Decorin as a prognostic biomarker in patients with malignant peripheral nerve sheath tumors

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Abstract. Malignant peripheral nerve sheath tumors (MPNSTs) are aggressive soft-tissue sarcomas. The prognosis of MPNSTs has been reported to differ among previous studies. However, there have been a number of reported prognostic biomarkers associated with MPNSTs. In the present study, a proteomics study was performed to discover the differential protein expression in patients with MPSNTs with different prognoses. The clinical data of 30 primary extremities of patients with MPNSTs, who underwent surgery at the Department of Hand Surgery, Huashan Hospital, Fudan University between January 2002 and December 2011, were acquired. A total of 16 patients succumbed to their diseases within 5 years, whereas 14 patients were disease-free for >5 years. Samples from the 9 patients who succumbed within 2 years were assigned to Group D, while samples from the 8 patients who were continuously disease-free for >5 years following diagnosis were assigned to Group L for the proteomics study. Label-free quantitative proteomics and mass spectrometry were performed to filtrate differential protein in patients with MPSNTs with different prognoses. Decorin was filtrated as a differential protein of note. The expression level of decorin was significantly lower in Group D compared with that in Group L (D/L=0.0948; P=0.0004). The result was verified by immunohistochemical staining in the 30 primary extremities of patients with MPNSTs. The 5-year survival rate of patients with positive expression of decorin was 78.57%, while the 5-year survival rate of patients negative for decorin expression was 18.75% (P=0.0014). Overall, a high level of decorin indicted a better prognosis in patients with MPNSTs. With further investigation, decorin may be a reliable prognostic biomarker for MPNSTs.

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Introduction

Malignant peripheral nerve sheath tumors (MPNSTs) are aggressive tumors that comprise 5-10% of all soft-tissue sarcomas (1). The extremities are the most common sites in which these tumors occur. The most common treatment for an MPNST is extended resection plus radiotherapy or chemotherapy (2). However, the prognosis for MPNSTs is generally poor, with a high rate of local recurrence and metastasis. The prognosis has been reported to differ among previous studies, with the 5-year survival rate ranging between 15 and 50% (1,3). Therefore, further investigation is required to identify potential predictive biomarkers for the prognosis of patients with MPSNTs.

The importance of tumor proteomics has recently become more recognized. However, to the best of our knowledge, the proteomic studies of MPNSTs are rarely reported in the literature, as they are rare in nature. Use of formalin-fixed paraffin-embedded (FFPE) tissues is a powerful resource for biomarker discovery, as it facilitates the long-distance exchange of samples, it is stable and biohazard-free, and it presents a limited number of ethical issues compared with the use of fresh tissues (4). Analyzing the FFPE tissue samples with a label-free quantitative proteomics approach has been reported to be an easy and effective method for investigation (5-7). To the best of our knowledge, research on MPNST samples using the aforementioned approach has not previously been reported in the literature.

In the present study, the FFPE tissue samples of patients with MPNSTs were obtained. A proteomics study on MPNST FFPE tissue samples with label-free quantitative proteomics and mass spectrometry was performed to discover the differential protein expressed in patients with MPSNTs with different

prognoses. Immunohistochemical staining was performed to verify the results of the present study.

Materials and methods

Sample collection. The clinical data of 30 primary extremities of patients with MPNSTs, who underwent surgery in the Department of Hand Surgery, Huashan Hospital, Fudan University, between January 2002 and December 2011, were acquired. The mean age of the patients was 49.06 years old, ranged from 11 to 71 years old, 8 patients were male while 9 were female. A total of 16 patients succumbed to their diseases within 5 years, whereas 14 patients had a survival rate of >5 years. The FFPE tissue samples of all these patients were obtained. The histological diagnosis of the tissues was reviewed by two senior pathologists. A total of 17 typical samples were divided into the following two groups: Group D, comprising of samples from 9 patients who succumbed within 2 years; and Group L, comprising of samples from 8 patients who were continuously disease-free for >5 years following diagnosis. The detailed clinical data are presented in Table I. Written informed consent was obtained from all patients or their family members. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the Helsinki declaration and its later amendments or comparable ethical standards. The present study was approved by the ethics committee of Huashan Hospital, Fudan University.

Label-free quantitative proteomics. Microtome sections (10-μm thick and 80-mm 2 wide) were cut from FFPE tissue blocks (10% formalin was used for 10 hr at room temperature) and deparaffinized by incubation in a graded series of xylene (100, 67 and 33%) for 10 min at room temperature prior to rehydration in a graded series of ethanol (100, 67 and 33%) for 10 min at room temperature. The tissue sections were scraped from the slides and then resuspended in SDT buffers (4% SDS, 100 mM DTT, 100 mM Tris-HCL, pH 7.6). All samples were incubated in the buffers at 100˚C for 20 min, and at 80˚C for 2 h with oscillation. The extracts were centrifuged for 30 min at 14,000 x g at 4˚C. Protein quantification was performed using the BCA (bicinchoninic acid) method. A total of 20 μg of each sample was obtained for SDS-PAGE (12%). Bands were clearly separated.

A total of 200 μg of each sample was solubilized in 100 mM dithiothreitol (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) using a boiling water bath for 5 min, and subsequently cooled down until it reached room temperature. A total of 200 μl uric acid (UA) buffer (urea, 8 M; Tris HCl, pH 8.0, 150 mM) was added, mixed and centrifuged for 15 min at 14,000 x g at 4˚C. A total of 200 μl UA buffer was added, centrifuged for 15 min at 14,000 x g and filtrated at 4˚C. Next, 100 μl indole-3-acetic acid (IAA, Sigma-Aldrich; Merck KGaA) in 50 mM UA was added, oscillated, kept in darkness for 30 min and centrifuged for 10 min at 14,000 x g at 4˚C. A total of 100 μl UA buffer was added and centrifuged for 10 min at 14,000 x g at 4˚C, repeated in duplicate. Subsequently, 100 μl dissolution buffer was added and centrifuged for 10 min at 14,000 x g at 4˚C and repeated twice. Lastly, a total of 40 μl trypsin buffer (5 μg trypsin in 40 μl dissolution buffer) was added, oscillated, kept at 37˚C for 16 h. A new collecting tube was changed and the sample was centrifuged for 10 min at 14,000 x g at 4˚C. The resulting peptides were collected as a filtrate. The peptide content was estimated by UV light spectral density at 280 nm. The result of OD280 peptide quantification of the two groups were >0.1, which means the effect of proteolysis was satisfied.

High-performance liquid chromatography and liquid chromatography-mass spectrometry (LCMS). A total of 2 μg of each enzymatic hydrolysis sample was obtained and LCMS analysis was performed. The system was used at room temperature. The desolvation gas was set to 500 l/h at a temperature of 350˚C. The cone gas was set to 25 l/h, and the source temperature was set to 120˚C. The liquid phase solution A was 0.1% formic acid acetonitrile water solution (2% acetonitrile), while the solution B was 0.1% formic acid acetonitrile aqueous solution (84% acetonitrile). Chromatographic Thermo Scientific EASY column (SC200; Thermo Fisher Scientific, Inc., Waltham, MA, USA) equipped with RP-C18 column (150 μm x100 mm) was balanced with 100% solution A. The samples were loaded onto Thermo Scientific EASY column SC001 traps equipped with RP-C18 column (150 μm x 20 mm) and separated by a chromatographic column with a 400 nl/min flow rate. The peptides generated from the digestion were eluted with the following binary gradients: Solution A and 0-45% solution B for 100 min, followed by 45-100% solution B for additional 12 min. The enzymatic hydrolysis sample was separated by capillary high performance liquid chromatography. MS was performed by Q-Exactive (Thermo Fisher Scientific, Inc.) for 120 min. The detection method was positive ions. Parent ion scan ranged between 300-1800 m/z.

Original files of LCMS/MS were imported into Maxquant software (version 1.3.0.5; https://www.biochem.mpg.de/5111795/maxquant). Label-free quantification was performed by using IBAQ, according to the Uniprot Human database (www.uniprot.org). The major parameters were as follows: Main search ppm, 6; missed cleavage, 2; MS/MS tolerance ppm, 20; de-isotopic, TRUE; enzyme, trypsin; database, uniprot_human_138560_20141014.fasta; fixed modification, carbamidomethyl (C); variable modification, oxidation (M), acetyl (protein N-term); decoy database pattern, reverse; iBAQ, TRUE; match between runs, 2 min; peptide false discovery rate (FDR), 0.01; and protein FDR, 0.01.

Immunohistochemical staining. Immunohistochemical staining was performed in 30 MPNST FFPE tissue samples to verify the chosen protein. Antibodies were acquired as follows: Anti-decorin (dilution, 1:50; cat. no. ab54728; Abcam). Two certified pathologists, who were blinded to the clinical data of the patients, performed the immunohistochemical staining. Samples were blocked with 10% goat serum (Thermo Fisher Scientific, Inc.) for 1hr at room temperature. The 5 μm-thick tissue sections were autoclaved in EDTA Antigen repair solution (Thermo Fisher Scientific, Inc.), and incubated with anti-decorin antibody at room temperature for 45 min. Immunostaining was performed using the biotin-free horseradish peroxidase enzyme-labeled polymer (SABC ready-to used antibody (sa1020, boster) Duration: 1:1,000 room temperature 1h of the Envision Plus detection system. (Leica Microsystems GmbH, Wetzlar, Germany) with a light microscope at x100 magnification. The results were based on the percentage of stained cells, <5 % was classified as negative, while others were classified as positive.

Table I. Clinical data of the patients with malignant peripheral nerve sheath tumors in Group L and Group D.

Patient no.	Age, years	Sex	Largest dimension of the tumor, cm	Tumor-involved nerve	Surgery	Adjuvant therapy	Local recurrence	Metastasis	Survival time following diagnosis, months	Status
1	70	Male	3	Ulnar nerve	Extended	No	No	No	>60	Disease-free
2	42	Female	4	Deep peroneal nerve	Extended	No	No	No	>60	Disease-free
3	41	Female	9	Median nerve	Extended	No	No	No	>60	Disease-free
4	11	Male	8	C5 nerve root	Extended	Radiotherapy	No	No	>60	Disease-free
5	55	Female	4	Radial nerve	Extended	Radiotherapy	No	No	>60	Disease-free
6	60	Female	6	Subcutaneous nerve	Extended	No	No	No	>60	Disease-free
7	59	Male	6	Median nerve	Extended	No	No	No	>60	Disease-free
8	53	Male	5.5	Median nerve	Extended	Radiotherapy	No	No	>60	Disease-free
9	50	Female	12	Brachial plexus	Subtotal	Chemotherapy	No	Yes	18	Succumbed to disease
10	71	Female	10	L2 nerve root	Subtotal	No	No	Yes	10	Succumbed to disease
11	67	Female	4	Subcutaneous nerve	Extended	Radiotherapy	No	Yes	3	Succumbed to disease
12	33	Male	8	Lateral cord	Extended	Radiotherapy	Yes	Yes	9	Succumbed to disease
13	60	Male	4.5	Sciatic nerve	Extended	No	No	Yes	4	Succumbed to disease
14	71	Female	1	Posterior tibial nerve	Extended	No	No	Yes	12	Succumbed to disease
15	23	Male	2	Suprascapular nerve	Extended	Radiotherapy	Yes	Yes	19	Succumbed to disease
16	42	Female	9	Radial nerve	Subtotal	No	No	No	2	Succumbed to disease
17	26	Male	7	Lower trunk	Extended	Radiotherapy	Yes	No	5	Succumbed to disease

Group L, succumbed within 2 years of diagnosis; Group D, continuously disease-free for >5 years following diagnosis.

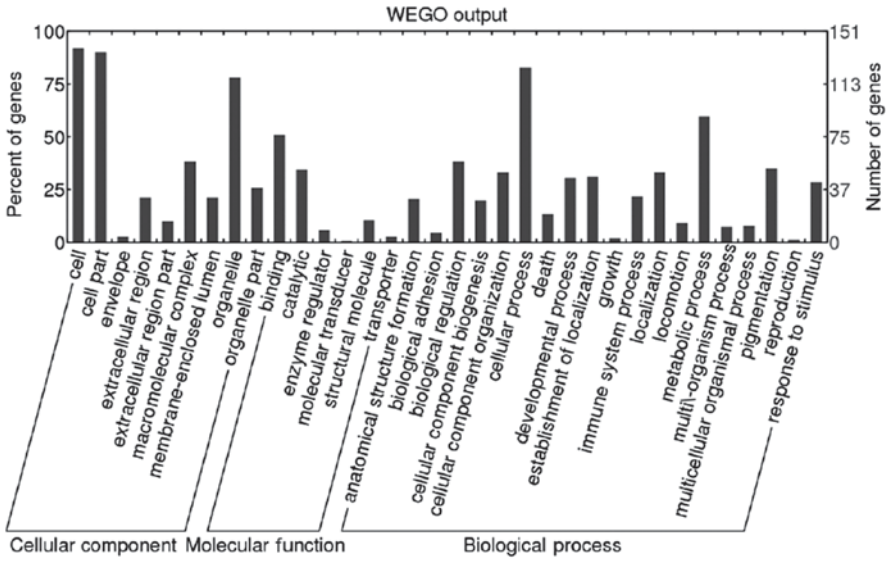

Figure 1. Result of Gene Ontology slim level 2, including the classification of biological process, molecular function and cellular component. WEGO, Web Gene Ontology Annotation Plot.

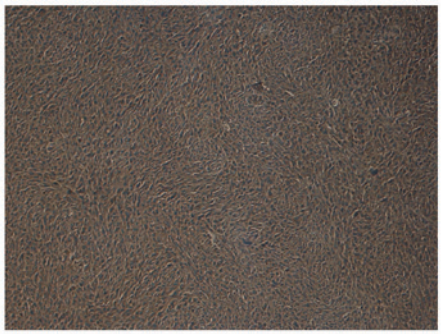

Figure 2. Representative image showing positive decorin expression in a patient with a malignant peripheral nerve sheath tumor (magnification, x100).

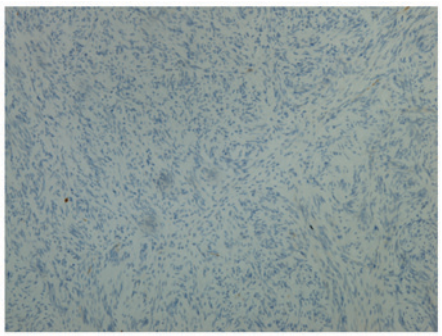

Figure 3. Representative image showing negative decorin expression in a patient with a malignant peripheral nerve sheath tumor (magnification, x100).

Statistical analysis. The data were analysed by Perseus (version 1.3.0.4; www.coxdocs.org). In the data analysis process, an unpaired Student's t-test was used to determine significant differences. P<0.05 was considered to indicate a statistically significant difference. All data are shown as mean ± SD (n=3).

Bioinformatics analysis. Bioinformatics analysis including Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genome (KEGG) was performed. GO analysis (http://www.geneontology.org/) was performed by Blast2GO (version 2.8.0) (8). KEGG pathways analysis was performed by KEGG Automatic Annotation Server (http://www.genome.jp/kegg/pathway.html) (9). The significant differential proteins were filtrated according to the following criteria: the ratio between Group L and Group D was >2.0 or <0.5, with a P-value of <0.05.

Results

Overview of quantitative proteomics. A total of 1,646 proteins were identified following protein extraction according to the previously described protocol. A total of 152 differential proteins were subsequently filtrated according to the following criteria: The ratio between Group L and Group D was >2.0 or <0.5, with a P-value of <0.05. There were 73 upregulated and 79 downregulated proteins in Group D compared with Group L.

Bioinformatics analysis. GO analysis (http://www.geneontology.org/) was performed by Blast2GO (version 2.8.0). A total of 151 (99.34%) differential proteins were annotated. Biological process, molecular function and cellular component were classified. The result of GO slim level 2 are presented as Web Gene Ontology Annotation Plot (WEGO) in Fig. 1.

A total of 167 KEGG pathways associated with 79 differential proteins were extracted by KEGG Automatic Annotation Server (http://www.genome.jp/kegg/pathway.html). Decorin, as an extinct differential protein associated with the malignant tumor, was filtrated. The level of decorin was significantly lower in Group D compared with that in Group L (D/L=0.0948; P=0.0004). Decorin was associated with the activation of the TGF-β signaling pathway. Decorin participated in 'cellular process', 'single-organism process', 'metabolic process', 'cellular component organization or biogenesis', and 'developmental process'.

Immunohistochemical staining. Immunohistochemical staining was performed in 30 MPNST tissue samples to verify the reliability of decorin. In Group L, decorin was positive in 11 patients (78.57%) and negative in 3 patients (21.43%). In Group D, 3 patients (18.75%) were positive for decorin and 13 patients (81.25%) were negative for decorin. Representative images of positivity and negativity for decorin in MPNST tissue samples are shown in Figs. 2 and 3. The 5-year survival rate of patients positive for decorin expression was 78.57%, while the 5-year survival rate of patients negative for decorin expression was 18.75%. The patients' 5-year survival rate with decorin positive expression was significantly higher than that with decorin negative expression (P=0.0014). According to these results, decorin may serve as a reliable prognostic biomarker for patients with MPNSTs. However, further investigations are required.

Discussion

The prognosis of MPNSTs has been reported to differ in the literature, with a 5-year survival rate ranging between 15 and 50% (1,3). The extremities are the most common site in which tumors occur. However, to the best of our knowledge, research focused on only extremity MPNSTs are extremely rare. In the present study, all the cases were primary extremity MPNSTs.

Investigations to examine the biomarkers for MPNSTs have been reported in the literature. Endo *et al* (10) reported that the inactivation of p14 (ARF), p15 (INK4b), and p16 (INK4a) genes indicated a poor prognosis in patients with MPNSTs. Bradtmöller *et al* (11) reported that the downregulation of phosphate and tensin homolog (PTEN) expression could contribute to malignant progression. Alaggio *et al* (12) reported that high expression of survivin correlated with a higher FNCLCC tumor grade and a lower survival probability in pediatric patients with MPNSTs. Fan *et al* (13) reported that the positive expression of E3 ubiquitin-protein ligase Mdm2 (MDM2) and tumor protein p53 (TP53) indicated a lower disease-free survival rate. Ikuta *et al* (14) reported that hyaluronan may serve as a useful marker in differentiating MPNSTs from neurofibromas, and in identifying patients with a poor prognosis. Wang *et al* (15) indicated that patients who were S-100 protein-negative had a higher recurrence rate and a lower survival rate in patients with spinal MPNSTs. Kolberg *et al* (16) reported that survivin (BIRC5), thymidine kinase 1 (TK1) and topoisomerase 2-α (TOP2A) were upregulated in patients with MPNSTs with a poor prognosis.

However, all these prognostic biomarkers were discovered by the method of quantitative polymerase chain reaction (PCR) and/or immunohistochemical staining, which do not have the ability to widely filtrate the differential proteins. Other proteomics methods, including iTRAQ and SILAC, are costly, time-consuming and not always feasible, as they are limited by the insufficient available tags for the simultaneous discrimination of multiple samples. Label-free quantitative proteomics avoids these defects and provides a reliable and convenient study method. To the best of our knowledge, the use of label-free quantitative proteomics in the examination of MPNSTs has yet to be reported in the literature. The present study used this method to filtrate differential protein in patients with MPNSTs with different prognoses.

Decorin is a major extracellular matrix protein and a member of the small leucine-rich proteoglycan family, which serves an important role in the biological process of development, tissue repair and tumor growth by regulating proliferation, differentiation, adhesion and migration (17). Decorin has been reported to be associated with lung (18), breast (19), liver (20), pancreatic (21), colon (22), bladder (23), prostate (24) and oral (25) cancer.

However, to the best of our knowledge, there are limited studies on decorin in soft-tissue tumors. Salomäki *et al* (26) reported that decorin was a biomarker for distinguishing between benign and malignant vascular tumors. Cates *et al* (27) reported that decorin may be used in the differential diagnosis between intramuscular myxoma and low-grade myxofibrosarcoma.

Only the study by Matsumine *et al* (28) reported the association of decorin with the prognosis of soft-tissue sarcoma. The study investigated 77 soft-tissue tumors, including only 4 MPNSTs, by PCR and immunohistochemical staining, and concluded that a reduced decorin level was a useful biomarker of aggressiveness. In the present study, the 5-year survival rate of patients with positive expression of decorin was 78.57%, while the 5-year survival rate of patients with negative expression of decorin was 18.75% (P=0.0014). Therefore, in accordance with the aforementioned study, high expression levels of decorin resulted in a good prognosis in patients with MPNSTs. The present study included a greatly enlarged sample size and was specifically aimed at MPNST; however, the underlying mechanism involved requires further investigation. In addition, it is important to point out a limitation to the present study. Due to the rarity of MPNSTs, the samples obtained for investigation were limited, which may impact the reliability of the results.

Overall, in the present study, label-free quantitative proteomics and mass spectrometry were used to analyze MPNST FFPE tissue samples. It was concluded that a high level of decorin indicates a better prognosis in patients with MPNSTs. With further investigations, decorin may serve as a reliable prognostic biomarker for MPNSTs. Furthermore, by using label-free quantitative proteomics and MS, additional prognostic biomarkers for MPNSTs may be identified in the future.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XJ and CC collected the clinical data, performed the experiment and wrote the manuscript. LC and TK analysed the data. CY designed the experiment and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the Helsinki declaration and its later amendments or comparable ethical standards. The present study was approved by the ethics committee of Huashan Hospital, Fudan University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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