



Published in final edited form as:

J Surg Res. 2007 October ; 142(2): 268–274.

SERUM PROTEIN PROFILING TO IDENTIFY HIGH-RISK NEUROBLASTOMA:

PRECLINICAL RELEVANCE OF BLOOD-BASED BIOMARKERS

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Abstract

Introduction—Development of early detection assays for advanced stage neuroblastoma (NB) remains elusive. We have previously shown serum protein profiling technologies can differentiate healthy from NB children. As various sources of patient related bias exist in serum proteins, we hypothesized a well controlled animal model may provide a better method to identify tumor blood-based markers during NB progression.

Methods—Tumors were induced in the left kidneys of nude mice by the injection of cultured human NB cells (10⁶). Sera was collected from control and tumor-bearing mice at 2, 4, and 6 weeks. Albumin-depleted sera was subjected to comparative proteomic profiling using two-dimensional gel electrophoresis. Paired samples at each time point were analyzed and differentially expressed serum proteins were identified by mass spectrometry. Additionally, sera proteomic analysis from children with Stage IV NB and healthy controls were performed.

Results—Overexpression of five mouse serum proteins [α_1 -acid glycoprotein, α_1 -antitrypsin, α_2 -macroglobulin, serum amyloid P-component, and serum amyloid A) were found only in NB-bearing mice. Changes in protein abundance were found to increase 2.5-fold ($p \leq 0.05$) between 2-, 4-, and 6-week mice. Underexpression of immunoglobulin kappa chain constant region (Ig κ -C) was observed in the sera of tumor bearing mice as compared to controls (2.5-fold, $p \leq 0.05$). Among NB patients, α_1 -acid glycoprotein, apolipoprotein A-IV, haptoglobin, and serum amyloid A were found to be upregulated.

Conclusions—We identified distinct acute phase proteins that show up-regulation in both an animal tumor model and high-risk NB patients. As these serum proteins have been recognized as markers of tumor progression and prognosis in human malignancies, the validation of these polypeptides may enable serum proteomic profiling to become a valuable tool for identifying high-risk NB.

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Keywords

Neuroblastoma; High-risk pediatric malignancy; Animal model; Serum proteomics; Acute phase proteins/response

INTRODUCTION

Neuroblastoma (NB) attracts attention among solid pediatric cancers in that approximately 50% of tumors present as metastatic (advanced-stage) disease and, as result are frequently associated with suboptimal prognosis and survival [1,2]. Because of these outcomes, screening programs were instituted with the hopes of decreasing mortality associated with high risk NB. Yet despite this early warning system for NB, the clinical survival of advanced stage disease has not changed over the last decade [3]. For these reasons, novel strategies are needed to advance the early detection of high-risk NB.

One area that promises to harbor effective biomarker applications in oncology is clinical proteomics. The utility of proteomics-based methodologies is such that it offers the potential of identifying signature patterns of multiple proteins specific to a particular cancer. Using these techniques, our previous work showed mass spectrometry-based protein profiles/pattern differences between serum samples among children with NB and non-cancer patients [4]. Nevertheless, a critical concern using serum-based analysis for biomarker detection are potential sources of bias that exist at the patient level (e.g., gender, age, genetics, environmental, dietary, and psychological factors) and the analytical level (blood collection/sample preparation and storage/handling methods) [5]. To address these concerns, we have undertaken the present study to better control these potential sources of variability.

In this study, we have implemented an established animal model of human NB that grows as a progressive intraabdominal solid tumor combined with proteomic technologies to identify serum markers of advanced stage NB. By elucidating differentially expressed serum proteins in an *in vivo* NB model that minimizes the bias of human studies, we provide clues to the molecular pathology of disease progression and provide the basis for developing a serum-based proteomic approach for finding early high-risk NB.

MATERIALS AND METHODS

Cell line and Mouse tumor implantation

The human NB cell line, SK-N-DZ, was grown as a monolayer in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and 10% fetal bovine serum at 37°C in 5% CO₂. Cells were harvested, counted (10⁶), and prepared according to Rowe et al [6].

The Indiana University Institutional Animal Care and Use Committee (IU IACUC) approved this protocol, and a total of 15 NCR female nude mice (4-6 weeks old) were used. After a 7-day acclimation period, mice were separated into 4 groups; Group 1 (control, n=6), Group 2 (2-week tumor, n=3), Group 3 (4-week tumor, n=3), and Group 4 (6-week tumor, n=3). Mice were anesthetized with intraperitoneal ketamine (50 mg/kg) and xylazine (5 mg/kg) and underwent tumor implantation (10⁶) in the left renal parenchyma as described by Rowe and co-investigators [6]. Controls for each experimental group (2 per group) underwent a sham operation and the left kidneys were injected (100 µl) with phosphate buffered saline (PBS).

Cardiac puncture, Serum collection, and Preparation

Cardiac puncture was performed to collect blood from each group according to the 2-, 4-, or 6-week time point. Mice were lightly anesthetized with inhaled halothane and a 1-CC syringe with 20-gauge needle (Becton Dickinson and Co., Franklin Lakes, NJ) was used to gain access to the heart. The mice were placed dorsally and the left chest wall was felt between the thumb and forefinger as to locate the beating heart. The needle of the syringe was lined up laterally above the chest wall in order to estimate how far the needle would need to be inserted into the chest to reach the heart. The needle was inserted laterally into the chest cavity, and advanced until the heart was punctured and blood began to rush into the syringe. The plunger of the needle was then gently aspirated as to collect blood from each cardiac cycle as possible and not collapse the heart. Using this technique, approximately 0.7 to 1.0 ml of blood was collected in 1.5 ml eppendorf tubes and placed on ice. Following the cardiac puncture the animal was euthanized per protocol approved by the IU IACUC. Blood was allowed to clot for 1 hour then immediately centrifuged at 2,500 rpm for 10 minutes at 4°C. Serum (0.3-0.5 ml) was separated into 100 µl aliquots and stored at -80°C until proteomic analysis.

Albumin-depletion of serum samples

The removal of high-abundance proteins from serum is a widely used initial step in biomarker discovery studies. Because serum albumin is a predominant protein in serum, we employed an albumin-depletion strategy prior to serum sample proteomic analysis. The albumin depletion approach we used is based on disposable (single-use) pre-packed albumin-specific binding resin columns (ProteoExtract® Albumin Removal Kit, Calbiochem, San Diego, Ca). The depletion procedure was performed at room temperature according to manufacturer's instructions.

Protein determination, Two-Dimensional Gel Electrophoresis (2D-PAGE), Image Analysis, Trypsin Digestion, and Mass Spectrometry

Total protein concentration in the depleted serum fractions was determined using the Bio-Rad protein assay (Bio-Rad) according to the manufacturer's instructions with bovine serum albumin (BSA) as standards. A total of 250 µg of protein was used for our analyses for both mice and human samples. The desired protein volume was subsequently lyophilized to 90% dryness in a speed-vac (ATR-Biotech). Samples were reduced with tris(2-carboxyethyl) (TCEP) and alkylated with 400 mM 4-vinylpyridine (4-VP). Protein samples were desalted using Protein Desalting Spin Columns (Peirce) and re-lyophilized in the speed-vac. Samples were re-suspended in rehydration buffer (7M Urea, 4% CHAPS, 0.2% Bio-Lytes (Bio-Rad), 2 mM tributylphosphine, 0.001% bromophenol blue). Proteomic methods consistent with isoelectric focusing (IEF), 2D-PAGE, image analysis (spot detection, quantification, and matching), in-gel trypsin digestion, and mass spectrometry were accomplished by previously described methods [7].

Human sample collection for serum proteomic analysis

Subjects were selected based on criteria approved by the Indiana University School of Medicine Institutional Review Board. Ten subjects were selected: five patients with Stage IV NB and five healthy children constituted control samples. The control group consisted of children who underwent an outpatient surgical procedure [thyroglossal duct cyst removals, hemangioma excision, orchipexy, inguinal hernia repairs, hydrocele repair, and cutaneous lesion excision (lipomas, dermoids, scar revisions, etc.)] at Riley Hospital for Children. Any evidence of prior malignancy (other than NB) or active/chronic infection classified as exclusion criteria for both groups in this study. Three milliliters of blood was collected in red top tubes from both groups and immediately centrifuged at 2,500 rpm for 15 minutes. Serum (1 ml) was separated into

100 μ l aliquots and stored at -80°C until proteomic analysis. Sera was prepared and analyzed as described for the mice sera.

RESULTS

***In vivo* growth of the human NB SK-N-DZ cell line xenografted into nude mice**

To search for early detection blood markers of NB, we utilized the animal NB model system as described by Rowe et al., which is characterized by an intraabdominal orthotopic tumor growth that shares many physical features attributed to clinical NB tumors [6]. SK-N-DZ was injected within the renal parenchyma of nude mice and allowed to grow for 2, 4, and 6 weeks. All mice survived tumor implantation and were alive at the time of blood collection. Mean tumor weight between the experimental mice was 0.46 ± 0.09 g (2 weeks) 3.21 ± 0.85 g (4 weeks) and 6.02 ± 1.09 g (6 weeks). Fig. 1 shows a 6-week implanted tumor in a nude mouse at necropsy. Gross examination of the tumor revealed a large bi-lobed, bulky mass which displaced abdominal structures and resided as a predominant intraperitoneal structure (Fig 1A). On extirpation of the tumor (weight 7.2 g), residual kidney parenchyma is observed (Fig 1B, arrow) as the NB tended to overgrow and replace renal tissue. In our model, mice injected with SK-N-DZ cells had no gross evidence of tumor metastasis to the liver or lungs as has been reported with the cell line SH-SY5Y [6].

Differential sera proteomic expression in nude mice implanted with human NB cells

At each designated time interval, blood was collected and albumin-depleted sera (250 μ g) was subjected to 2D-PAGE to generate serum protein profiles of healthy control and NB mice. Fig. 2 shows representative 2D-protein sera maps depicting tumor growth at 2, 4, and 6 weeks after injection of SK-N-DZ. Using these protein expression profile gels from PBS treated (control) and NB-induced mice at 2, 4, and 6 weeks, we analyzed and identified differentially expressed proteins using computer-assisted spot detection software (Phoretix 2D Evolution) between Coomassie blue stained control and tumor gels. To better define proteins that were associated with tumor progression, we concentrated on proteins whose expression was greater than 2.5-fold in each successive time interval. Similarly, proteins that had successive decline greater than 2.5-fold from 2 to 6 weeks were also selected. Unique polypeptides were identified by MALDI-TOF mass fingerprinting.

By comparing serum proteins profiles of healthy nude mice and nude mice injected with NB cells, we identified 6 proteins that exhibited detectable and statistically significant quantitative changes ($p < 0.05$); 5 were up-regulated and 1 was down-regulated in tumor-bearing mice (Table 1). Overexpressed proteins included α_1 -acid glycoprotein (AGP), α_1 -antitrypsin, α_2 -macroglobin, serum amyloid P (SAP), and serum amyloid A (SAA). Categorically, these proteins represent major acute phase proteins (APPs) found in serum. In contrast, we identified immunoglobulin kappa chain constant region (Ig κ -C) as a protein whose expression decreased in tumor-bearing mice over 6-weeks. Alternatively, Ig κ -C appeared to be strongly induced in healthy controls as compared to NB mice. Taken together, these results imply circulating levels of specific APPs correlate with NB progression and serum Ig κ -C changed in response to NB growth.

Sera protein profiles in patient samples with Stage IV NB

In order to correlate whether the identified mice serum proteins had clinical significance, we analyzed the relative abundance of serum proteins using the proteomic methods listed from a total of 10 patient samples between Stage IV NB ($n=5$) and healthy children ($n=5$). Fig. 3 shows a representative 2D-PAGE pattern obtained using 250 μ g of protein among NB and control sera. Within our set of 10 patients, we identified five spots which were differentially expressed between cancer and healthy children. The spots were subsequently selected and identified by

MALDI-TOF. The altered plasma proteins are summarized in Table 2. They were classified as members of specific families, and they were found to include APPs and immune-related proteins. The APPs were α_1 -acid glycoprotein (AGP), apolipoprotein A-IV (ApoA-IV), haptoglobin (HP), and serum amyloid A (SAA). These were observed to be increased in the advanced stage NB samples as compared to controls. Similarly to the mice results, we showed immunoglobulin kappa chain constant region (Ig κ -C) to be decreased in cancer sera. The expression of these polypeptides were statistically significant ($p \leq 0.05$). In all, these preliminary results suggest sera from advanced stage NB express a similar acute phase response as the orthotopic mouse NB model and thus may play an important role in the progression of this malignancy.

CONCLUSION

Challenges exist in the discovery of early detection high-risk NB specific biomarkers. Advances in high-throughput technologies, such as gene arrays and proteomic profiling, are impacting the ways in which the next-generation cancer biomarkers are found. Furthermore, because genetic mutations lead to distinct expression changes at the protein level, we have taken considerable interest in protein pattern proteomics as a tool to detect high-risk NB. Using protein pattern recognition methods, we have previously shown discriminatory evidence distinguishing serum proteins between children with NB from healthy patients [4]. Yet confounding sources of bias associated with serum proteomics such as patient demographic (age, gender, etc.) and sample collection, preparation, and storage methods have raised concerns over serum protein profiling of disease [5]. In order to address these issues, we used a preclinical animal model of human NB combined with a proteomic approach to identify differentially expressed serum proteins that may be associated with NB progression.

We found 6 serum proteins that were uniquely expressed in a growing animal model of human NB. Five proteins were successively upregulated during the examined time interval (2-, 4-, and 6-weeks); α_1 -acid glycoprotein (AGP), α_1 -antitrypsin (ATT), α_2 -macroglobulin (A2M), serum amyloid P (SAP), and serum amyloid A (SAA). As these serum proteins belong to the family of APPs in mice, the clinical significance of each was evaluated in relation to tumor growth. All proteins identified have links to cancer progression [8-16] and we demonstrated an increase of each of these APPs in tumor-bearing mouse sera (Figure 2). The serum immunoglobulin protein Ig κ -C was found to decrease in mice harboring NB as compared to controls. Surprisingly, aberrant expression of Ig κ -C in cancer cells and tissues has been reported [17, 18]. While we did not observe this trend in the present study, further work is needed to clarify the reduction of this serum Ig with regards to NB tumor progression.

As the animal results represent attractive proteins from which more accurate diagnostic biomarkers may be found for high-risk NB, we compared serum protein profiles from children with Stage IV NB and healthy controls. Our data demonstrate the differential expression of 5 proteins; α_1 -acid glycoprotein (AGP), apolipoprotein A-IV (ApoA-IV), haptoglobin (HP), and serum amyloid A (SAA). We also show the relative decrease of Ig κ -C in NB patient samples compared to healthy children as observed in the animal NB model. With regards to the upregulated serum proteins, all represent APPs. AGP is a normal component of human blood and has strong associations as a cancer progression marker and immunomodulation [8,9, & 19]. ApoA-IV is a 46 kD plasma glycoprotein with limited associations with cancer; Kawakami and colleagues showed ApoA-IV to increase in sera from hepatocellular cancer after radiofrequency ablation [20]. HP is a well characterized APP with increasing evidence as a cancer biomarker [21-25]. Lastly, SAA is an acute phase apolipoprotein associated as a marker of cancer progression [26-30]. In NB, Combaret et al identified high SAA levels were detected in patient sera with poor prognosis [31]. Taken altogether, the overabundance of APPs in

relation to malignancy suggests serum proteomic profiling of the APP response is a promising method to enable early detection of high-risk NB.

Despite corroboration of APPs to cancer, the expression of these common serum proteins in our tumor progression model has limitations. For instance, Koomen et al identified a similar plasma protein response for pancreatic cancer. While showing promising sensitivity, the low specificity of their results was plagued by the fact that APPs are not specific for cancer [32]. Nevertheless, as APPs may not be NB-specific signatures for advanced disease, it has been postulated that APPs may exert a tumor-mediated immune suppression. Reports have implicated APPs acting as non-specific blocking factors protecting tumors against the host's immunological attack [33-35]. This notion is indirectly supported by our results which show overexpression of serum APPs in an animal NB progression model and preliminary human NB samples. Whether the APPs are host induced by tumor secreted cytokines or directly produced by the tumor remains to be confirmed. As it is intriguing to surmise that APPs are expressed for tumor protection, this may simply reflect a host inflammatory response.

In this study, we describe the application of proteomic techniques to identify NB-associated serum markers. By screening an orthotopic nude mouse model of human NB for candidate serum biomarkers and examining their presence in the sera of Stage IV NB patients, we identified 6 serum proteins in the progressive mouse model and 5 serum proteins in the human NB subjects. With the exception of Ig κ -C, all proteins represent APPs. Because these represent common serum proteins, it has been proposed that the dysregulated cellular processes leading to advanced-stage cancer lead to the up-regulation of these high-level APPs [36]. As deciphering these neoplastic pathways is paramount, strategies for early detection of advanced-stage NB will likely include both gene array profiling along with serum proteomics. This study demonstrates the use of a preclinical model to build signature serum protein profiles in NB progression. The implications from the preliminary human samples underscore the significance of clinical proteomics that may lead to improved outcomes and reduced recurrence associated with high-risk NB.

ACKNOWLEDGEMENTS

The authors would like to thank both the Vera Bradley Foundation and the A.N.N.A. foundation (Indianapolis, IN) for their continuous support of our research. Additionally, we are grateful to Drs. Jessica Kandel and Jianzhong Huang (Columbia University, Babies & Children's Hospital of New-York) for instruction and methodologies related to the animal model of neuroblastoma.

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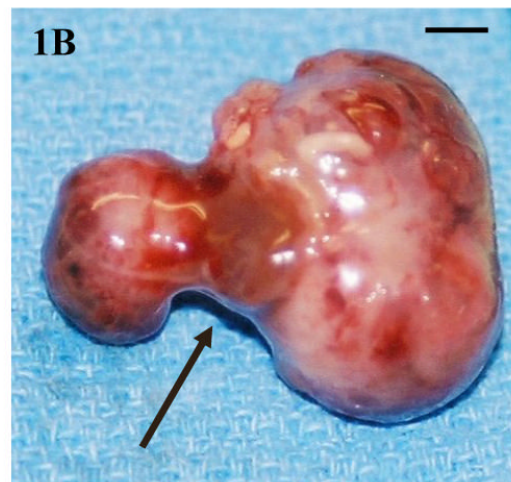
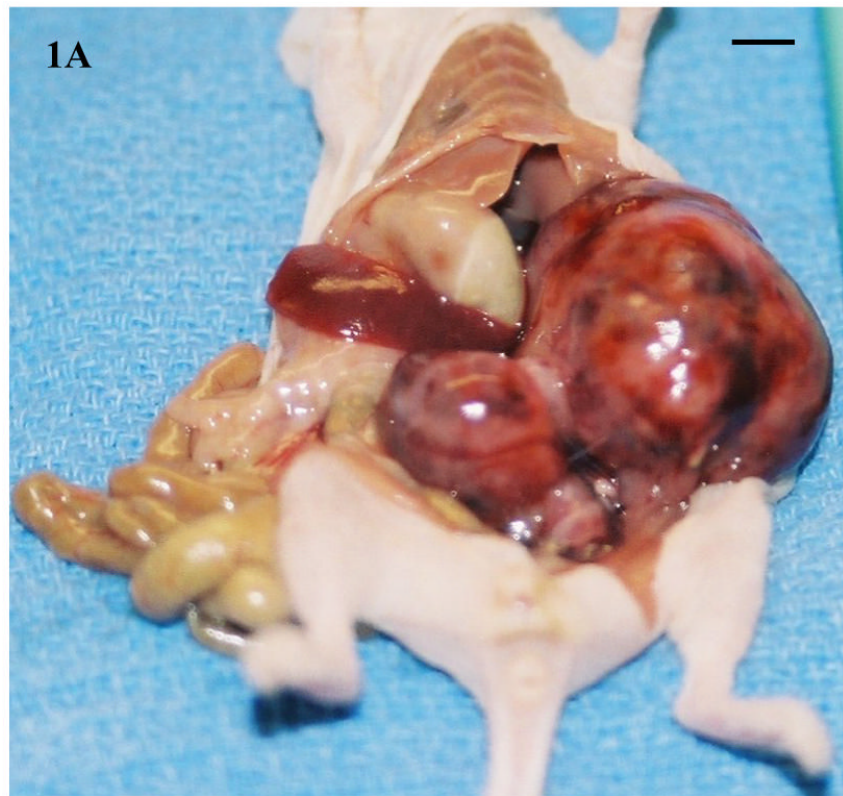


Figure 1. NCR-nude mouse implanted with SK-N-DZ human neuroblastoma (NB) cell line at 6-weeks

Fig 1A shows a large intra abdominal tumor at necropsy. On removal of tumor, a large bi-lobed mass is appreciated (Fig1B). Weight of tumor was 7.2 g. An arrow shows area of residual renal parenchyma. Bar represents 0.5 cm.

Comparison of the 2D PAGE Patterns from Xenograft NB Mice Sera

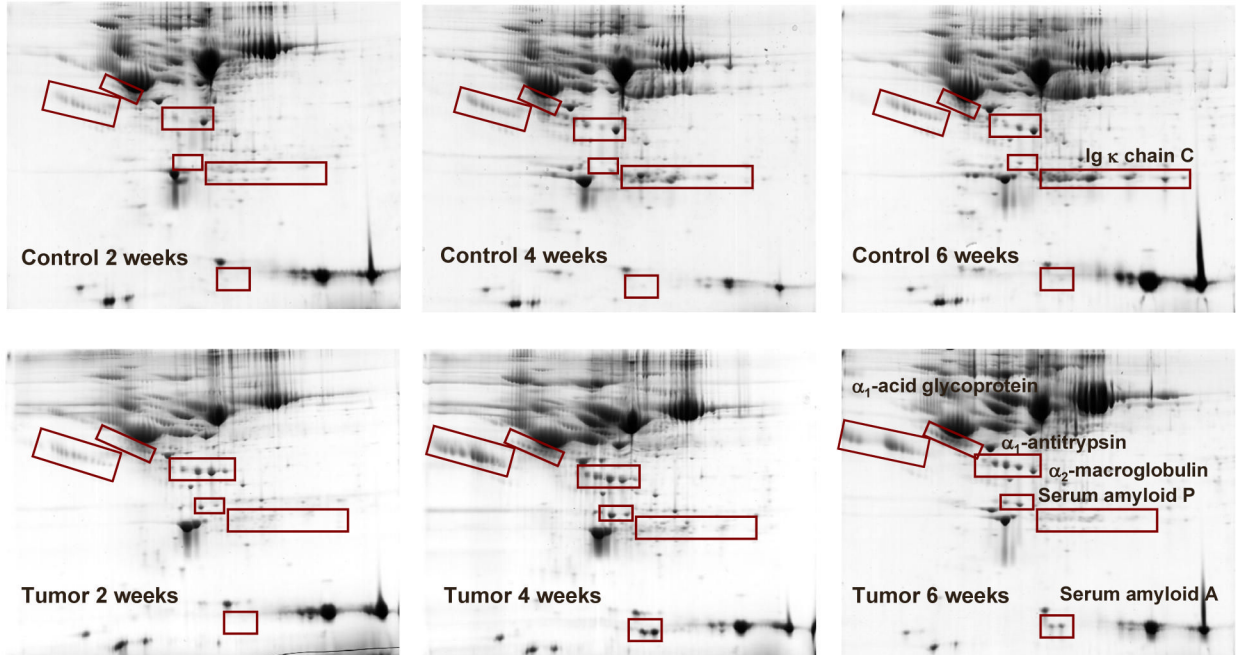


Figure 2. Proteomic profiling maps of orthotopic NB mouse sera versus healthy controls
 NCR-nude mice were implanted with NB (10^6) and allowed to grow for 2, 4, and 6 weeks. At each time interval, sera was collected via cardiac puncture. Sera was depleted of albumin and subjected to 2D-PAGE. The detection of protein changes and identities was accomplished using gel analysis software and mass spectrometry as described in the Materials and Methods. The top panel shows sera from control mice (kidneys were injected with PBS). The bottom panel shows sera collected from NB implanted mice at 2, 4, and 6 weeks. The identification of proteins in the control mice (top panel) or tumor-bearing mice (bottom panel) indicates proteins that increased 2.5-fold during the observed time interval.

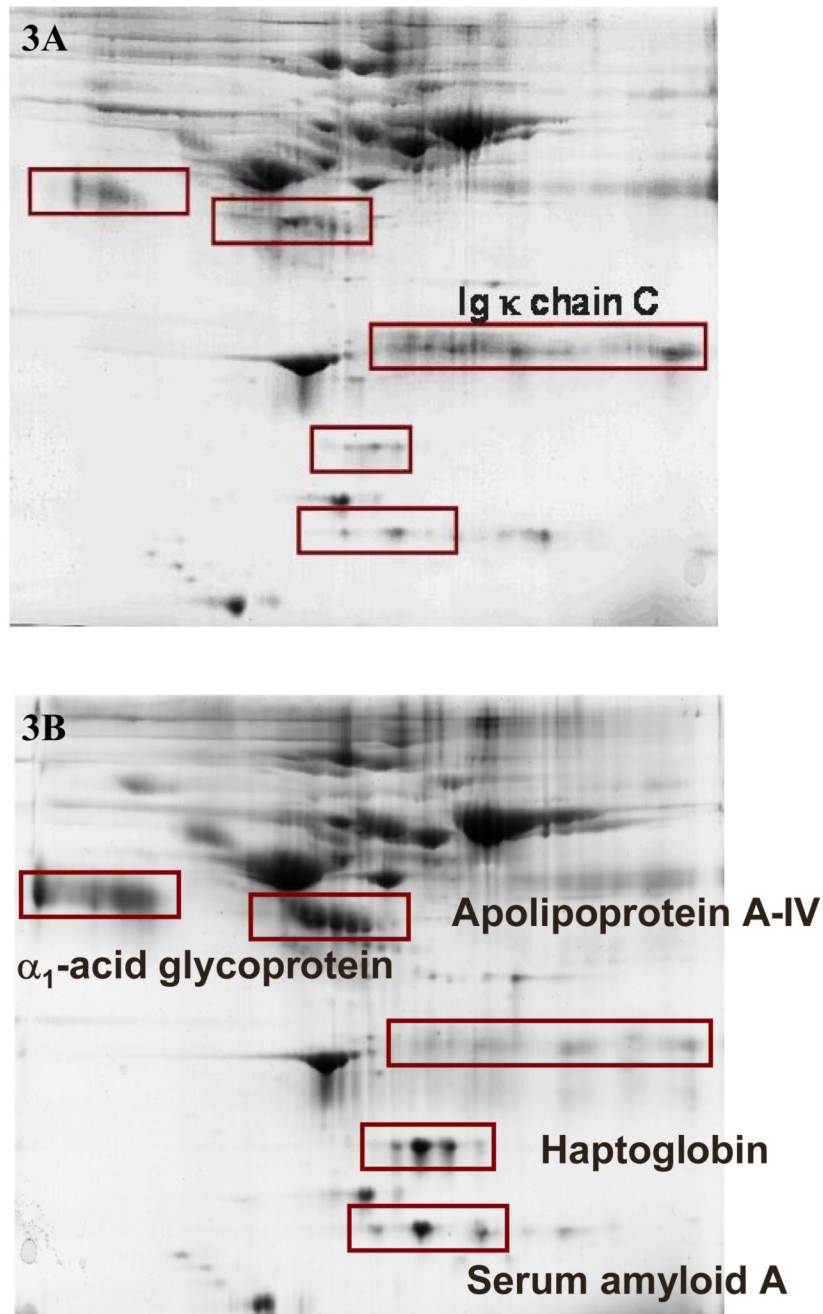


Figure 3. Clinical correlation of sera protein profiles from patients with Stage IV NB and healthy children

Blood was obtained from 10 patients; 5 with Stage IV NB and 5 healthy controls. Sera was subjected to proteomic methodologies as described for the mouse NB model. Fig 3A shows a representative healthy control sera protein profile from 5 human samples. Fig 3B shows a representative advanced stage NB protein profile from 5 children with NB. Unique proteins that were identified were statistically significant (greater than 2.5-fold expression, $p \leq 0.05$).

Table 1
Differentially expressed proteins in NB animal progression model

Mouse Serum Proteins				
Protein Identification	Accession Number	Molecular Weight (kDa)	pI	Function
Ig κ chain C	P01837	11.71	Undefined	Secreted immunoglobulin
α_1 -acid glycoprotein	P07361	21.87	5.28	Acute-phase protein
α_1 -antitrypsin	P22599	43.47	5.32	Acute-phase protein
α_2 -macroglobin	Q61838	134.73	6.12	Acute-phase protein
serum amyloid P	P12246	23.87	6.38	Acute-phase protein
serum amyloid A	P05367	11.60	5.89	Acute-phase protein

Table 2
Differentially expressed proteins in human Stage IV NB serum samples compared to healthy controls

Human Serum Proteins				
Protein Identification	Accession Number	Molecular Weight (kDa)	pI	Function
Ig κ chain C	P01837	11.71	Undefined	Secreted immunoglobulin
α_1 -acid glycoprotein	P07361	21.87	5.28	Acute-phase protein
apolipoprotein A-IV	P06727	43.40	5.18	Acute-phase protein
haptoglobin	P00738	43.34	6.13	Acute-phase protein
serum amyloid A	P05367	11.60	5.89	Acute-phase protein