# Two-dimensional Gel Electrophoresis Using Immobilized pH Gradient Strips and Flamingo™ Fluorescent Gel Stain Identified Non-nuclear Proteins Possibly Related to Malignant Tumour Progression

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**Abstract.** *The understanding of tumour progression is one of the most important strategies to conquer tumour. QR-32 is a regressive murine fibrosarcoma cell line, and QRsP-11 is a progressive malignant tumour cell clone derived from QR-32. Ina recently published study a differential display analysis for the cytoplasmic proteins was shown by using two-dimensional gel electrophoresis (2-DE) making full use of isoelectric focusing capillary gels and Coomassie brilliant blue R-250 staining. Furthermore, a differential display analysis of the nuclear proteome for QR-32 and QRsP-11 was performed. The present study shows a non-nuclear proteomic differential display analysis, using 2-DE making full use of immobilized pH gradient strips and Flamingo™ fluorescent gel stain, between QR-32 and QRsP-11 to identify particular proteins which may be involved in malignant progression. In QRsP-11 25 proteins were up-regulated, including hypoxia up-regulated protein 1, and 6 were down-regulated compared with QR-32. These results suggest that the identified non-nuclear proteins showing different expression between QR-32 and QRsP-11 possibly related to malignant tumour progression.*

Tumour development and progression are the most crucial features of malignant tumours for patients who are expected to recover from cancer. Progressive tumour cells show rapid growth, invasiveness and metastatic capacity compared with regressive benign tumour cells. Progressive and regressive tumour models of murine fibrosarcoma cells (QR-32 clone and QRsP-11 clone) have been established (1). QR-32 is weakly tumourigenic and non-metastatic. QRsP-11 is a progressive malignant tumour cell clone derived from QR-32.

Proteomics is the comprehensive analysis of the total protein complement of a genome. The combination of two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) is a popular method of proteomics. The technique of 2-DE makes it possible to separate proteins according to both their charges in isoelectric focusing (IEF) gels and their weight in sodium dodecyl sulfate (SDS) gels. The 2-DE technique has unique advantages for examining the expressions of thousands of proteins simultaneously (2). We have reported many proteomic studies of QR-32 and QRsP-11 cells by using 2-DE. A differential display analysis for the expression of nuclear proteins between QR-32 and QRsP-11 showed 8 nuclear proteins, differentially regulated, including zing finger protein ZXDC in QRsP-11 compared with QR-32. However, from nuclear proteomic analysis, it was difficult to elucidate the whole mechanism of tumour progression (3). The proteomic differential display analysis for the expression of cytoplasmic proteins in QR-32 and QRsP-11 showed 11 protein spots, differentially regulated, including heat shock protein (HSP)-90 in QRsP-11 compared with QR-32 (4). However, since the identification of more proteins related to tumour progression is necessary, in the present study, immobilized pH gradient (IPG) strips and Flamingo™ fluorescent gel stain were used in place of IEF capillary gels and Coomassie brilliant blue R-250 staining to identify weakly expressed non-nuclear proteins.

## Materials and Methods

*Tumour cell lines and culture condition.* QR-32 and QRsP-11 are murine fibrosarcoma cell lines, which were established at Hokkaido University, the origin and characteristics of which have been

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described previously (4-6). Briefly, QR-32 cells are unable to grow when injected subcutaneously in normal C57Bl/6 mice and they spontaneously regress in normal syngeneic mice. QRsP-11 cells were obtained from the tumours which arose in mice after subcutaneous co-implantation of QR-32 cells with gelatin sponge, and showed strong tumorigenicity. They were cultured in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, non-essential amino acids and L-glutamine, at 37˚C, in a humidified 5% carbon dioxide-95% air mixture. We used these cell lines passaged less than 15 times culture after the cells had been sent to our laboratory.

*Sample preparation*. The non-nuclear proteins from QR-32 and QRsP-11 were extracted by means of NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instruction (6).

*Two-dimensional gel electrophoresis (2-DE)*. Isoelectric focusing (IEF) was performed in an IPGphor 3 IEF unit (GE Healthcare, Buckinghamshire, UK) on 11 cm, immobilized, pH 3-10 linear gradient strips (BIO RAD, Hercules, CA, USA) at 50 μA/strip, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a precast polyacrylamide gel with a linear concentration gradient of 5-20% (BIO RAD), run at 200 V (7, 8).

*Fluorescent gel staining*. After 2-DE and fixing the gels were stained with a fluorescent gel staining, Flamingo™ Fluorescent Gel Stain (BIO RAD), overnight. Stained gels were washed with ultra-pure water (Wako Pure Chemical Industries, Osaka, Japan) three times (9, 10).

*Image analysis and spot picking*. Expression levels of the protein spots were quantified with Progenesis SameSpot software (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK) (7, 9), and the differences in expression between QR-32 and QRsP-11 were analysed statistically by ANOVA test. 2-DE analysis was repeated three times. After statistical analysis, the gels were re-stained with See Pico™ (Benebiosis Co., Ltd, Seoul, Korea) (11), and the selected spots whose expression was significantly different between QRsP-11 and QR-32 were picked up for the mass spectrometry (MS) analysis.

*High-performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS)*. An Agilent 1100 LC/MSD Trap XCT (Agilent Technologies, Palo Alto, CA, USA) was used for HPLC and MS/MS. Protein identification was performed in the Agilent Spectrum MILL MS proteomics workbench against the Swiss-Prot protein database search engine (http://kr.expasy.org/sprot/) and the MASCOT MS/MS Ions Search engine (http:// www.matrixscience.com/search_form_select.html). The criteria for positive identification of proteins were as follows: filter by protein score≥10.0, and filter peptide by score≥8, % scored peak intensity (% SPI) >70. The Spectrum Mill workbench can search MS/MS spectra using an MS/MS ion search (12-14).

## Results

*Detection of up- and down-regulated non-nuclear protein spots in 2-DE gels*. The cytoplasmic protein marker glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected, but the nuclear protein marker lamin B1 could not be detected in the non-nuclear fraction of either QR-32 or QRsP-11 (data not shown). Figure 1A and B show 2-DE patterns of non-nuclear fractions from QR-32 and QRsP11, respectively. More than 600 protein spots were detected. Expression of 25 proteins appeared to be up-regulated, and that of 6 appeared to be down-regulated in QRsP-11 compared with QR-32.

*Identification of up- and down-regulated protein spots*. These 31 spots were picked up and identified by using Agilent 1100 LC/MSD Trap XCT LC-MS/MS system. The results are shown in Table I.

## Discussion

This proteomic differential display analysis by MS and 2-DE using IPG strips and Flamingo™ fluorescent gel stain in place of IEF capillary gels and Coomassie brilliant blue R-250 staining for non-nuclear proteins from regressive murine fibrosarcoma cell clone QR-32 and its derived malignant progressive cell clone QRsP-11 showed 6 down-regulated and 25 up-regulated non-nuclear proteins in QRsP-11 compared with QR-32 cells.

Hypoxia up-regulated protein 1, an endoplasmic reticulum chaperone, is essential for the maintenance of cellular viability under hypoxia and is reported to be overexpressed in tumour cells. Namba *et al*. reported that up-regulation of this protein reduces the antitumor activity of celecoxib, a cyclooxygenase-2 (COX-2)-selective non-steroidal anti-inflammatory drugs (NSAID), by inhibiting apoptosis (15). Stojadinovic *et al*. reported that it was up-regulated in some invasive breast tumours and its up-regulation appeared to be associated with indicators of poor prognosis and metastasis (16). This protein was up-regulated in QRsP-11 cells here.

Heat-shock 70 kDa (HSP70) protein 4 is one of several heat-shock 70 kDa proteins (17). Some reports showed the relation of HSP70 proteins to cancer progression. Garg *et al*. reported that HSP70-2 expression was associated with early spread and progression of urothelial carcinoma of bladder cancer and that HSP70-2 may be a potential therapeutic target for bladder urothelial carcinoma (18). Ramp *et al*. reported that HSP70-mediated inhibition of apoptosis seems to be of minor importance for carcinogenesis and tumor progression in renal cell carcinoma cells (19). This protein was also up-regulated in QRsP-11 cells here.

It has also been reported that HSP90 is thought to be a very important molecule for cancer progression (10, 20). Some inhibitors have been reported and therapies using them are also being carried out (21). HSP90 was also up-regulated in QRsP-11. These reports from other groups support our results here.

Alpha-enolase is an isoenzyme of enolase, a key protein catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway. Takashima *et al*. reported that expression of alpha-enolase correlated

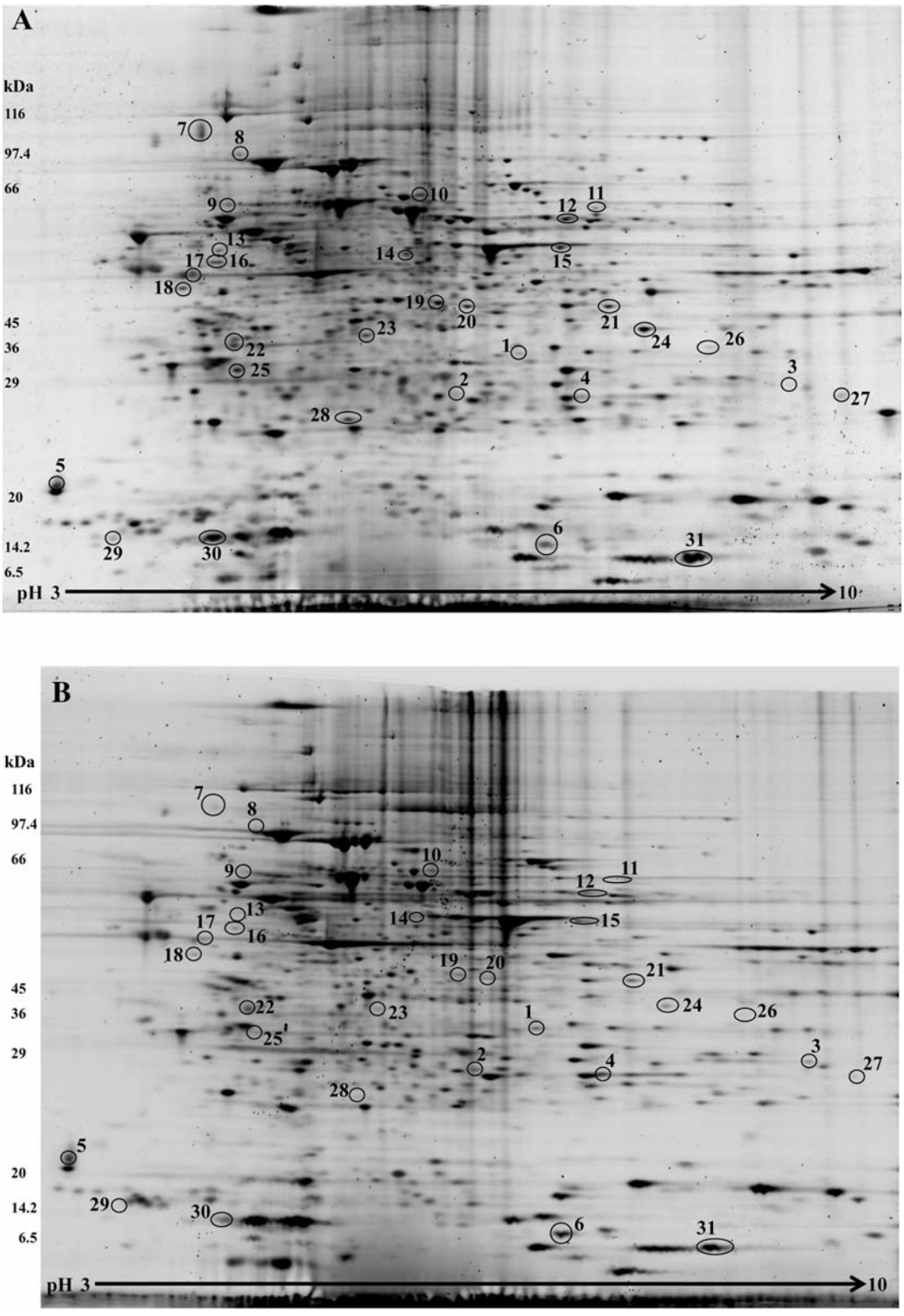


Figure 1. *2-DE patterns of non-nuclear proteins. Panels (A) and (B) show the 2-DE patterns of the non-nuclear fraction of QRsP-11 and QR-32, respectively. Proteins (80 μg) were separated by 2-DE using a pH 3-10 gradient in the first dimension, followed by second-dimensional separation on a precast polyacrylamide gel with a linear concentration gradient of 5-20% stained with Flamingo™ Gel Stain. The intensity of spots 1-6 decreased, while spots 7-31 increased in QRsP-11.*

Table I. *Identification of up- and down-regulated non-nuclear proteins in QRsP-11 compared to QR32. Spot numbers refer to those shown in Figure 1. These spots were cut out and were subsequently identified by MS.*

| Spot | Protein name | Accession No. | Theoretical | | Distinct peptides | Sequence coverage (%) | MS/MS search score | Change in QRsP-11 (fold) |
|---|---|---|---|---|---|---|---|---|
| | | | *p*I | Mr | | | | |
| | | | | | | | | Down-regulated |
| 1 | Pyrroline-5-carboxylate reductase 1, mitochondrial | Q922W5 | 6.36 | 32,373.6 | 10 | 40 | 142.76 | 2.0 |
| 2 | Proteasome subunit alpha | Q9QUM9 | 6.35 | 27,372.6 | 1 | 5 | 14.78 | 2.1 |
| 3 | Proline synthetase co-transcribed bacterial homolog protein | Q9Z2Y8 | 8.37 | 30,048.8 | 2 | 10 | 26.16 | 1.9 |
| 4 | Triosephosphate isomerase | P17751 | 6.90 | 26,712.8 | 9 | 46 | 141.83 | 1.6 |
| 5 | Calmodulin | P62204 | 4.09 | 16,837.7 | 5 | 54 | 72.65 | 1.5 |
| 6 | Cystatin-B | Q62426 | 6.82 | 11,045.7 | 3 | 26 | 41.48 | 1.9 |
| | | | | | | | | Up-regulated |
| 7 | Hypoxia up-regulated protein 1 | Q9JKR6 | 5.12 | 111,181.6 | 1 | 1 | 14.00 | 2.9 |
| 8 | Heat-shock 70 kDa protein 4 | Q61316 | 5.15 | 94,133.1 | 2 | 3 | 22.94 | 2.6 |
| 9 | Heat-shock protein 90-beta 1 | P08113 | 4.74 | 92,476.2 | 3 | 4 | 43.86 | 2.0 |
| 10 | T-complex protein 1 subunit alpha B | P11983 | 5.82 | 60,449.0 | 10 | 24 | 140.95 | 2.0 |
| 11 | Pyruvate kinase isozymes M1/M2 | P52480 | 7.17 | 57,845.2 | 3 | 9 | 45.71 | 1.8 |
| 12 | Pyruvate kinase isozymes M1/M2 | P52480 | 7.17 | 57,845.2 | 10 | 28 | 134.26 | 1.6 |
| 13 | Cytoskeleton-associated protein 4 | Q8BMK4 | 5.46 | 63,692.4 | 4 | 8 | 55.47 | 1.8 |
| 14 | Alpha-enolase | P17182 | 6.37 | 47,141.1 | 11 | 37 | 170.94 | 2.2 |
| 15 | Alpha-enolase | P17182 | 6.37 | 47,141.1 | 7 | 20 | 100.46 | 1.5 |
| 16 | Vimentin | P20152 | 5.06 | 53,687.9 | 4 | 10 | 55.08 | 2.3 |
| 17 | Heat-shock protein 90-alpha | P07901 | 4.93 | 84,788.3 | 2 | 3 | 29.96 | 2.3 |
| 18 | Reticulocalbin-1 | Q05186 | 4.70 | 38,113.2 | 3 | 10 | 32.15 | 1.8 |
| 19 | Pyruvate kinase isozymes M1/M2 | P52480 | 7.17 | 57,845.2 | 5 | 12 | 76.40 | 2.1 |
| 20 | Pyruvate kinase isozymes M1/M2 | P52480 | 7.17 | 57,845.2 | 5 | 12 | 80.20 | 1.7 |
| 21 | Annexin A1 | P10107 | 6.97 | 38,734.5 | 3 | 12 | 44.54 | 1.5 |
| 22 | Annexin A5 | P48036 | 4.83 | 35,752.6 | 8 | 28 | 108.00 | 1.8 |
| 23 | Transitional endoplasmic reticulum ATPase | Q01853 | 5.14 | 89,322.3 | 5 | 7 | 70.18 | 1.8 |
| 24 | Glyceraldehyde-3-phosphate dehydrogenase | P16858 | 8.43 | 35,810.2 | 10 | 40 | 157.23 | 1.9 |
| 25 | 60 kDa Heat-shock protein, mitochondrial | P63038 | 5.91 | 60,955.8 | 12 | 26 | 155.28 | 5.0 |
| 26 | Voltage-dependent anion-selective channel protein 2 | Q60930 | 7.44 | 31,733.0 | 3 | 12 | 36.34 | 1.7 |
| 27 | Glutathione S-transferase Mu 1 | P10649 | 7.72 | 25,970.2 | 2 | 12 | 30.58 | 2.4 |
| 28 | Adenylate kinase isoenzyme 1 | Q9R0Y5 | 5.67 | 21,539.7 | 3 | 16 | 45.01 | 3.7 |
| 29 | 60S Acidic ribosomal protein P2 | P99027 | 4.42 | 11,651.0 | 3 | 63 | 43.33 | 2.3 |
| 30 | Thioredoxin | P10639 | 4.80 | 11,675.5 | 4 | 31 | 48.08 | 1.6 |
| 31 | 10 kDa Heat-shock protein, mitochondrial | Q64433 | 7.91 | 10,962.8 | 6 | 74 | 73.80 | 1.6 |

positively with tumor size and venous invasion (16). In response to up-regulated alpha enolase expression, the fibrinolytic system might be inordinately accelerated, and increased local fibrinolysis may contribute to cancer cell invasion and metastasis.

Vimentin is a type III intermediate filament in cells of mesenchymal origin, and acts as a scaffolding protein to stabilise connective tissues and cells. Many reports have shown that vimentin plays important roles in the epithelial-to-mesenchymal transition, invasion and metastasis (22, 23).

Reticulocalbin-1 is a member of the family of $Ca^{2+}$-binding proteins. This molecule is localized in the endoplasmic reticulum and functions in the secretory pathway of cells. Liu *et al*. reported that reticulocalbin was up-regulated in highly invasive breast cancer cell line compared with poorly invasive cell line (24). QRsP-11 cells are very invasive and metastatic. The result of up-regulation of alpha-enolase, vimentin and reticulocalbin-1 in QRsP-11 cells here supports their specific features.

Annexin A1 is a member of the annexin superfamily. It plays a role in membrane fusion and exocytosis. Links between the up-regulation of annexin A1 and progression of breast cancer have been reported (25).

Although these results demonstrated that some of proteins identified in present study may have a functional potentiality of tumor progression, further studies still necessary.

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