Proteomic Analysis for Nuclear Proteins Related to Tumour Malignant Progression: A Comparative Proteomic Study Between Malignant Progressive Cells and Regressive Cells

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Abstract. *Tumour development and progression consists a series of multiple changes in gene expression. Progressive tumour cells acquire more aggressive properties manifested by rapid growth, invasiveness and metastatic ability, as well as increased genetic instability leading to multiple genetic alterations. Therefore, it is crucial to identify the possible intracellular and extracellular molecular mechanisms that accelerate tumour progression, in particular to identify nuclear proteins which interact with DNA. Nuclear proteomics provides an opportunity to qualitatively and quantitatively examine protein effectors that contribute to cellular phenotype. This study performed a differential display analysis for the expression of nuclear proteome between regressive tumour cell clone QR-32 and malignant progressive tumour cell clone QRsP-11 using two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS). Eight nuclear proteins whose expressions were different between QR-32 and QRsP-11 cells were identified. Seven of those protein spots, zing finger protein ZXDC, lamin-A/C, far upstream clement-binding protein 1, heterogeneous nuclear ribonucleoprotein K, heteroge-neous nuclear ribonucleoprotein A/B and guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1, were down-regulated in QRsP-11, while one protein, nucleolin, was up-regulated in QRsP-11.*

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Tumour development and progression can be said to be the most crucial and serious features of malignant tumours, since progressive tumour cells show rapid growth, invasiveness and metastatic capacity compared with regressive tumour cells. Inflammation is recognised as a risk factor for human cancer progression (1). Some reports have shown a possible relationship between inflammation and carcinogenesis in organs such as the oesophagus, stomach, pancreas, liver, bile duct and colon (2, 3). Regressive and inflammation-associated progressive tumour models of murine fibrosarcoma cells (QR-32 clone and QRsP clone) have been established by Okada *et al*. (4-6). QR-32 is a clone of QR clones, which are weakly tumourigenic and non-metastatic. QR-32 cells regress spontaneously after injection of up to 2×10^5 cells subcutaneously or 1×10^6 cells intravenously in normal syngeneic mice. However, they grow progressively when they are subcutaneously co-implanted with gelatin sponge. Many culture tumour lines (QRsP) have been established, and they have the ability to grow progressively in mice, even in the absence of gelatin sponge. QRsP clones are more tumourigenic and metastatic malignant tumour cells than QR-32 cells.

Proteomics approaches are useful in investigating the difference between tumour regressive QR-32 and progressive QRsP, because they can identify qualitative and quantitative changes of proteins. In recent study, a proteomic differential display analysis was performed for the expression of cytoplasmic proteins in QR-32 and QRsP-11 by using two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) (7). Nine protein spots were up-regulated in QRsP-11 cells and identified as calreticulin precursor, tropomyosin 1 alpha chain, annexin A5, heat shock protein (HSP)90-alpha, HSP90-beta, phosphatidylethanolamine-binding protein and peroxiredoxin 2, and two protein spots

were down-regulated in QRsP-11 cells and identified as acidic leucine-rich nuclear phosphoprotein 32 family member E and hepatoma-derived growth factor (7).

Many important events such as replication and transcription occur in the nucleus, for which the nucleus needs many proteins. It is important to understand what kind of proteins are coordinated and regulated in the nucleus in order to understand the molecular mechanism of tumour progression.

The objective of this study was to identify, by protemics methods, the nuclear proteins whose expressions are different between QR-32 and QRsP-11 using 2-DE and MS. The comparison of the differential expression of proteins between single-cell-originated benign tumour cells and their derived malignant progressive tumour cells is beneficial for detecting important factors in tumour progression due to their very close genetic backgrounds.

Materials and Methods

Tumour cell lines and culture condition. The origin and characteristics of QR-32 and QRsP-11 murine fibrosarcoma cell lines used in this study have been described previously (8, 9, 10). Briefly, a transplantable fibrosarcoma BMT-11 was induced by 3-methyl-cholanthrene in a C57BL/6 mouse, and a tumourigenic clone BMT-11 cl-9 was subsequently isolated by limiting dilution. BMT-11 cl-9 cells were exposed *in vitro* to quercetin, giving rise to a number of random subclones. These subclones are unable to grow when injected subcutaneously (1×10^5 cells) in normal C57Bl/6 mice and they spontaneously regress in normal syngeneic mice. These variants were named QR clones, representing quercetin-induced regressive tumour. The QR-32 clone, which is one of the variant cell clones, and which form aggressive tumours upon co-implantation with a gelatin sponge was used in this study. Several tumour cell lines were obtained from the tumours which arose in mice after subcutaneously co-implantation of 1×10^5 QR-32 cells with gelatin sponge (Spongel, Yamanouchi Pharm. Co. Ltd. Japan). These lines were named QRsP-1, -2, -3, -4 *etc*. QRsP-11, one of the QRsP clone cells, was used as a progressor in this study. QR-32 and QRsP-11 were cultured in Eagle's minimum essential medium supplemented with 10% FBS, sodium pyruvate, non-essential amino acids and L-glutamine, at 37˚C, in a humidified 5% carbon dioxide –95% air mixture.

Sample preparation. The 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. Extracted nuclear protein fraction and cytoplasmic protein fraction were concentrated by using microcon YM-3 (Millipore Corporation, Bedford, MA, USA). Their protein concentration was determined by Lowry's method (11).

Two-dimensional gel electrophoresis (2-DE). Eighty μg of protein was used for each 2-DE. For the first dimension, 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. Samples were mixed with 200 μl of rehydration buffer (8 M urea, 2% CHAPS, 0.01% bromophenol blue, 1.2% Destreak reagent (GE Healthcare) and 0.5% IPG buffer (GE Healthcare) and loaded into the IPGphor strip holder (GE Healthcare). IEF was performed in the following voltage program: rehydration for 10 h (no voltage), a stepwise increase from 0 to 500 V for 4 h, 500 to 1.000 V for 1 h, 1,000 to 8,000 V for 4 h, a linear increase from 8,000 V for 20 min, and a final phase of 500 V from 20,000 to 30,000 Vh. In the second dimension, SDS-PAGE was performed on a precast polyacrylamide gel with a linear concentration gradient of 5-20% (BIO RAD), run at 200 V (12).

Fluorescent gel staining. Agitation was carried out at all stages. After 2-DE, the gels were subjected to fixing solution with 40% ethanol and 10% acetic acid for 2 h. The gels were stained with a fluorescent gel staining, Flamingo™ Fluorescent Gel Stain (BIO RAD) overnight. Stained gels were washed with Milli-Q water three times (13).

Image analysis and spot picking. The positions of the protein spots on the gels were recorded by using the ProEXPRESS 2D Proteomic Imaging System (PerkinElmer Inc, Waltham, MA, USA). Expression levels of the proteins were quantified by analysing the intensity of each spot with Progenesis PG200 software (Nonlinear Dynamics Ltd Newcastle Upon Tyne, UK) (13). The differences in expression between QR-32 and QRsP-11 were analysed statistically by the Student's *t*-test. 2-DE analysis was repeated three times. After statistical analysis, the gels were re-stained with See Pico™ (Benebiosis Co, Ltd, Seoul, Korea), and the selected spots whose expression was significantly different between QR-32 and QRsP-11 were cut and removed for the MS analysis.

In-gel digestion. The See Pico dye was removed from the gel piece by rinsing three times in 60% methanol, 50 mM ammonium bicarbonate, and 5 mM DTT for 15 min. The sample in the gel piece was reduced twice in 50% ACN, 50 mM ammonium bicarbonate, and 5 mM DTT for 10 min. The gel piece was dehydrated in 100% ACN twice for 30 min, and then rehydrated with an in-gel digestion reagent containing 10 μg/ml sequencing-grade-modified trypsin (Promega, Madison, WI, USA) in 30% ACN, 50 mM ammonium bicarbonate, and 5 mM DTT. This procedure for in-gel digestion was performed overnight at 30˚C. The samples were lyophilized overnight with the use of Labconco Lyph-lock 1L Model 77400 (Labconco, Kansas, MO, USA). Lyophilized samples were dissolved in 0.1% formic acid (14).

LC-MS/MS. Samples dissolved in 0.1% formic acid were centrifuged at 21,500 g for 5 min and the supernatant was stored at –80˚C as samples for MS until use. An Agilent 1100 LC/MSD Trap XCT (Agilent Technologies, Palo Alto, CA, USA) was used for HPLC and MS/MS. 25 μof each sample was applied and separated on a column (Zorbax 300SB-C18, 75 μm, 150 mm, Agilent Technologies). The Agilent 1100 capillary pump was operated under the following conditions: Solvent A: 0.1% formic acid; Solvent B: ACN in 0.1% formic acid. Column flow: 0.3 μl/min, primary flow: 300 μl/min. Gradient: 0-5 min 2% B, 60 min 60% B. Stop time: 60 min. 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

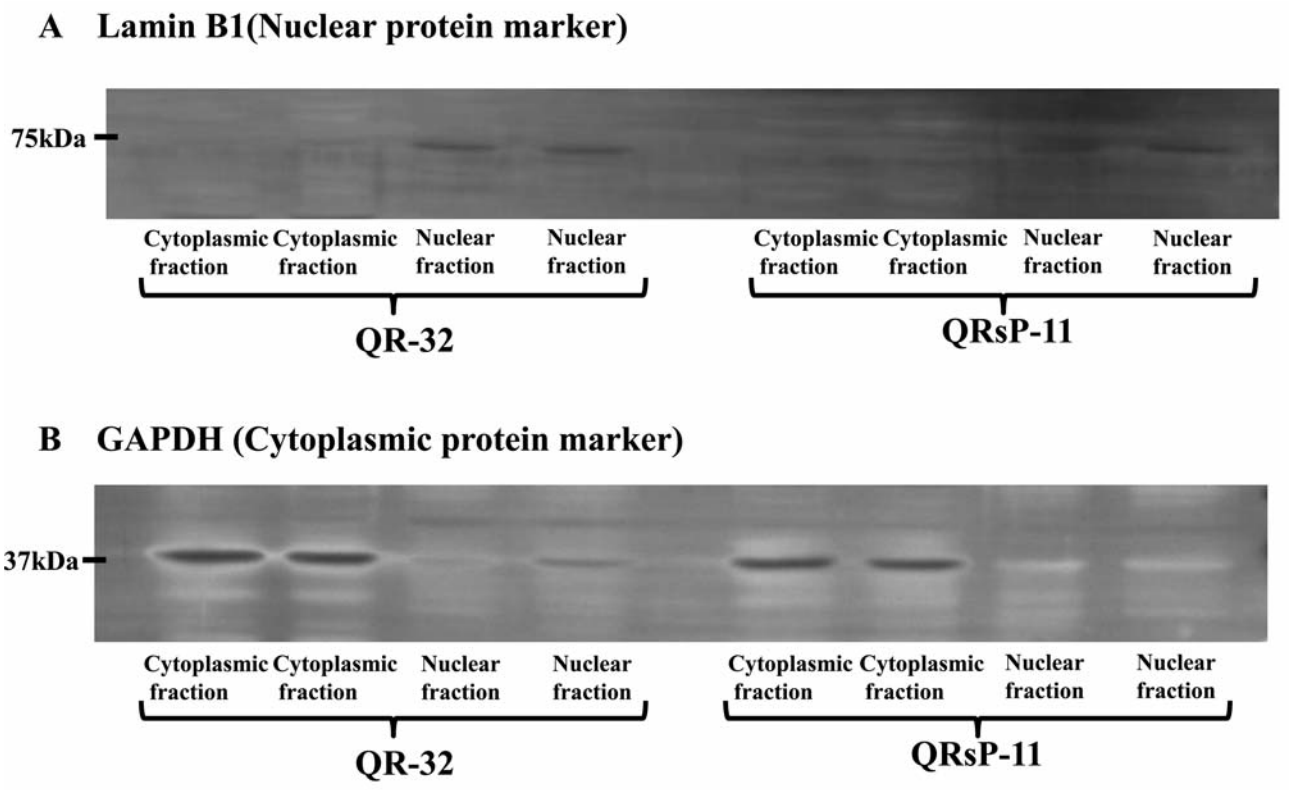

Figure 1. *Highly purified sample of nuclear fraction. In panel (A), lamin B1 of a nucleolar protein marker was detected only in nuclear extraction. In panel (B), GAPDH of a cytoplasmic protein marker was detected only in the cytoplasmic fraction, but not in the nuclear fraction.*

proteins were as follows; filter by protein score>10.0, and filter peptide by score>8, % scored peak intensity. The Spectrum Mill workbench can search MS/MS spectra using an MS/MS ion search (15, 16).

Western blot analysis. After SDS-PAGE, each gel ws transferred electrophoretically onto PVDF membranes (immobilon; Millipore, Bedford, MA, USA) and blocked overnight at 4˚C with TBS containing 5% skimmed milk. Primary antibodies were anti-GAPDH affinity purified goat polyclonal antibody raised against a peptide mapping within an internal region of GAPDH of human origin (dilution range 1: 200) and anti-lamin B1 affinity purified goat polyclonal antibody raised against a peptide mapping at the carboxy terminus of lamin B of human origin (dilution range 1: 200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). For each, membranes were incubated with primary antibody for 1h at room temperature, washed three times with TBS containing 0.05% Tween-20 and one time with TBS, and then incubated with horseradish peroxidase-conjugated secondary antibody (dilution range 1:5.000; Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA) for 1 h at room temperature, and developed with a chemifluorescence reagent (ECL Plus Western Blotting Detection Reagents; GE Healthcare). The immunoreactive protein bands were then obtained by using the ProEXPRESS 2D Proteomic Imaging System (PerkinElmer Inc) (17).

Results

Separation of nuclear and cytoplasmic proteins from QR-32 and QRsP-11 cells. To yield a highly pure nuclear fraction, nuclear proteins were extracted by using NE-PER nuclear and cytoplasmic extraction reagent kit. As shown in Figure 1A, nuclear protein marker of lamin B1 was detected in nuclear fraction of both QR-32 and QRsP-11, but not detected in cytoplasmic fraction of both QR-32 and QRsP-11. Figure 1B shows that GAPDH, a cytoplasmic protein marker, could not be detected in the nuclear fraction, but was detected in cytoplasmic fraction. This suggested that this preparation of the nuclear fraction resulted in a highly pure detection.

Detection of up-regulated or down-regulated nuclear protein spots in 2-DE gels and identification of them. Protein expression was assessed in each three samples of QR-32 nuclear fraction, QRsP-11 nuclear fraction, QR-32 cytoplasmic fraction and QRsP-11 cytoplasmic fraction. Figure 2A and B show 2-DE patterns of nuclear fractions, and Figure 2C and D show 2-DE patterns of cytoplasmic fractions. The nuclear and cytoplasmic fractions show quite

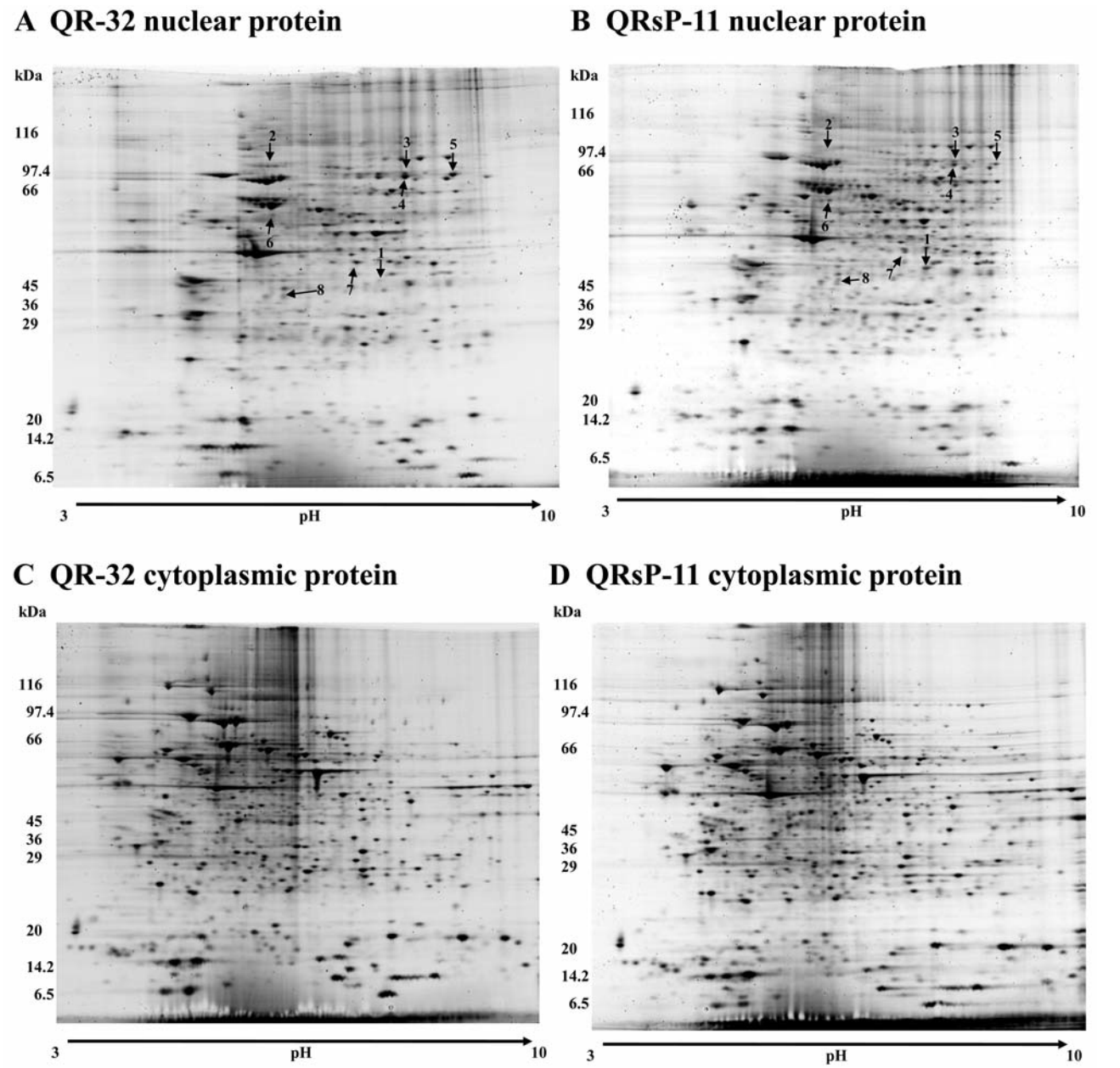

Figure 2. *2-DE patterns of nuclear fraction. Panels (A) and (B) show the 2-DE patterns of the nuclear fraction of QR-32 and QRsP-11, respectively. Panels (C) and (D) show the 2-DE patterns of the cytoplasmic fraction of QR-32 and QRsP-11, respectively. Proteins (80 μg) were separated by 2-DE using a 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. Spot 1 increased, while spots 2-8 decreased in QRsP-11.*

different 2-DE patterns. More than 300 protein spots were visualised on the 2-DE gels, and the differences in spot intensities between QR-32 and QRsP-11 were compared visually for each gel. Seven spots appeared to be down-regulated in QRsp-11 compared to QR-32, but only one spot appeared to be up-regulated in QRsp-11 compared to QR-32 (Figure 2A, B). These eight spots, which were clearly identified clearly using Agilent 1100 LC/MSD Trap XCT, were cut out from the each gel, digested and lyophilized for MS analysis. These results were shown in Table I. The expressional comparisons of each spot by fluorescent staining are shown in Figure 3.

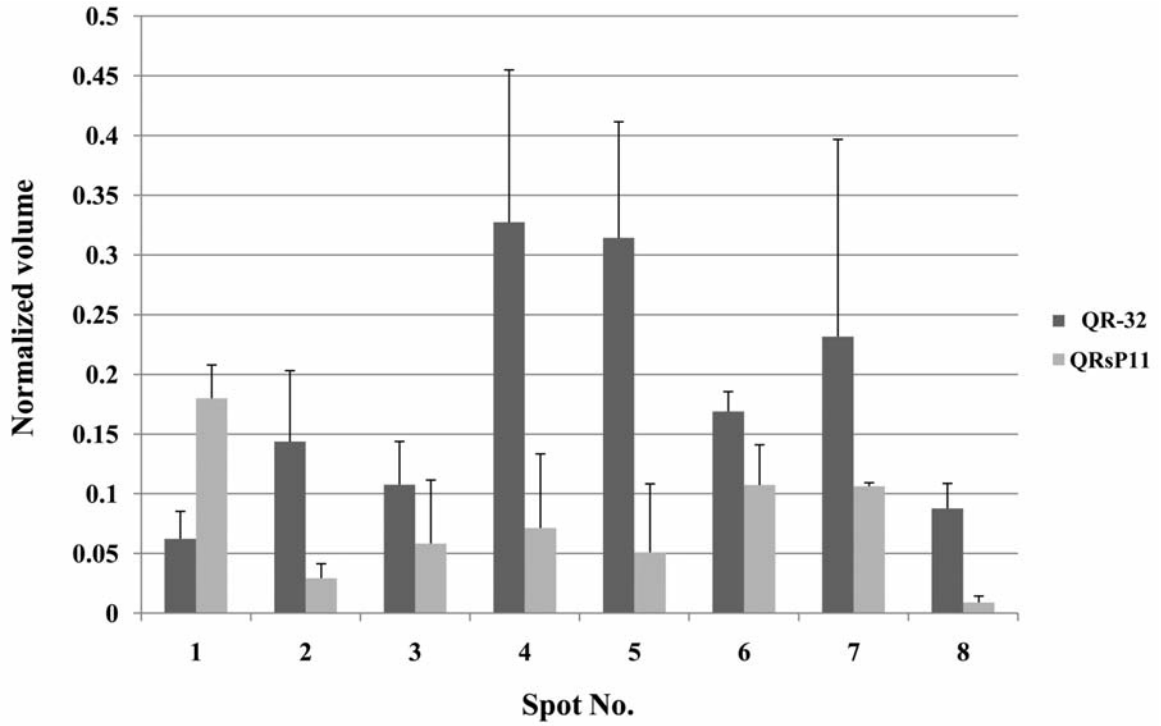

Figure 3. *The comparison of each spot between QR-32 and QRsP-11. Volume analysis for eight protein spots. Protein levels of the spots were quantified by analyzing normalized volume, the intensity and the area of each spot in the total of them. The differences in expression between QR-32 and QRsP-11 were analyzed by Student's t-test. The data are represented as mean+SD. The spot numbers correspond to those in Figure 2A and B. All data between QR-32 and QRsP-11 are significant ($p<0.05$).*

Discussion

This study reports a nuclear proteomic differential display analysis of regressive murine fibrosarcoma cell clone QR-32 and its derived malignant progressive cell clone QRsP-11, using 2-DE and MS to identify nuclear proteins that may play roles in tumour progression. Seven down-regulated nuclear protein spots in QRsP-11 cells were identified compared with QR-32 cells. These proteins were identified to be zing finger protein ZXDC, lamin-A/C, far upstream clement-binding protein 1, heterogeneous nuclear ribo-nucleoprotein K, heterogeneous nuclear ribonucleoprotein A/B and guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1. There was only one up-regulated protein in QRsP-11. which was identified as nucleolin.

Nucleolin is a major nucleolar protein of exponentially growing eukaryotic cells, which is directly involved in the regulation of ribosome biogenesis and maturation. It is found associated with intranucleolar chromatin and pre-ribosomal particles, and it induces chromatin decondensation by binding to histone H1. It is thought to play a role in pre-rRNA transcription, ribosome assembly, cell proliferation and growth (18, 19). Many reports have shown a relationship between up-regulation of nucleolin and tumourigenesis and tumour progression. Mourmouras *et al*. reported that an altered nuclear nucleolin expression seemed to accompany melanoma progression (20), while Grinstein and Wernet reported that nucleolin was abundant not only in proliferating cancerous cells but also in cancer stem cells, and high levels of nucleolin expression were related to poor clinical prognosis for certain types of cancer (21). Matrix-metalloproteinases (MMPs), which are able to degrade extra cellular matrix components, are crucial in metastasis. Fahling *et al*. reported that the elevated binding of nucleolin to the 3' untranslated regions of MMP-9 mRNA may be important for the increased efficiency of MMP-9 translation (22). Furthermore Yang *et al*. showed that binding of up-regulated nucleolin to the proliferating cell nuclear antigen (PCNA) inhibited nucleotide excision repair directly, and it contributed to carcinogenesis (23).

Zinc finger protein ZXDC cooperates with the class II transactivator CIITA to promote transcription of MHC class I and MHC class II genes (24). From the viewpoint of tumour immunosurveillance, down-regulation of MHC class I and II may be a cause of carcinogenesis and malignant progression of tumour (25).

Lamins are components of the nuclear lamina, a fibrous layer on the nucleoplasmic side of the inner nuclear membrane, which is thought to provide a framework for the nuclear envelope and may also interact with chromatin. Roth *et al*. performed

Table I. *Identification of nuclear proteins which are differentially expressed between QR-32 and QRsP-11.*

Spot no.	Protein name	Accession no.	Theoretical *p*I	Theoretical Mr	Distinct peptides	Sequence coverage (%)	MS/MS search score	Change in QRsP-11 (fold change)
1	Nucleolin	P09405	4.69	76,723.4	1	4	12.88	Increase (2.888)
2	Zinc finger protein ZXDC	Q8C8V1	7.94	90,755.6	1	2	11.75	Decrease (0.204)
3	Lamin-A/C	P48678	6.54	74,237.9	3	7	42.27	Decrease (0.542)
4	Far upstream element-binding protein 1	Q91WJ8	7.73	68,539.9	3	6	36.08	Decrease (0.218)
5	Far upstream element-binding protein 1	Q91WJ8	7.73	68,539.9	3	6	36.08	Decrease (0.218)
6	Heterogeneous nuclear ribonucleoproteinK	P61979	5.39	50,976.5	3	6	39.66	Decrease (0.635)
7	Heterogeneous nuclear ribonucleoproteinA/B	Q99020	7.69	30,831.4	2	7	24.55	Decrease (0.459)
8	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	P62874	5.60	37,377.2	2	7	21.84	Decrease (0.103)

Spot numbers refer to those in Figure 2A and B. Eight discrete changes in protein expression by 2-DE gels in QR-32 and QRsP-11 cells. These spots were cut out and 8 spots were subsequently identified by MS. Spots shown increased or decreased by more than 1.5-fold ($p<0.05$).

differential expression proteomics study on a human syngeneic cellular *in vitro* progression model of the colorectal adenoma-to-carcinoma sequence, and showed down-regulation of lamin A/C (26). Hutchison *et al*. reported the relation of up-regulated lamin A/C and progression of colorectal cancer (27, 28).

In QRsP-11 cells, two down-regulated protein spots having same molecular weight and different isoelectric points were identified as far upstream element-binding protein-1 (FBP-1). FBP-1 binds to an upstream element of the c-myc promoter and regulates the *c-myc* mRNA level. It is thought to act both as activator and repressor of transcription (29). However, two reports showed up-regulation of FBP-1 in hepatocellular carcinoma cells (30, 31).

Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is a member of the hnRNP family. This family has several different cellular roles such as transcription, mRNA shuttling, RNA editing and translation. Although Zhang *et al*. reported down-regulation of hnRNP K in a human colorectal cancer cell line LoVo having higher metastatic potential compare with SW480 having lower metastatic potential (32), several reports showed up-regulation of hnRNP K in colorectal cancer, oral squamous cell carcinoma and prostate cancer (33, 34). Though this discrepancy was not demonstrated in this study, the down-regulation of FBP-1 and hnRNP K in QRsP-11 is an interesting event.

Heterogeneous nuclear ribonucleoprotein A/B (hnRNP A/B) is a transcriptional repressor which binds to CArG box motifs, single-stranded and double-stranded DNA, and RNA, and it forms the core of the ribonucleoprotein complex that associates with nascent transcripts in eukaryotic cells (35). Gao *et al*. reported that binding of hnRNP-A/B to the osteopontin promoter significantly decreases osteopontin promoter activity, mRNA levels and protein expression of osteopontin. These results inhibited osteopontin dependent metastatic behavior, motility, and invasion of cancer cells (36).

The current study could not prove whether over-expression of nucleolin is associated with progression, because of lack of functional and structural information in nucleolin. However, it is suggested that these proteins have potential as functional proteins in progressive fibrosarcoma cells induced by foreign body-promoted inflammatory cells.

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