

Identification of GRP75 as an Independent Favorable Prognostic Marker of Neuroblastoma by a Proteomics Analysis

Wen-Ming Hsu,¹ Hsinyu Lee,^{4,5} Hsueh-Fen Juan,^{4,6} Yu-Yin Shih,^{4,7} Bo-Jeng Wang,⁷ Chien-Yuan Pan,^{4,5} Yung-Ming Jeng,² Hsiu-Hao Chang,³ Meng-Yao Lu,³ Kai-Hsin Lin,³ Hong-Shiee Lai,¹ Wei-Jao Chen,¹ Yeou-Guang Tsay,⁸ Yung-Feng Liao,^{5,7} and Fon-Jou Hsieh⁹

Abstract **Purpose:** Neuroblastoma (NB) is a heterogeneous neoplasm. Detailed biological discrimination is critical for the effective treatment of this disease. Because the tumor behavior of NB is closely associated with the histologic state of differentiation, we thus aimed to identify novel differentiation-associated markers of NB with prognostic implication. **Experimental Design:** A human NB cell line SH-SY5Y was used as a model system to explore potential biomarkers for the differentiation of NB by proteomic analyses. Seventy-two NB tumor tissues were subsequently investigated by immunohistochemistry to validate the correlations between the expression of a novel prognostic marker, various clinicopathologic and biological factors, and patient survival. **Results:** Using two-dimensional differential gel electrophoresis, we found a total of 24 spots of proteins in SH-SY5Y cells whose expression was enhanced following differentiation. Glucose-regulated protein 75 (GRP75) was unambiguously identified as one of the five proteins that were dramatically up-regulated following differentiation. Immunohistochemical analyses of 72 NB tumor tissues further revealed that positive GRP75 immunostaining is strongly correlated with differentiated histologies ($P < 0.001$), mass-screened tumors ($P = 0.016$), and early clinical stages ($P < 0.001$) but inversely correlated with MYCN amplification ($P = 0.010$). Univariate and multivariate survival analyses showed that GRP75 expression is an independent favorable prognostic factor. **Conclusions:** The present findings clearly showed that our proteomics-based novel experimental paradigm could be a powerful tool to uncover novel biomarkers associated with the differentiation of NB. Our data also substantiate an essential role of GRP75 in the differentiation of NB.

Neuroblastoma (NB) is the most common extracranial solid tumor of infancy and childhood with an incidence of 8.0 per million per year (1), and this tumor arises from primitive neuroepithelial cells of the neural crest and occurs most

frequently in the adrenal gland (2). NB is quite a heterogeneous tumor and presents a broad clinical and biological spectrum ranging from highly undifferentiated tumors with very poor outcomes to the most differentiated benign ganglioneuroma or NB with high probability of spontaneous regression and hence favorable prognosis. At least two categories of NB have been identified (3, 4). One, the favorable NB, is associated with young age and early stage at diagnosis, triploid karyotypes with whole chromosome gains, and excellent clinical outcome despite minimal or no therapy. The other, the unfavorable NB, is associated with older age and advanced stage and pseudodiploid karyotypes with structural changes including deletion of 1p or 11q, unbalanced gain of 17q, and/or amplification of the MYCN proto-oncogene. Patients with unfavorable NB usually have a very poor outcome despite multimodality therapies including bone marrow transplantation. Furthermore, we have also shown previously that the biological characteristics of NB tumors would significantly influence the surgical decision (5). We showed that gross total resection, a highly risky operation and controversial treatment for NB, of the primary tumor could carry favorable outcome only in a specific group of NB with certain biomarkers (5). These lines of evidence suggest that a more detailed understanding of the clinical and biological characteristics of NB is imperative for the selection of appropriate therapeutic intervention on these tumors to achieve effective treatment without unnecessary complications.

Authors' Affiliations: Departments of ¹Surgery, ²Pathology, and ³Pediatrics, National Taiwan University Hospital and National Taiwan University College of Medicine; ⁴Department of Life Science and Institutes of ⁵Zoology and ⁶Molecular and Cellular Biology, National Taiwan University; ⁷Institute of Cellular and Organismic Biology, Academia Sinica; ⁸Institute of Biochemistry and Molecular Biology and Proteomics Research Center, National Yang-Ming University; and ⁹Graduate Institute of Clinical Medicine, National Taiwan University College of Medicine, Taipei, Taiwan

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Y.-G. Tsay, Y.-F. Lao, and F.-J. Hsieh contributed equally to this work.

Requests for reprints: Fon-Jou Hsieh, Graduate Institute of Clinical Medicine, National Taiwan University College of Medicine, 7 Chung-Shan South Road, Taipei 100, Taiwan. Phone: 886-2-23562614; Fax: 886-2-23934197; E-mail: fjhshieh@ntu.edu.tw.

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Translational Relevance

The present findings clearly showed that our proteomics-based novel experimental paradigm could be a powerful tool to uncover novel biomarkers associated with the differentiation of neuroblastoma. Our present study shows the establishment and application of a novel proteomics-based experimental paradigm for the identification of NB tumor biomarkers. Evaluation of GRP75 expression in tumor tissues of NB may provide complementary prognostic information for further subclassification of these tumors, which in turn may help to determine the most appropriate strategy of treatment. Furthermore, because the expression of GRP75 strongly correlates with the differentiation of NB, GRP75 allows itself as a potential target of the treatment of NB. Together, the present data delineate a good example for the employment of proteomic approaches in the identification of potential biomarkers of cancers and recognize the potential of GRP75 as a therapeutic target of NB.

Although the pathogenesis of NB remains obscure, it has been suggested that aberrant regulation of cellular differentiation could contribute to the development of this cancer (6). Coincided with this concept, the prognosis of NB patients has been shown to be closely associated with the histologic grade of differentiation in tumors from NB patients (7). Most biomarkers for favorable outcome in NB, such as HNK-1, Trk-A, H-Ras, glucose-regulated protein 78 (GRP78), and calreticulin, are associated with the differentiation of NB cells (8–12). Here, we employ a proteomic approach to look for novel biomarkers for cellular differentiation that could play a pivotal role in the pathogenesis of NB. Recent advances in proteomic technologies have allowed the identification of proteins whose amounts and states could be altered by the disease process in a high-throughput manner, making proteomic analysis one of the most valuable tools for defining biomarkers of various tumors (13, 14).

The present study successfully identified GRP75 (74 kDa; pI 5.6) as a novel biomarker of NB. GRP75 is a member of heat shock protein 70 family and is first cloned from the cytoplasmic fraction of normal mouse fibroblast (15). In addition to its primary residence in mitochondria, GRP75 is also present at endoplasmic reticulum, plasma membrane, cytoplasmic vesicles, and cytosol (16). It involves in cellular functions ranging from stress response, intracellular trafficking, antigen processing, control of cell proliferation, differentiation, and tumorigenesis (17). Our data have confirmed that the expression of GRP75 in NB tumor tissues strongly correlates with differentiated histologies and predicts a favorable patient's outcome. GRP75 could thus play a critical role in the differentiation of NB.

Materials and Methods

Experimental procedures for cell culture and treatment, protein extraction and Cy dye labeling, two-dimensional differential gel electrophoresis (DIGE) and imaging analysis, liquid chromatography-

tandem mass spectrometry analysis, and transfection of a small interfering RNA targeting GRP75 are included in the Supplementary Data.

Western blot analysis

Two-dimensional Western blot. To show the specifically enhanced expression of GRP75 after the differentiation of NB cells, proteins extracted from SH-SY5Y cells treated with DMSO (0.1%) or all-*trans* retinoic acid (ATRA; 10 μ mol/L) for 5 days were resolved by two-dimensional electrophoresis. Proteins were blotted onto a nitrocellulose membrane electrophoretically and incubated with a goat anti-GRP75 antibody (1:200; Santa Cruz Biotechnology). The immunoreactive spots were visualized with ECL Plus Western Blotting Detection System (Amersham Biosciences).

One-dimensional Western blot. The details of protein extraction from cells or tumor tissues, electrophoresis, and immunoblotting have been described previously (11, 18). The antibodies used and their dilution were as follows: goat anti-GRP75 antibody (1:200; Santa Cruz Biotechnology), rabbit anti-glyceraldehyde-3-phosphate dehydrogenase antibody (1:1,000; Santa Cruz Biotechnology), mouse anti-neuron-specific enolase (1:1,000; Millipore), and horseradish peroxidase-conjugated secondary antibodies (1:5,000; Santa Cruz Biotechnology). The expression levels of GRP75, calreticulin, and neuron-specific enolase were assessed by densitometry and normalized with those of glyceraldehyde-3-phosphate dehydrogenase, the internal control. Data were shown as the mean \pm SD of the averages of triplicate measurements from three independent experiments.

Indirect immunofluorescence staining and confocal microscopy

SH-SY5Y cells were grown on coverslips and incubated in DMEM/Ham's F-12 containing 2% fetal bovine serum followed by the treatment with DMSO (0.1%) or ATRA (10 μ mol/L) for 5 days. Cells were then fixed by 4% paraformaldehyde in PBS at room temperature for 60 min and permeabilized by 10% formaldehyde in PBS containing 0.1% Triton X-100 at room temperature for 10 min. Detergent-permeabilized cells were blocked with PBS/2% bovine serum albumin at room temperature for at least 1 h and were incubated with a goat anti-GRP75 antibody in PBS/0.1% bovine serum albumin at 4°C overnight. Following extensive washes with PBS, cells were incubated with an Alexa Fluor 488 donkey anti-goat IgG in PBS/0.1% bovine serum albumin at room temperature for 1 h. Following the removal of unbound antibodies by washes with PBS, the costaining of actin was done by repeating the staining procedure using a mouse anti-actin antibody as the primary antibody and an Alexa Fluor 647 goat anti-mouse IgG as the secondary antibody in the procedure described above. Nuclei were visualized by 4',6-diamidino-2-phenylindole. Fluorophore-conjugated antibodies and 4',6-diamidino-2-phenylindole were obtained from Invitrogen. Immunofluorescence images were obtained by using a Leica TCS-SP5 laser scanning confocal microscope. The fluorescence intensity of GRP75 was quantitated by Leica Application Suite Advanced Fluorescence and analyzed by Student's *t* test. Data were shown as the mean \pm SD of the average fluorescence per cell from five different viewing areas. The numbers of cells that were subject to fluorescence quantitation were 279 for DMSO-treated cells and 413 for ATRA-treated ones, respectively.

ATRA-induced neuronal differentiation of NB cells

SH-SY5Y human NB cells that were transfected with a GRP75-specific small interfering RNA or a nonspecific one (Mock) were then treated with 10 μ mol/L ATRA or vehicle alone (0.1% DMSO) in DMEM/Ham's F-12 containing 10% fetal bovine serum followed by incubation at 37°C for 3 days. Treated SH-SY5Y cells were harvested and lysed by PLB (Promega) containing the Complete protease inhibitor cocktail. Clarified lysates containing equivalent amounts of proteins were resolved by SDS-PAGE. The expression levels of calreticulin and neuron-specific enolase, two markers for the neuronal differentiation

of NB cells (12, 19), were visualized by Western blotting using specific antibodies. The level of glyceraldehyde-3-phosphate dehydrogenase was also determined as a protein load control.

To quantify neurite outgrowth of NB cells induced by ATRA, 150 or more transfected SH-SY5Y cells, whose dendritic trees were relatively isolated and did not have discontinuities in their dendritic trees, were chosen for quantification of neurite length. Image for Windows was the morphometric program used to measure the neurite length.

Patients and treatment

In a period of 14 years (from December 1990 to December 2004), 72 histologically proven NB patients with complete follow-up were enrolled in this study. Eight of these 72 patients were diagnosed by mass screening of urinary vanillyl-mandelic acid without any symptoms. This group of patients carried a male-to-female ratio of 40:32 and a median age at diagnosis of 2.5 years (range, 0-11.5 years). The majority of tumors (41 cases) were originated primarily from the adrenal gland and others from retroperitoneum (18 cases), mediastinum (6 cases), neck (4 cases), and pelvis (3 cases). Based on the criteria of the International Neuroblastoma Pathology Classification, the differentiating status of the tumor histology was categorized into undifferentiated NB, differentiating NB (including poorly differentiated subtype), and ganglioneuroblastoma (GNB), distinguishable by the percentage and degree of differentiation of the NB cells (11, 20, 21). Both differentiating NB and GNB were pooled as differentiated NB for the survival analysis. The distinction of tumor staging was based on the International Neuroblastoma Staging System (22). MYCN status of the tumor tissue was evaluated by fluorescence *in situ* hybridization analysis of formalin-fixed, paraffin-embedded tissues or fresh tumor single cells (23, 24). Patients were treated with surgery only or a combination of multiple modalities including chemotherapy, radiotherapy, and/or autologous bone marrow transplantation according to the patient's risk grouping (25). The median follow-up after diagnosis was 48.0 months with a range of 1 to 168 months, and the overall 5-year survival rate in this cohort was 53.9%.

Immunohistochemical staining

To validate the expression of GRP75 in NB, 72 formalin-fixed, paraffin-embedded tumor tissues that were obtained before chemotherapy were evaluated by immunohistochemical staining with a standard streptavidin-biotin method (11). Briefly, the paraffin sections (5 μ m) of tumors were dewaxed, rehydrated, and incubated with a goat anti-GRP75 antibody (1:100) at 4°C overnight. Antibody-reactive

sections were allowed to interact with the N-Histofine Simple Stain MAXPO (Nichirei) for 30 min at room temperature followed by color development using diaminobenzidine. The nuclei were counterstained with hematoxylin. One ganglioneuroma tumor with consistent GRP75 expression by immunohistochemistry was used as a positive control. Nonimmunized goat serum was used to rule out nonspecific immunostaining. Tumors with various differentiating histologies were included in each staining. The immunoreactivity of GRP75 was assessed by one pathologist who was blinded to the clinical backgrounds of the patients. A specific blocking peptide containing the reactive epitope of the anti-GRP75 antibody was used to compete the binding of anti-GRP75 for the determination of its specificity.

Statistical analysis

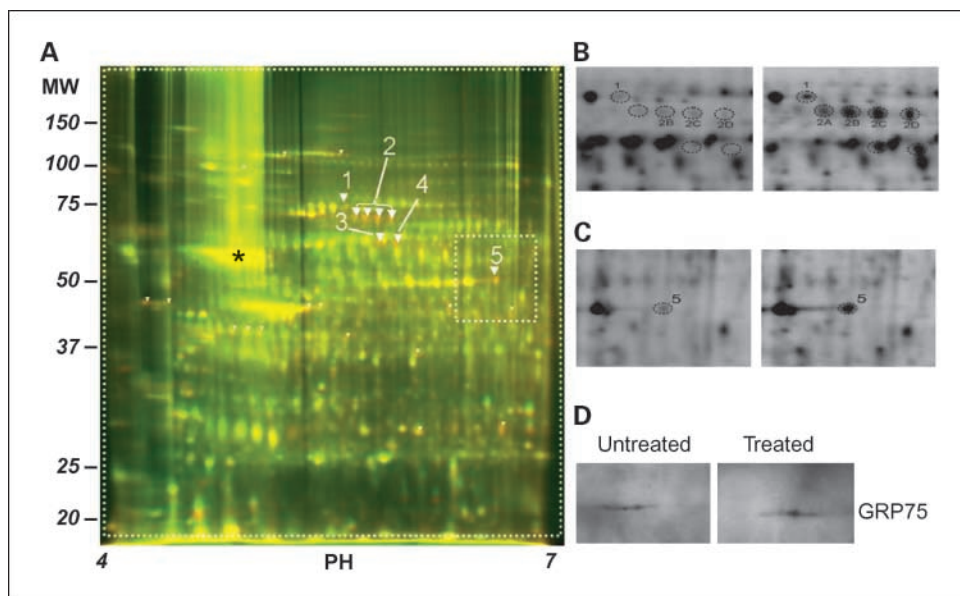
SPSS 10.0 for Windows software was used to perform the statistical analyses. The significance of the variations between the data resulted from different treatments was analyzed by Student's *t* test. Associations between pairs of categorical variables were assessed with Pearson's χ^2 test. Survival probabilities in various subgroups were estimated using the Kaplan-Meier method and analyzed by log-rank tests. The influence of each variable on survival was assessed by the multivariate Cox proportional hazard model. All statistical tests were two-sided, and those with a $P \leq 0.05$ were considered to be statistically significant.

Results

Identification of differentiation-associated proteins in NB cells by two-dimensional DIGE

SH-SY5Y cells treated with ATRA for 5 days showed morphologic characteristics of differentiation with prominent neurite extension as well as increased expression of calreticulin and neuron-specific enolase (data not shown). The proteins extracted from untreated and differentiated cells were stained with Cy3 and Cy5, respectively, and analyzed by two-dimensional DIGE. A total of 24 orange/red spots that indicate protein candidates with enhanced expression after differentiation were highlighted for the entire cellular proteome (Fig. 1A, arrowheads). The amounts of most proteomic constituents were not significantly altered in differentiated cells versus undifferentiated ones, suggesting that the increased expression of those protein candidates is a specific response to ATRA-induced

Fig. 1. A, differential two-dimensional proteomic analysis of NB cells before and after differentiation. Red spots, increased expression after cell differentiation; green spots, decreased expression after cell differentiation; yellow spots, unchanged expression after cell differentiation. B, area containing the spots 1 to 4 is enlarged to show the circular areas in undifferentiated cells (left) and differentiated cells (right) for assessment of the respective fluorescent signals. C, area containing the spots 5 is enlarged to show the circular areas in undifferentiated cells (left) and differentiated cells (right) for assessment of the respective fluorescent signal. D, GRP75 expression in undifferentiated and differentiated NB cells evaluated by two-dimensional Western blot.



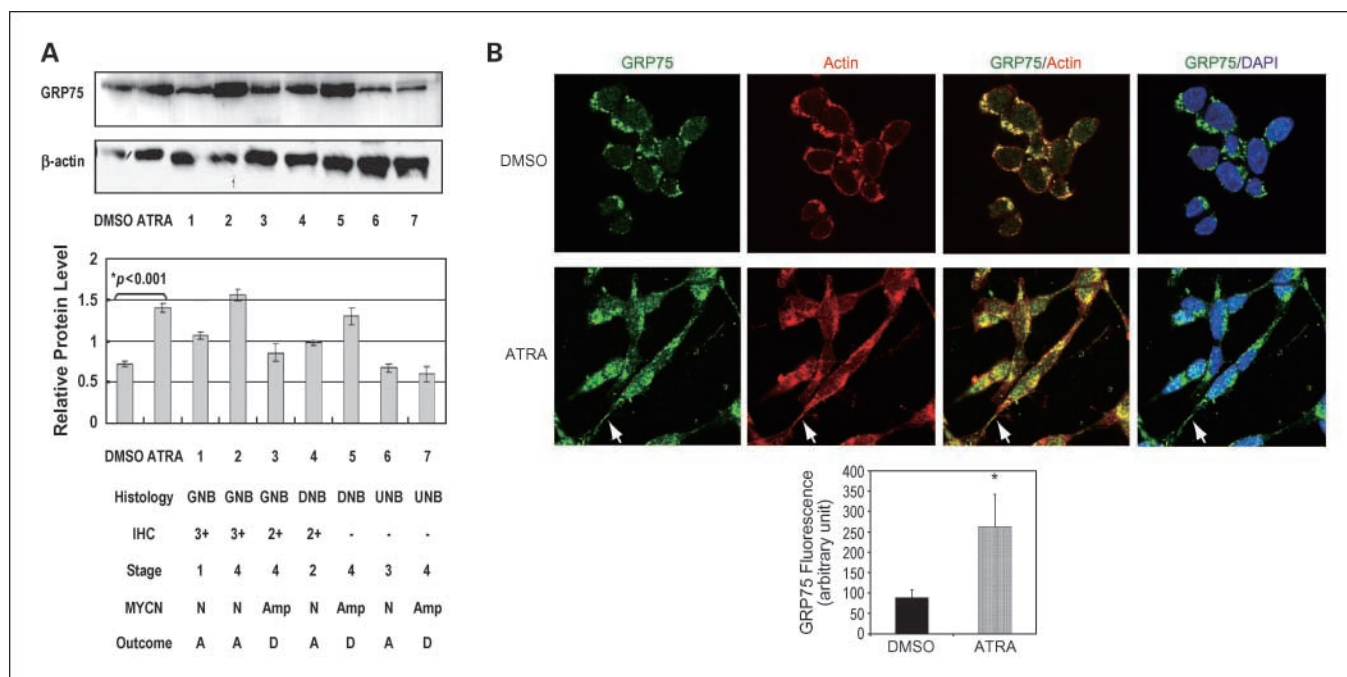


Fig. 2. A, GRP75 protein levels evaluated by Western blot in SH-SY5Y cells treated with DMSO or ATRA and in 7 NB tumors (cases 1-7). The histopathology, GRP75 immunohistochemical staining (IHC) grading, clinical stage, MYCN status, and patient outcome of the NB tumors are depicted for comparison. DNB, differentiating NB; UNB, undifferentiated NB; N, nonamplified; Amp, amplified; A, alive; D, dead. B, immunolocalization and immunofluorescence intensity of GRP75 in undifferentiated and differentiated NB cells.

differentiation. Among these protein spots, eight of the most abundant ones were analyzed for the extent of the increases. The fluorescent signals of a protein spot in different proteomes were determined as signal percentages in respective proteomes (Supplementary Table S1). Using this approach, we estimated that the signal percentages of tubulin, one of the most intensely stained proteins (Fig. 1A, asterisk), remained largely unchanged, comprising 9.8% and 9.2% of the total signals in untreated and differentiated cells, respectively. We found that the fold of change for these up-regulated proteins during differentiation ranged from 1.7 to 2.7 (Fig. 1B and C; Supplementary Table S1). The above protein spots were also subject to liquid chromatography-tandem mass spectrometry analyses to reveal their identities. Because four of the eight protein spots (Fig. 1A, spots 2A, 2B, 2C, and 2D) share similar results, these eight spots actually represented a total of five proteins, including GRP75 (spot 1), heat shock protein 2 (spots 2A, 2B, 2C, and 2D), protein disulfide isomerase A3 precursor (spot 3), subunit 1 β of TCP1-containing chaperonin, (spot 4), and Eno1 protein (spot 5) (Supplementary Table S1). Our data also revealed that the expression of these proteins during differentiation was a moderate increase and not likely an all-or-none phenomenon. Among these identified proteins, GRP75 is a member of heat shock protein 70 family and has been suggested to play a functional role in the control of tumorigenesis (17). In addition, we have shown previously that GRP78, another member of the heat shock protein 70 family, is a favorable prognostic marker for NB (11). These findings prompted us to examine the differential expression and the function of GRP75 in NB, validating the efficiency of proteomic approach in identifying key proteins involved in the differentiation of NB cells.

Enhanced GRP75 expression in differentiated NB cells

To confirm the specifically enhanced expression of GRP75 after differentiation, we performed two-dimensional Western blotting by using a specific anti-GRP75 antibody. In the lysates from both undifferentiated and differentiated cells, several protein spots of 75 kDa and pI 5 to 6 were found to be specifically reactive with this anti-GRP75 antibody (Fig. 1D). Our data also showed that the expression of GRP75 is significantly increased after cell differentiation, in accordance with the results obtained from two-dimensional DIGE analysis. Using one-dimensional Western blotting analysis, we further quantitatively showed that protein levels of GRP75 in ATRA-treated NB cells are increased approximately 1.8-fold on differentiation (Fig. 2A, ATRA versus DMSO control, $P < 0.001$, t test).

Dramatic change in the subcellular distribution of GRP75 during the induced differentiation of NB cells

Differential subcellular distribution of GRP75 that is concomitant with cellular immortalization has been observed in normal versus cancerous cells (16), prompting us to examine the subcellular localization of GRP75 in undifferentiated and differentiated NB cells. Using immunofluorescence staining, we found that ATRA-treated SH-SY5Y cells show a significant increase in the staining intensity of GRP75 (an average of 262 ± 80 fluorescence unit per cell) with a pan-cytoplasmic distribution, whereas those treated with DMSO show a lower level of GRP75 (an average of 88 ± 19 fluorescence unit per cell) with a juxtannuclear pattern (Fig. 2B). In differentiated cells, the overall staining intensity of GRP75 became prominent, consistent with the notion that enhanced GRP75 expression correlates with the differentiation of NB. The neuritic localization, as

defined by actin staining, of GRP75 can also be vividly observed in ATRA-treated cells (Fig. 2B, *arrows*), implicating a possible function of GRP75 in neurite formation.

Correlation between the differential expression of GRP75 and the differentiation status of NB tumors

The expression levels of GRP75 protein in seven NB tumor tissues, including three GNB, two differentiating NB, and two undifferentiated NB, were evaluated by Western blot analyses. Consistent with the data obtained with cultured NB cells, we found that tumors displaying better differentiation (GNB or differentiating NB) exhibit higher expression levels of GRP75 protein in general (Fig. 2A). However, there was a lack of clear statistical correlation between histologic grading and the protein expression of GRP75 evaluated by Western blotting. This discrepancy probably reflected the cellular heterogeneity in tumor tissues (12, 26). To circumvent this problem, we performed immunohistochemical staining of GRP75 in NB tumor tissues to assess the GRP75 expression in individual cells. We found that positive GRP75 staining could be specifically observed in ganglion cells of a ganglioneuroma tumor tissue and seems to display a pancytoplasmic distribution (Fig. 3A, 1, *arrows*), whereas the protein expression of GRP75 was negligible in Schwannian stromal cells. The specificity of the anti-GRP75 antibody for immunohistochemistry was confirmed by a competition study, which showed that the GRP75 immunostaining was blocked completely by the specific

peptide (Fig. 3A, 2). In addition to the mature ganglion cells, neuroblastic cells with various differentiating states also showed positive GRP75 immunostaining in the cytoplasm (Fig. 3A, 4-6, *arrows*), whereas undifferentiated neuroblastic cells usually showed negative staining (Fig. 3A, 3). Immunohistochemistry thus indicates a more specific method to assess the GRP75 protein expression in the neuroblastic cells among a heterogeneous NB tumor.

GRP75 expression and clinicopathologic and biological factors

For further understanding the association between GRP75 protein expression and cellular differentiation as well as its clinical significance, we then extended our studies to a larger patient population using immunohistochemical evaluation of GRP75 expression in NB tumor samples. These NB tumors were classified based on their immunoreactivity of GRP75 into four categories: “-” (no expression, no stained cells or only isolated single stained cells seen), “1+” (weak expression, 10-35% of neuroblastic cells stained), “2+” (moderate expression, 35-70% of neuroblastic cells stained), and “3+” (strong expression, >70% of neuroblastic cells stained; Fig. 3A, 3-6). Tumors were assigned into negative GRP75 protein expression (“-” in immunoreactivity) and positive GRP75 protein expression (“1+,” “2+,” or “3+” in immunoreactivity) for the statistical analysis. We found that immunoreactivities (1+ to 3+) of GRP75 could be detected in 34 of the 72 NB (47.2%). The intensity and percentage of positive GRP75 immunostaining

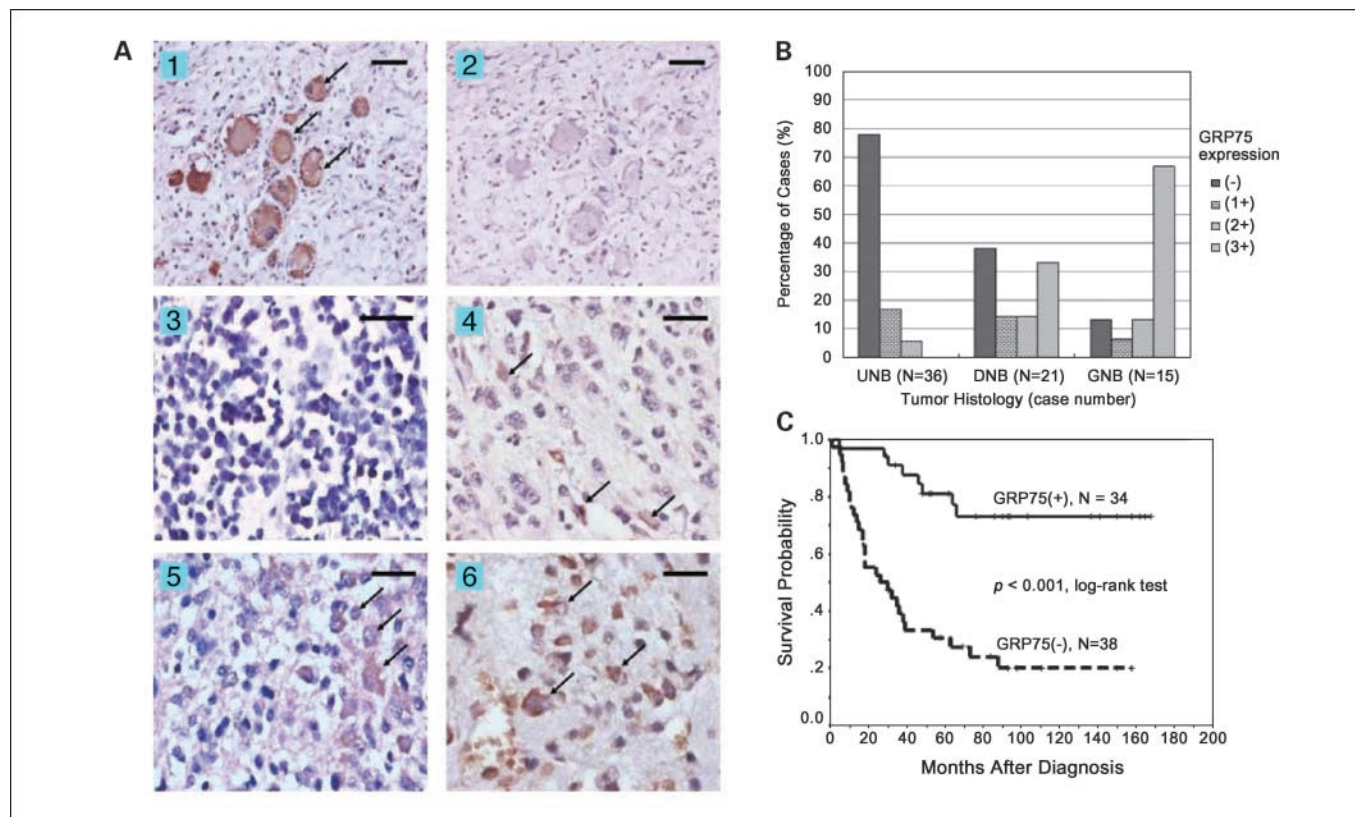


Fig. 3. Immunohistochemical analysis of GRP75 expression in NB. A1, positive control GRP75 immunostaining of a ganglioneuroma; A2, positive control GRP75 immunostaining blocked by the specific peptide; A3, “-” GRP75 immunostaining; A4, “1+” GRP75 immunostaining; A5, “2+” GRP75 immunostaining; A6, “3+” GRP75 immunostaining. Bar, 100 μ m. B, percentage distribution of GRP75 expression in undifferentiated NB, differentiating NB, and GNB. C, Kaplan-Meier survival analysis according to the expression of GRP75 in 72 NB patients.

correlated strongly with the differentiation of tumor histology. As the tumor histology displayed a differentiation pattern, the intensity and percentage of positive GRP75 immunostaining increased simultaneously (Fig. 3B; $P < 0.001$). The relationship between the GRP75 protein expression and the clinicopathologic and biological variables of NB was summarized in Table 1. In addition to histologic grade of differentiation, positive GRP75 immunostaining also correlated strongly with tumors detected by mass screening ($P = 0.016$) as well as early clinical stages (stages I, II, and IVS; $P < 0.001$). Furthermore, there was an inverse correlation between GRP75 protein expression and the unfavorable biomarker of MYCN amplification ($P = 0.010$).

Patients with positive scores for GRP75 expression showed favorable clinical outcomes

Kaplan-Meier analysis showed that patients with positive GRP75 protein expression had a significantly better predictive 5-year survival rate than patients with negative GRP75 protein expression (81.2% and 30.7% respectively; Fig. 3C; $P < 0.001$, log-rank test). Univariate analysis showed that, in addition to GRP75 expression, patient's age ≤ 1 year, early clinical stages (stage I, II, or IVS), and differentiated histology (including differentiating NB and GNB) also correlate with a better survival, whereas MYCN amplification predicts a very poor outcome (Table 2). Multivariate analysis by the Cox proportional hazard model showed that, in addition to clinical stage and MYCN status, GRP75 expression is also an independent prognostic factor (Table 2).

To understand the significance of GRP75 expression in the prognostic discrimination, the effect of GRP75 protein expression on patient survival was further analyzed against

tumor histology, clinical stage, and MYCN status. Positive GRP75 expression clearly predicted a better survival in patients with either undifferentiated or differentiated histologies of NB (Fig. 4A and B; $P = 0.002$ and 0.025 , respectively). Because most patients with early-stage NB (stages I, II, and IVS) showed positive GRP75 protein expression with favorable outcomes, they could not be discriminated by GRP75 expression for their prognosis. Nevertheless, in patients with advanced-stage diseases (stage III or IV), the prognosis could be clearly distinguished by GRP75 protein expression (Fig. 4C; $P = 0.005$). Although 7 of 15 patients (46.7%) died of their disease in the category of advanced stages and positive GRP75 protein expression, 28 of 33 patients (84.8%) died in the category of advanced stages and negative GRP75 expression. The prognosis of NB patients with MYCN amplification could not be distinguished by GRP75 expression due to the low frequency of positive GRP75 protein expression and very poor outcome for these patients. For the patients without MYCN amplification, positive GRP75 protein expression clearly predicted a favorable outcome (Fig. 4D; $P < 0.001$). Whereas only 6 of 31 (19.4%) patients with normal MYCN copy number and positive GRP75 protein expression died of their disease, 16 of 25 (64%) patients with normal MYCN copy number and negative GRP75 protein expression died of their disease. Together, our data clearly showed that altered GRP75 expression is a signature of differentiated NB tumors.

Discussion

Our present study shows the establishment and application of a novel proteomics-based experimental paradigm for the

Table 1. GRP75 expression and clinicopathologic and biological characteristics of NB

Variable	Cases	Positive GRP75 expression (%)	P*
Mass screening			
Yes	8	7 (87.5)	0.016
No	64	27 (42.2)	
Sex			
Male	40	20 (50.0)	0.598
Female	32	14 (43.8)	
Age at diagnosis (y)			
≤ 1	22	14 (63.6)	0.064
> 1	50	20 (40.0)	
Clinical stage			
I	6	5 (83.3)	<0.001 †
II	13	10 (76.9)	
IVS	5	4 (80.0)	
III	10	2 (20.0)	
IV	38	13 (34.2)	
Primary tumor site			
Adrenal	41	19 (46.3)	0.863
Extra-adrenal	31	15 (48.4)	
Tumor histology			
Undifferentiated NB	36	8 (22.2)	<0.001
Differentiating NB	21	13 (61.9)	
GNB	15	13 (86.7)	
MYCN			
Amplified	16	3 (18.8)	0.010
Nonamplified	56	31 (55.4)	

* χ^2 test.

† Stages I, II, and IVS versus stages III and IV.

Table 2. Clinicopathologic and biological factors affecting survival rate

Variable	Univariate analysis		Multivariate analysis	
	Relative risk (95% confidence interval)	P	Relative risk (95% confidence interval)	P
Age at diagnosis (y): >1 vs ≤1	3.667 (1.425-9.488)	0.007	1.143 (0.372-3.511)	0.816
Clinical stage: advanced (III and IV) vs early (I, II, and IVS)	14.654 (3.501-61.337)	<0.001	8.551 (1.527-47.869)	0.015
MYCN: amplified vs nonamplified	6.216 (2.971-13.004)	<0.001	3.088 (1.275-7.478)	0.012
GRP75 expression: negative vs positive	5.369 (2.438-11.824)	<0.001	2.705 (1.070-6.838)	0.035
Histology: undifferentiated vs differentiated*	2.247 (1.151-4.385)	0.018	1.009 (0.446-2.285)	0.983
Primary tumor site: adrenal vs extra-adrenal	1.591 (0.807-3.135)	0.180	1.046 (0.472-2.317)	0.911

*Including differentiating NB and GNB.

identification of NB tumor biomarkers. Using this approach, we have successfully identified and validated a new prognostic factor GRP75 that correlates with the clinical outcomes of NB patients. Presently, a common strategy for the identification of clinical biomarkers has been to find the differentially expressed proteins in tumor tissues but not in nontumor counterparts by using proteomic analysis for the identification of new biological markers of cancers (14). However, this strategy is not suitable for the study of NB due to the following two reasons. Firstly, there is usually no nontumor

counterpart in a NB surgical specimen for comparison. Secondly, NB represents a heterogeneous spectrum of tumors ranging from undifferentiated to mature histologies. Accordingly, the protein expression profiles from the tumor tissues could be highly varied from patient to patient, and there is no signature expression profile for a group of tumors with different clinical features. To circumvent these problems, we have resorted to a human NB cell line SH-SY5Y for the proteomic analyses. This human NB cell line established from NB patient's tumor tissue represents a group of relatively

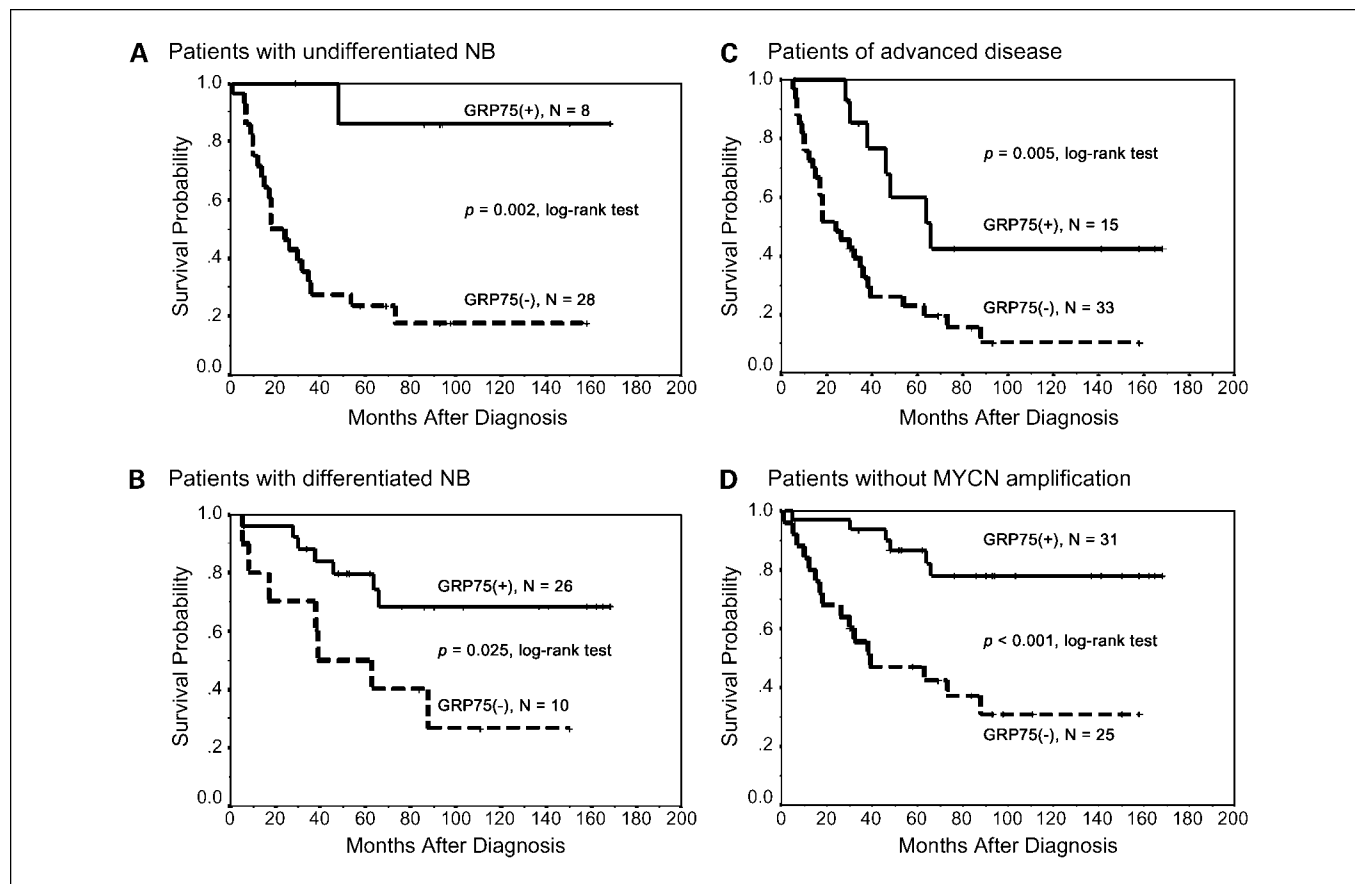


Fig. 4. Kaplan-Meier survival analysis according to the expression of GRP75 in (A) patients with undifferentiated NB, (B) patients with differentiated NB (including differentiating NB and GNB), (C) NB patients of advanced stages (stage III and IV), and (D) NB patients without MYCN amplification.

homogeneous cells with stable gene expressions recapitulating nicely the *in vivo* biological characteristics. In addition, these NB cells possess a great potential to differentiate *in vitro*, which is coupled well with the clinical tumor behavior (4, 6, 8). Therefore, the NB cell line can serve as an ideal model for clinical investigations.

We employ a systematic proteomic approach by using two-dimensional DIGE analysis to identify differentially expressed proteins between these proteomes derived from SH-SY5Y cells before and after ATRA-induced differentiation (27). Our screening results in the identification of GRP75 as a novel biomarker associated with the differentiation of NB. The enhanced expression of GRP75 in SH-SY5Y cells after differentiation depicted by two-dimensional DIGE is quantitatively confirmed by Western blotting (two-dimensional and one-dimensional) and visualized by immunofluorescence staining (Figs. 1 and 2). Due to the component heterogeneity of the NB tumors, the lack of clear correlation between the histologic grade of differentiation and the protein levels of GRP75 was observed in whole tumor lysates (Fig. 2A; refs. 12, 26). This discrepancy was then resolved by specifically assessing the GRP75 expression in NB cells by immunohistochemical analyses. The results show that the intensity and percentage of GRP75 immunostaining indeed correlates well with the histologic grade of differentiation and the clinical stage of tumors (Fig. 3; Table 1). Most significantly, positive GRP75 immunostaining strongly correlated with the clinical factors of tumors detected by mass screening as well as early clinical stages (Table 1). Interestingly, NB tumors that are either at early clinical stages or detected by mass screening are inclined to spontaneous differentiation or regression (4). Altogether, our results favor that the expression of GRP75 correlates preferentially with the differentiation of NB cells both *in vitro* and *in vivo*.

Survival analysis has shown that positive GRP75 expression predicts a favorable prognosis in NB patients independent of age, clinical stage, tumor histology, or MYCN status. In addition, although GRP75 expression correlates well with histologic grade of differentiation, its expression predicts a favorable prognosis in patients with either undifferentiated or differentiated histologies of NB (Fig. 4A and B). These findings suggest that GRP75 could negatively regulate the growth of NB by promoting its differentiation. Furthermore, although clinical stage and MYCN status are two well-recognized most important clinical and biological prognostic factors for NB (3, 25), NB patients with advanced stages (stages III and IV, 48 of 72 patients, 5-year survival rate of 33.7%) and absence of MYCN amplification (56 of 72 patients, 5-year survival rate of 68.8%) are actually two large group of prognostic heterogeneity. Additional factors are required for further discrimination of these two groups of patients. Although GRP75 expression cannot differentiate the prognostic difference among NB patients with early stages or those with MYCN amplification, our analyses show that positive GRP75 expression predicts a favorable prognosis among NB patients with advanced stages as well as those with tumors carrying normal MYCN copy number (Fig. 4C and D). Thereby, assessing GRP75 expression in NB may provide complementary prognostic information in addition to clinical stage and MYCN status, which in turn may help to determine the most appropriate therapy for the NB patients.

It has been shown that GRP75 is up-regulated in various cancer tissues, cancer cell lines, as well as immortalized cell lines, suggesting that up-regulation of GRP75 may contribute to tumorigenesis (28). However, our present study establishes a novel role of GRP75 in NB by showing that up-regulation of GRP75 is closely correlated with the differentiation of NB cells both *in vitro* and *in vivo*. GRP75 is initially known as mortalin due to its presence in the cytosolic fraction of mortal cell phenotype (15). Subsequent studies revealed that normal mortal cells exhibit a pancytoplasmic distribution of GRP75, whereas immortal cells display a perinuclear distribution (29). It has also been shown that nondividing cells express higher levels of GRP75 than dividing cells do (30). In addition, normal brain neurons and ganglion cells express high levels of GRP75 in the cytoplasm (30, 31). It is thus plausible that GRP75 could contribute to the nondividing, fully differentiated phenotype of normal neuronal and ganglion cells. Consistent with this notion, our present data show that the expression of GRP75 is evident in the cytoplasm of the mature ganglion cells or differentiating NB cells in the tumor tissue and that differentiated SH-SY5Y cells exhibit a pancytoplasmic and neuritic distribution of GRP75 (Figs. 2 and 3). Whether GRP75 localized at different intracellular compartments can play distinctive cellular functions is not clear. Nevertheless, our data show for the first time that the change in the intracellular distribution of GRP75 coincides with the development of neuronal phenotypes of differentiated NB cells.

A recent study has shown that GRP75 and GRP78, another favorable prognostic marker of NB (11), could bind to receptor for hyaluronan-mediated motility (RHAMM) with an associated down-regulation of RHAMM in Jurkat cells (32). The GRP75/78-RHAMM complex could then bind to the microtubules to stabilize the microtubules in the interphase and prevent the depolymerization of microtubules for the progression of mitosis (32). The essential role of RHAMM in neurite extension has been suggested (33), and the expression of RHAMM has been linked to the progression and metastasis of a variety of cancers (34). Our preliminary data show that SH-SY5Y cells treated with ATRA do exhibit reduced expression of RHAMM, correlating with enhanced neuronal differentiation (data not shown). The possibility thus exists that the pancytoplasmic GRP75 in differentiating NB cells, along with GRP78, may prevent these cells from engaging into mitosis by binding with and down-regulating RHAMM while promoting the neurite formation simultaneously. In addition, although GRP75 can predict the outcome of NB patients independent of other clinicopathologic factors, GRP75 expression is not an independent prognostic factor when GRP78 expression is also placed into the multivariate Cox regression model (data not shown). This finding suggests a possible synergistic correlation between these two heat shock protein 70 family members, GRP75 and GRP78, in affecting NB tumor behavior.

In summary, our study showed that by using a systematic proteomic analysis of NB cells we are able to identify GRP75 as a new prognostic factor of NB. Expression of GRP75 correlates well with the differentiation of NB cells both *in vitro* and *in vivo*. Evaluation of GRP75 expression in tumor tissues of NB may provide complementary prognostic information for further subclassification of these tumors, which in turn may help to

determine the most appropriate strategy of treatment. Furthermore, because the expression of GRP75 strongly correlates with the differentiation of NB, GRP75 allows itself as a potential target of the treatment of NB. Together, the present data delineate a good example for the employment of proteomic approaches in the identification of potential biomarkers of cancers and recognize the potential of GRP75 as a therapeutic target of NB.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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