Original article

Apoptosis of non-tumor cells contributes to increased serum cytochrome c level in a neuroblastoma xenograft model

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Background Neuroblastoma (NB) is one of the most common malignant solid tumors of childhood. It is still not clear whether the apoptosis of tumor cells or the non-tumor cells contributes to the increase of concentration of cytochrome c (Cyt c) in the serum of the cancer patients. The aim of this research was to identify the source of the Cyt c in the serum when the tumor grows up by subcutaneous inoculation of human NB cells into nude mice.

Methods We subcutaneously inoculated human NB cells (KP-N-NS) into nude mice and collected the sera of tumor-bearing mice (n=14) and control mice (n=25) 4 weeks later in order to screen for and identify differentially expressed proteins in the serum. Differentially expressed proteins in the serum were screened by surface-enhanced laser desorption/ionization-time-of-flight (SELDI-TOF) mass spectrometry.

Results The relative intensity of a protein having a mass-to-charge ratio (m/z) of 11 609 was 3338.37±3410.85 in the tumor group and 59.84±40.74 in the control group, indicating that the expression level of this protein in the tumor group was 55.8 times higher than that in the control group. Serum proteins were separated and purified by high-performance liquid chromatography (HPLC). Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was performed to produce peptide mass fingerprints (PMFs). Spectrum analysis and a database search revealed that the highly expressed protein (m/z=11 605.4) from the serum of tumor-bearing mice was the mouse Cyt c.

Conclusions Increased concentration of Cyt c in the serum of tumor-bearing nude mice might be partially attributed to the secretion of this protein by non-tumor cells.

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Neuroblastoma (NB) is one of the most common malignant solid tumors of childhood with the incidence rate of 10.9 cases per million people in Europe.[1] NB accounts for about 28% of all cancers diagnosed in European and US infants. However, very few serum markers have been identified for NB detection in clinical diagnosis.

Surface-enhanced laser desorption/ionization-time-of-flight (SELDI-TOF) mass spectrometry is suitable for clinical diagnosis and mass screening of disease-related biomarkers, because it needs only a small amount of sample and results can be obtained within a short time.[2,3] Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) is suitable for the detection of compounds and biological macromolecule and is a useful proteomic analysis method for identification of proteins or biomarkers through sequence alignment of peptide mass fingerprints (PMFs) to those in a database.[4]

The differentially expressed proteins in the serum have been screened and identified in various tumors for the early diagnosis and prognosis evaluating of the tumor. A good many of differential proteins, including cytochrome c (Cyt c), have been isolated and identified. When the concentration of Cyt c in the serum of the cancer patients increases with no tumor specificity, it is usually thought to be released from the apoptotic cells. It is still not clear whether the apoptosis of tumor cells or the non-tumor cells contributes to the increase of concentration of Cyt c in the serum of the cancer patients. In this research, the human NB cells were subcutaneously inoculated into nude mice to identify the source of the Cyt c in the serum when the tumor grows up. In this study, we compared the expression levels of serum proteins between tumor-bearing mice and controls. The aim of this study was to identify and determine the source of these differentially expressed proteins.

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METHODS

Cell culture and nude mice animal experiments

Fifty female nude mice, aged 3–4 weeks, were randomly divided into 2 groups of 25 mice each, for both the tumor group and the control group. NB cells were inoculated after a 1-week adaptation to the environment. All of the experimental procedures in this study were approved by the Ethics Committee at the First Affiliated Hospital of Zhengzhou University.

The human NB cell line KP-N-NS was grown in a medium supplemented with 10% fetal bovine serum (FBS) at 37°C in the presence of 5% CO_2. Tumor cells in the log phase were harvested, centrifuged, and resuspended in a new medium to a final concentration of about 1×10^7 cells/ml. A 0.1-ml aliquot of the cell suspension (1×10^6 cells) was inoculated subcutaneously in the groin of nude mice; mice of the control group were injected with the same volume of cell culture medium.

Collection of serum samples

Four weeks after inoculation with cancer cells, serum samples from tumor-bearing mice and control mice were collected. After euthanization by ketamine injection, the chest of a mouse was immediately dissected, and the heart was pierced to collect blood. The volume of blood samples ranged from 0.3 ml to 0.6 ml. Blood samples were centrifuged at 3000 r/min for 10 minutes and then stored at –80°C.

Screening for differentially expressed proteins by SELDI-TOF mass spectrometry

Serum samples from the tumor and control groups were thawed on ice for 30 to 60 minutes and centrifuged for 5 minutes at 10 000 r/min at 4°C. A 5-μl aliquot of serum was added to 10 μl of U9 denaturing solution and vortexed at 600 r/min for 30 minutes at 4°C. Each U9-treated serum sample was diluted to 200 μl with a binding buffer and vortexed at 600 r/min for 2 minutes at 4°C. The samples were loaded onto a WCX2 ProteinChip (ID 1170324864). Before testing the sample chip, the accuracy of the SELDI mass spectrometry system was calibrated to less than 0.1% with an All-in-One protein standard. Raw data were collected by using the ProteinChip Software 3.2 (Ciphergen Biosystems, USA), with the laser intensity set to 185, sensitivity to 7, and maximum detection limit at a mass-to-charge ratio (m/z) of 100 000. Optimized data ranging from m/z 2000 to m/z 20 000 were collected.

Data processing

All data were analyzed using the ProteinChip Software 3.2. Spectra were externally calibrated and normalized to the total ion current. The noise of each spectrum was filtered by discrete wavelet transformation with the biomarker wizard of the ProteinChip Software 3.2 and the Zhejiang University Cancer Institute-ProteinChip Data Analysis System (ZUCI-PDAS, China). The baseline was subtracted and peak clusters were completed using second-pass peak selection (signal to noise ratio >2). The minimum threshold of cluster analysis was set at 10%. The m/z values within the 0.3% mass accuracy window were considered equal between replicates.

Since the efficiencies in distinguishing the sample for each m/z peak were significantly different, the ion intensity for the m/z of each protein of interest was analyzed by the Wilcoxon rank sum test. *P* values of each peak represented the difference between groups, and $P < 0.05$ was considered significantly different.

The non-linear support vector machines (SVM) classifier was used to compare mass spectrometry data for each sample. Differentially expressed proteins were further selected as candidates for protein markers. In this study, the SVM classifier was based on the radial-basis-function kernel type, with the γ parameter set to 0.6 and the penalty function set to 19.

Statistical analysis

After noise filtering and clustering analysis, spectra of different groups were analyzed by a *t*-test, with α set to 0.01 to determine the different proteins between the 2 groups.

Serum protein separation by high-performance liquid chromatography (HPLC)

The serum samples of the tumor-bearing group and the control group were thawed on ice. A 100-μl aliquot of the serum was diluted with 400 μl of ultrapure water, and 500 μl of acetonitrile (ACN) was added to precipitate the high-abundance proteins from the serum. The mixture was incubated at –20°C for 30 minutes and then centrifuged at 13 000 r/min for 10 minutes. The supernatant was transferred to a new tube and dried for 20 minutes in the SPD SpeedVac refrigerated vapor trap (Thermo Fisher Scientific, USA). The freeze-dried samples were separated by HPLC (C18 column: 5 μm 300GEA-C18-254605-0806236, SunChrom, Germany). A total of 34 tubes of protein solutions were collected at different time points. Purified protein solutions were dried by SPD SpeedVac and reduced to 20 μl for storage.

Determination of differentially expressed serum proteins

A 1.5-μl aliquot of the protein sample solution separated by HPLC was premixed with 1.5 μl α-cyano-4-hydroxycinnamic acid (CHCA) for deposition on the MALDI target plate and air-drying. Cyt c (12 361.96 Da) + CHCA and insulin (5734.51 Da) + CHCA were used as calibration controls. The target plates were loaded in the MALDI-TOF spectrometer for testing, and the peaks of the target protein with m/z 11 605.4 were tracked in the MALDI-TOF spectrum.

Identification of proteins by liquid chromatography coupled with tandem mass spectrometry

Differentially expressed proteins determined by MALDI-TOF were selected for further identification. A

20-μl aliquot of the corresponding protein fractions was mixed with 60 μl of 8 mol/L urea and vortexed to facilitate solubility. After the addition of 0.8 μl of 1 mol/L dithiothreitol (DTT), samples were incubated for 1 hour at room temperature; following the addition of 3.2 μl of 1 mol/L iodoacetamide (IAM), samples were incubated for 45 minutes in the dark. Another 3.2-μl aliquot of 1 mol/L DTT was added, and samples were incubated at room temperature for 30 minutes. The mixture was further diluted with 400 μl of 50 mmol/L NH_4HCO_3 to adjust the final urea concentration to 1 mol/L and the pH to 8.0. The mixture was digested overnight at 37°C with 0.08 μg trypsin, and the reaction was stopped by adding 0.2% trifluoroacetic acid (TFA). The digested protein sample was injected onto the liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) column. The PMFs produced by LC-MS/MS was compared to those in the SEQUEST in the Bioworks database (Thermo Fisher Scientific).

MALDI-TOF-MS-MS data processing and database searching

Launch-pad v2.4 software (Shimadzu Corporation, Japan) was used for data acquisition and processing. The post-source decay (PSD) analysis was performed using the Mascot sequence query (*http://www.matrixscience.com/search_form_select.html*) and the NCBI protein database, which allowed for up to 1 missed cleavage, and a peptide tolerance of ±0.3 Da (monoisotopic peak). The m/z values were monoisotopic.

RESULTS

Tumor xenograft and survival in nude mice

Seventeen of 25 (68%) nude mice in the tumor group developed tumors, ranging in diameter from 6 mm to 10 mm. Four weeks after subcutaneous inoculation of tumor cells, there was obvious weight loss in the tumor-bearing mice, and 3 mice died of cachexia in the fourth week. The remaining 14 tumor-bearing nude mice and control mice survived.

Serum protein profiling by SELDI-TOF mass spectrometry

Seventeen serum samples from the tumor group and 25 samples from the control group were detected by SELDI. The relative intensity of the peaks was analyzed by the Wilcoxon rank sum test. One peak with *P* value less than 0.001 was highly expressed in the tumor group and the m/z was 11 609. The relative expression levels of protein corresponding to the m/z 11 609 peak were 3338.37±3410.85 in the tumor group and 59.84±40.74 in the control group (P <0.001) (Figure 1).

Protein separation and MALDI-TOF analysis

Serum proteins from tumor-bearing nude mice were separated by HPLC, and 34 tubes of proteins were collected and sent for MALDI detection. The results of

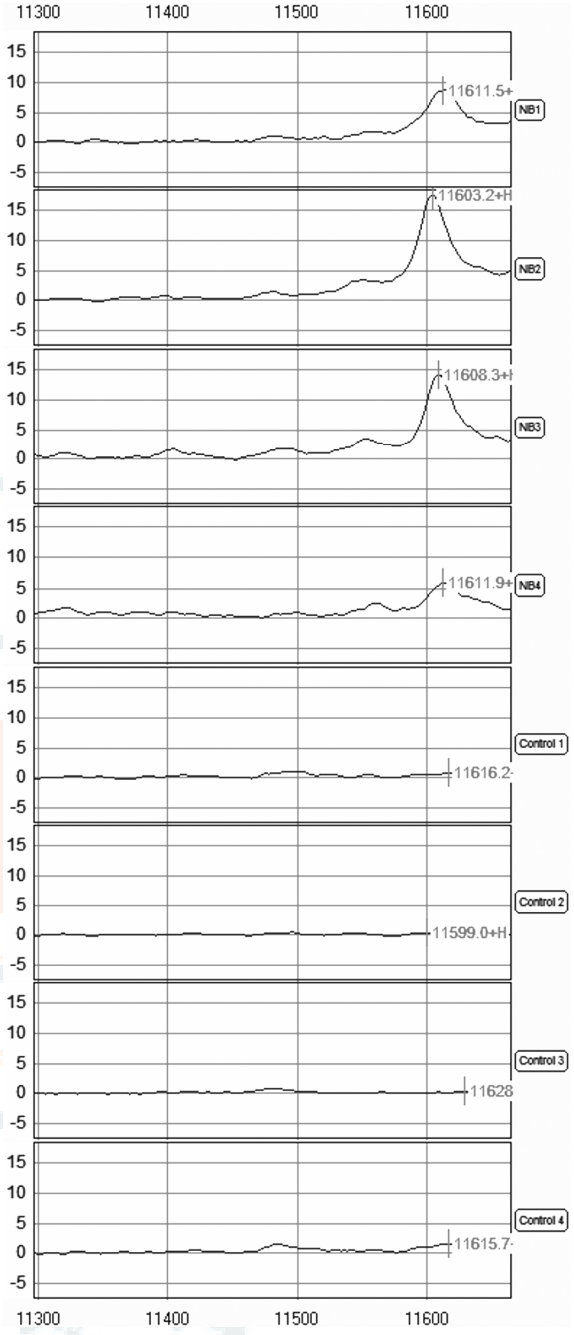

Figure 1. The sera protein content with m/z of 11 609 in the tumor-bearing mice was 55.8 times higher than that of mice in the control group.

MALDI revealed that the protein with m/z=11 606.2 was in the 26th tube (Figure 2).

Identification of target protein

The target protein was digested with trypsin, and the resulting peptide mixture was detected by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). The protein peaking at 11 606.2 was found to have 16 fragments with different amino acid sequences (Table 1; Figure 3). According to the sequence alignment with a database, the peptide sequences were determined to match the murine Cyt c protein（score: 160.28; coverage: 63.81).

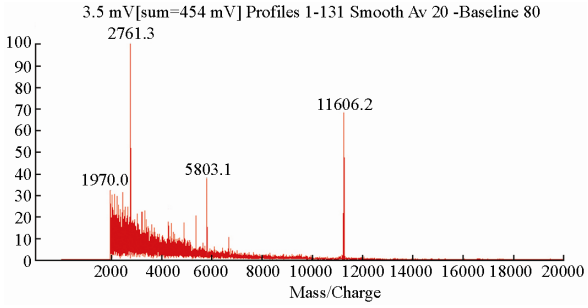

Figure 2. The MALDI result of the separated protein with m/z=11 606.2

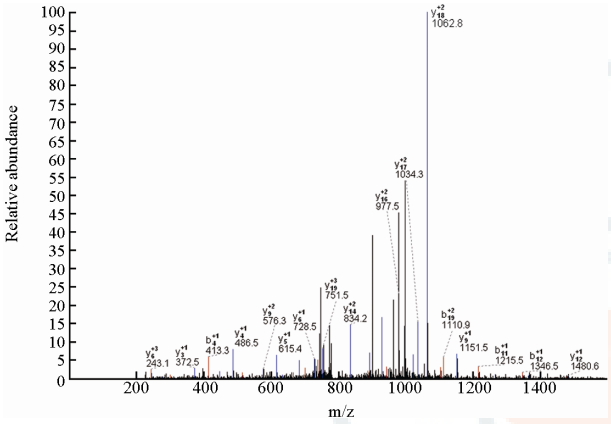

Figure 3. One PMF of murine cytochrome C protein. The amino acid sequence of the peptide was K.NKGITWGEDTLMEYLENPKK.Y.

Table 1. Identification of potential protein biomarkers with identified peptides and covered sequence

Peptides identified	Peptide (Hits)	Score	Coverage (%)
R.ADLIAYLK.K	13/14	2.61	0.18
K.MIFAGIKK.K	12/14	2.67	0.28
K.M*IFAGIKK.K	13/14	2.75	0.21
K.TGPNLHGLFGR.K	18/20	3.04	0.53
K.GERADLIAYLK.K	15/20	2.74	0.47
K.TGQAAGFSYTDANK.N	19/26	4.49	0.33
K.HKTGPNLHGLFGR.K	23/48	3.60	0.31
R.KTGQAAGFSYTDANK.N	23/28	5.48	0.53
K.TGQAAGFSYTDANKNK.G	23/30	5.01	0.50
R.KTGQAAGFSYTDANKNK.G	34/64	5.00	0.48
K.GITWGEDTLMEYLENPK.K	22/32	5.49	0.36
K.GITWGEDTLM*EYLENPK.K	21/32	5.58	0.61
K.GITWGEDTLMEYLENPKK.Y	24/34	5.08	0.64
K.GITWGEDTLM*EYLENPKK.Y	19/34	4.86	0.48
K.NKGITWGEDTLMEYLENPK.K	21/36	3.58	0.62
K.NKGITWGEDTLMEYLENPKK.Y	30/76	5.52	0.53

m/z: 11 606.2; Protein name: Cytochrome c, somatic [Mus musculus].

DISCUSSION

Cyt c is a cytochrome oxidase present in the inner mitochondrial membrane under normal physiological conditions.[5] It is not only a peripheral component of the electron transport chain in mitochondria but is also involved in apoptosis.[6,7] Extensive studies have been performed to reveal the role of Cyt c during apoptosis in many clinical conditions such as tumors, systemic inflammatory response syndrome (SIRS), and multiple organ dysfunction syndrome (MODS).[8] The serum level of Cyt c is elevated in patients with cancer, myocardial infarction, apoptosis-related liver disease, and influenza virus-induced encephalopathy.[9,10] Serum Cyt c is believed to be of considerable clinical importance in predicting disease-related abnormal apoptosis. When apoptosis is triggered, Cyt c is released from the mitochondria, and it accumulates in the cytoplasm. Translocated Cyt c binds to Apaf-1 and serves as a component of the caspase-9 complex. Activation of the caspase cascade leads to apoptosis.[11]

Cyt c can also be released from the cytoplasm to the extracellular environment. For example, in patients with SIRS or MODS, there is a persistent increase of serum Cyt c due to increased release from damaged cells rather than clearance from the kidneys and liver. Therefore, the serum level of Cyt c plays a role in determining the degree of organ damage and predicting the prognosis of patients with SIRS and MODS.[12] Moreover, the Cyt c level in patients with liver disease correlates with the extent of liver damage and the number of apoptotic liver cells, indicating that Cyt c is released from apoptotic cells.[13] These results show that Cyt c is a marker of cell membrane leakage, but it may also be released from other apoptotic cells. In the late stage of apoptosis, cell membrane leakage facilitates the release of cytoplasmic proteins that cause secondary necrosis.[14,15] During cancer chemotherapy, the level of Cyt c increases rapidly in the serum of patients because of increased cell death.[16]

The amino acid sequences of the Cyt c among different living things are some conservative, but they are different between *homo sapiens* and mice. The identified amino acid sequences in this research corresponded with the mice source Cyt c. If the Cyt c was released only due to the apoptosis of the tumor cells, the Cyt c in the serum should be human source Cyt c. The increased Cyt c in the mice serum in this research were mice source Cyt c, which proved they came from the non-tumor cells of mice. In our study, the serum concentration of Cyt c was significantly higher in nude mice inoculated with human NB cells than in the control group mice; this finding suggested that the increased serum Cyt c levels in tumor-bearing mice was not only due to apoptosis of tumor cells but may also be due to its release from non-tumor cells.

Previous studies have shown that when NB cells and Chang human hepatocytes (HC) were co-cultured, apoptosis regulation changes.[17] HCs upregulated the expression of Bcl-2 in NB cells and increased the tolerance of NB cells to apoptosis. In contrast, there was a 20-fold increase in apoptosis of HCs, which was mediated by NB cells through upregulated tumor necrosis factor (TNF) and Fas/Fas-L expression levels.[17] Therefore, it is reasonable to hypothesize that NB cells increase apoptosis of their surrounding cells or tissues, causing non-cancerous cells to release Cyt c and thus

increasing the serum concentration of Cyt c.

On the other hand, apoptosis affected not only the tumor cells but also other tissues surrounding or distant from the tumor foci in patients with MODS due to cachexia, particularly those with advanced cancer. In our study, 3 nude mice died of cachexia 4 weeks after inoculation with tumor cells, indicating that MODS occurred in some mice at this time and that apoptotic or necrotic non-tumor cells in the body released Cyt c.

Additionally, the apoptosis of neuronal cells could increase along with an increase in the concentration of Cyt c in the cell culture medium.[18] When apoptosis of tumor cells occurred in the tumor-bearing nude mice, the extracellular concentration of Cyt c increased, resulting in the apoptosis of non-tumor cells. This may be another reason for the increase in levels of serum Cyt c.

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