Proteomic Analysis Showed Down-regulation of Nucleophosmin in Progressive Tumor Cells Compared to Regressive Tumor Cells

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Abstract. *Important strategies against cancer are based on the understanding of the mechanisms of tumor progression. To elucidate alterations regarding tumor progression, we have performed proteomic differential display analysis for the expression of intracellular proteins in the regressive murine fibrosarcoma cell clone QR-32 and the progressive malignant tumor cell clone QRsP-11, derived from QR-32, by means of combination of two-dimensional gel electrophoresis (2-DE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS), and we have previously reported on relevant results. However, besides the protein spots which we already reported, we identified three more particular spots of interest. In the present study, two-dimensional western blot analysis demonstrated a significantly lower expression of three isoforms of nucleophosmin in progressive, compared to regressive cell clones. These results suggest that the down-regulation of the identified nucleophosmin proteins in QRsP-11 cells compared to QR-32 cells is possibly related to tumor malignant progression.*

The most crucial features of malignant tumors are unpredictable development and progression. Progressive tumor cells show rapid growth, unrestricted proliferation activity, serious invasiveness and disorderly metastatic capacity, compared to regressive benign tumor cells. Okada *et al*. have established progressive and regressive murine fibrosarcoma tumor models (QR-32 clone and QRsP-11 clone) (1, 2). The regressive clone QR-32 is a weakly tumorigenic and non-metastatic cell clone. The progressive clone QRsP-11, on the other hand, is a progressive malignant tumor cell clone derived from QR-32. After injection of 1×10^6 cells intravenously, or up to 2×10^5 cells subcutaneously, in normal syngeneic mice, QR-32 cells regress spontaneously. However, when they are subcutaneously co-implanted with gelatin sponge, they grow progressively. After these progressively-growing cells were established as cell lines (QRsP), they had the ability to progressively grow in mice, even in the absence of gelatin sponge. The characteristic feature of QRsPs as malignant tumor cell clones is that they are more tumorigenic and metastatic, and QRsP-11 is one such QRsP clone.

The aim of this study was to identify the differentially expressed proteins between the clones QR-32 and QRsP-11. The comparison of the differential expression of proteins between benign tumor cells of single-cell origin and their derived malignant tumor cells is beneficial in detecting various important factors in inflammation-associated tumor progression. We have reported many proteomic studies of QR-32 and QRsP-11 cells by using two-dimensional gel electrophoresis (2-DE) (3-5). The differential display analysis for the expression of nuclear proteins between QR-32 and QRsP-11 showed eight nuclear proteins to be differentially-regulated, including zing finger protein ZXDC, in QRsP-11 compared with QR-32 cells (4). The proteomic differential display analysis for the expression of cytoplasmic proteins in QR-32 and QRsP-11 cells showed 11 spots for differentially regulated proteins, including heat-shock protein (HSP)-90 in QRsP-11 compared with QR-32 cells (3). Our recent 2-DE analysis of QR-32 and QRsP-11 cells showed three spots for down-regulated proteins in the progressive malignant tumor cell clone QRsP-11, compared to QR-32 cells, which were not identified in previous studies. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) identified these

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three spots as nucleophosmin. In the present study, we investigated these three spots by means of 2-D western blot analysis with an antibody against nucleophosmin.

Materials and Methods

Tumor cell lines and culture conditions. QR-32 and QRsP-11 are murine fibrosarcoma cell lines which were established at the Hokkaido University, the origin and characteristics of which have been previously described (1-6). Briefly, QR-32 cells are unable to grow when injected subcutaneously in C57Bl/6 mice and they spontaneously regress in syngeneic mice. QRsP-11 cells were obtained from the tumors which arose in mice after subcutaneous co-implantation of QR-32 cells with gelatin sponge, and show 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. We used these cell lines passaged fewer than 10 times in culture after the cells had been sent to our laboratory from the Division of Cancer Pathobiology, Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan.

Sample preparation. Cells were homogenized in lysis buffer [50 mM Tris-HCl (pH 7.5), 165 mM sodium chloride, 10 mM sodium fluoride, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% NP-40] on ice. After centrifugation at 21,500 ×*g* for 30 min at 4˚C, the supernatants were used for sample analysis (7).

Two-dimensional gel electrophoresis (2-DE). Eighty micrograms of protein were used for each 2-DE. For the first dimension, isoelectric focusing (IEF) was performed in an IPGphor 3 IEF unit (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK) on 11 cm, immobilized, pH 3-10 linear gradient strips (Bio Rad, Hercules, CA, USA) at 50 μA/strip. Samples were dissolved in 200 μl of rehydration buffer [8 M urea, 2% CHAPS, 0.01% bromophenol blue, 1.2% Destreak reagent (GE Healthcare)] and loaded into the IPGphor strip holder (GE Healthcare). IEF was performed using 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 4h; 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, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a pre-cast polyacrylamide gel with a linear concentration gradient of 5-20% (Bio Rad), ran at 200 V (8).

Fluorescent gel staining. After 2-DE, the gels were washed with ultrapure water three times, and then fixed in 40% ethanol and 10% acetic acid solution for 2 h. The gels were stained with a fluorescent gel staining, Flamingo™ Fluorescent Gel Stain (Bio-Rad), overnight. Stained gels were washed with ultrapure water (Wako Pure Chemical Industries, Osaka, Japan) three times (9, 10).

Image analysis and spot picking. The gels were scanned using a ProEXPRESS 2D Proteomic Imaging System (Perkin Elmer, Waltham, MA, USA). Expression levels of the protein spots were quantified with the Progenesis SameSpot software (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK) (7, 9), and the differences in expression between QR-32 and QRsP-11 cells were analyzed statistically by ANOVA test, with $p<0.05$ being considered significant. Two-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 cells were picked-up for MS analysis.

In-gel digestion. The See Pico™ dye was removed by washing three times in 60% methanol, 0.05 M ammonium bicarbonate, and 0.005 M DL-dithiothreitol (DTT) for 15 min. The sample in the gel piece was reduced twice in 50% acetonitrile (ACN), 0.05 M ammonium bicarbonate, and 0.005 M DTT for 10 min. The gel pieces were dehydrated twice in 100% ACN for 30 min and incubated with an in-gel digestion reagent containing 10 μg/ml sequencing-grade modified trypsin (Promega, Madison, WI, USA) in 30% ACN, 0.05 M ammonium bicarbonate, and 0.005 M 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) (7). Lyophilized samples were then dissolved in 0.1% formic acid (12).

LC-MS/MS. Peptide sequencing of identified protein spots was performed using an Agilent 1100 LC-MSD Trap XCT (Agilent Technologies, Palo Alto, CA, USA). Proteins were identified in an Agilent Spectrum Mill MS proteomics workbench against the Swiss-Prot protein database search engine (http://kr.expasy.org/sprot/) and MASCOT MS/MS Ions Search engine (http//www.matrixscience.com/search_form_select.html). Standards/parameters for induction of candidate proteins were set as follows: filter by protein score >10.0, and filter peptide by score >8.0. The Spectrum Mill workbench searched for MS/MS spectra, using an MS/MS ion search (13, 14).

Western blot analysis. Fifteen micrograms of whole-cell lysates were subjected to electrophoresis on 10% SDS polyacrylamide gels, and then transfered onto polyvinylidene fluoride (PVDF) membranes (immobilon; Millipore, Bedford, MA, USA). After blocking overnight at 4˚C with Tris-buffered saline (TBS) containing 5% skimmed milk, the membranes were incubated with primary antibody against B-23 (mouse monoclonal antibody to nucleophosmin/NPM1; concentration 0.2 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) at 4˚C overnight, and then incubated with the secondary antibody conjugated with horseradish peroxidase (dilution 1:10,000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 1 h at room temperature after washing three times with TBS containing Tween-20 and once with TBS. Membranes were then treated with a chemilluminescent reagent (ImmunoStar Long Detection; Wako, Osaka, Japan) and proteins were detected by using Image Reader LAS-1000 Pro (Fujifilm Corporation, Tokyo, Japan). Mouse monoclonal antibody against α-tubulin (dilution 1:200; Calbiochem, San Diego, CA, USA) was used for normalization of the proteins from western blot analysis.

Two-dimensional western blot analysis. Eighty micrograms of samples were separated on 2-DE gels and then transfer onto PVDF membranes at 90 mA for 78 min. The membranes were blocked overnight at 4˚C with TBS containing 5% milk. Membranes were incubated with the primary antibody to NPM at 4˚C overnight and then incubated with the secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. After washing three times with TBS containing Tween-20 and once with TBS, membranes were then treated with a chemilluminescent reagent and imaged using Image Reader LAS-1000 Pro.

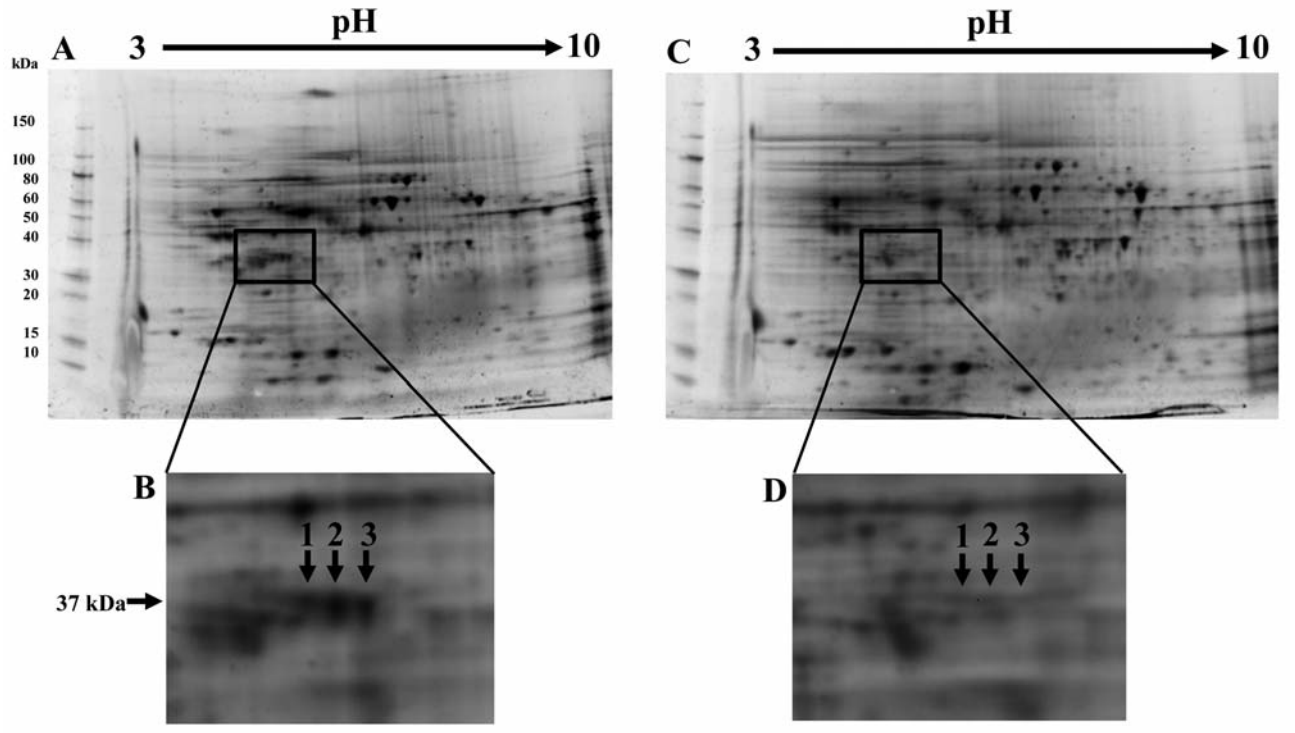

Figure 1. *Comparison of spots between QR-32 (A, B) and QRsP-11 (C, D) cells. In the square, arrows indicate three protein spots (numbered 1-3). These three spots were found to be weaker on 2-DE gels of QRsP-11 (D), compared to QR-32 (B).*

Table I. *Identification of the protein spots of down-regulated expression in QR-32 and QRsP-11 cells.*

Spot	Distinct peptides	MS/MS search score	Coverage (%)	Mass (Da)/pI	Accession no.*	Protein
1	8	106.10	28	32560.2/4.62	Q61937	Nucleophosmin
2	7	107.89	35	32560.2/4.62	Q61937	Nucleophosmin
3	4	60.09	23	32560.2/4.62	Q61937	Nucleophosmin

*Accession no. for SwissProt data base.

Results

Detection of protein spots on 2-DE gels. Many protein spots were visualized on the 2-DE gels. Among these, three protein spots (numbered 1-3) were found to be significantly weaker on 2-DE gels of QRsP-11 cells compared to QR-32 cells (Figure 1).

Identification of proteins by LC-MS/MS. Each spot provided a good spectrum of amino acids upon LC-MS/MS analysis, and was identified as NPM. MS and MS/MS spectra of trypsin-digested spots are shown in Figure 2 and Table I.

Western blot analysis. Regressive murine fibrosarcoma cell clone QR-32 exhibited a strong band of NPM, but its expression was very faint in progressive malignant cell clone QRsP-11 (Figure 3).

Two-dimensional western blot analysis of nucleophosmin isoforms. Three pairs of QR-32 and QRsP-11 were analyzed by 2-D western blotting analysis. Using a specific antibody against nucleophosmin, the spots were confirmed as being three NPM isoforms (Figure 4). The different isoforms were found to be significantly down-regulated in QRsP-11 cells compared to QR-32 cells.

Discussion

Nucleophosmin (also known as B23, NO38, or numatrin) is frequently conserved in vertebrates and widely distributed

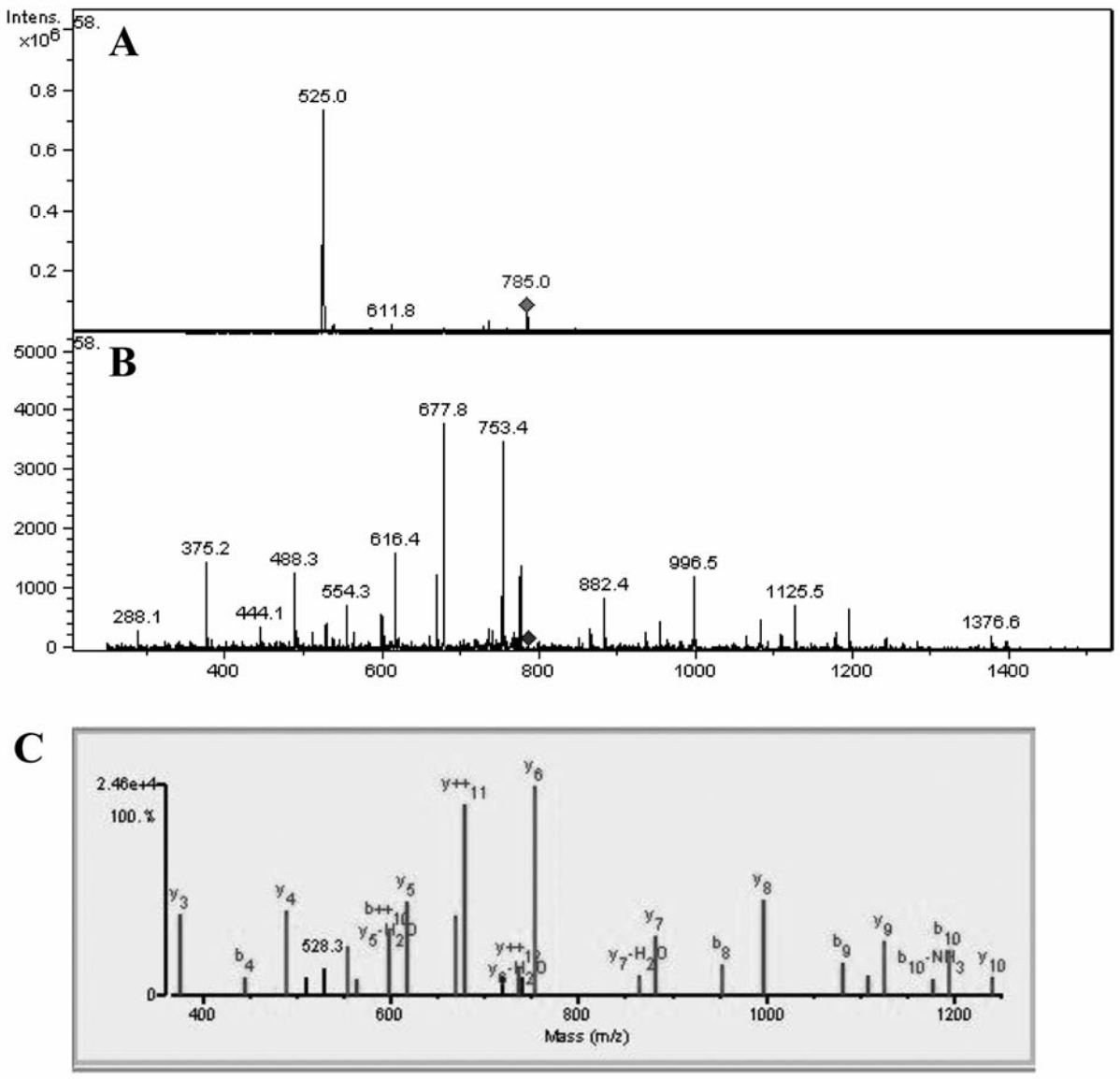

Figure 2. *Mass spectrometry (MS) and MS/MS spectra of trypsin-digested spot 1, shown in Figure 1. A: MS spectra of trypsin-digested spots; nucleophosmin precursor ion m/z is 785.0. B and C: MS/MS spectrum of a precursor ion with m/z 785.0 marked by a diamond in (A). The MS/MS spectrum identifies the partial tryptic peptide [K]VDNDENEHQLSLR[T] from nucleophosmin, processed with a spectrum Mill workbench.*

among different species, with a molecular weight of 35 to 40 kDa. In the human and rat, NPM exists at least with two isoforms, NPM1 and NPM1.2 (B23.1 and B23.2, respectively), which are generated from a single gene *via* alternate splicing (15). However, in mice, only NPM1 exists.

NPM is a non-ribosomal nucleolar phosphoprotein, implicated in cancer, which shuttles continuously between the nucleus and the cytoplasm (16). NPM is a multifunctional protein that is indispensable for various cellular processes including centrosome duplication, ribosome biogenesis, cell-cycle progression, apoptosis, transformation and genomic stability (17-20). Importantly, NPM displays several interrelated nucleolar functions that contribute to cell growth. Therefore, many researchers have focused their attention on these NPM activities and numerous attempts have been made to elucidate its role in cancer cells. Although the role of NPM in oncogenesis has been an object of study for a long time, this is still controversial because its precise role is complex.

NPM protein levels are generally correlated with mitogen-induced cell proliferation, and NPM fusion proteins are found in several malignancies (21). NPM would be expected to be a

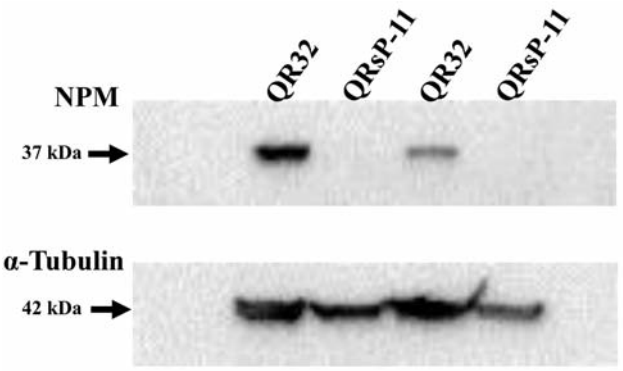

Figure 3. *Western blot analysis of nucleophosmin (NPM). Figure shows the band of NPM (37 kDa) and α-tubulin (42 kDa), as a loading control, in QR-32 and QRsP-11 cells.*

protein with proto-oncogenic activity, and in fact the NPM gene is a direct transcriptional target of the *c-MYC* proto-oncogene transcription factor (22). Moreover, NPM protein levels are elevated in dividing cells and cancer cells (23), but tend to decrease when cells differentiate and stop growing (18). Therefore, we focused our attention on NPM protein levels to possibly elucidate tumorigenesis in the present study.

From many reports, NPM has been reported to be up-regulated, mutated, re-arranged and deleted in cancer cells. Many studies have reported that NPM overexpression is related to carcinogenesis and tumor progression in prostate (24), gastric (25), colon (23), ovarian (26), hepatocellular (27) and urinary bladder carcinomas (28). Grisendi *et al*. reported that NPM was frequently up-regulated in carcinomas and was involved in chromosome translocation in hematological malignancies, where it forms fusion proteins with different partners (16). Overexpression of NPM has also been reported to suppress p53-mediated growth arrest and apoptosis, following exposure of cells to UV radiation or hypoxia (29, 30).

On the other hand, there are opposing reports for the studies described above. Li *et al*. reported that NPM protected cells from apoptotic cell death induced by diverse stresses through a mechanism involving inhibition of the p53 tumor-suppressor protein (31). They pointed-out that NPM might stabilize p53, a major regulator of apoptosis, or promote its degradation during nucleolar stress (31, 32). Our finding in this study supports their theory. There are conflicting reports on NPM levels (gain or loss of expression) and the effect of NPM on p53 activity.

The potent activity of p53 as inducer of cellular apoptosis and cell-cycle arrest demands tight control of its function. The major mode for provoking p53 action is DNA damage, which leads to the interaction between murine double minute 2 homolog (MDM2) and p53, increased p53 stability and activity (33). p53 activity is also induced by cellular oncogenes, such as *RAS* and *MYC* (34), and viral proteins such as viral cyclin (K cyclin) expressed by Kaposi's sarcoma-associated herpesvirus (35). *RAS* and *MYC* increase the levels of alternative reading frame (ARF), which binds MDM2 and translocates it to the nucleoli, relieving the negative regulation of p53 by MDM2 (36-38).

The stress response pathway that correlates with p53, p14 alternate reading frame (ARF) and MDM2 plays a central role in mediating cellular responses to oncogene activation, and genomic instability, and induces various forms of DNA damage. Recent studies pointed out that invalidation of the pathway results in many kinds of cancer, and may be crucial for tumor progression. One of the triggers of cell-cycle arrest and apoptosis induced by DNA damage is p53. It works as a transcription factor that serves as a point of convergence for various stress signals, such as DNA damage, oncogene activation and hypoxia-induced factor, all of which are common features of cancer cells. Activation of the p53 pathway centers on regulation of p53 protein stability and function (38). MDM2 is a p53 antagonist consisting of a nucleoplasmic and nucleolar really interesting new gene (RING)-finger protein that targets p53 degradation and inhibits p53 transcriptional activity through the proteasome (39). MDM2 also interacts with other tumor suppressor proteins, such as p14ARF, retinoblastoma protein, and promyelocytic leukemia protein (40-45). MDM2 expression is induced by p53 and establishes a feedback mechanism that prevents the protein from accumulating under normal conditions (46).

Several reports demonstrated that NPM actually stabilized p53 through direct interaction with the tumor suppressor and indirect action by inhibition of MDM2 (32, 47). Therefore, we hypothesized that the malignant alteration of tumor might be closely related to the specific ability of NPM in influencing the stabilization of p53 interacting with MDM2.

NPM is known to translocate between the nucleolus and nucleoplasm in response to cytotoxic drugs and genotoxic stress, such as inhibition of RNA polymerase I, DNA intercalating agents, and UV damage (48-50). NPM and MDM2 interact or co-localize increasingly, consequently to divergent cellular stresses, such as UV damage, proteasome inhibition, and expression of apoptosis-inducing viral proteins. Colombo *et al*. reported that high levels of ectopic NPM were seen to activate p53 and trigger p53-mediated growth arrest and cellular senescence in normal mouse embryo fibroblasts (MEFs), while promoting a slight stimulation of growth in *p53*-null MEFs (47). They pointed out that the effect of NPM in suppressing the early stages of tumorigenesis may be related to its role in maintaining genome stability and ensuring DNA integrity (47). Kurki *et al*. reported that p53 stabilization was augmented by rapid UVC-induced nucleoplasmic translocation of NPM and an increase in nucleoplasmic p53–NPM and MDM2–NPM

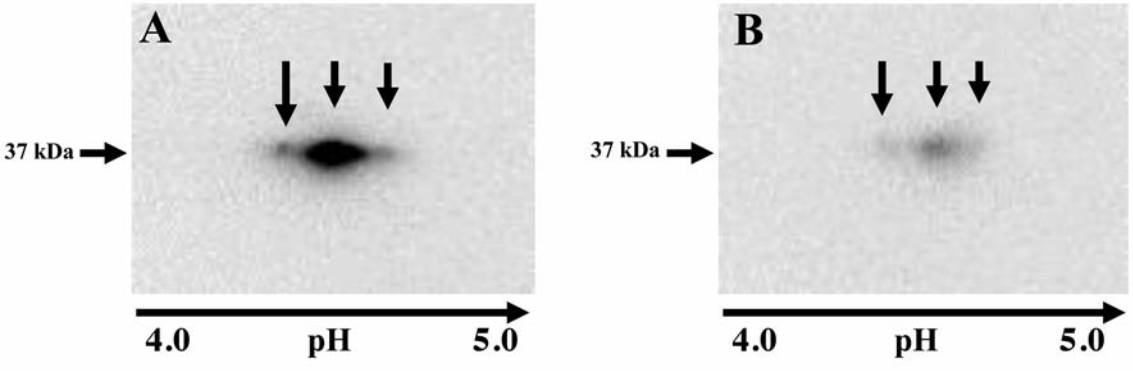

Figure 4. *Two-dimensional western blot analysis of nucleophosmin. Three spots of nucleophosmin can be seen in samples from QR-32 (A) and QRsP-11 (B) cells.*

complexes (32). They pointed-out that the dynamic re-organization of the complexes allows for early p53 stabilization and possibly assists in its modifications, such as sumoylation, commencing the subsequent stabilization effects. High levels of p53 competed for the interaction of NPM and MDM2 and depletion of NPM increased p53–MDM2 complex formation (32).

NPM can bind to p53 and regulate its stability and activity (47). Moreover, Kurki *et al*. reported that NPM tended to form complexes with MDM2 and may be unable to bind to and dissociate pre-formed p53–MDM2 complexes (32). They also indicated that the interaction of NPM with MDM2 was independent of p53 and is reduced *in vitro* by high levels of p53 (32). This suggests competition between p53 and MDM2 for binding sites on NPM.

The multiple interactions of NPM with cellular proteins suggest that it can act as a platform for protein interactions, affect their stabilization, or allow protein modifications to take place. Therefore, NPM could stabilize the complex formation between p53, MDM2, and other proteins needed for post-translational modifications in response to cellular stimulation. NPM might affect p53 stabilization through inhibition of its negative regulator, MDM2. Therefore, the depletion of NPM might cause the regressive tumor cells QR-32 to acquire progressive malignancy.

In this study, we performed 2-DE and MS to identify intracellular proteins expressed differentially in QR-32 and QRsP-11 cells, and we identified the intracellular protein NPM, whose expression differed between these two clones. The expression of NPM was down-regulated in the progressive tumor cells compared to the regressive tumor cells. It is possible that the tumor cells may acquire the malignant phenotypes in the course of progression *via* the dual function in regulation of p53 activity: by inhibiting p53 degradation through NPM interactions with MDM2, and by assembling complexes that are needed for p53 modifications, augmenting its activation and stabilization. Reduction of NPM might cause the stabilization of p53 and result in tumor progression.

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