Immunoseroproteomic profiling in autoantibody to ENO1 as potential biomarker in immunodiagnosis of osteosarcoma by serological proteome analysis (SERPA) approach

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ABSTRACT

Osteosarcoma (OS) is the most common highly malignant primary solid bone tumor. Despite its relatively low incidence among cancers, it remains one of the most harmful primary malignant tumors in childhood and adolescence. It is now evident that serum autoantibodies against tumor-associated antigens (TAAs) could be used as serological cancer biomarkers in types of cancers. Serological proteome analysis (SERPA) approach was applied to profile anti-TAA autoantibody response in sera from patients with OS and normal human, as well as explore difference between this response. This approach can detect autoantibodies that could serve as clinical biomarkers and immunotherapeutic agents. Enzyme-linked immunosorbent assay (ELISA) and Western blotting were further used to validate the level of identified TAAs. ENO1 as a 47KD TAA in OS was identified and characterized by SERPA. Analysis of 172 serum samples with OS, osteosarcoma (OC), and normal human sera (NHS) by ELISA showed higher frequency of anti-ENO1 autoantibodies in OS sera compared to others. Interestingly, decrease of ENO1 immunoreactivity was observed in most patients after treatments, which may imply a potential association between anti-ENO1 autoantibody titers and disease progression. Nine of twelve sera reacted strongly against purified ENO1, but three reacted weakly against purified ENO1, which indicated 75.0% sera with positive optimal density values from ELISA were consistently positive in Western blotting. The expression of ENO1 in OS tissues was evaluated by immunohistochemistry in tumor microarray. ENO1 was one of the autoantibodies that elicited autoimmune responses in OS and can be used as biomarkers in immunodiagnosis and progression of OS.

Introduction

Osteosarcoma (OS), a bone tissue produced by malignant osteoblasts, is the most common and highly malignant primary bone tumor produced by primordial bone forming mesenchymal cells. It mainly occurs in the adolescent long bone period with a high degree of malignancy. Nevertheless, like all other sarcomas, the incidence of OS was less than 1% of all cancers with annual incidence approximately two to four persons/ million. The American Cancer Society estimated that there were about 1,000 new cases in the United States each year, of which about 400 were children and adolescents. Furthermore, OS is the most common bone cancer occurred in children and adolescents aged 10–20. Although the incidence of OS is relatively low, it is highly malignant, overlooked and mistaken for benign or traumatic consequences. The initial symptoms of OS are usually nonspecific and the onset is subtle; however, the progress is developing rapidly. Approximately, 20% of OS patients can detect metastasis at diagnosis usually in lung. It is still one of the most harmful primary malignant tumors leading to high amputation rate, disability rate, and mortality. In addition, the tumor size and metastasis detected at diagnosis always predict that the outcome is worse.

In the past decades, many studies have shown that the expression of abnormal proteins is often accompanied by the occurrence and development of tumor tissues, some of which can enter the blood circulation system and be recognized by the immune system. This kind of protein is called tumor-associated antigens (TAAs), which can further trigger the production of autoantibodies. This notion has come from evidence that anti-TAA autoantibodies are immunological "sentinels" of potential molecular events related to tumorogenesis. What is more, autoantibodies show increased levels in very early stage of carcinogenesis and can be stable with high levels in sera even though the levels of corresponding antigen were low even after removal of these antigens. These advantages of autoantibodies have aroused more and more researchers to use these autoantibodies against TAAs as serological cancer biomarkers. In addition, in recent years, great progress has been made in the study of humoral immunity to
TAAs, such as the potential utility of TAAs and anti-TAA autoantibodies systems as cancer biomarker tools to detect cancers early, monitor cancer progression, discover new therapeutic targets, and design personalized therapeutic interventions.\(^{22}\)

At present, emerging studies on the molecular markers or pathways on OS have shown that these molecules play key roles in the occurrence and prognosis of OS, which may be used to predict the possibility of OS diagnosis or micro-metastases as well as the response to chemotherapy. In addition, these pathways may become potential targets of new OS chemotherapeutic drugs. However, although there are many genes and corresponding protein products such as HER\(^2\), p53,\(^{24}\) and HSPs,\(^{25}\) considered as biomarkers of OS, it is generally recognized that they are still insufficient for early cancer diagnosis, individualized therapeutic interventions guiding, and outcome prediction. It is worth noting that since no molecular diagnostic and/or prognostic markers have yet been established in clinical, risk stratification is mainly based on initial stage of the disease and the response to chemotherapy. Therefore, it is necessary to develop a new diagnostic and prognostic indicator such as TAAs to detect these drug-resistant tumors as soon as possible, so as to adopt more active treatment methods in advance to improve the prognosis.

As recent advances in proteomic technology have thrust, the bone cancer field into the era of proteomic approaches to the identification of serum biomarkers for the early, noninvasive diagnosis of cancer and for monitoring tumor progression. A well-established immuno-seroproteomic approach known as serological proteome analysis (SERPA) was used to profile the serum autoantibody repertoire from cancer patients to identify their target TAAs for the serological diagnosis and management of OS.\(^{26,27}\)

**Methods**

**Sample collection**

Ninety-five sera samples were collected from 52 patients with OS (including 24 serial serum samples, 2–5 samples were collected at the time of diagnosis, before and after surgery without other treatment in the progression of OS, ranging 0 to 400 days after diagnosis). After informed consent been signed, all samples were collected through IRB-approved protocols from the two collaborative institutions, namely Henan Luoyang Orthopedic Hospital (HLOH) in China and Institutional Human Subject Review Boards of the University of Texas at El Paso. These samples were used to establish proteomic profiles and evaluate the targeted TAAs. Twenty-eight age-matched anonymous osteochondroma (OC) sera samples from HLOH were used as benign bone tumor controls. In controlling the potential impact of nonspecific host response on sera proteomic profiles, OC patients were probably more appropriate than normal subjects because both OS and OC patients share primary tumors with similar anatomic location and histological type. In addition, 49 age and sex-matched normal human sera (NHS) from normal human donors were collected from HLOH annual physical examination in people without clinical evidence of cancers or autoimmune diseases. After the collection of blood, taking half an hour to allow the blood to clot by leaving it undisturbed at room temperature. Remove the clot by centrifuging at 1500 × g for 10 min. The serum supernatant was collected and stored at −80°C until further use.

**Cell line culture and extract**

U2-OS (ATCC® HTB96TM) and Saos-2 (ATCC® HTB8TM) cell lines were purchased from the American Type Culture Collection (Manassas, VA) and cultured following specific protocol. The cell lines were cultured with 5% CO\(_2\) at 37°C using ATCC formulated McCoy’s 5A Medium Modified (Cat# 302007), supplemented with 15% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 units/mL streptomycin. After cells covered 90% of 175-cm\(^2\) Falcon tissue culture flasks, they were rinsed once with McCoy’s 5A without FBS. Trypsin-EDTA (Cat# 25200056, Gibco, Carlsbad, CA) was used to remove cells from the flask, which were harvested in a 15-mL centrifuge tube for further study.

**One-dimensional gel electrophoresis (1-D) and Western blotting**

In order to screen autoantibody-positive sera, U2-OS and Saos-2 cells were lysed directly in 1 × Laemmli’s sample buffer and boiled for 10 min. After centrifugation to remove insoluble fraction, samples were loaded onto 4–15% Mini-PROTEAN™TGX Stain-FreeTM Protein Gels (Cat# 4568081, Bio-Rad, Hercules, CA), and then transferred onto 0.45 μm nitrocellulose (NC) membrane (Cat# 16201115, Bio-Rad, Hercules, CA) for Western blotting. The NC membrane was stained with Ponceau S to confirm transfer efficacy of the protein and cut into 0.1-inch-wide stripes. After blocked with 3% nonfat milk prepared in 1 × Phosphate-Buffered Saline containing 0.05% Tween-20 (PBST) for 1 h at room temperature (RT), the NC membrane was incubated with sera at a dilution of 1:200 with 1.5% nonfat milk in 1 × PBST. Goat anti-human IgG-HRP (Cat# sc-2453, Santa Cruz Biotechnology, CA, 1:10000 dilution) was used as secondary antibody with a dilution of 1:10000 with 1.5% nonfat milk in 1 × PBST for 30 min at RT. The positive bands were detected with SuperSignal™ West Pico Chemiluminescent Substrate (Prod# 34080, Thermo Fisher Scientific Inc., IL). Quantitative assessment of band molecular weight was performed by Image Lab statistical software (Bio-Rad, CA, USA).

**Antibody absorption**

Through 12% SDS-PAGE gel, Saos-2 cell lysates were separated and then transferred onto NC membrane, to purify the band-specific autoantibody from sera. The NC membrane around 47-kDa was cut-off from the whole one, cut into small pieces, which were incubated with individual serum containing auto-antibody against 47-kDa autoantigen at 4°C overnight. The membranes were subsequently washed three times with PBST.
(5 min once). 100-mM glycine solution, pH 3.0 was used to elute the autoantibodies bound on the membrane, which was immediately neutralized with 1 M Tris-HCl, pH 8.0.

**Indirect immunofluorescence (IIF)**

Indirect immunofluorescence assay was performed on Saos-2 cell chamber slides. Saos-2 cells were seeded in 6 well plate at a density of 6,000 cells/well and incubated overnight. After washed with PBS for three times, the cells were fixed with acetone and methanol (ratio 3:1) at −20°C. The cells were blocked with 0.5% BSA for half an hour at RT, followed by washed five times with PBS. PBS and normal human sera were used as blank and negative controls, respectively. Antikeratin antibody (AKA) human sera were used as positive control. The sera were diluted at 1:80 in PBS (pH 7.4) and incubated with the slides for half an hour at RT. After extensive washing, the slides were incubated with Goat anti-human IgG (H + L) Secondary Antibody, FITC (Cat# H10301, Thermo Fisher Scientific Inc., IL) as secondary antibody diluted 1:1000 in PBS for 1 h at RT. Before adding a drop of mounting media containing 1.5 µg/mL 4',6'-diamidino -2-phenylindole (DAPI) (Cat# H-1200, Vector Laboratories Inc. Burlingame, CA) to prevent photobleaching, the slides were washed three times with PBS, then were examined under Laser Scanning Confocal Microscopy (LSCM) Olympus FV3000 at 400X magnification. All images were acquired under the same conditions. Ambiguous results were considered negative. The immunostaining pattern was compared with available experimental gene/protein characterization data in the UniProtKB/Swiss-Prot database and other experimental evidence for location described in scientific literature for consistency.

**Two-dimensional gel electrophoresis (2-DE) and Western blotting analysis**

U2-OS and Saos-2 were cultured and lysed in rehydration buffer (Cat# 1632106, Bio-Rad, Hercules, CA). The supernatant containing protein was collected after vortexed vigorously for 1.5 h at RT and be centrifuged at 16000 × g for 0.5 h at 4°C. The protein concentration was measured by Bradford assay. In the first dimensional (1-D) Western blotting, a total of 150 µg protein was mixed with rehydration buffer. Three pH 3–10, 7 cm isoelectric focusing strips were performed at 50 mA per gel, 300 V for 0.5 h, followed by 8000 V for 2.5 h, and additional 8000 V for 5 h. The strips were used for the second dimensional (2-D) Western blotting by using 15% SDS-PAGE gel. Staining one gel of three with 0.1% Coomassie blue R-250. After destained, the spots on were visualized. Then, transferring protein of the other two gels onto NC membrane, respectively, was blocked with 3% nonfat milk for 2 h. Then, these two NC membranes were incubated with the mixture of 11 representative OS sera and a pool of 5 NHS used as control at a dilution of 1:200 with 1.5% nonfat milk. Goat anti-human IgG-HRP (Cat# sc-2453, Santa Cruz Biotechnology, CA, 1:10000 dilution) was used as secondary antibody with a dilution of 1:10000 with 1.5% nonfat milk in 1 × PBST for 30 min at RT. Positive bands/spots were detected with enhanced chemiluminescence (ECL) substrate (Thermo Fisher Scientific Inc., IL). Targeted gel spots were excised from the Coomassie blue-stained gel, after which mass spectrometry was used to identify the protein spots.

**Mass spectrometry (MS) and protein identification**

The targeted gel spots excised from a Coomassie blue-stained preparative gel were further to be identified by MS. MS and MS/MS data for protein identification were obtained by using a MALDI-TOF-TOF instrument. Instrument parameters were set using the 4000 Series Explorer software (Applied Biosystems). The MS spectra were recorded in reflector mode in a mass range from 800 to 4000 with a focus mass of 2000. The TOF/TOF calibration mixtures (AB SCIEX) were used to calibrate the spectrum to a mass tolerance within 10 ppm. The MS spectra were processed using TOF-TOF Series Explorer software (V4.0, AB SCIEX). At least 1000 laser shots were typically accumulated with a laser pulse rate of 400 Hz in the MS mode, whereas in the MS/MS mode, spectra up to 2,000 laser shots were acquired and averaged with a pulse rate of 1,000 Hz. For MS calibration, autolysis peaks of trypsin ([M + H] + 842.5100 and 2211.1046) were used as internal calibrates, and the most intense ion signals (up to 10) were selected as precursors for MS/MS acquisition, excluding the trypsin autolysis peaks and the matrix ion signals. Peptide mass fingerprinting (PMF) and MS/MS queries were performed by using the Mascot search engine 2.2 (Matrix Science, Ltd.) embedded into GPS-Explorer Software 3.6 (Applied Biosystems) on the database of NCBI_Triticum and UniProt_viridiplantaee with the following parameter settings: 100 ppm mass accuracy, trypsin cleavage one missed cleavage allowed, carbamidomethylation set as fixed modification, oxidation of methionine was allowed as variable modification, MS/MS fragment tolerance was set to 0.4 Da. A GPS Explorer protein confidence index ≥ 95% were used for further manual validation.

**Enzyme-linked immunosorbent assay (ELISA)**

Purified ENO1 protein (Cat# E-6126, Sigma-Aldrich, St. Louis, Missouri) was diluted to a final concentration of 1.0 µg/ml in PBS and coated onto a Clear Flat-Bottom Immuno Nonsterile 96-Well Plates, which were then incubated overnight at 4°C. After blocked with 2% BSA, the plates were washed three times with 1 × PBST. Human sera were diluted at 1:100 in the antigen-coated wells. After washed five times with 1 × PBST, goat anti-Human IgG (H + L) Secondary Antibody, HRP and the substrate (1 mg/ml 2,2-azino-bis [3-ethylbenzthiazoline-6-sulfonic acid] with 0.005% hydrogen peroxide in citrate buffer, pH 4.6) were used as detecting reagents. The optical density (OD) was measured at 405 nm using an automated plate reader. All serum samples were assayed in duplicate, and all positive sera were further confirmed by Western blotting.
**Immunohistochemistry (IHC)**

Immunohistochemistry was performed using commercially available bone disease spectrum and normal bone tissue microarray (TMA). OS and OC tissue microarray contained 51 cases of OS, 27 OC, plus 2 bone marrow tissue, with single core per case. Normal bone tissue microarray contained 11 cases of bone tissue, 1 OS, and duplicate cores per case. After the tissues dewaxing, endogenous peroxide was blocked by 3% hydrogen peroxide in methanol, and antigen was retrieval by microwave done with a Trilogy Pretreatment Solution. Avidin/Biotin blocking kit was used to block and TMA slides were incubated with mouse monoclonal anti-EN01 antibody (Cal# sc-101513, santa cruz biotechnology, Inc) at concentration (1:200 dilution). Biotinylated secondary antibody with HRP was used as detecting system, and DAB Substrate Kit (3,3’-diaminobenzidine) used as detecting reagents. The tissues were counterstained with hematoxylin and fixed with Richard-Allan Scientific™ Signature Series Bluing Reagent and dehydroyized with different concentrations of ethanol and citrisolvent. Finally, the slides were mounted with per-mount mounting medium and observed under brightfield microscopy. In short, five representative 100X and 400X magnification fields were randomly selected for each patient for histology evaluation. Positive rate (PR) and staining intensity (SI) were used to describe the expression based on the number and staining intensity of positively stained cells in the tissue samples. The evaluation of staining intensity was as follows: “-” for negative, score 0; “+” for low expression, score 1; “++” for moderate expression, score 2; “+++” for high expression, score 3. The percentage of cells was divided into four levels, 1 (up to 25%), 2 (25%-50%), 3 (50%-75%), 4 (75%-100%). It was defined positive if the total score of PR and SI was greater than 2.

**Function analysis of differentially expressed proteins**

Gene ontology (GO) annotation was performed to better understand the biological functions of these differentially expressed proteins. Go over-representation test of the selected genes (corresponding to the identified proteins by SERPA from U2-OS and Sao-2 cell lines) were performed by using the cluster Profiler package against genome annotation for human (org.hs.eg.db) database in R (version 3.4.2). Only GO from genes with e-value ≤1e-10 were accepted for database search.

**Statistical analysis**

Mean and standard deviation was used to describe continuous variables when the data were normally distributed. If the OD value of anti-EN01 autoantibody was not normally distributed, nonparametric Mann–Whitney U-tests was used to compare the level of autoantibody between two groups, and nonparametric Kruskal–Wallis test were used to compare differences of anti-EN01 autoantibody among multiple groups. The cutoff value of the optical density (OD) was designated as the mean absorbance of the NHS controls plus three standard deviations (mean+3SD) for determining a positive reaction. Chi-square test or Fisher’s exact test was used for statistical analysis of classified data. Related-samples Wilcoxon signed rank test was employed to analyze the serial assay of anti-EN01 by ELISA along the disease progression. Receiver operating characteristic (ROC) was used to evaluate the diagnostic value of sera anti-EN01 autoantibody. P value was two-sided, lower than 0.05 was considered statistically significant. All statistical analysis was performed using IBM SPSS Statistics 24.0.

**Results**

**Screening of patients’ sera for the presence of autoantibodies to potential TAAs**

We found that some of the samples contained autoantibodies reacting with one or more cellular antigens. As high as 96.2% of autoantibodies were detected in the sera of OS, which were significantly higher than that of normal human sera (30.6% and 32.7%; Table 1). In contrast, there was no significant association between OC and NHS group. Western blotting analysis of five representative sera from each of the groups against these two cells extracted proteins was shown in Figure 1. Interestingly, 38.5% (20/52), 30.8% (16/52), 28.8% (15/52), 26.9% (14/52), 15.4% (8/52), 13.5% (7/52), 13.5% (7/52), and 11.5% (6/52) OS sera were identified by 1-D Western blotting analysis containing antibodies against unknown cellular protein antigens from U2-OS cell extracted proteins around 47kD, 33kD, 60kD, 54kD, 37kD, 27kD, 17kD, and 29kD, respectively. No reactivity with the 60kD, 37kD, 27kD, 17kD, and 29kD proteins were detected in 49 normal human sera. For the 1-D Western blotting analysis with Saos-2 cellular protein, 48.1% (25/52), 36.5% (19/52), 30.8% (16/52), 28.8% (15/52), 26.9% (14/52), 21.2% (11/52), 17.3% (9/52), 15.4% (8/52), 11.5% (6/52), and 11.5% (6/52) OS sera were identified containing antibodies against unknown cellular protein antigens around 47kD, 37kD, 66kD, 56kD, 33kD, 60kD, 29kD, 27kD, 17kD, and 50kD, respectively. Additionally, no reactivity with the 37kD, 66kD, 33kD, 29kD and 17kD proteins were found in 49 normal human sera. Seven identified protein bands (47kD, 33kD, 60kD, 37kD, 27kD, 17kD, and 29kD) were overlapped between these two cell lines. Moreover, further analysis of the autoantibody positivity to 27kD and 50kD Saos-2 cellular antigens in OS showed no significant difference with NHS. The most common immunoreactivity band was around the 47 KD region (38.5% and 48.1% in U2-OS and Saos-2 cell proteins), which implied that the targeting TAAs could be focus on the protein with molecular weight around 47kD.

**Table 1. Frequency of autoantibodies in sera from patients with OS, OC, and normal human responses to the antigens extracted from U2-OS and Saos-2 cell lines in Western blotting.**

| Serum samples       | No. tested | Frequency of autoantibodies against cellular protein antigens from U2-OS cell | Frequency of autoantibodies against cellular protein antigens from Saos-2 cell |
|---------------------|------------|--------------------------------------------------------------------------------|--------------------------------------------------------------------------------|
| Osteosarcoma        | 52         | 94.2% (49/52)*                                                                 | 96.2% (50/52)*                                                                 |
| Osteochondroma      | 28         | 50.0% (14/28)                                                                 | 64.3% (18/28)                                                                 |
| Normal human        | 49         | 30.6% (15/49)                                                                 | 32.7% (16/49)                                                                 |

*P value relative to NHS: P < 0.001.
**Indirect immunofluorescence (IIF) microscopy**

The specific positive reaction serum of positive rate of OS, normal control screened by 1-D WB were selected to further verify the candidate osteosarcoma related antigen autoantibody by IIF. Immunofluorescence staining pattern of Saos-2 cells showed the 47-kDa proteins were mainly in the cytoplasm. Immunofluorescence analysis of Saos-2 cells confirmed that the sera containing 47-kDa antigen antibodies appeared to have the cytoplasmic and perinuclear staining patterns (Figure 2).

**Identification of candidate TAAs by SERPA approach**

Eleven representative sera were selected from OS patients with common strong immunoreactivity in the 1-D Western blotting. The NC membranes were incubated with these 11 representative OS sera and a pool of 5 NHS used as control. Images of immunoblots of Two-dimensional gel electrophoresis (2-DE) analysis gels exposed on films were overlaid and digitally matched with images of the corresponding Coomassie Blue-stained reference 2-DE gels (Figure 3). Protein spots in stained 2-DE gels corresponding to the immunoreactive spots
Figure 2. The pattern of 47-kD proteins revealed by immunofluorescence analysis of Saos-2 cell substrate. Immunofluorescence was done using whole sera or sera purified with 47-kD antigen. Three representative OS sera (C, E, G) were used, and one normal human serum (A) was used as control. Left panel (A, C, E, G): whole sera; right panel (B, D, F, H): sera purified with 47-kD antigen.
were then processed for MALDI-TOF/TOF MS analysis and the resulting MS/MS spectra were analyzed by the MASCOT search engine using the NCBI and UniProt database. Interestingly, alpha-enolase (ENO1) was identified as the top hit from the 47kD spot recognized by 10 of the 11 selected OS sera and without immunoreaction with NHS pool. SERPA analysis of other immunoreactive spots with other region recognized by these 11 reactive OS sera revealed additional candidate TAAs involved in different molecule functions. As shown in Table 2, a total of 20 proteins from U2-OS and Saos-2 cell lines hits were identified from the analysis of 2-DE spots recognized by these 11 highly reactive OS sera compared with the NHS pool. Moreover, five of them (ENO1, GAPDH, TPI1, DENND4A, and TUBA1C) were identified successfully from both of two cell lines. The 20 identified proteins were predicted to be involved in 48 different biological processes, to possibly have 10 kinds of molecular functions, and to involve eight categories of cellular components (Figure S1).

**Prevalence of autoantibody to ENO1 in OS, OC, and normal individuals**

After identifying several sera from our initial OS patient test cohort contained autoantibodies to ENO1, we used the full-length recombinant ENO1 protein as coating antigen in ELISA to evaluate the levels of anti-ENO1 autoantibody in the sera of patients with OS and controls, to determine the frequency of these autoantibodies in a validation cohort of OS sera (n = 95), as well as a non-OS control cohort of OC (n = 28) and NHS (n = 49). The receiver operating characteristic (ROC) curves discriminated between OS and OC from NHS groups of anti-ENO1 autoantibody with AUCs of 0.853, P < .001 (OS vs NHS) and 0.711, P < .001 (OC vs NHS), respectively (Figure 4 A, B, and C). 17 sera from OS patients showed a significant higher frequency (12/52, 23.1%) and levels of autoantibodies to ENO1 compared to OC sera (1/28, 3.6%, P < .05) and NHS (1/49, 2.0%, P < .01), suggesting that they are OS-related (Figure 4 D and E).
Evaluation of anti-ENO1 autoantibody level in OS patients along the disease progression

Since 67 serial sera samples from 24 OS patients were obtained at a wide range of time period (ranging 0 to 400 days after diagnosis), we questioned whether the levels of anti-ENO1 autoantibody might change with the progress of the disease. The presence of autoantibodies to ENO1 was assessed by ELISA in patients collected continuously at three time points of diagnosis, before surgery and after surgery. The OD value of before and after surgery was the average OD value of serum collected at a particular time point after surgery during a month period (mean, 4.15 months). Unexpectedly, although there was no signification association of anti-ENO1 autoantibody titer at these three time points (Figure 4 F), decrease of ENO1 immunoreactivity was observed in most patients along the disease progression (Figure 4 G, H, and I).

Expression of ENO1 by Western blotting

Western blotting analysis showed that all the 12 sera samples reacted with different signal densities against purified ENO1. Nine of twelve sera reacted strongly against purified ENO1, but three reacted weakly against purified ENO1 in Western blotting. By contrast, both of 10 randomly selected OC and NHS sera show no immunoreactivity or very weak reaction signal against purified ENO1 by Western blotting (Figure 6).

Discussion

Autoimmune phenomena manifested as autoantibodies to cellular components have been described in many types of cancers. The objective of this study was to identify and characterize autoantibodies and the targeted antigens as biomarkers in OS, and further analyze the frequency and specificity of autoantibodies in sera from patients with OS. To screen the
autoantibody-positive sera, we initially have tested 52 sera from OS patients, 28 sera from OC patients, and 49 age-sex matched normal human sera, for the presence of autoantibodies to the TAAs from extracted protein antigens from U2-OS and Saos-2 culture cells in 1-D Western blotting and by IIF.

Our observation that sera from OS patients showed a stronger immunoreactivity against both U2-OS and Saos-2 (94.2% and 96.2%) cellular proteins by 1-D Western blotting than sera from OC (50.0% and 64.3%) and NHS (30.6% and 32.7%) under identical experimental conditions, which was consistent with the proposed notion of this project that OS may exhibit different molecular phenotypes that could influence differential anti-tumor immune responses. Seven identified protein bands (47 kD, 33 kD, 60 kD, 37 kD, 27 kD, 17 kD, and 29 kD) were observed from both cell lines. Moreover, further analysis of the autoantibody positivity to 27 kD and 50 kD Saos-2 cellular antigens in OS showed no significant difference with NHS. Intriguingly, among 52 sera from patients with OS, 38.5% (20/52) and 48.1% (25/52) of sera contained autoantibody against a protein migrating around 47 kD region on SDS-PAGE gel of U2-OS and Saos-2 cellular proteins, respectively. We may narrow the focus on the identified protein with molecular weight around 47 kD.

Particularly, the immunofluorescence staining pattern of Saos-2 cells showed the unknown 47 kD proteins were predominantly in the cytoplasm and were not found in the nuclear fraction, which is consistent with monoclonal ENO1 antibody staining in these cells in our previous studies,26 in U2-OS as well as MCF7 in other studies indicating mainly localization to plasma membrane and cytosol and in addition localized to the nucleoplasm.31 The immunoseroproteomics profiling led to the identification of 20 potential TAAs of 56 protein spots targeted by serum autoantibodies in OS patients compared with the NHS pool. Especially, ENO1 (11 of 12) had a higher immunoreactive frequencies in sera with OS. Moreover, five of them (ENO1, GAPDH, TPI1, DENND4A, TUBA1C) were
identified successfully from both of two cell lines. As a nuclear variant of ENO1, c-myc promoter-binding protein-1 (MBP-1) located at nucleus, known to be recognized by anti-ENO1 antibodies, which could explain the nucleus staining in IIF of whole sera. DENND4A and MBP1 both are MYC binding nuclear proteins with different function. MBP1 binds to the myc promoter and downregulates c-myc transcription.\textsuperscript{32,33} DENND4A, whose synonym is IRLB, contains a tripartite DENN domain, a nuclear localization signal, two PPRs, and a calmodulin-binding domain.\textsuperscript{34} DENND4A promotes the exchange of GDP to GTP, converting inactive GDP-bound Rab proteins into their active GTP-bound form, may bind to ISRE-like element (interferon-stimulated response element) of MYC P2 promoter.\textsuperscript{35,36}

Of 20 identified proteins, the molecular and cellular functions of all proteins have been documented in literatures, and several proteins were reported relating to cancer in previous studies. For instance, in addition to ENO1, the protein hits included key chaperone such as Protein/nucleic acid deglycase DJ-1 (PARK7), 60kD heat shock protein (HSPD1), protein disulfide-isomerase A6 (PDIA6), Calreticulin (CALR), Nucleophosmin (NPM1), Heat shock protein beta-1

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**Figure 5.** Evaluation of ENO1 protein expression in normal, osteosarcoma, and chondrosarcoma tissues by immunohistochemistry. (A, B) Negative staining of ENO1 expression in representative normal bone tissue at 100X and 400X magnification, respectively; (C, D) Positive staining of ENO1 expression in osteosarcoma tissue at 100X and 400X magnification, respectively; (E, F) Positive staining of ENO1 expression in chondrosarcoma tissue at 100X and 400X magnification, respectively.

**Figure 6.** Western blotting analysis with representative positive sera in ELISA. Representative sera from patients with OS (lanes 1-3), OC patients (lanes 4-6) and normal human sera (lanes 7-9) probed against purified ENO1 showed differences in immunoreactivity by Western blotting. 9 of 12 (75.0%) sera with positive OD values were consistently positive in Western blotting.
(HSPB1), Additional candidate TAAs identified by our SERPA approach have been associated with Metal-binding, Hydrodase, Transferase, Isomerase, DNA-binding, cell motility, GTP-binding and so on. ENO1 and GAPDH have been revealed to be “moonlight” as plasminogen receptors, contributing to the conversion of plasminogen to plasmin and promoting extra-cellular matrix remodeling with ensuing increased cancer cell migration and metastasis.\textsuperscript{37–39} In glioma tumors, ENO1 was overexpressed to promote migration and tissue invasion,\textsuperscript{40} while its secretion from prostate cancer stromal cells was also found to improve cell migration.\textsuperscript{41} One group illustrated that a small molecular inhibitor of ENO1 reduced pancreatic cancer cell invasion and migration properties,\textsuperscript{42} suggesting that this cytoplasmic enzyme played a significant role in aggressive disease progression. The overexpression of these metabolic enzymes on the surface of tumors could break immune tolerance, making them targets of humoral responses. Of interest, anti-ENO1 autoantibodies may also assuage tumor metastasis by interfering with plasminogen binding, thus resulting in reduced tumor growth, migration, and invasion.\textsuperscript{43} This makes ENO1 and other cytoplasmic enzymes likely a contributor to plasminogen-binding attractive candidates for anti-cancer immunotherapy. ENO1 promotes cellular functions related to tumor aggressiveness, including increased glycolysis, activation of oncogenic signaling pathways, chemoresistance, and cell proliferation, migration, invasion, and metastasis.\textsuperscript{44} The high expression and targeting of ENO1 in a variety of human cancers make it an attractive cancer biomarker candidate and therapeutic target.

Perhaps, the greatest challenge in the investigation of serological study in OS is the serum samples collection, due to its low incidence rate all around the world. In addition, it is less evident that the incidence of the disease has an uneven geographic distribution worldwide. We fortunately collected 95 serum samples from 52 OS patients including 24 patients with serial serum samples obtained at the time of diagnosis or at different time points along the disease progression since 2014. Based on our extensive experience in characterizing autoantibody responses in cancer, we determined the frequency of autoantibodies to the identified potential TAAs in patients with different clinical stages of OS, benign bone tumor, as well as in age and sex-matched normal individuals without any no cancer diagnosis. Our results of ROC curves analysis of anti-ENO1 autoantibody indicate it predominately discriminates the OS group from OC as well as can differentiate OC patients from normal individuals. Sera from OS patients showed a significantly higher frequency and levels of autoantibodies to ENO1 compared to OC sera and NHS, suggesting that they are OS-related. In addition, 9 of 12 sera reacted strongly against purified ENO1, but 3 weakly against purified ENO1, which indicated 75.0% sera with positive OD values were consistently positive in Western blotting. These results revealed heterogeneity across several patients in their anti-ENO1 serum immunoreactivity.

The true clinical value/advantage of using serum autoantibodies as a biomarker has been recently called into question. Unfortunately, many published studies on TAA identification have failed to ascertain the relevance of the TAA to the tumor progression or disease prognosis. We consequently examined whether anti-ENO1 autoantibody levels might change along the disease progression or even prognosis. The presence of autoantibodies to ENO1 was assessed by ELISA over time collected serially at three time points: diagnosis, before surgery, and after surgery. The OD value of before and after surgery was the average OD determined on serum that had been collected at a particular time point after surgery during a month period (mean, 4.15 months). Interestingly, although there was no significant association of ENO1 titers at these three time points, decrease of ENO1 immunoreactivity was observed in most patients along the disease progression, which may imply a potential association between changes in anti-ENO1 autoantibody titers and disease progression. This may attribute to the lack of adequate and sufficient serial samples for further in-depth analysis.

Anti-TAA autoantibodies might display heterogeneity in epitope recognition within a given antigen. We have found an incomplete correlation in the detection of anti-ENO1 antibodies between the different immunoassays (ELISA, Western Blotting, and IIF). We proposed that the immunoreactivity to ENO1 in OS could be indicative of aberrant expression of this protein in certain OS tumors and performed the IHC by using commercially available tissue microarrays. We observed that all the osteosarcoma and chondrosarcoma specimens expressed the ENO1 protein, while on normal bone tissues samples in the array did express the protein. Most of these showed strong cytoplasmic and sporadically nuclear positivity (56 of 73, 76.7%). Unfortunately, because osteosarcoma tissue specimens were not available in large numbers for this study, there was no significant correlation between immunostaining density and histological characteristics including clinical stage and TNM grading. The predominant elevation of ENO1 expression in OS compared to normal bone tissue suggested that this protein might be upregulated during OS carcinogenesis. This remains to be confirmed in a more complete analysis of ENO1 expression in other types of bone tumor OS at different stages.

To the best of our knowledge, this is the first study to report that certain patients with OS produce autoantibodies to ENO1 and that this protein is notably highly expressed in OS tissues. The identification and characterization of novel OS-associated autoantibodies targeting ENO1 from the glycolytic and plasminogen pathways provide a new promising tool to fine-tune early OS diagnosis and management using minimally invasive methods, as well as enhance our understanding of the immune system’s role during OS tumorigenesis. Moreover, anti-ENO1 autoantibody level was potentially associated with disease progression in this subset of patients, implying that this marker may be a further tool not only for diagnosing OS but also for disease progression or even prognosis. Taken together, our results suggested that the autoantibody to ENO1 might be considered as a potential serological biomarker for OS.

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Data availability statement
The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Disclosure statement
The authors report no conflict of interest.

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