

The analysis of chemotherapy resistance in human lung cancer cell line with microchip-based system

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Abstract Microchip-based systems have many desirable characteristics and can be used in much cellular biochemical analysis. Glucose-regulated protein 78 (GRP78), an endoplasmic reticulum chaperone, has a critical role in chemotherapy resistance of some cancers. This work aimed at analyzing the correlation between the expression of GRP78 and an anticancer drug, topoisomerase II inhibitor-VP-16, in human lung cancer cell line NCI-H460 using this microchip-based system. The cells were cultured on a PDMS chip, the expression of GRP78 at both protein and mRNA levels for the cells under the condition with or without the induction of A23187 were assayed by immunofluorescence and chip electrophoresis, respectively. Then

the cells were treated by VP-16, percentages of apoptosis and the cycle distributions of the cells were detected by flow cytometry. The cells cultured on the PDMS attached and spread well to micro-channels with high viability. Compared with the non-induced cells, the expression of GRP78 at both protein and mRNA levels for the A23187-induced cells were increased greatly. After treatment by VP-16, the percentage of apoptotic cells decreased nearly threefold for the A23187-induced cells in contrast to the non-induced cells ($13.15 \pm 3.84\%$ versus $34.03 \pm 11.45\%$), and the cells distributed in S phase reduced dramatically ($11.96 \pm 1.27\%$ versus $20.76 \pm 3.05\%$) whereas in G₁ phase increased greatly ($74.16 \pm 0.95\%$ versus $57.06 \pm 4\%$). GRP78 is correlated to the resistance to VP-16 in human lung cancer cell line. The microchip-based system has the potential application and feasibility for cell culture and its functional research.

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1 Introduction

Microchip-based systems have many desirable characteristics, such as lower consumptions of reagents and samples, smaller space requirements, and shorter analysis time. Applications of these systems, widely known as micro total analysis systems (μ -TAS) or lab-on-a-chip, have been spreading rapidly (Aurox et al. 2002; Reyes et al. 2002; Tudos et al. 2001; Verpoorte 2002). Many applications, including flow-injection analyses, solvent extractions, and micro-reactors have been demonstrated (Hisamoto et al. 2004; Sato et al. 2003a, b). In these systems, full advantage

was taken of the scale merits of the micro-space, i.e. a short diffusion distance, a large specific interface area, a rapid and efficient reaction. Microchip techniques also appear to provide some advantages for cellular biochemical analysis systems, because the scale of the liquid micro-space inside a microchip is fitted to the size of the cells. Rapid and sensitive immunoassay systems for protein analyses with these systems have been demonstrated as well (Hosokawa et al. 2007; Herr et al. 2007; Kazuhiko et al. 2007).

Lung cancer is the most common carcinoma among men worldwide with respect to both incidence and mortality and represents an increasing problem among women (Devesa et al. 2005); still, survival rates are poor. Tumor resistance in patients receiving chemotherapy represents a major problem in treatment of lung cancer. An important mechanism of chemotherapy resistance may result from the influence of tumor cell niche that occur naturally in solid tumors (Pelletier et al. 1990; Shen et al. 1987). Glucose-regulated protein-78 (GRP78), a representative endoplasmic reticulum chaperone in the tumor cell niche, plays an important role in the resistance of chemotherapy in many tumors including breast, ovarian, colon and bladder cancer. However, little is known about the function of GRP78 in human lung cancer chemotherapy resistance.

The conventional methods for the *in vitro* detection of GRP78 mainly include immunoprecipitation, Western blot, gel electrophoresis on the cells cultured in flasks (Ramsay et al. 2005; Reddy et al. 2003; Jeon et al. 2000). These processes involve a relatively long time, troublesome liquid-handling procedures and large quantities consumption of reagent; some require labor-intensive purification of the protein and complex analysis steps.

In this work, a novel microchip-based system was used to detect the expression of GRP78 at protein and mRNA levels in a human non-small cell lung cancer cell line NCI-H460 through immunofluorescence and electrophoresis analysis. Herein, a highly potent ER stress inducer, known agent of calcium ionopore-A23187, was used as the inducer of GRP78 (Ramsay et al. 2005). Meanwhile, the correlation between GRP78 and the resistance to an anti-cancer drug, topoisomerase II inhibitor-VP-16, including its possible mechanism of the resistance was established. In addition, the potential application of the microchip system on the research of cell culture and its function had been investigated.

2 Materials and methods

2.1 Fabrication of PDMS chip and PMMA chip

The microchip used for cell culture and analysis was made from polydimethyl siloxane (PDMS, Sylgard 184, Dow

Corning, Midland, MI) according to standard soft lithography methods (Dertinger et al. 2001; Duffy et al. 1998). Briefly, the master molds were fabricated by patterning negative photoresist (SU82075, Microchem, MA) by photolithography. The PDMS micro-device was fabricated by replica molding PDMS against the masters. Inlets for the fluids were punched out of the PDMS using a sharpened needle. The surface of the PDMS sheet and a glass slide were activated with the air plasma and brought together for irreversible bonding.

The microchip used for electrophoresis was made from poly(methyl methacrylate) (PMMA Dalian Institute of Chemical Physics, Chinese Academy of Sciences) according to a reported method (Zhou et al. 2005). Dimensions and layouts of the PDMS and PMMA chips were shown in Fig. 1.

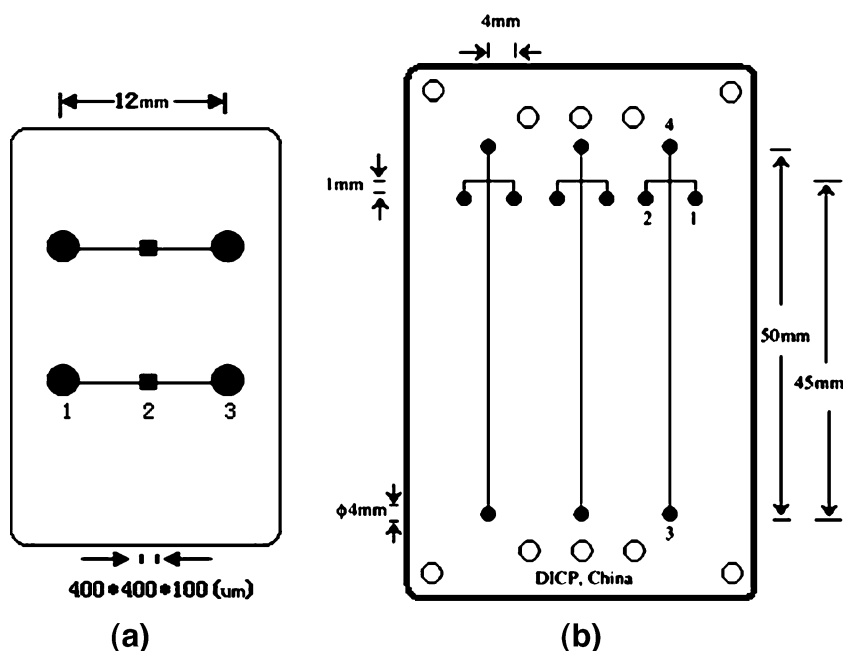
2.2 Cell culture on PDMS chip

Human non-small cell lung cancer cell line NCI-H460 was obtained from American Type Culture Collection (Manassas, VA, USA). Prior to cell culture, the PDMS microchip was treated as follows: First, the chamber was washed with 0.1 M NaOH, 0.1 M HCl, and water, successively, for 1 h each in an ultrasonic bath. Next, the chip was autoclaved at 120°C for 15 min. The culture chamber was filled with 0.01% poly-L-lysine solution (Sigma-Aldrich Co., St. Louis, MO) for 1 h in order to coat its inner surface. The channel was washed with sterilized water before introducing the cells. The cells were seed into the micro-chamber at the density of around 1×10^5 cells per milliliter and incubated at 37°C in 5% CO₂ atmosphere. The cells have to attach themselves to the wall of the culture chamber to grow, and poly-L-lysine can bind cells to the chamber wall tightly. The culture medium was RPMI 1640 (Sigma-Aldrich Co, St. Louis, MO, USA) supplemented with 5% fetal bovine serum (FBS) and 100 µg/ml kanamycin and was changed periodically every day to supply enough nutrition to the cells. The viabilities of the cells were detected by a Trypan blue exclusive assay, i.e., after incubation for 24 h, the cells were exposed to 0.2% Trypan blue solution and immediately observed under an inverse light microscope.

2.3 Immunofluorescence assay of GRP78 protein on PDMS chip

When grown to 60% confluence, the cells were exposed to A23187 (Sigma Chemical Co, Taufkirchen, Germany) at different concentrations (0, 1, 2, 4 and 6 µM) for 24 h. The time point study of A23187 has been performed previously (Yun et al. 1995). Then the cells were washed with PBS for twice, fixed with 4% paraformaldehyde in PBS for 10 min,

Fig. 1 Dimension and layouts of the chips used in this work. (a) PDMS chip for cell culture: (1) inlet; (2) chamber of cell culture; (3) outlet. (b) PMMA chip for the electrophoresis: (1) injection waste; (2) sample; (3) waste; (4) buffer



permeabilized in PBS containing 0.1% Triton X-100 and 5% bovine serum albumin for 30 min before the detection of GRP78 with immunofluorescence. The media with anti-GRP78 (C-20) goat polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a dilution of 1:1,000 were introduced into the culture chamber and the cells were incubated overnight at 4°C. After being washed with PBS for three times, the cells were stained with anti-goat FITC green-conjugated secondary antibody (Santa Cruz Biotechnology, Inc) at a dilution of 1:100 at 37°C for 30 min. Cell images were subsequently captured with fluorescence microscope and analyzed using Northern exposure image analysis/archival software (Mississauga, Ontario, Canada).

2.4 Electrophoresis assay of GRP78 mRNA on PMMA chip

To evaluate the effect of A23187 on the induction of GRP78 mRNA, the RNA for induced and non-induced cells was extracted and the PCR products were analyzed by a PMMA chip electrophoresis. After 24 h incubation with A23187 at different concentrations as mentioned above, the cells on PDMS chip were harvested and total RNA was extracted. RT-PCR was performed with RNA PCR Kit (AMV, Ver.3.0, TaKaRa, Japan) according to the manufacturer’s instructions as previously reported (Wang et al. 2002). The “housekeeping” gene, β -actin was used as an internal control to confirm the equal loading in each experiment and amplified from the same cDNAs. The primers specific for GRP78 and β -actin are shown in Table 1.

According to the previous report (Zhou et al. 2005), electrophoresis on PMMA chip was performed. As shown

in Fig. 1(b), Every GRP78 PCR product was loaded into the sample reservoir and then was injected by applying a voltage of 400 V/cm at the sample waste and grounding the sample reservoir for 30 s. The buffer and waste reservoirs had no potentials applied since the low diffusion coefficients of DNA fragments in the polymer sieving medium resulted in no substantial increase in the injection plug length or deterioration of separation channel. During separation the relative potentials were switched to 0, 0.65, 0.3, and 0.3 kV at the buffer, waste, sample, and sample waste reservoirs, respectively. The micro-channels were rinsed with distilled water after each run. No pretreatment of the channel walls was done before separation. A LIF detection system was applied to the electrophoresis microchip.

2.5 Anti-cancer drug VP-16 test on PDMS chip

Twenty-four hours after seeding, the viabilities of the cells either non-induced or A23187-induced at a concentration of 2 μ M were assayed after the treatment by anti-cancer drug VP-16. VP-16 at concentration of 30 μ M was introduced into the culture chambers respectively, after 6 h incubation, the drug media were discarded and the cells were cultured

Table 1 Characteristics of primers used for conventional RT-PCR

Gene	Sequence of primers	Product (bp)
GRP78	5' GATAATCAACCAACTGTTAC 3' 5' GTATCCTCTTCACCAGTTGG 3'	577
β -actin for GRP78	5' TCGTCACCAACTGGGACGACATGG 3' 5' GATCTTGATCTTCATTGTGCTGGG 3'	750



Fig. 2 The NCI-H460 cells cultured on the PDMS chip spread and attached to the micro-channels well after 24 h incubation

in new media for 48 h further before the harvest for the analysis of apoptosis and cell cycle by flow cytometry (FAC star; BD Biosciences).

2.6 Analysis of apoptosis

The cells treated by VP-16 were trypsinized from the PDMS chip, washed twice with ice-cold PBS (pH 7.4), and re-suspended in $1 \times$ binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl_2) at a concentration of 1×10^6 cells per milliliter. One hundred microliters of cell suspension was transferred to 5 ml plastic tubes, 5 μl of annexin V-fluorescein isothiocyanate (PharMingen) and 5 μl propi-

dium iodide (PI) were added. The cells were gently vortexed and incubated in the dark at room temperature for 25 min. 400 μl of binding buffer was added to each tube, and annexin V staining was analyzed within 1 h by flow cytometry (FAC star; BD Biosciences). Cells negative for both PI and annexin V staining are live cells, annexin V positive staining cells are early apoptotic cells, and PI positive and annexin V positive staining cells are primarily cells in late stages of apoptosis. The experiments were repeated two to three times.

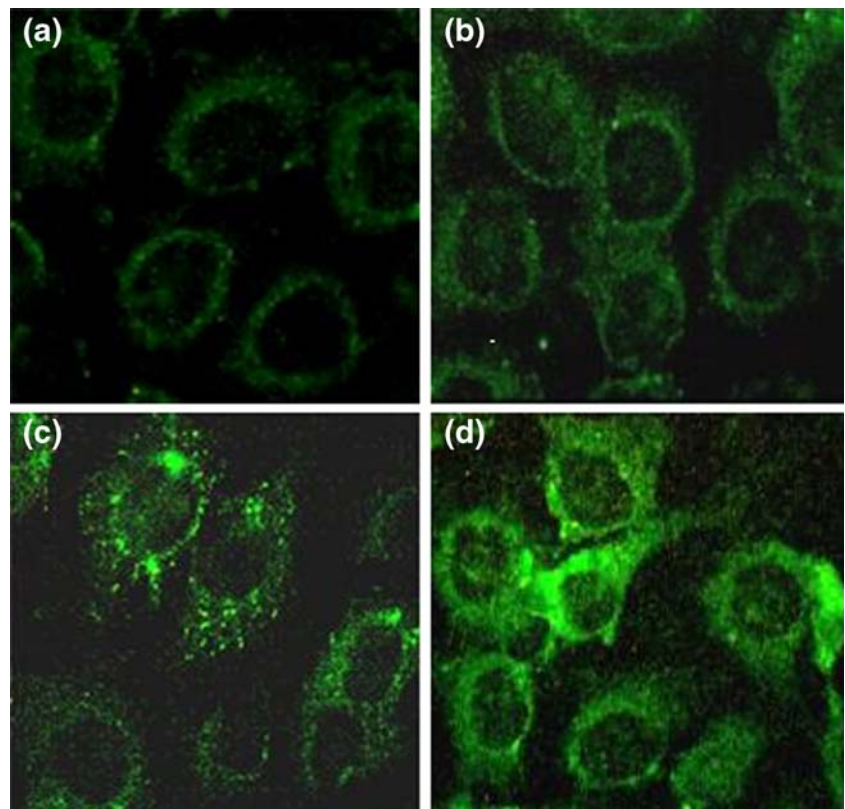
2.7 Cell cycle analysis

Following seeding, exponentially growing cells treated with VP-16 were trypsinized and fixed in 70% ethanol. The fixed cells were treated with PBS containing 0.1% (v/v) Triton X-100, 0.2 mg/ml DNase-free RNase, and 20 g/ml PI for 30 min at room temperature. The cell cycle distributions were analyzed by a fluorescence-activated cell sorting (FACS) scan flow cytometry analysis (FAC star; BD Biosciences) and data were analyzed with ModFit software. The cell cycle distribution measurements were repeated three to five times.

2.8 Statistical analysis

The statistical analyses were carried out with SPSS 12.0 for Windows software. Student's *t* test and χ^2 test (chi-square

Fig. 3 The immunofluorescence detection of GRP78 in NCI-H460 cell line with the induction of A23187 for 24 h at different concentration [(a) 0, (b) 1, (c) 2, (d) 4 μM ; 400 \times]



test) were used to compare results of flow cytometry and analysis of PCR product, respectively. A value of $p < 0.05$ was regarded as statistically significant.

3 Results

3.1 Cell culture on PDMS chip

As shown in Fig. 2, the cells cultured on the PDMS chip spread and attached to the micro-channels well and grew up to confluence after 24 h incubation. Trypan blue exclusion assay showed less than 1% of cells died. The viability and the morphology of the cells suggested that the PDMS chip provides a suitable environment for cell maintenance and the cells could be used in other assays further. In each experiment, the PDMS chip had been successfully employed as a substrate for growth of the adhered cells.

3.2 Expression of GRP78 protein

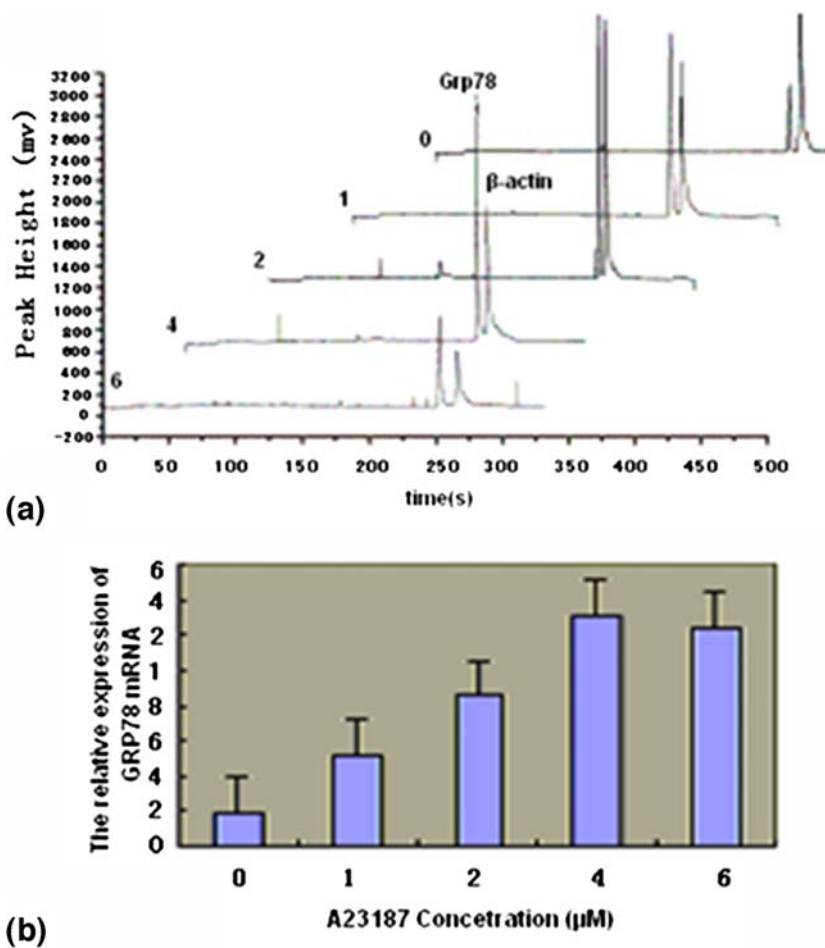
To examine the effect of A23187 on GRP78 protein expression in NCI-H460 cell line, we carried out an immu-

nofluorescence assay on the cells treated with various concentrations of A23187 ranging from 0 to 6 μM for 24 h. As it is shown in Fig. 3, GRP78 displayed a perinuclear, reticular pattern of distribution in the cells. Compared to the non-induced cells, the intensity of the fluorescence for the induced cells was clearly strong. Furthermore, with the increasing of A23187 concentrations, the intensity became stronger obviously. All these meant A23187 could induce the expression of GRP78 at protein level effectively in NCI-H460 cell line.

3.3 Expression of GRP78 mRNA

To analyze whether the expression of GRP78 mRNA corresponded to the GRP78 protein induced by A23187 in NCI-H460 cell line, total cellular RNA was prepared from the different disposed cells and assayed by microchip electrophoresis. Similar to the results of immunofluorescence assay, A23187 caused an elevation of GRP78 mRNA as well. Furthermore, GRP78 mRNA showed a nearly A23187 dose-dependent increase ranging from 1 to 6 μM of the concentrations (Fig. 4), causing two to sevenfold elevation, compared to the non-induced cells (control, $p < 0.05$).

Fig. 4 The results of microchip electrophoresis of GRP78 RT-PCR products for the non-induced or A23187-induced cells for 24 h at different concentration (1, 2, 4, 6 μM) evaluated by the ratio of peak height GRP78/Peak height β -actin (a) Electrophoregram (b) Bar graph



3.4 Correlation between overexpression of GRP78 and drug-resistance to VP-16

In order to know whether the elevation of GRP78 induced by A23187 could lead to the development of drug resistance to VP-16 in NCI-H460 cell line, the non-induced and A23187-induced cells were exposed to VP-16, and cell viabilities were measured using flow cytometry. As shown in Fig. 5, the percentage of apoptotic cells in the non-induced group was about threefold as compared with that of the A23187-induced group ($34.03 \pm 11.45\%$ versus $13.15 \pm 3.84\%$), which were statistically significant difference ($p < 0.05$). These results suggested that GRP78 overexpression could suppress VP-16 induced apoptosis in NCI-H460 cell line, which meant GRP78 had the correlation to the resistance to VP-16 in human lung cancer.

3.5 Cell cycle distribution

The cell cycle distributions determined by flow cytometry showed that there was a great increase in G_1 phase ($74.16 \pm 0.95\%$ versus $57.06 \pm 4\%$) and a dramatic reduction in S phase ($11.96 \pm 1.27\%$ versus $20.76 \pm 3.05\%$) for the A23187-induced cells compared with the non-induced cells ($p < 0.05$, Table 2). Combined with the expression of GRP78, it indicated that GRP78 overexpression did alter the cell cycle distribution significantly ($p < 0.05$), and this also suggested that GRP78 caused a lower apoptosis and higher survival may through the G_1 arrest for the lung cancer cells when challenged with VP-16.

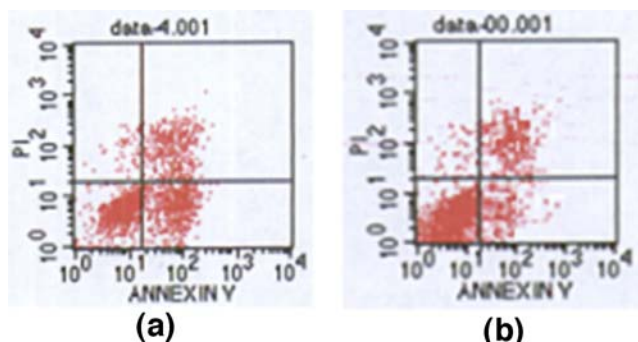


Fig. 5 Measurement of apoptotic cells under VP-16 (30 μ M) treatment for 6 h determined by FACS analysis. (a) Cells of A23187 non-induced group; (b) cells of A23187-induced group at concentration of 2 μ M. The cell labeled with annexin V and PI. Viable cells were those with low annexin or no annexin and PI staining (lower right panel). Early stage apoptotic cells were represented by high annexin and high PI staining (lower right panel), later stage apoptotic cells were represented by high annexin (upper right panel), and necrosis cells were represented by high PI and low annexin staining (upper left panel)

Table 2 Cell cycle distribution and percentage of apoptosis for the non-induced and A23187-induced cells ($x \pm s$) %

Group	Cell cycle distribution			Percentage of apoptosis
	G_0/G_1	S	G_2/M	
A23187-induced cells	74.16 ± 0.95	11.96 ± 1.27	3.96 ± 2.14	13.15 ± 3.84
A23187 non-induced cells	$57.06 \pm 4^*$	$20.76 \pm 3.05^*$	11.29 ± 3.05	$34.03 \pm 11.45^*$

* $P < 0.01$, compared with A23187-induced cells

4 Discussion

We were to detect the expression of GRP78 and evaluate the relationship between overexpression of GRP78 and resistance to anti-cancer drug VP-16 in human lung NCI-H460 cell line with microchip-based system. The investigation revealed two novel observations: First, the expression of GRP78 at both protein and mRNA levels were elevated by the induction of calcium ionophore A23187. Under these conditions, the induction of GRP78 could protect the cells from apoptosis induced by anti-cancer drug VP-16. Second, the results obtained by the microchip-based system were in good correlation with those by the conventional methods of Western blot and agarose gel electrophoresis for cells cultured in flasks showing more advantages over the conventional methods (Wang et al. 2007). These observations suggested that overexpression of GRP78 was correlated to chemotherapy resistance to VP-16 in human lung cancer NCI-H460 cell line and the microchip-based system as a novel technology could be applied to the study of protein and mRNA assay.

In this study, the PDMS and PMMA chips were used for the cell culture and the assay of the GRP78 protein and mRNA expression. Having the characteristic of optically transparent and biocompatible with high gas and oxygen permeability, the two kinds of chips were often utilized for biochemical analysis. PDMS and PMMA chips have special advantages respectively (Torisawa et al. 2007; Sato et al. 2003a, b and Liao et al. 2007). Our results of immunofluorescence assay for GRP78 protein on PDMS chips involved simple liquid-handling procedures, low reagent consumption and high throughput compared with

Table 3 Comparison of the specificity of slide and PDMS chip platforms in immunofluorescence analysis

Platform	Slide	PDMS chip
Sample tested one time	One	Two
Antibody consumption for one sample	50 μ l	5 μ l
Operation	Complex	Simple

the assay on the conventional slide platform (especially for consumption of the expensive antibody, Table 3). Furthermore, the whole processes from cell culture to the detection of GRP78 protein were done on a PDMS chip continuously and conveniently. Thus PDMS chips might be the ideal platform for cell-based biochemical studies involving fluorescent protein assay. Meanwhile, our results of electrophoresis on PMMA microchip for GRP78 mRNA also showed many advantages, such as time-saving, simple operation and higher sensitivity in contrast to conventional slab gel electrophoresis as reported before (Wang et al. 2007). For example, the separation time for the GRP78 products on the PMMA microchip electrophoresis was 275 s whereas it was 2 h in the gel electrophoresis; the signal of PCR product detectable on PMMA microchip electrophoresis system was at 1:50 dilution yet it was extremely faint and could easily be overlooked by the slab gel electrophoresis for the same dilution. All these indicated that the new, cheap, disposable and highly sensitive PMMA microchip electrophoresis technique should be deserved to be used in the diagnosis of cancer and other clinical diseases.

Our results of apoptosis and cycle distributions for the cells treated by VP16 also showed that overexpression of GRP78 in NCI-H460 rendered the cells more resistance to VP-16 through the change of distribution of cell cycle. But how does GRP78, an ER lumen protein, protect cells from DNA damage imposed by the topoisomerase inhibitors VP-16? Topoisomerase II α is an ATP-dependent nuclear enzyme that plays important roles in DNA replication and chromosome segregation by its ability to change the topological structure of DNA. Topoisomerase inhibitors such as VP-16 interact with the enzyme to stabilize topoisomerase-DNA complex, blocking strand-passing activity, thereby resulting in DNA breakage (Froelich-Ammon and Osheroff 1995). It had been proposed that the chaperone function of GRP78 could affect growth factor processing, creating a cell proliferation block to escape drug killing that only occurs in cycling cells (Tomida and Tsuruo 1999). Since VP-16 targets S phase cells (Brewer et al. 1999) and here we confirmed that the induction of A23187 dramatically reduced the percentage of S phase cells, we proposed that GRP78 might render the cells resist to VP-16-induced apoptosis through altering the cell cycle distribution in NCI-H460 cell line. This may also offer a therapeutic target in human lung cancer.

5 Conclusion

In summary, this study demonstrated that overexpression of GRP78 induced by calcium ionophore A23187 directly conferred resistance to VP-16 induced apoptosis in human

lung carcinoma NCI-H460 cell line through the G₁ arrest. Simple liquid-handling procedures, low reagents consumption, highly sensitive and rapid analysis for the protein and mRNA were attained by the microchip-based system. This system was valuable in the cell culture and the detection of cellular components and deserved to be studied further.

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