P-glycoprotein-mediated acquired multidrug resistance of human lung cancer cells in vivo

Y Abe1,2, Y Ohnishi3, M Yoshimura, E Ota1,2, Y Ozeki2, Y Oshika1,2, T Tokunaga1, H Yamazaki1, Y Ueyama1,3,4, T Ogata1, N Tamaoki1 and M Nakamura1,4

1Department of Pathology, Tokai University School of Medicine, Bohseidai, Isehara-shi, Kanagawa 259-11; 2Department of Surgery II, National Defense Medical College, Namiki 3-2, Tokorozawa-shi, Saitama 356; 3Central Institute for Experimental Animals, Nogawa 1430, Kawasaki-shi, Kanagawa 213; 4Kanagawa Academy of Science and Technology (KAST) Sakado 3-2-1, Takatsu-ku, Kawasaki-shi, Kanagawa 213; 5Automated Multiphasic Health Testing and Services, Tokai University School of Medicine, Bohseidai, Isehara-shi, Kanagawa 259-11, Japan.

Summary We examined whether the increased expression of P-glycoprotein (P-gp) encoded by the human multidrug resistance gene MDR1 is related to the acquired multidrug resistance of lung cancer in vivo. We estimated the chemosensitivity of lung cancer xenografts (LC-6, adenocarcinoma; Lu-24, small-cell cancer) by calculation of relative tumour growth (T/C%, treated/control) in vivo, based on statistical significance determined by the Mann-Whitney U test (P<0.01, one-sided). MDR1 gene expression levels were evaluated by reverse transcription–polymerase chain reaction (RT–PCR) assay. P-gp production and P-gp localisation were examined by Western blotting and by immunohistochemical analysis respectively. LC-6 and Lu-24 were initially sensitive to both vincristine (VCR, 1.6 mg kg⁻¹); LC-6, 45%; Lu-24, 39%) and doxorubicin (DOX, 12 mg kg⁻¹; LC-6, 26%; Lu-24, 27%) in vivo. VCR-resistant variants (LC-6R, 66% and Lu-24R, 68%) selected with VCR (0.4 mg kg⁻¹ × 9) significantly acquired cross-resistance to DOX (LC-6R, 55% and Lu-24R, 55% respectively). RT–PCR assay showed increased levels of MDR1 expression in LC-6R and Lu-24R with stable MDR1 expression levels. P-gp expression levels were elevated, and the percentage of P-gp-positive tumour cells increased in both LC-6R and Lu-24R. These results suggest that P-gp/MDR1 overexpression is related to acquired multidrug resistance in lung cancer in vivo.

Keywords: P-glycoprotein; lung neoplasm; xenograft; acquired drug resistance

Lung cancer is generally treated by a combination of therapeutic protocols using cisplatin, vinca alkaloids, doxorubicin (DOX) and etoposide (VP-16) (Brittan et al., 1988; Williams, 1989; Hansen, 1992). However, the failure of chemotherapy as a result of cellular drug resistance is still a major problem in the treatment of lung cancer. Especially, development of acquired drug resistance in tumours initially sensitive to chemotherapy is a major issue in the treatment of lung cancer patients.

Mechanisms of multidrug resistance were analysed in various human neoplastic cell lines resistant to anti-cancer agents in vitro (Chen et al., 1986, 1990; Ueda et al., 1987). Selection of cells resistant to lipophilic compounds (DOX, vinca alkaloids, podophyllotoxins and colchicine) results in the development of cross-resistance to other related drugs (Fojo et al., 1985). This classical multidrug resistance phenomenon is known to be related to the overexpression of P-glycoprotein (P-gp) encoded by the human multidrug resistance gene (MDR1) (Gros et al., 1986). Recently, atypical multidrug resistance induced by overexpression of multidrug resistance-associated protein (MRP) has been reported in lung cancer cells in vitro (Cole et al., 1992; Versantvoort et al., 1992; Zaman et al., 1993).

Our previous clinicopathological studies have not shown intrinsic multidrug resistance in non-small-cell lung cancer (NSCLC) to be related to P-gp/MDR1 (Abe et al., 1994a). However, certain pulmonary adenocarcinomas revealed significantly increased MDR1 expression. Many authors have also reported drug resistance mechanisms associated with P-gp in lung cancer (Lai et al., 1989; Volm et al., 1991; Holzmaier et al., 1992). We did not find multidrug resistance to be intrinsically related to MDR1 overexpression in lung cancer xenografts (including LC-6 and Lu-24) in vivo (Abe et al., 1994b). However, it has not been clarified whether acquired multidrug resistance is related to increased levels of MDR1 expression in human cancer cells in vivo.

In this study, we selected VCR-resistant variants from human NSCLC (LC-6R) and small-cell lung cancer (SCLC, Lu-24R) xenografts in vivo, and evaluated whether these VCR-resistant xenografts showed cross-resistance to DOX by chemosensitivity tests in vivo. The expression levels of P-gp/MDR1 were also analysed before and after selection in these xenografts. We also examined the gene expression levels of miscellaneous factors associated with multidrug resistance including MRP, topoisomerase IIα (Topo IIα) and glutathione-S-transferase-π (GST-π) in the xenografts. We discuss here the hypothesis that acquired multidrug resistance is induced by the increased expression of P-gp/MDR1 in lung cancer in vivo.

Materials and methods

Human lung cancer xenografts

Two human xenografts (LC-6, NSCLC, adenocarcinoma; Lu-24, SCLC, oat-cell type) were originally established at the Central Institute for Experimental Animals (Kanagawa, Japan) from primary lung cancer materials from patients who had received no anti-cancer chemotherapy. The tumour xenografts were maintained by serial subcutaneous transplantation in nude mice (BALB/c-nu/nu, Clea Japan, Tokyo), and used at 10–20 passages in this study. Xenograft specimens obtained from mice sacrificed under deep anaesthesia were frozen and stored at −80°C until analysed. Tumour xenografts were also prepared for routine histopathological analyses.

The drug-sensitive epidermoid carcinoma cell line, KB-3-1, and its resistant derivative, KB-8-5, were cultured in Dulbecco’s modified Eagle’s minimal essential medium supplemented with 5% fetal bovine serum (FBS) at 37°C in a fully humidified 95% air, 5% carbon dioxide atmosphere.
Establishment of VCR-resistant xenografts in vivo

The human NSCLC (LC-6) and SCLC (Lu-24) xenografts were sensitive to the maximum tolerated doses (MTDs) of both VCR and DOX in vivo. We selected VCR-resistant xenografts, LC-6R and Lu-24R, from LC-6 and Lu-24, respectively, by serial passage in mice and by administration of VCR (0.4 × 9 mg kg⁻¹) in vivo, according to our previous report (Abe et al., 1993). No significant morphological differences were noted between parental and VCR-resistant xenografts.

In vivo chemosensitivity test

VCR (Shionogi, Osaka, Japan), DOX (Kyowa Hakko Kogyo, Tokyo), cisplatin (Nihon Kayakki, Tokyo), mitomycin (Kyowa Hakko Kogyo) and cyclosporin A (CysA, Sand, Tokyo) were purchased from the sources shown. All drugs were dissolved in saline and used for in vivo chemosensitivity tests.

We performed in vivo chemosensitivity tests on the lung cancer xenografts (LC-6, LC-6R, Lu-24 and Lu-24R) according to the procedures reported previously (Inaba et al., 1988, 1989). Six female mice (BALB/c-nu/nu; 6–15 weeks old) bearing xenografts (tumour volume: 100–300 mm³) were given the MTD of VCR (1.6 mg kg⁻¹) or DOX (12 mg kg⁻¹). The tumour volume (V) was calculated by the equation, \[ V = \frac{1}{2} \times A \times B, \]
in which A and B are the experimental measurements in mm of length and width, respectively. Growth of the tumour xenografts was measured by the relative tumour volume (RV), which was expressed as \[ RV = \frac{V_{t}}{V_{0}}, \]
in which \( V_{t} \) is the tumour volume at day 14 and \( V_{0} \) is the initial tumour volume when the treatment was started (day 0). The effects of the drugs were represented by RV of the xenografts, and the T/C% values were defined as the ratio of the RV of the treated xenografts to controls after drug administration. Animal experiments were carried out in accordance with the guidelines established by the Central Institute for Experimental Animals.

We examined the effects of prior inoculation with the P-gp inhibitor CysA on the sensitivity to anti-cancer agents in the xenografts in vivo, according to our previous report (Abe et al., 1996). Nude mice bearing tumour xenografts were treated with VCR (0.4 mg kg⁻¹) or DOX (12 mg kg⁻¹) 3 h after intravenous administration of CysA (50 mg kg⁻¹). VCR was used at the low concentration of 0.4 mg kg⁻¹ in this study because we had certified in advance that co-administration of high doses of VCR (1.6 mg kg⁻¹) was fatal for the mice with CysA.

Reverse transcription–polymerase chain reaction (RT–PCR) assay

Total cellular RNA specimens were prepared from frozen materials (Sambrook et al., 1989). The expression levels of MDR1 transcripts were determined by the modified RT–PCR procedure described previously (Noonan et al., 1990), using the following primers: sense, AACGTTAGTGACAAAAGGCCTCG, nucleotides 2041–2046; antisense, GGGTAAGACATGTGAAAAACAA, nucleotides 2260–2283 (Abe et al., 1994a). The primer sequences were derived from exon 16 and exon 18, respectively, separated by introns to prevent amplification of contaminating genomic DNA. We avoided amplification of contaminating murine MDR gene transcripts in the tumour xenografts by using the above primers specific for the human MDR1 gene (Abe et al., 1994a,b). RT–PCR with these primers amplified a 243 bp fragment of MDR1 cDNA. We estimated MDR1 expression level in comparison with that of the housekeeping gene β2-microglobulin (β2m).

Northern blot analyses

Total cellular RNA samples (20 μg) fractionated through agarose gels were blotted onto nitrocellulose membranes (Gene Screen Plus, New England Nuclear), and the blots were hybridised with 32P-labelled MDR1 cDNA probe (kindly supplied by Dr I Pastan, National Cancer Institute, Bethesda, MD, USA). The level of MDR1-specific transcript expression (4.2 kb) was evaluated in comparison with that of the housekeeping gene β-actin (2.2 kb).

Levels of expression of Topo Iα, GST-π and MRP genes were also evaluated in the xenografts by Northern blot analysis. Complementary DNAs (Topo Iα, Dr T Ando; rat GST-π cDNA, Dr A Sugioka through the Japanese Cancer Research Resources Bank) were used. A human MRP cDNA was prepared by PCR amplification of the fragment corresponding to nucleotides 240–503 from KB8-5 cells (Ota et al., 1995). We evaluated expression of each gene-specific transcript (Topo Iα, 4.6 kb; GST-π, 0.7 kb; MRP, 6.5 kb).

Western blotting

Solubilised samples were separated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (10% polyacrylamide; 250 mM Tris, 250 mM glycine, pH 8.3; 0.1% SDS; for 42 min, at 200 V) according to the method of Laemmli (Friedlander et al., 1989). The proteins were electrophoretically transferred to nitrocellulose membranes (Bio-Rad, 0.45 μm pore size) in 48 mM Tris, 39 mM glycine, 0.0375% SDS, 20% methanol at 0.8 mA cm⁻² for 1 h. The membranes were probed with a monoclonal anti-human P-gp antibody (C219, CIS Bio International; 0.2 μg ml⁻¹, 1 h) after blocking non-specific binding with 10% non-fat dried milk overnight at 4°C. The blots were then incubated with biotinylated anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA, USA), followed by incubation with streptavidin–peroxidase complex (Vector). Peroxidase-labelled blots were then examined by the enhanced chemiluminescence method (Amersham).

Immunohistochemistry

P-gp-positive tumour cells were analysed immunohistochemically with anti-P-gp polyclonal antibody Ab-1 (Oncogene Science) (Toth et al., 1994). Tumour sections were serially incubated with Ab-1, peroxidase-conjugated F (ab')₂ of donkey anti-rabbit IgG (Amersham), rabbit monoclonal peroxidase–antiperoxidase complex (Dako) and peroxidase-conjugated F(ab')₂ fragments. The products were visualised with 3,3-diaminobenzidine tetrahydrochloride.

Results

In vivo drug sensitivity

The growth rates of human lung cancer xenografts in the chemosensitivity tests are shown with relative tumour volume (Figures 1–3). Evaluation as 'sensitive' was defined based on statistical significance determined by the Mann–Whitney U-test (P<0.01, one-sided) (Abe et al., 1994b).

LC-6 was sensitive to the MTD of both VCR and DOX (Figure 1a), and LC-6R selected in vivo by VCR was resistant to VCR and acquired cross-resistance to DOX (Figure 1a). Lu-24 was also initially sensitive to the MTD of both VCR and DOX (Figure 1b), and Lu-24R selected by VCR was resistant to VCR and acquired cross-resistance to DOX (Figure 1b). Table 1 shows T/C% values of each xenograft in vivo on day 14 after drug administration. The T/C% values of LC-6 exposed to the MTD of VCR (45%) and DOX (26%) were significantly lower than those (66% and 55%) of LC-6R. The T/C% values of Lu-24 to the MTD of VCR (39%) and DOX (27%) were also significantly lower than those of Lu-24R (68% and 55%).

This acquired drug resistance in LC-6R was circumvented by co-administration of CysA (Figure 2). The acquired drug resistance to VCR of LC-6R was reversed by co-administration of CysA (T/C%: 90% to 38%), which when administered alone showed no anti-cancer effect. The
acquired cross-resistance of LC-6R to DOX was also circumvented by co-administration of CysA (T/C%: 55% to 15%). CysA did not apparently affect the growth of LC-6, when it was administered with or without anti-cancer drugs (data not shown).

The changes in responsiveness to non-P-gp-mediated anti-cancer agents (cisplatin and mitomycin) were not significantly different between LC-6 and LC-6R, while LC-6R showed a 3-fold greater susceptibility to mitomycin C (Table 1).

MDR1 expression

Northern blots showed no apparent MDR1 expression in LC-6, LC-6R, Lu-24 or Lu-24R xenografts. Semi-quantitative RT–PCR assay showed no MDR1 expression in LC-6 or Lu-24 xenografts (Abe et al., 1994b). LC-6R and Lu-24R with acquired cross-resistance, however, showed increased levels of MDR1 expression compared with the sensitive parent xenografts LC-6 and Lu-24 (Figure 3). The xenografts LC-6R and Lu-24R serially transplanted into nude mice (four generations) without VCR showed no marked fluctuations in the levels of MDR1 expression.

P-gp production

The VCR-resistant xenografts, LC-6R and Lu-24R, showed increased production of P-gp protein by Western blotting in comparison with the respective parent xenografts (LC-6 and Lu-24 respectively; Figure 4). Immunohistochemical analysis with anti-P-gp polyclonal antibody (Ab-1) also revealed marked increases in the number of P-gp-positive tumour cells in LC-6R and Lu-24R compared with their respective parental xenografts (Figure 5), whereas LC-6 and Lu-24 xenografts showed no P-gp-positive tumour cells.

**Table 1**

| Group | Number of xenografts | P-gp-positive xenografts | Levels of P-gp protein |
|-------|----------------------|--------------------------|------------------------|
| LC-6  | 12                   | 0                        | 0.1                    |
| LC-6R | 12                   | 12                       | 0.6                    |
| Lu-24 | 12                   | 0                        | 0.0                    |
| Lu-24R| 12                   | 12                       | 0.5                    |

**Figure 1** (a) Growth rates of LC-6 and LC-6R in the in vivo chemosensitivity test. (b) Growth rates of Lu-24 and Lu-24R in the in vivo chemosensitivity test. Each group included six nude mice bearing tumour xenografts. ■, untreated control; ●, maximum tolerated doses (MTDs) for vincristine (VCR) treatment, 1.6 mg kg⁻¹; ▲, MTDs for doxorubicin (DOX) treatment, 12 mg kg⁻¹. Evaluation as 'sensitive' was strictly defined, based on statistical significance determined by the Mann–Whitney U-test (P<0.01, one-sided). Asterisks (*) indicate significant differences between control and treated groups.

**Figure 2** Growth rate of LC-6R in the in vivo chemosensitivity test with cyclosporin A (CysA). Each group included six nude mice bearing tumour xenografts. Evaluation as 'sensitive' was strictly defined based on statistical significance determined by the Mann–Whitney U-test (P<0.01, one-sided). Asterisks (*) indicate a significant difference between control and treated groups: ■, untreated control; ●, VCR (0.4 mg kg⁻¹) treatment; ▲, treated with only CysA, 50 mg kg⁻¹; ●, VCR (0.4 mg kg⁻¹) treatment with CysA (50 mg kg⁻¹); ▲, treated with DOX (12 mg kg⁻¹) and CysA (50 mg kg⁻¹).
**Table 1** *In vivo* chemosensitivity test [T/C% value at day 14 (U-test)]

| Tumour | LC-6 | LC-6R | Xenograft | Lu-24 | Lu-24R |
|--------|------|-------|-----------|-------|--------|
| VCR    | 45±6 (+) | 66±15 (-) | 39±8 (+) | 68±15 (-) |
| DOX    | 26±5 (+) | 55±12 (-) | 27±6 (+) | 55±13 (-) |
| VCR+CysA | 38±8 (+) |       |           |       |        |
| DOX+CysA |       | 15±3 (+) |           |       |        |
| CDDP   | 23±4 (+) | 13±3 (+) |           |       |        |
| MMC    | 1±0 (+) | 3±1 (+) |           |       |        |

Relative tumour volume (RV) = \( V_{14}/V_0 \), where \( V_{14} \) is tumour volume at day 14 and \( V_0 \) is the initial value at the beginning of treatment (day 0). T/C%, growth ratio of the relative volume of the treated xenografts to controls (untreated) on day 14 of treatment (VCR, 1.6 mg kg\(^{-1}\); DOX, 12 mg kg\(^{-1}\); CDDP, 7 mg kg\(^{-1}\); MMC, 1.7 mg kg\(^{-1}\)). U-test, significance of differences were estimated by the Mann-Whitney U-test \((P<0.01, \text{one-sided}; +, \text{significant}; --, \text{not significant})\).

**Figure 3** MDRI expression in the tumour xenografts determined by reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR revealed a 243 bp fragment of MDRI cDNA. (a) Lane 1, KB8-5 xenograft; lane 2, KB3-1 xenograft; lane 3, LC-6; lanes 4–6, LC-6R serially passaged. (b) Lane 1, KB8-5 xenograft; lane 2, KB3-1 xenograft; lane 3, Lu-24; lanes 4–5, Lu-24R serially passaged.

**Figure 4** P-gp production in the tumour xenografts. Western blotting was performed with the anti-human P-gp monoclonal antibody, Ab-1. These VCR-selected multidrug-resistant xenografts (LC-6R and Lu-24R) showed enhanced production of P-gp.

**Figure 5** Localisation of P-gp. Immunohistochemical analysis was performed with anti-P-gp polyclonal antibody, Ab-1. The multidrug-resistant xenografts (a, LC-6R; and b, Lu-24R) contained P-gp positive cancer cells (arrow).

**Topo IIx, GST-\(\pi\) and MRP gene expression**

No significant changes were observed in MRP, Topo II\(\pi\) or GST-\(\pi\) gene expression between parent xenografts (LC-6 and Lu-24) and their corresponding drug-resistant derivatives (LC-6R and Lu-24R) by Northern blot analysis (data not shown).

**Discussion**

Many studies using human tumour cell lines have revealed that multidrug resistance mechanisms are correlated to the overexpression of P-gp/MDRI in vitro (Chen et al., 1990; Roninson, 1991). It has, however, not been clearly demonstrated whether acquired multidrug resistance is influenced by P-gp/MDRI overexpression in human cancers in vivo (Starling et al., 1990).

The VCR-resistant variants (LC-6R and Lu-24R) selected in vivo from drug-sensitive xenografts (LC-6 and Lu-24) showed cross-resistance to DOX, and the drug resistance to VCR and DOX of LC-6R was overcome by co-administration of the P-gp inhibitor, CysA. RT-PCR assay showed increased levels of MDRI expression in LC-6R and Lu-24R, whereas no marked changes were seen in the expression of other miscellaneous drug resistance-related factors (Topo II\(\pi\),
GST-π and MRP (Zwelling et al., 1990; Nakagawa et al., 1990; Cole et al., 1992). In LC-6R and Lu-24R, P-gp expression levels were elevated and P-gp-positive tumour cells increased. These results supported the hypothesis that acquired multidrug resistance is induced by increased P-gp protein/MDR1 expression in human lung cancer xenografts.

Western blotting showed small amounts of P-gp in LC-6 and Lu-24, whereas a highly sensitive RT-PCR assay revealed no MDR1 expression in these sensitive xenografts. In this RT-PCR assay, we selected primers which were specific for human MDR1 and did not amplify the murine mdr gene. Immunohistochemical analysis showed no P-gp-positive tumour cells in these sensitive xenografts. Therefore, the signals seen in LC-6 and Lu-24 might have included non-specific reactions to murine P-gp-related molecules probably in the stromal elements by Western blotting with murine monoclonal anti-P-gp antibody, C219.

Several studies have shown that NSCLC with neuroendocrine properties expresses high levels of P-gp/MDR1 (Lai et al., 1989), while some authors reported that the expression levels of P-gp/MDR1 in lung cancer were not so high (Fojo et al., 1987; Goldstein et al., 1989). On the other hand, we reported enhanced MDR1 expression in a limited number of pulmonary adenocarcinomas (Abe et al., 1994a). However, it has not been demonstrated conclusively whether acquired multidrug resistance in lung cancer is related to P-gp/MDR1 overexpression in vivo. The results presented here strongly support the hypothesis that acquired multidrug resistance is related to the increased expression of P-gp/MDR1 in pulmonary adenocarcinoma and small-cell lung carcinoma in vivo.

The multidrug-resistant xenografts expressed MDR1 at lower levels than the in vitro multidrug-resistant carcinoma line, KB8-5. It is very important to determine how MDR1 expression levels can induce the multidrug resistance of tumour cells in human lung cancer in vivo. Previously, we suggested that in vivo sensitivity assays more accurately reflect drug resistance as a result of low-level MDR1 overexpression in the human epidermoid carcinoma KB line (Abe et al., 1996). Reduced levels of MDR1 expression might be related to P-gp-mediated multidrug resistance in vivo compared with that in vitro.

It is difficult to determine whether the observed multidrug resistance phenotype was caused by the clonal selection of intrinsically P-gp-positive cancer cells or the activated production of P-gp in resistant cancer cells (Chaudrey et al., 1993; Chen et al., 1994; Brock et al., 1995). The multidrug-resistant xenografts, LC-6R and Lu-24R, used in this study showed stable MDR1 expression during four serial passages without exposure to VCR. Immunohistochemical analysis revealed definite P-gp-positive cancer cells in multidrug-resistant xenografts, whereas no P-gp-positive tumour cells were detected in the parental xenografts. It is impossible to conclude from these results whether the observed multidrug resistance was owing to clonal selection of P-gp-expressing cells or the activated production of P-gp.

Recently, the mechanism of atypical multidrug resistance in lung cancer by MRP has been discussed (Cole et al., 1992). Previously we demonstrated the clinical relevance of MRP overexpression in the intrinsically multidrug-resistant NSCLC, especially in pulmonary squamous cell carcinoma (Ota et al., 1995). We are also currently engaged in studies to determine the relevance of MRP in the acquired multidrug resistance phenotype in pulmonary squamous cell carcinoma xenografts.

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