-PCR analysis (examples illustrated in Figure 1) indicated that 86.7% (26/30) of tumour specimens expressed *mrp1* mRNA, whereas 4 did not. Approximately 17% (5/30) of specimens expressed *mdr1* gene transcripts, 25 specimens did not. As summarised in Table III (B), four tumours studied (13.3%) expressed both *mdr1* and *mrp1* mRNAs while 10% (3/30) expressed neither gene transcripts. No significant correlation was found between expression of *mdr1* or *mrp1* mRNAs and the clinicopathological characteristics available.

Comparison of protein and mRNA results. As summarised in Table IV (A), cross-comparison of mRNA and protein results indicated that 4.3% of NSCLC specimens expressed both *mdr1* gene transcripts and MDR1 P-gp protein; a further 4.3% expressed the mRNA, but apparently did not translate this into protein; while 30.4% did not express either MDR1 mRNA or protein. This analysis, however,

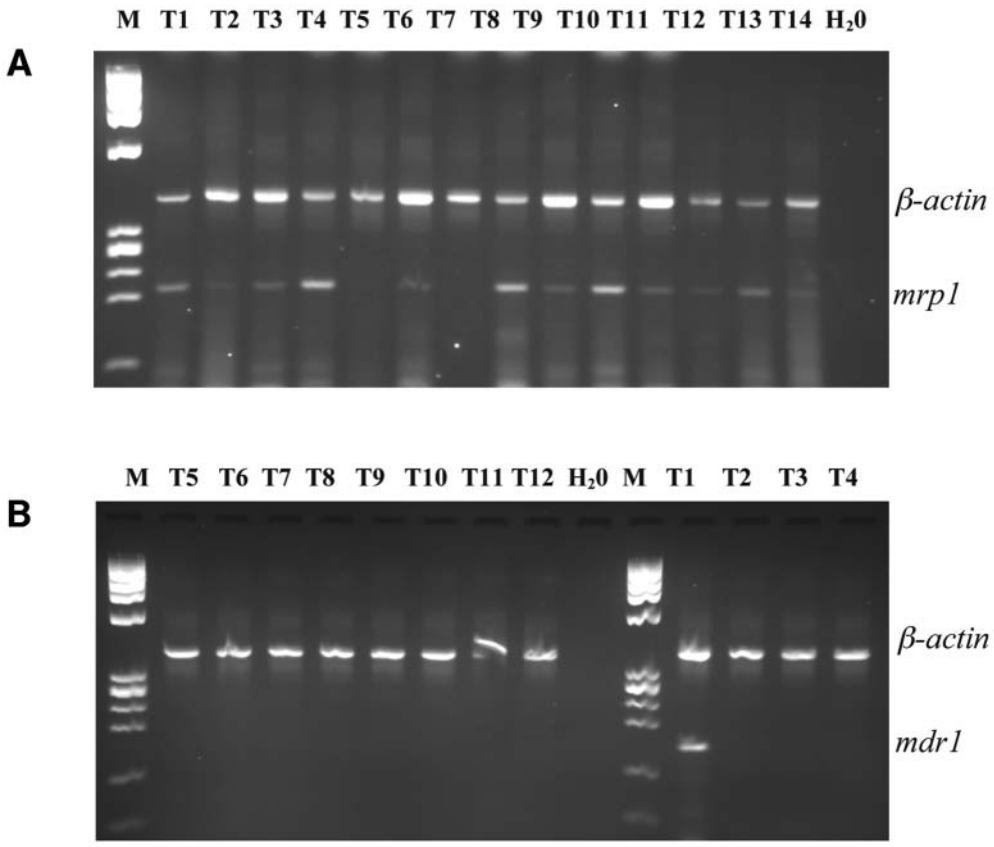

Figure 1. *Example results following (A) mrp1 and (B) mdr1 RT-PCR co-amplification with β-actin in NSCLC specimens. H_2O replaced RNA as negative control; M=molecular weight marker.*

suggests that almost 61% of cases had no detectable *mdr1* mRNA, but expressed MDR1 P-gp protein. Chi-squared analysis showed no significant ($p=0.636$) correlation between *mdr1* mRNA and MDR1 P-gp protein expression in the NSCLC specimens analysed in this study.

In the case of MRP-1, ~17% of NSCLC were found to co-express *mrp1* mRNA and MRP-1 protein, with 8.3% not expressing MRP-1 at all (Table IV (B)). Approximately 71% of specimens were found to transcribe, but not to translate, MRP1; while 4.2% of cases had detectable levels of MRP-1 protein, but the corresponding gene transcripts could not be detected following 30 cycles of RT-PCR. No significant ($p=0.569$) correlation was found between *mrp1* mRNA and MRP-1 protein expression.

Table IV. *(A) Comparison of MDR1 P-gp mRNA and protein expression.*

% Cases	MDR1 P-gp$^-$	MDR1 P-gp$^+$
mdr1 mRNA$^-$	30.4	60.9
mdr1 mRNA$^+$	4.3	4.3

(B). Comparison of MRP-1 mRNA and protein expression.

% Cases	MRP-1$^-$	MRP-1$^+$
mrp1 mRNA$^-$	8.3	4.2
mrp1 mRNA$^+$	70.8	16.7

Discussion

Resistance to chemotherapy is a major obstacle in the clinical management of lung cancer. While, at the time of diagnosis, most small cell lung carcinomas (SCLCs) are responsive to chemotherapy, the majority of NSCLCs (which constitute ~85% of all lung cancers) are intrinsically resistant to a broad spectrum of chemotherapeutic agents (7-8, 20-21). Although this multiple drug resistance is likely to be a multi-factorial event, not all possible mechanisms contributing to this resistance are yet defined. A better understanding of the gene expression changes involved in, and the level of change contributing to (*i.e.* whether it is at the mRNA and/or protein level), this phenomenon may help improve on choices of adjuvant or *neo*-adjuvant therapy.

Drug efflux pumps have been implicated in conferring drug resistance on cancer cells both *in vitro* and *in vivo* and potential modulators of such pumps have been described by ourselves (22) and others (23). As described earlier, previously reported studies in NSCLC generally focussed on analysis of MDR1 or MRP1 gene expression individually – or, in a few cases, both pumps – at either the mRNA or protein level (see Table I). However, with the exception of a study by Berger *et al.* (7), where a very limited number (4/116) of specimens were analysed using RT-PCR in addition to immunohistochemistry, co-analysis of MDR1 and MRP1 mRNA and protein in the same NSCLC specimens has not been investigated. In the study reported here, tumour specimens were bisected and processed for subsequent mRNA or protein analysis.

Results from our studies indicate that MDR1 and MRP1 drug efflux pumps are commonly expressed in NSCLC. Expression of one, or both, proteins was detected in almost 74% of cases, with corresponding mRNAs detected in most (90%) cases. Overall, results from previous studies of MDR1 and MRP1 expression, although somewhat conflicting, seem to suggest MRP-1 expression to be the predominant efflux pump expressed in NSCLC. This is in agreement with our mRNA analysis, where the majority (almost 87%) of cases were found to express *mrp1*. However, immunohistochemical analysis on the same specimens indicated MDR1 P-gp to be the predominant efflux pump.

These results may suggest that the level (mRNA or protein) of expression control may differ for MDR1 and MRP1 in NSCLC; however, it must be considered that mRNA and protein levels for MDR1 and MRP1, respectively, did not correlate significantly. While it is not unexpected that genes may be transcribed, but not translated (as found in ~4% of cases with MDR1 and 71% of cases for MRP1), the fact that almost 61% of NSCLC apparently expressed MDR1 P-gp protein, with no corresponding *mdr1* mRNA detectable, is unexpected. Possible explanations for this may include differing sensitivities of the methods of analysis (*i.e.* RT-PCR for mRNA *versus* immunohistochemistry for protein). Another possible explanation may be that the two regions of the tumours studied by these methodologies, after bisection, may have had different efflux pump profiles – although in a study of 30 specimens it seems unlikely that such a bias would exist in so many cases.

Conclusion

The results from this study indicate that expression of drug efflux pumps (MDR1 P-gp and/or MRP-1) is common in NSCLC and so potentially could contribute, at least in part, to the chemo-resistant nature of this cancer. Future studies of larger cohorts of NSCLC cases, including the use of increased sensitivity methods (*i.e.* qPCR) for mRNA analysis and, if possible, analysing proteins and mRNAs isolated from the same cell populations, should assist in clarifying conflicting results between mRNA and protein expression and should contribute to our understanding of drug efflux pump involvement in NSCLC drug resistance.

Acknowledgements

This work was supported by funding from Ireland's Higher Educational Authority Programme for Research in Third Level Institutions (PRTLI) Cycle 3 and Dublin City University Research Fellowship.

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Received December 19, 2006
Revised February 13, 2007
Accepted February 20, 2007