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Identification And Characterization Of Tumor-Associated Antigens (taas) And Anti-Taas Autoantibodies As Biomarkers In Immunodiagnosis Of Human Osteosarcoma By Serological Proteome Analysis (SERPA)

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IDENTIFICATION AND CHARACTERIZATION OF TUMOR-ASSOCIATED
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BIOMARKERS IN IMMUNODIAGNOSIS OF HUMAN OSTEOSARCOMA BY
SEROLOGICAL PROTEOME ANALYSIS (SERPA)

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Jitian Li

2017

Dedication

This dissertation is dedicated to my family. All of my achievements would not have been reached without their constant patience, love, and support.

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by

JITIAN LI, M.D., M.P.H.

DISSERTATION

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The University of Texas at El Paso
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of the Requirements
for the Degree of

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THE UNIVERSITY OF TEXAS AT EL PASO

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Abstract

Osteosarcoma (OS) is the most common highly malignant primary solid bone-tumor. Despite its relatively low incidence rate among overall cancers, it remains one of the most harmful primary malignant tumors in childhood and adolescence. Although some tumor markers like mutant p53 can be potentially used as biomarker to detect OS, its extensive association with the clinical outcome is poorly understood. The establishment of a methodology to identify patient with early stage of OS remains to be investigated. It is now evident that serum autoantibodies against tumor-associated antigens (TAAs) could be used as serological cancer biomarkers in types of cancers, which derives from the notion that anti-TAA autoantibodies are considered as immunological “sentinels” underlying molecular events associated with tumorigenesis. New insights into molecular and cellular biology and the differential diagnosis of cancers have also been obtained. Moreover, customized TAA arrays significantly increase sensitivity/specificity and provide a great promise for the early detection of cancer, monitoring cancer progression, discovery of novel therapeutic targets, and designing personalized therapeutic interventions.

During the past decade, proteomic approaches, such as serological proteome analysis (SERPA), have been used to identify the repertoire of immunoreactive proteins in various diseases. Recent several years, we have used this approach to extensively screen sera from patients with certain types of cancer such as hepatocellular carcinoma (HCC), esophageal squamous cell carcinoma (ESCC), Prostate Cancer (PCa) and Lung Cancer (LC), and sera from patients with pre-cancer conditions such as liver fibrosis to identify and characterize the potential TAAs.

We used SERPA to profile anti-TAA autoantibody responses in sera from patients with Osteosarcoma (OS), and Normal Human, and explore differences of these responses. This approach can detect autoantibodies to TAAs that could serve as clinical biomarkers and immunotherapeutic agents. Sera from OS, Osteochondroma (OC) and Normal Human Sera (NHS) were probed by immunoblotting against cellular proteins extracted from U2-OS and Saos-2 cell lines, with OS sera showing stronger immunoreactivity. MALDI-TOF/TOF Mass Spectrometry

(MS) analysis of immunoreactive protein spots revealed that several OS sera contained autoantibodies to a number of proteins, particularly to alpha-enolase (ENO1). Analysis of 172 serum samples from patients with OS, OC and NHS by Enzyme-Linked Immunosorbent Assay (ELISA) showed higher frequency of anti-ENO1 autoantibodies in OS sera compared to others. Interestingly, descent of ENO1 immunoreactivity was observed in most patients after treatments, which may imply a potential association between anti-ENO1 autoantibody titers and disease progression. The expression of ENO1 in Osteosarcoma tissues was evaluated by immunohistochemistry (IHC) in Tumor Microarray (TMA). We observed the cumulative positive rate of autoantibodies against seven selected TAAs identified from SERPA (ENO1, NPM1, GAPDH, TPI1, HSP60, PDLIM1, STMN1) in OS reached 90.4%, significantly higher than that in normal control sera. These results support the central hypothesis of this proposed project that "customized" TAA arrays constitute promising and powerful tools for enhancing the serological detection of OS. Together, our intriguing findings demonstrate that ENO1 is one of autoantigens that elicit autoimmune responses in OS and can be used as biomarkers in immunodiagnosis and progression of OS.

Key Words: Osteosarcoma (OS), Tumor-associated antigen (TAA), Serological proteome analysis (SERPA), Alpha-enolase, Cancer early detection, Immunodiagnosis.

Running Title: Immunoseroproteomic profiling in human Osteosarcoma.

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Chapter 1: Introduction

Osteosarcoma (OS), characterized by the production of osteoid material by malignant osteoblastic cells, is the most common highly malignant primary bone-tumor deriving from primitive bone-forming mesenchymal cells¹. Nevertheless, it is, like all other sarcomas, a low incidence rate less than 1% of all cancers diagnosed in the United States². The American Cancer Society estimates that approximately 1,000 new cases arise in the USA each year and about 400 of these are in children and teens. In addition, OS is the most frequent bone cancer occurring in children and adolescents aged 10–20 years³. Despite its relatively low incidence rate among overall cancers, OS is highly malignant and can be often neglected on misidentification with benign lesions or trauma contributing to fatal consequences since the initial symptoms of the disease are commonly quite nonspecific and subtle in onset, and furthermore, it progresses aggressively and approximately 20% of patients with OS have detectable metastases at diagnosis usually developing in the lung⁴⁻⁷. It remains one of the most harmful primary malignant tumors in childhood and adolescence, responsible for a high rate of amputation, disability and death. Current OS therapies are inadequate, with five year survival rate of ~60% even after pre- and post-operative neo-adjuvant chemotherapy and excision of operable lesions⁸. Moreover, tumor size and metastases detectable at diagnosis always portend a worse outcome^{5,9}. Thus, a critical need in the diagnosis and management of OS is to determine an optimal combination of clinical biomarkers that could detect tumors early with high specificity/sensitivity and with limited invasiveness, and that could accurately predict which diagnosed patient will develop aggressive tumors requiring treatment.

Over the last few decades, many studies demonstrated that these autologous cells developing to tumors contain self-antigens and its abnormal exposure or presentation of these antigens recognized by the immune system, and further trigger the production of autoantibodies that have been generally termed anti-TAA autoantibodies against these cellular antigens. This notion has come from evidence that anti-TAA autoantibodies are immunological “sentinels” underlying molecular events associated with tumorigenesis¹⁰⁻¹². What is more, they exhibit

increased levels in very early stages of carcinogenesis¹³ and are observed stable with high level in patients' sera despite low levels of the corresponding antigens¹⁴ or even after removal of these antigens^{15,16}. Such benefits of these autoantibodies have triggered a growing enthusiasm for applying these autoantibodies against tumor-associated antigens (TAAs) as serological cancer biomarkers. Moreover, recent years have seen an enormous increase in efforts to humoral immunity to TAAs for addressing specific clinical issues such as the potential utility of TAAs and anti-TAA autoantibodies systems as cancer biomarker tools for the early detection of cancer, monitoring cancer progression, discovery of novel therapeutic targets, and designing personalized therapeutic interventions¹⁷.

At present, the emerging studies of the molecular markers or pathways on OS have denoted these molecules as a key role in OS tumorigenesis and prognostication, which may be exploited to predict specific outcomes such as the likelihood of diagnosis or micrometastases at diagnosis and response to chemotherapy. In addition, these pathways could also be potential targets for new OS chemotherapeutic agents. However, in spite of the availability of a plethora of genes and their protein products like HER2¹⁸, p53¹⁹, HSPs²⁰, which may be considered as OS biomarkers, it is widely recognized that their uses with the available clinical information is still insufficient for early cancer diagnosis and for guiding individualized therapeutic interventions and predicting outcomes. 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, non-invasive diagnosis of cancer and for monitoring tumor progression. One approach includes direct profiling of human cancer sera, using two-dimensional gel electrophoresis (2-DE) system and mass spectroscopy, to identify distinctive protein signatures characteristic of different tumor types^{6,21-24}. A second approach, which is the focus of this dissertation, is the exploitation of the serum autoantibody repertoire from cancer patients for the identification of TAAs and the design of customized TAA arrays for the serological diagnosis and management of OS^{25,26}.

1.1 Osteosarcoma

1.1.1 Epidemiology and etiology

In spite of primary bone cancer, bone is one of the most common sites for tumor metastasis, in particular for breast and prostate cancers, and bone metastases are frequently associated with intractable bone pain, pathological fractures, nerve compression and hypercalcemia due to osteolysis²⁷. Approximately 1,500 new sarcomas of bone are recorded in the USA per year while 93,000 new cases of lung carcinoma and 88,000 new cases of breast carcinoma are diagnosed⁸. Among various types of bone tumors, OS is the most frequent highly malignant bone-tumor comprising about 20% of primary bone sarcomas. Although it is a low incidence rate less than 1% of all cancers diagnosed in the USA², approximately 1,000 new cases arise each year. The incidence of OS has always been considered to be higher in males than in females²⁸ and it was a rate of 5.4 per million persons per year in males while 4.0 per million in females according to the recent Surveillance, Epidemiology, and End Results (SEER) data in 2008²⁹. With respect to ethnicity, the more recent data from SEER indicate that OS occurs more frequent in Asians/Pacific Islander and in Hispanics²⁸.

There are two age-specific peaks in frequency in OS. Most of these occur in young patients, with a peak manifestation during the second and third decade of life. The two types of malignant bone cancers that predominate in children and adolescents are osteosarcoma and Ewing sarcoma, which represent about 56% and 34% of bone cancers, respectively³⁰. In the United States, approximately 400 new cases of OS are diagnosed younger than 20 years old annually, coinciding with the pubertal growth spurt³⁰. The second peak, slightly increasing from the fourth decade, has its top after the sixth decade. And cases of primary conventional osteosarcoma may arise in older patients, but as age increases, secondary osteosarcoma is more likely. Such tumors develop in patients who have had a preexisting lesion or disease in the bone, such as Paget disease and radiation-induced sarcomas disease³¹. The diagnosis of secondary OS shows significance in terms of prognosis and expected response to treatment; secondary OS does not respond well to chemotherapy and has a worse outcome than that of primary conventional osteosarcoma³¹.

OS can appear in any bone, while the most common sites are near the metaphyseal growth plates of the long bones of the extremities. It frequently localizes in the femur (42%, with 75% of these tumors in the distal femur), the tibia (19%, with 80% of these tumors in the proximal tibia), and the humerus (10%, with 90% of these tumors in the proximal humerus)³².

To date, the etiology of OS is still largely unknown, however a number of linkage studies have implicated an interaction of environmental insults and genetic susceptibility. Several studies demonstrate the etiology of OS involves epidemiologic and environmental factors, and genetic impairments. Currently, the limited well-known risk factors associated with the development of OS comprise ionizing radiation, alkylating agents, Paget's disease, hereditary retinoblastoma, the Li-Fraumeni familial cancer syndrome, and other chromosomal abnormalities³². Among them, exposure to radiation is the only proven exogenous risk factor but with a long interval (10–20 years)³³. Additionally, OS can also develop in response to genetic predisposition conferred by RB1 or tumor protein p53 mutations. For example, RB1 mutations predispose to OS in the teen years, long after an infant's retinoblastoma is cured. Cumulative OS incidence following retinoblastoma has been estimated as 7% at 20 years^{34,35}. Discoveries regarding the etiology of osteosarcoma will enable patients to avoid the causes and prevent its occurrence, however, presently the causes of OS are not completely known. Therefore, the molecular pathways or markers of OS will potentially reveal the forming and developing of OS, which also promises some new effective treatments for this disease.

1.1.2 Diagnosis

With the advancing modern treatment protocols that combine chemotherapy and surgery in the last two decades, although survival rate increased from 10-20% up to 60-70%, there is still a need for convincing early detections and efficient therapeutic alternatives in case of non-responders to chemotherapy, relapsed patients and patients with metastasis. In fact, more than 30% of patients with OS will succumb to metastatic disease³⁶. Clinically, the diagnosis of OS requires a combination of clinical presentation, radiologic studies, and pathologic tissue evaluation. The

initial clinical symptom of OS is frequently pain in the affected area, which may also be associated with localized soft tissue swelling or limitation of motion in the adjacent joint. Pain usually occurs after strenuous exercise or a trauma and usually lasts for around 3 months before diagnosis with progressing over time, and commonly following with swelling with a hard painful mass in the affected region¹. However, the initial symptoms of the disease are often quite nonspecific and subtle in onset, which often does not draw the patient's attention. Most OS are diagnosed at a relatively advanced stage of the disease during which the presence of sclerosis and the malignant nature of the disease are clearly evident⁵. In addition, the typical radiographic features have been extensively illustrated, but diagnosis is frequently overlooked when the tumor presents as a variant or early in its development. Rarely, however, OS may be detected before aggressive features are manifested on radiographs, and such OS can be neglected on misidentification with benign lesions⁴ or trauma⁶. Beyond the clinical symptoms and radiographs, the histological examination of the biopsy specimens is still preferred by many orthopedic oncologists for the diagnosis of OS. Despite its invasive procedure and burdens to the patients, the accuracy of diagnosis may vary among different sample collections and different observers, making the clinical prediction questionable. Additionally, inappropriately performed biopsies are a frequent cause of misdiagnosis, amputation and local recurrence, and are associated with reduced survival⁸. As a result more emphasis should be placed on early diagnosis not only attempting to deliver effective chemotherapy to small localized disease but also crucially important to reduce the extent of local resection minimizing disability enhancing the long-term survive rate and improving the quality of life of patients³⁷. Furthermore, such limitations of the current diagnosis approach have been suggested that a critical unmet need in the diagnosis and management of OS is the development of reliable non-invasive biomarkers for early diagnosis of OS.

1.1.3 Classification and staging

OS can be divided into two major categories: primary tumors that occur de novo in otherwise normal bone, comprising the most common OS: conventional OS (also called classic,

intramedullary or central), and secondary tumors that develop in abnormal bone in the setting of a preexisting benign lesion, irradiated bone, retinoblastoma, or Paget disease of bone³². Conventional OS has three histologic subtypes: fibroblastic, chondroblastic, and osteoblastic OS. In all three subtypes, there is production of osteoid (immature bone matrix) by malignant spindle-shaped sarcoma cells³¹.

Staging is classified based on the aggressive grade and the extensive and the spread levels of OS. According to the Musculoskeletal Tumor Society Staging System³⁸ and Enneking System³⁹, which are widely used in clinical, tumor stages have been classified based on tumor grade (I, low grade; II, high grade), tumor extension (A, intraosseous involvement; B, extraosseous extension) and the presence of distant metastases (III)³⁸, as reported in Table 1. Most conventional OSs present as stage IIB tumors which is non-metastatic tumor with an associated soft tissue mass³⁹.

Table 1: Surgical staging of bone sarcomas³⁸.

Stage	Grade	Site	Metastasis
IA	Low	Intracompartmental	No
IB	Low	Extracompartmental	No
IIA	High	Intracompartmental	No
IIB	High	Extracompartmental	No
III	Any	Any	Regional or distant

Definitions: I, low grade; II, high grade; A, intraosseous involvement; B, extraosseous extension, III, the presence of distant metastases.

The more recent version (the seventh edition) of the American Joint Committee on Cancer (AJCC) staging system is similar to the MTS Staging System, but it incorporates into the system the size of the tumor and the presence of skip metastases⁴⁰ (Table 2). Additionally, it more specifically subdivided stages I and II into A and B categories depending on tumor size being greater or less than 8 cm in any dimension, rather than intra- or extra-compartmental. Moreover,

it has the extra stage IV which is divided into IV-A, describing pulmonary metastases, and IV-B, describing other metastases.

Table 2: American Joint Committee on Cancer staging system for bone tumors⁴⁰.

Stage	Tumor	Nodes	Metastasis	Grade
IA	T1	N0	M0	G1, 2 low grade, GX
IB	T2	N0	M0	G1, 2 low grade, GX
	T3	N0	M0	G1, 2 low grade, GX
IIA	T1	N0	M0	G3, 4 high grade
IIB	T2	N0	M0	G3, 4 high grade
III	T3	N0	M0	G3, 4
IVA	Any T	N0	M1a	Any G
IVB	Any T	N1	Any M	Any G
	Any T	Any N	M1b	Any G

Definitions: T1, tumor 8 cm or less in greatest dimension; T2, tumor more than 8 cm in greatest dimension; T3, discontinuous tumors in the primary bone site; N0, no regional lymph node metastasis; N1, regional lymph node metastasis; M0, no distant metastasis; M1a, lung; M1b, other distant sites; G1, well differentiated (low-grade); G2, moderately differentiated (low-grade); G3, poorly differentiated; G4, undifferentiated; GX, grade cannot be assessed.

1.1.4 Treatment

The 5-year survival rate of patients with OS was 10–20% before the 1970s when treatment was mainly limb amputation⁴¹. Due to the rapid development of surgical techniques and the application of radiotherapy and/or effective systemic chemotherapy over the past thirty years, approximately two-thirds of children with non-metastatic OS are cured with the combination of surgery and chemotherapy, which has made limb salvage procedures a safe alternative to amputation and led to an increase in disease-free and overall survival rates^{39,42}. Generally, after initial diagnosis, patients usually receive multi-agent preoperative chemotherapy and then surgical

extirpation of the primary tumor in the affected bone followed by postoperative chemotherapy⁴³. The response of the preoperative chemotherapy is measured by the extent of necrosis in the resected tumor, which is considered as good when $\geq 90\%$ tumor necrosis could be found whereas response is considered as poor when $< 90\%$ tumor necrosis are found⁴⁴. However, unfortunately, in spite of modifications in postoperative chemotherapy, the poor responders to the preoperative chemotherapy often associate with have a worse outcome and a high risk of developing metastasis compared to patients who have a good response to chemotherapy⁴³. In fact, the long term survival rate of these poor responders has not been improved in the past 30 years⁴⁵. Therefore, it is essential to develop a novel focus on identification of diagnostic and prognostic indicators to detect these resistant tumors as early as possible, so that more aggressive therapy can be used upfront to improve the outcomes.

1.2 Tumor-associated antigens (TAAs) and anti-TAAs autoantibodies

It is now evident that cancer sera comprise autoantibodies that react with a unique group of autologous cellular antigens called TAAs^{10,46,47}. The types of cellular proteins that induce these autoantibody responses are quite varied and include the tumor suppressor p53^{48,49}, oncogene products such as HER-2/neu and ras⁵⁰, proteins that protect mRNAs from degradation such as p62⁵¹ and CRD-BP⁵², onconeural antigens⁵³, differentiation-antigens such as tyrosinase and the cancer/testis antigens⁵⁴, and anti-apoptotic proteins such as surviving⁵⁵ and LEDGF⁵⁶. Elements contributing to produce such autoantibodies are not fully understood but the available studies believe that many of the target antigens are cellular proteins whose aberrant regulation or overexpression could lead to tumorigenesis, such as p53^{48,49}. In terms of the mRNA binding protein p62, known as a fetal protein absent in adult tissues, immunogenicity appears to be related to abnormal expression of p62 in tumor cells⁵⁷. The immune system in certain cancer patients appears to have the capability of sensing these abnormalities in self-antigens and responding by producing autoantibodies⁵⁸. An emerging concept is that autoantibodies associated with a specific type of cancer are directed against aberrantly regulated or activated protein components of

molecular pathways involved in the malignant transformation process in that particular type of cancer⁵⁹. Taken together, anti-TAAs autoantibodies might be regarded as reporters implying aberrant cellular mechanisms in tumorigenesis¹⁰. In recent years, research on humoral immunity to TAAs has received significant attention, and investigators are now beginning to address specific clinical questions such as the potential utility of TAA-autoantibody systems as early cancer biomarker tools to monitor therapeutic outcomes, or indicators of disease prognosis¹⁷.

1.3 Tumor-Associated Antigens (TAAs) in OS

Albeit the reports concerning the repertoire and function of TAAs in OS are still limited, growing evidence suggests that these tumors might express several diagnostic and/or therapeutic targets. The numerous TAAs summarized in Table 3 have been described in previous studies. In human OS, HER2/erbB-2 is overexpressed in 40-45% and correlates with poor prognosis, early pulmonary metastases and poor response to preoperative chemotherapy^{60,61}. Although expression of the p53 protein has been reported in OS, unexpectedly, the following several studies did not find a specific correlation between p53 expression and the clinical outcome, suggesting that p53 fails as a marker for OS^{19,62}. Several previous reports had shown that P-glycoprotein expression may associated with an increased risk for chemotherapy resistance⁶³⁻⁶⁶. Afterwards, HSP27 was reported that the overexpression in human OS is related with poor prognosis⁶⁷. Sudo et al. suggested that melanoma-associated antigen (MAGE) family members are expressed in substantial numbers of OS as tumor-rejection antigens in a major histocompatibility class-I-restricted manner⁶⁸. And in sarcoma cell lines, HSP72 has been shown to be selectively expressed on their cell surface thereby overcoming protection and acting as the target for natural killer cells⁶⁹, which correlates with a good response to neoadjuvant chemotherapy⁷⁰. The GD2 ganglioside was found overexpressed in OS⁷¹ and later, some groups demonstrated that anti-GD2 antibody therapy improves survival in high-risk neuroblastoma⁷². Two tumor-rejection antigens SART1⁷³ and SART3⁷⁴ were reported to be expressed in OS, in which the results suggest that these proteins and their derived peptides could be molecules appropriate for use in specific immunotherapies for

HLA-A24⁺ patients with OS or malignant fibrous histiocytosis. The homogeneous expression of the B7-H3 (8H9 antigen) on cell membrane makes it an attractive candidate for targeted immunotherapy⁷⁵. Additionally, the human METCAM/MUC18 (melanoma antigen/MUC18), a cell adhesion molecule, was shown overexpressed in OS, and played a central role in the metastasis of OS and suggested that targeted inhibition of this antigen by ABX-MA1 might be a novel immunotherapeutic approach in the management of OS⁷⁶. Furthermore, this study also concluded that anti-MUC18 antibodies could inhibit the development of OS metastases in a preclinical model⁷⁶. In the past few years, association of CXCR4 expression with initial metastases⁷⁷, SAA associated with type of tumor and high-risk OS development⁴⁵ and CLUAP1 potentially useful as a prognostic/diagnostic marker for OS⁷⁸ has been suggested. Jacobs et al. reported that all 9 OS tissue samples expressed *MAGE-1*, 2, 8, and 8 of 9 expressed NYESO-1, cancer testis antigens or cancer germline genes, in their cancer germline gene expression in pediatric solid tumors studies⁷⁹. Like the other tumors, survivin is also overexpressed in OS as an anti-apoptotic molecule, and several investigators has revealed that survivin overexpression is associated with poor clinical outcome and it may be used as an independent predictor of survival for OS patients⁸⁰. More recently, Maehara et al. showed that the level of midkine expression, known as a heparin-binding growth factor midkine, correlates with the prognosis of patients with OS, and anti-midkine functional antibodies effectively inhibit growth of OS cells *in vitro*⁸¹.

In the recent past, several studies have attempted to identify novel targets for immunotherapy exploiting humoral and cellular immune responses against OS, although their precise role in cell biology remains unclear. Among them, two serological antigens OSAA-3 and OSAA-5 were identified exclusively by serum from OS patients but not by serum from normal individuals, implying that the immune responses to these two antigens were OS-associated⁸². Papillomavirus binding factor (PBF) was identified by derived cDNA library screening with autologous tumor-reactive CD8⁺T cells, which was overexpressed in most OS and might contribute to peptide-based vaccination and/or adoptive antigen-specific T-cell therapy of patients with OS and other bone and soft tissue tumors⁸³. The report by Rouleau et al. showed that low

level of endosialin/tumor endothelial marker 1 (TEM1) in normal tissues and the high levels measured in several sarcoma subtypes, suggesting that TEM1 may be a suitable target protein for selective therapeutic intervention⁸⁴. In more recent years, the researchers found that interleukin 11 receptor alpha (IL11R α)⁸⁵ and fibroblast activation protein (FAP)⁸⁶ were selectively expressed in OS patients compared with healthy groups indicating that they may play roles in tumor development and progression in OS.

On the other hand, the recognition that human tumors stimulate the production of autoantibodies against TAAs has opened a new chapter in cancer biology focusing on the possibility that autoantibodies could be exploited as serological tools for the early diagnosis and management of cancer²⁵. The autoantibody titers against HSP60 have been reported to be increased in patients with OS but no correlation with clinical performance was found⁸⁷. One year later, the other group reported that such immunoreactivity against HSP90 might be of predictive value in human OS since the presence of anti-HSP90 autoantibodies correlates with a good response to neoadjuvant chemotherapy and their absence correlates with the occurrence of metastase⁸⁸.

Upon the current limited information, it seems that the autoantibodies in OS appear to be directed preferentially against proteins that participate in tumorigenesis and are highly expressed in bone tumors. Unfortunately, many published studies on TAA identification have failed to further detail this association. Additionally, since no molecular diagnostic and/or prognostic markers have yet been clinically established, risk stratification is largely based on the initial stage of the disease and on the response to chemotherapy. It would be essential to develop a novel focus on identification of diagnostic and prognostic indicators to detect these resistant tumors as early as possible, so that more aggressive therapy can be used upfront to improve the outcomes.

Table 3: Identification of TAAs or anti-TAA autoantibodies in OS analyzed in multiple studies.

Target antigens	Description	Observation in OS	Ref.
HER2	Oncogene	Correlates with poor prognosis for patients with OS.	60,61

P53	Tumor suppressor	Fails as a marker in OS because of no significant correlation between p53 expression and the clinical outcome and response to chemotherapy.	19,62
P-glycoprotein	ATP-binding cassette (ABC) transporters	Increased risk for chemotherapy resistance.	63-66
HSP27	Heat shock protein 27, protein chaperone and antioxidant	Correlates with poor prognosis for patients with OS.	67
MAGE	Melanoma antigen family A	Expressed in substantial numbers of OS in a major histocompatibility class-I-restricted manner.	68
HSP72	Heat shock protein 70 family and a chaperone protein	Correlates with a good response to neoadjuvant chemotherapy.	69,70
GD2	Disialoganglioside GD2, a sialic acid-containing glycosphingolipid	Overexpressed in OS.	71,72
SART1, SART3	Squamous cell carcinoma antigen recognized by T cells, tumor-rejection antigens	Potentially used in specific immunotherapies HLA-A24 ⁺ patients with OS or malignant fibrous histiocytosis.	73,74
B7-H3	58 KD glycosylated tumor-associated protein antigen	Potential molecules for use in specific immunotherapies for HLA-A24 ⁺ patients with OS or malignant fibrous histiocytosis.	75
Melanoma antigen MUC18	MCAM (melanoma cell adhesion molecule) and as CD146 (endothelial antigen)	Correlates directly with tumor progression and metastatic potential.	76
CXCR4	Chemokine receptor type 4, an alpha-chemokine receptor	Potentially used as a prognostic factor and as a predictor of potential metastatic development in OS.	77
SAA	Serum amyloid A, a family of apolipoproteins associated with high-density lipoprotein (HDL)	Increased SAA levels associated with type of tumor and high-risk OS development.	45
CLUAP1	Clustering associated protein 1	Potentially used as a prognostic/diagnostic marker and/or for a target of immunotherapy of OS.	78
MAGE 1,2,8	Melanoma-associated antigen (MAGE)	Expressed <i>MAGE-1</i> , 2, 8 in all 9 OS tissue samples.	79
NY-ESO-1	New York esophageal squamous cell carcinoma 1 (NY-ESO-1), a cancer-testis antigen	Expressed NY-ESO-1 in 8 of 9 OS tissue samples.	79

Survivin	Inhibitor of apoptosis	Potentially used as an independent predictor of survival for OS patients.	80
Midkine	Heparin-binding growth factor	Correlates with the prognosis of patients with OS.	81
OSAA-3 and OSAA-5	Unknown	Potentially used as candidates for diagnosis and targets for immunotherapy in OS.	82
PBF	Papillomavirus binding factor (PBF)	May contribute to peptide-based vaccination and/or adoptive antigen-specific T-cell therapy of patients with OS and other bone and soft tissue tumors.	83
TEM1	Tumor endothelial marker 1 (TEM1), prototypical member of a family of genes expressed in the stroma of tumors.	Potentially used as a target protein for selective therapeutic intervention.	84
IL-11R α	Interleukin-11 receptor alpha-chain	May represent a new therapy for patients with OS pulmonary metastases.	85
FAP	Fibroblast activation protein	Might be considered as a novel therapeutic target against this cancer.	86
Anti-HSP60 antibody	Autoantibodies against heat shock protein 60	Increases of anti-HSP60 antibodies at the time of first diagnosis of OS.	87
Anti-HSP90 antibody	Autoantibodies against heat shock protein 90	Correlates with a good response to neoadjuvant chemotherapy and their absence correlates with the occurrence of metastases.	88
Anti-MUC18 antibody	Autoantibody against MUC18	Inhibits the development of OS metastases in a preclinical model.	76
Anti-midkine	Autoantibody against midline	Inhibits growth of OS cells <i>in vitro</i> .	81

1.4 Identification of TAAs

The study of the humoral response to infectious diseases and chronic diseases, such as cancer, is important for many reasons, including understanding the host response to disease, identification of protective antigens, vaccine development, and discovery of biomarkers for early diagnosis and prognosis. While several approaches have been used during the past decades for the

identification of TAAs, the most successful have been the serological screening of cDNA expression libraries and phage display libraries and more recently proteomics-based approaches. One of the approaches is the utilization of serum antibodies from cancer patients to immunoscreen cDNA expression library to identify TAAs in cancer, and some of these identified TAAs may have potential diagnostic and prognostic values in cancer. In early studies, we examined sera from cancer patients by Western blotting, using extracts of cultured tumor cells as source of antigens, or by indirect immunofluorescence on fixed tumor cells. Using these techniques we identified sera with high-titer reactivity to putative TAAs, and subsequently used the antibodies in these sera to isolate antigen DNA sequences from cDNA expression libraries (Fig. 1). In this manner, several novel TAAs, including p62⁵¹ and p90⁸⁹ were identified in our previous studies. Subsequently, several novel, as well as previously defined TAAs⁹⁰ were identified with cancer sera using a methodology called SEREX (Serological analysis of recombinant cDNA expression libraries), which is essentially a modification of a previous method⁹¹. The rationale behind SEREX is that intracellular proteins which are involved in carcinogenesis are provoking autoantibody responses and therefore autoantibodies can be used to immunoscreen cDNA expression libraries to isolate, identify and characterize proteins that might potentially be involved in malignant transformation (Fig. 2). In cases of OS, novel as well as previously defined TAAs have been identified using the SEREX method, including CLUAP1⁷⁸, OSAA-3 and OSAA-5⁸².

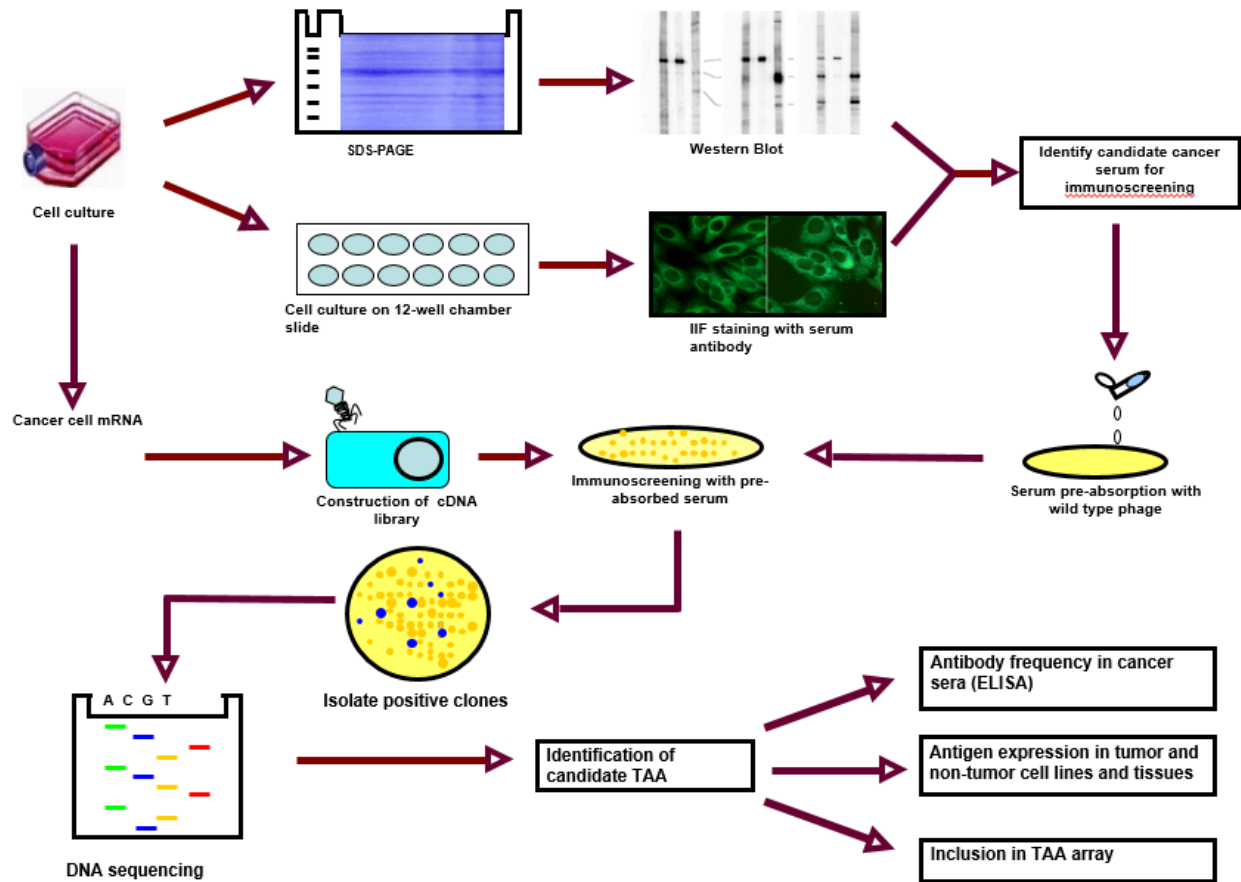


Figure 1: TAAs identification exploiting cDNA expression library immunoscreening. In brief, the sera from cancer patients were initially examined using extracts of cancer culture cells as source of antigens in Western blotting and by indirect immunofluorescence (IIF) on whole cells. With these two techniques, we identify sera which have high-titer fluorescent staining or strong signals to cell extracts on Western blot and subsequently use the antibodies in these sera to isolate cDNA clones from cDNA expression libraries. Additionally, the isolate positive clones were performed by mass spectrometry to further confirm the results from cDNA expression library screening.

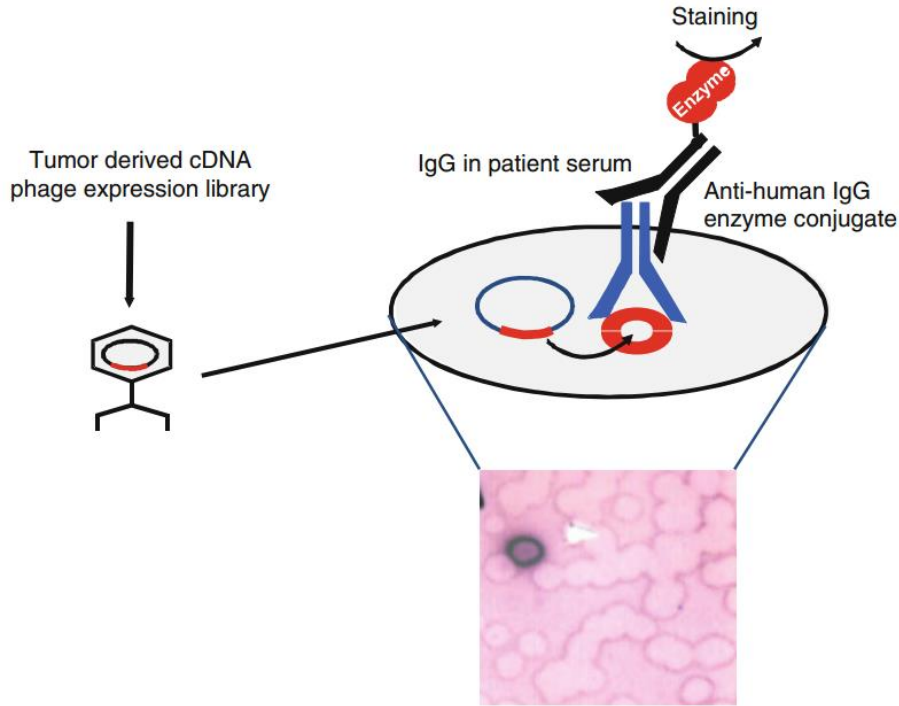


Figure 2: Serological analysis of recombinant cDNA expression libraries (SEREX)⁹².

1.5 Cancer immunoseroproteomics

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, non-invasive diagnosis of cancer and for monitoring tumor progression. One approach includes direct profiling of human cancer sera, using two dimensional gel electrophoresis (2-DE) system and mass spectroscopy, to identify distinctive protein signatures characteristic of different tumor types^{6,21-24}. Previously, we provided one of the first examples of the characterization and validation of the protein biomarker signature of primary bone cancer by using SELDI-TOF-MS to profile sera from OS patients with and without bone metastases and a cluster of unique proteins in the profile of patients with bone metastases were identified as discrete isoforms of SAA⁴⁵. A second approach, focused in our current studies, is the exploitation of the serum autoantibody repertoire from cancer patients for the identification of TAAs and the design of customized TAA panels or arrays for the

serological diagnosis and management of OS^{25,26}. This approach has been termed as “serological proteome analysis” (SERPA)⁹³.

Our previous studies have demonstrated that the SERPA approach can be applied to many models of disease. Recent several years, we have used this approach to extensively screen sera from patients with certain types of cancer such as hepatocellular carcinoma (HCC)⁹⁴ and esophageal squamous cell carcinoma (ESCC)⁹⁵, Prostate Cancer (PCa)^{96,97} and Lung Cancer (LC)^{98,99} and sera from patients with pre-cancer condition such as liver fibrosis¹⁰⁰ to identify and characterize the potential TAAs. In our laboratory, this protocol was developed specifically for screening immune sera to support the development and characterization of TAAs. A brief description of the SERPA approach we have used to identify and characterize TAAs is shown in Fig.3. Briefly, the sera from cancer patients were initially examined using extracts of culture cells as the source of protein antigens in One-dimensional gel electrophoresis (1-D) Western blotting and by indirect immunofluorescence (IIF) on whole cells. With these two techniques, we identified sera that have high-titer fluorescent staining or strong signals to cell extracts on (1-D) Western blotting and narrow the targeting proteins on specific molecular weight bands, and subsequently used the antibodies in these sera as probes in a proteomic approach to isolate potential TAAs. Cell extracts of cultured cancer cells was applied onto the first dimension isoelectrofocusing gel (1D-IEF), and subsequently loaded onto the second-dimension gel (2DE-SDS-PAGE). The proteins were transferred to the nitrocellulose membrane or visualized by Coomassie blue staining (or silver staining). After immunoblotting (2-DE Western blotting) with cancer sera and benign tumor sera or normal human sera (as controls), a number of protein spots of interest were excised from the 2-DE gels, digested by trypsin, and subsequently analyzed by mass spectrometry (MS). In subsequent studies, we will use several approaches such as Enzyme-linked Immunosorbent Assay (ELISA), 1-D Western blotting and immunohistochemistry (IHC) with tissue arrays to comprehensively characterize and validate the identified the TAAs and anti-TAA autoantibodies systems that are potentially useful in cancer immunodiagnosis, and then evaluate the sensitivity and specificity of different antigen-antibody systems as markers in certain type of cancer for

further developing “TAA array” systems for cancer diagnosis, prediction, and for following the response of patients to treatment.

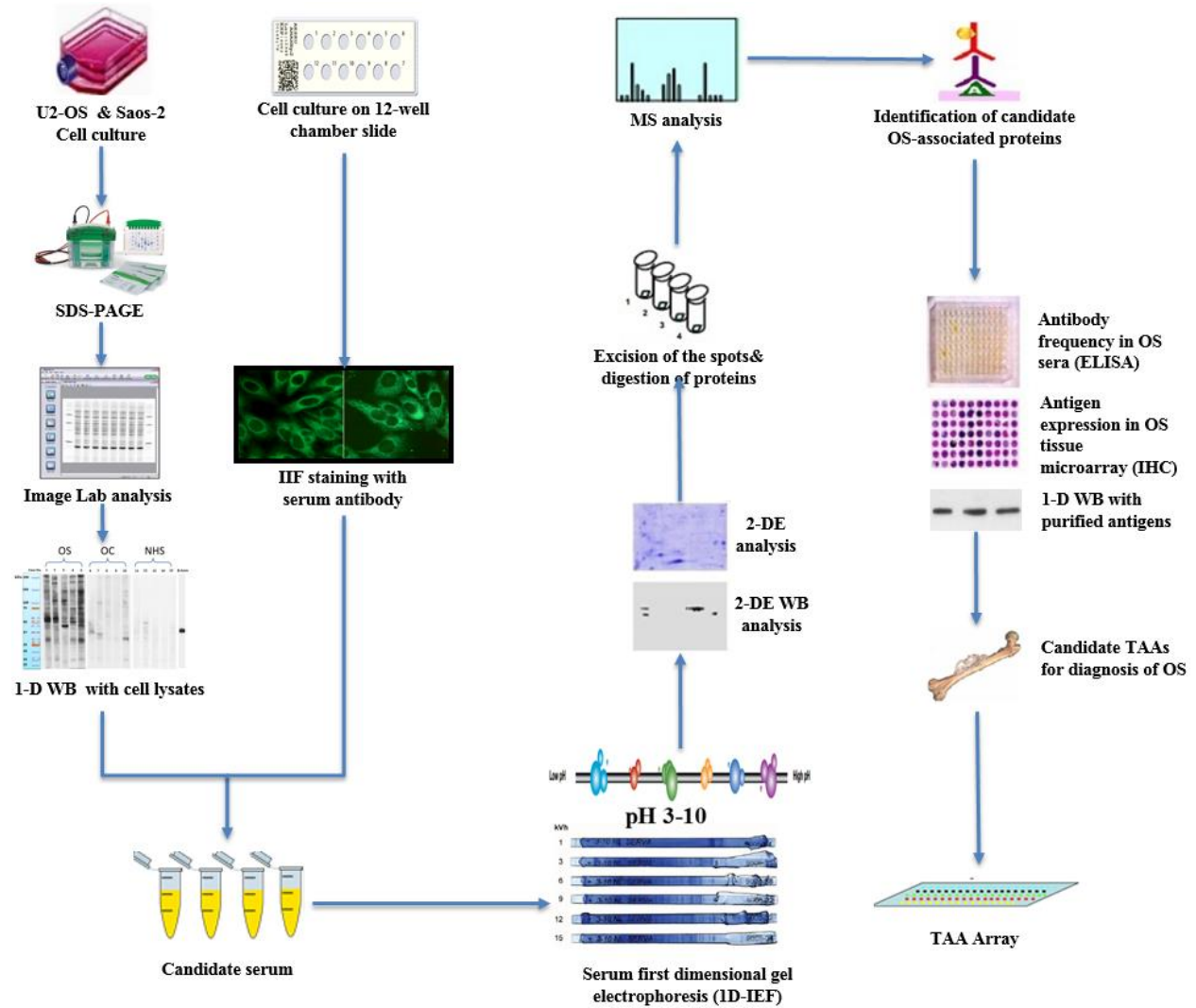


Figure 3: Schematic representation of identification and validation of TAAs using serological proteome analysis (SERPA) approach. In brief, the sera from OS patients and controls were initially examined using extracts of culture cells as source of antigens in Western blotting and by indirect immunofluorescence (IIF) on whole cells. With these two techniques, we identify sera which have high-titer fluorescent staining or strong signals to cell extracts on Western blotting and narrow the targeting proteins on specific molecular weight bands, and subsequently use the antibodies in these sera as probes in immunoproteomic screening. Cell extracts of cultured human cells was also applied onto the first dimension gel (isoelectrofocusing gel), and subsequently loaded onto the second-dimension gel (2-DE-SDS-PAGE). The proteins were transferred to the nitrocellulose membrane or visualized by silver staining or Coomassie brilliant blue staining. After immunoblotting with OS sera and control sera, a number of protein spots of interest were excised from the 2-DE gels, digested by trypsin, and subsequently analyzed by mass spectrometry (MS). In subsequent studies, we will characterize the identified cellular proteins that are potential biomarkers in OS.

1.6 TAA mini-arrays and cancer immunodiagnosis

Interest in the use of anti-TAA autoantibodies as serological markers for cancer diagnosis derives from the recognition that these antibodies are generally absent, or present in low frequency in normal individuals and in non-cancer conditions²⁶. Their persistence and stability in the serum of cancer patients is an advantage over other potential markers, including the TAAs themselves, which are released by tumors but rapidly degraded or cleared after circulating in the serum for a limited time⁵⁹. Furthermore, the widespread availability of methods and reagents to detect serum autoantibodies facilitates their characterization in cancer patients and assay development. However, compared with autoimmune diseases, where the presence of a particular autoantibody may have diagnostic value, cancer-associated autoantibodies, when evaluated individually have little diagnostic value. This is mainly due to their low frequency, sensitivity, and specificity. Such drawback has been observed in our previous study and we found it can be overcome by using mini-arrays of properly selected TAAs, and that different types of cancer may require different TAA arrays to achieve the sensitivity and specificity required to make immunodiagnosis a feasible adjunct to tumor diagnosis and even prognosis⁴⁷.

Our pioneering findings provide evidence that detection of autoantibodies in cancers can be substantially enhanced by using a mini-array of several TAAs as target antigens^{47,101,102}. For

instance, this mini-array comprised 14 full-length recombinant proteins expressed from cDNAs encoding surviving, CAPER α , RalA, p62, Koc, MDM2, cyclin B1, p53, 14-3-3 ζ , p90, IMP1, c-Myc, NPM1 and p16 was customized for detection of HCC. The antibody frequency to any of these individual TAA was variable, ranging from 5.6% to 21.1% in HCC¹⁰³. However, with the successive addition of TAAs to a final total of 14 TAAs, there was stepwise increase of positive antibody reactions up to 69.7% in HCC¹⁰³. The fact is that antibodies to any individual antigen such as anti-p53, anti-p62 or anti-c-Myc do not reach levels of sensitivity which could become routinely useful in diagnosis^{47,101-103}. These data indicate that the combination of multiple TAAs might yield higher sensitivity for serological diagnosis of cancer. On the other hand, the data also suggest that in the selection of different TAA-antibody systems, some of the TAAs may turn out to be more specific for a certain type of cancer. It is conceivable that specific autoantibody profiles can be identified with the help of customized TAA arrays and that the results could be beneficial for differential diagnosis of specific types of cancer.

1.7 Dissertation project hypothesis

Our previous studies have showed that detection of autoantibodies in cancer patients can be substantially improved exploiting mini-arrays of selected TAAs as target antigens. These studies support the central hypothesis of this proposed project that "customized" TAA arrays constitute promising and powerful tools for enhancing the serological detection of OS. Implementation of TAA arrays in immunoscreening programs for cancer diagnosis, or as tools for monitoring cancer progression and guiding therapeutic interventions, requires maximizing their sensitivity and specificity. This can be achieved through the identification of novel TAAs and design of optimal TAA arrays to discriminate serologically OS from benign bone tumors or other cancers and diseases.

1.8 Dissertation project specific aims and rationale

1. Identify and characterize novel TAAs in OS using SERPA approach. The rationale is that intracellular proteins involved in bone carcinogenesis provoke autoantibodies that can be used as probes in proteomic analysis to isolate, identify, and characterize potential oncoproteins.

2. Validate the potential value of novel identified TAAs as OS biomarkers. This will be achieved by: a) Determine whether the autoantibodies to the TAAs identified in Aim 1 are specific to serum from OS patients with different clinical stages or are also detected in serum from patients with the related but benign Osteochondroma (OC) and normal individuals, and b) examining the expression of the TAAs in clinical bone tissue specimens and a panel of bone cell lines.

3. Design customized TAA mini-arrays that could be used to enhance autoantibody detection in OS. The sensitivity and specificity of these arrays will be tested against OS sera, benign bone tumors or other cancers and controls.

Chapter 2: Materials and Methods

2.1 Serum samples

Ninety five serum samples from 52 OS patients including 24 patients with serial serum samples collected at least two to five samples obtained at the time of diagnosis and at different time points along the disease progression. All samples are collected through IRB-approved protocols from the two collaborative institutions, namely Henan Luoyang Orthopedic-Traumatological Hospital (HLOH) in China and Institutional Human Subject Review Boards of the University of Texas at El Paso, after informed consent had been signed. These samples were used to create proteomic profiles and evaluate targeted TAAs. Twenty-eight serum samples from age-matched anonymized OC patients was also from HLOH were used as benign bone tumor controls. We chose age-matched OC as a benign control because it is the most common benign bone tumor and these samples were readily available in our study. In terms of controlling for potential impact of nonspecific host response on serum proteomic profiles, OC patients are probably more appropriate than normal subjects because both OS and OC patients share primary tumors that have similar anatomic location and tissue type. In addition, 49 age and sex matched normal serum samples from normal human donors were collected from HLOH annual physical examination in people with no clinical evidence of OS as well. The normal donor serum samples were pooled used as a normal control in 2-DE Western blotting and individually tested in 1-D Western blotting. After collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature. This usually takes 15-30 minutes. Remove the clot by centrifuging at 1,000-2,000 $\times g$ for 10 minutes in a refrigerated centrifuge. The serum supernatant is collected and divided into aliquots and stored at -80 °C until use.

Table 4: Characteristics of patients with Osteosarcoma (OS), Osteochondroma (OC) and Normal Human (NH)

	OS (n=52)	OC (n=28)	NH (n=49)
Age			
Mean, SD	24.7, 14.2	17.2, 12.0	24.0, 13.1
Range	4-64	4-52	4-59
Gender (%)			
Male	36 (69.2)	19 (67.9)	31 (63.3)
Female	16 (30.8)	9 (32.1)	18 (36.7)
Clinical stage (%)			
Stage I-II	38 (73.1)		
Stage III-IV	10 (19.2)		
Unknown	4 (7.7)		
Histologic type (%)			
Osteoblastic	36 (69.2)		
Fibroblastic	6 (11.5)		
Osteoblastic	4 (7.7)		
Others	6 (11.5)		
Anatomic site (%)			
Femur	27 (51.9)		
Tibia	15 (28.8)		
Fibula	3 (5.8)		
Knee	3 (5.8)		
Others	4 (7.7)		
Tumor size (%)			
<5 cm (%)	16 (30.8)		
≥5 cm (%)	36 (69.2)		
Grade (%)			
1	42 (80.8)		
3	4 (7.7)		
Unknown	6 (11.5)		

2.2 Cell line and cell extracts

U2-OS (ATCC® HTB-96™) and Saos-2 (ATCC® HTB-8™) cell lines were purchased from the American Type Culture Collection (Manassas, VA) and culture following the specific protocol as provided. The cell lines were maintained in a humidified atmosphere with 5% CO₂ at 37 °C using the supplier's prescribed ATCC-formulated McCoy's 5A Medium Modified (Cat# 30-2007), supplemented with fetal bovine serum (FBS) to a final concentration of 15%, and 100 units/mL penicillin and 100 units/mL streptomycin. Cells grown in 175-cm² Falcon tissue culture flasks were allowed to reach 80-90% confluence. Then, cells were rinsed once with McCoy's 5A

without FBS and removed from the flask by incubating them with a solution containing trypsin-EDTA (Cat# 25200056, Gibco, Carlsbad, CA), and harvested in a 15 mL centrifuge tube for further study. U2-OS and Saos-2 cells are lysed directly in 1X Laemmli's sample buffer (Cat# 1610737, Bio-Rad, Hercules, CA) and boiled each cell lysates in sample buffer at 100°C for 5 min. Lysates can be aliquoted and stored at -20°C for future use.

2.3 Identified recombinant proteins and antibodies

Purified ENO1 protein (Cat# E-6126, Sigma-Aldrich, St. Louis, Missouri) was acquired from Sigma. Full length protein GAPDH (ab77109), TPI1 (ab100826), HSP60 (ab78430), PDLIM1 (ab177676) and STMN1 (ab87492) were purchased from AbcamInc (Cambridge, MA). Commercially available antibodies used in this study included mouse anti-beta actin (Cat# sc-4778, Santa Cruz Biotechnology, CA, 1:1000 dilution), goat anti-mouse IgG-HRP (Cat# sc-2005, Santa Cruz Biotechnology, CA, 1:5000 dilution), rabbit polyclonal antibody to ENO1 (Cat# A1033, Neo Scientific, Cambridge, MA).

2.4 Expression and purification of identified recombinant proteins

NPM1 construct GFP-NPM1 WT (plasmid ID: 17578) from Addgene Inc. (Cambridge, MA, USA) was subcloned into the pET28a vector to express the fusion protein with N-terminal 6×His tags. The recombinant NPM1 protein expressed in *Escherichia coli* BL21 (DE3) was purified using nickel column chromatography.

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

To screen the autoantibody-positive sera, U2-OS and Saos-2 cells were lysed directly in 1X Laemmli's sample buffer and boiled for 10 min. After the removing of the insoluble fraction by centrifuge, samples were loaded onto 4–15% Mini-PROTEAN®TGX Stain-Free™ Protein Gels (Cat# 4568081, Bio-Rad, Hercules, CA), which was then transferred onto 0.45 µm nitrocellulose membrane (Cat# 16201115, Bio-Rad, Hercules, CA) for Western blotting. The membrane was stained with Ponceau S to confirm the transfer efficacy of the proteins and then the membrane was cut into 0.1-inch wide stripes. After blocking with 3% nonfat milk prepared in 1×

Phosphate Buffered Saline (PBS), containing 0.05% Tween-20 (PBST), for 1h at RT, the nitrocellulose 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 1X 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).

2.6 Indirect immunofluorescence (IIF) microscopy

Indirect immunofluorescence assay was performed on HEp-2 antinuclear antigen tissue slides (Cat# AN-1012, MBL, Des Plaines, IL). Normal human sera and PBS were used as negative and blank controls, respectively, and Sjogren's Syndrome A Control (SS-A) human sera was used as positive control. The sera were diluted at 1:40 in PBS, pH 7.4 and incubated with the slides for 30 min at RT. After extensive washing, the slides were incubated with Goat Anti-Human IgG (H+L) Secondary Antibody, FITC (Cal# H10301, Thermo Fisher Scientific Inc., IL) as secondary antibody diluted 1:1000 in PBS for 1h at RT. The slides were washed three times with PBS before adding a drop of mounting media containing 1.5 µg/mL 4',6'-diamidino-2-phenylindole (DAPI) (Cal# H-1200, Vector Laboratories Inc. Burlingame, CA) to prevent photobleaching. The slides were then examined under fluorescence microscopy, Leica DM1000 (Leica Microsystems, Houston, TX), at 400×magnification. Images were acquired using the software QCapture 290.1 (Qimaging, Burnaby, BC, Canada). All images were acquired under identical conditions. Ambiguous results were considered negative. The immunostaining patterns is compared for consistency with available experimental gene/protein characterization data in the UniProtKB/Swiss-Prot database and other experimental evidence for location described in scientific literature^{104,105}.

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

U2-OS and Saos-2 cells were directly lysed in rehydration sample buffer (8M Urea, 50 mM dithiothreitol (DTT), 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.2% carrier ampholytes) as provided by Bio-Rad Laboratories (Cat# 1632106, Bio-Rad, Hercules, CA) and were vortexed vigorously for 90 mins at room temperature (RT). Insoluble substances were removed by centrifuge at 16,000×g or 30 min at 4 °C. Supernatant was collected and protein concentration was measured by the Bradford Protein Assay (Cat# 500-0201, Bio-Rad, Hercules, CA). For the first dimensional gel electrophoresis (1D-IEF) analysis, a total of 200 µg protein was mixed with rehydration buffer containing a trace bromophenol blue prepared in proteomics-grade water and applied on a pH 3–10, 7-cm isoelectric focusing (IEF) strip (Cat# 163-2002, Bio-Rad, Hercules, CA). IEF was performed at a current of 50 mA per gel, 300 V for 30 min, followed by 8000 V for 2.5 h, and additional 8000 V for 5 h. Strips were immediately stored at –80 °C for the second dimensional gel electrophoresis (2-DE) analysis. For the second dimensional electrophoresis, 15% SDS polyacrylamide gels (SDS-PAGE) were used. Proteins were transferred onto nitrocellulose membrane for 2-DE Western blotting analysis or stained with 0.1% Coomassie blue R-250 prepared in 40% methanol/10% acetic acid. The spots were visualized using PD Quest 2-DE analysis software (Bio- Rad Laboratories, Hercules, CA) as described in the manufacturer's manual and also in our previous study^{94,95,100}.

2.8 Serological proteomic analysis (SERPA)

For 2-DE Western blotting, the proteins on 2-DE gel were directly transferred into nitrocellulose membrane and following the same protocol as described above. A schematic representation of protein biomarker identification in OS is summarized in Fig 3. Membrane was stained with Ponceau S to confirm the transfer efficacy of the proteins and several reference circles were drawn around prominent protein bands/spots on the stained nitrocellulose membranes to serve as orientation and alignment marks. These marks were essential for accurate digital overlay of immunoreactive spots in the film of the nitrocellulose membrane (from analysis 2DE gel)

probed with human sera with corresponding protein spots in the Coomassie-stained reference 2-DE gel. Molecular weight markers on the sides of the 2D gels also facilitated the alignment. The reference and analysis 2-DE gels were run in the same apparatus under identical conditions to ensure accurate alignment of protein spots. After blocking with 3% nonfat milk prepared in 1× PBS, containing 0.05% Tween-20 (PBST), for 1h at RT, the nitrocellulose membranes were incubated with 11 representative OS sera and a pool of 5 NHS used as control 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 1X PBST for 30 min at RT. The positive bands were detected with Enhanced Chemiluminescence (ECL) substrate (Thermo Fisher Scientific Inc., IL).

2.9 Sample preparation for mass spectrometry (MS)

The targeted gel spots were excised from a Coomassie blue-stained preparative gel and then washed with HPLC grade water, followed by destained with acetonitrile (ACN) for 15 mins to remove Coomassie blue staining, and dried in a vacuum centrifuge (Vacufuge, Eppendorf, Westbury, NY) as described previously¹⁰⁶. After the reduction (with 5 mM DTT) of disulfide bounds and alkylation (with 10mM iodoacetoamide) of cysteine residues, digestion will be performed by addition of 12.5 ng/μL of sequencing-grade trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate containing 5 mM CaCl₂. Following the enzymatic digestion overnight at 37°C, the peptides will be extracted with 25mM ammonium bicarbonate in 50% ACN, followed by 5% formic acid (FA) in 50% ACN solution. After removal of acetonitrile in a vacuum centrifuge (Vacufuge, Eppendorf, Westbury, NY), the sample will be desalted by C18 bead ziptips (POROS R2, Applied Biosystems, Framingham, MA)¹⁰⁷, and dried out by in a vacuum centrifuge before mass spectrometry analysis.

2.10 Identification of candidate TAAs by mass spectrometry (MS)

MS and MS/MS data for protein identification were obtained by using a MALDI-TOF-TOF instrument (5800 proteomics analyzer; Applied Biosystems). 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 1,000 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 2,211.1046) 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.

2.11 Protein identification

Peptide mass finger printing (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 (downloaded on 3/4/2015; 51829 sequences) and UniProt_viridiplantae (downloaded on 4/7/2015; 2872433 sequences) 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 $\geq 95\%$ were used for further manual validation.

2.12 Enzyme-linked immunosorbent assay (ELISA)

ELISA procedures were performed essentially as indicated in previous studies^{51,102,108,109}. Briefly, purified recombinant proteins were diluted in PBS to a final concentration of 1.0 $\mu\text{g/ml}$ and coated onto a Clear Flat-Bottom Immuno Nonsterile 96-Well Plates (Cal# 3455, Thermo Fisher Scientific Inc., IL), which were then incubated overnight at 4 °C. Human sera diluted at 1:100 were incubated in the antigen-coated wells. Goat anti-Human IgG (H+L) Secondary Antibody, HRP (Cal#H10307, Thermo Fisher Scientific Inc., IL, 1:4000 dilution) 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 (SpectraMax 190; Molecular Devices, Sunnyvale, CA). All serum samples were assayed in duplicate and all positive sera were further confirmed by Western blotting.

2.13 Immunohistochemistry (IHC)

Immunohistochemical study was performed using commercially available bone disease spectrum and normal bone tissue microarray (TMA) (Cat# OS802a&BO244d, US Biomax, Inc.) Osteosarcoma and chondrosarcoma tissue microarray contains 51 cases of osteosarcoma, 27 chondrosarcoma, plus 2 bone marrow tissue, and single core per case. And normal bone tissue microarray contains 11 cases of bone tissue, 1 osteosarcoma, and duplicate cores per case. The tissues were deparaffinized, endogenous peroxide was blocked in 3% hydrogen peroxide in methanol, and microwave antigen retrieval was done using a Trilogy Pretreatment Solution (Cat# 920P-10, Cell Marque, Rocklin, CA). Blocking was done using Avidin/Biotin blocking kit (Cat# 928B-00, Cell Marque, Rocklin, CA) and TMA slides were incubated with polyclonal anti-ENO1 antibody at concentration (1:200 dilution). Biotinylated secondary antibody, CytoScan™ HRP Detection System (Cat# 951D-10, Cell Marque, Rocklin, CA), and DAB Substrate Kit (3,3'-diaminobenzidine) were used as detecting reagents (Cat# 957D-20, Cell Marque, Rocklin, CA). The tissues were counterstained with hematoxylin (Cat# 930D-10, Cell Marque, Rocklin, CA), fixed by Richard-Allan Scientific™ Signature Series Bluing Reagent (Cat# 7301, Thermo Fisher Scientific Inc., IL) and dehydrolyzed with different concentration of ethanol and Citrisolvent. Finally, the slides were mounted with permount mounting medium and observed under brightfield microscopy (Leica LDM7000, S/N393985, Leica Microsystems Inc., US). Briefly, five representative 100X and 400X magnification fields for each patient were randomly selected 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 sum of positive rate core and staining intensity score was used to estimate the antigen expression in each sample, in which the final score, <4 was defined as low/negative expression, whereas ≥ 4 was defined as high/positive expression¹¹⁰. The protein expression was compared for consistency with available experimental gene/protein characterization data in types of cancers from the Pathology Atlas^{105,111}.

2.14 Function analysis of differentially expressed proteins

Gene ontology (GO) annotation was performed to better understanding 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)^{112,113}. Only GO from genes with e-value $\leq 1e-10$ for database search was accepted.

2.15 Statistical analysis

Data regarding the different immunoreactivity of the sera were summarized by mean of OD value. Due to the sera autoantibodies against some TAAs were not normally distributed, nonparametric Mann-Whitney U tests were used to compare differences of autoantibody levels between two groups, and nonparametric Kruskal-Wallis test were used to compare differences of antibody levels among multiple groups. χ^2 tests were used to compare the differences of frequency between two groups and among multiple groups. Related-samples Wilcoxon signed rank test was employed to analyze the serial assay of anti-ENO1 autoantibody by ELISA along the disease progression. The receiver operating characteristic (ROC) analysis of single-variable was conducted for each anti-TAA for the distinguishing of OS from controls, leading to estimates of area under the curve (AUC) with 95% confidence interval (CI). The optimal cut-off value (CV) for the optical density (OD) of an ELISA for determining a positive reaction was designated as the mean absorbance of the NHS controls plus three standard deviations (mean+3SD). Statistical

analysis was carried out in SPSS software, version 24.0. Differences were considered statistically significant by significant level 0.01 or 0.05.

Chapter 3: Results

3.1 Screening of patients' sera with OS, OC and normal individuals for the presence of autoantibodies to potential TAAs.

In this initial study, we have tested 52 sera from OS patients (age 4-64 years), 28 sera from OC patients (age 4-52 years) and 49 age-sex matched normal human individuals, for the presence of autoantibodies to the TAAs from extracted protein antigens from U2-OS and Saos-2 culture cells in 1-D Western blot and by indirect immunofluorescence (IIF). We found that some of the patients' sera contained autoantibodies reacting with one or more cellular antigens (Fig. 4&6). As shown in Table 5, autoantibodies were detected in up to 96.2% sera from patients with OS, which were significantly higher than that in normal human sera (30.6% and 32.7%). In contrast, there is no significant association between OC and NHS group, which implies that the OS sera may encompass more specific autoantibodies than OC sera against the proteins extracted from either U2-OS or Saos-2 cell lines. Western blotting analysis of five representative sera from each of the cohort groups against these two cell extracted proteins are shown in Fig. 4&6. 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), 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. As shown in Fig. 5, 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), 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 (Fig. 7). Seven identified protein bands (47KD, 33KD, 60KD, 37KD, 27KD, 17KD, 29KD) were overlapped between these two cell lines. Moreover, further analysis of the autoantibody positivity to 27 KD and 50KD Saos-2 cellular

antigens in OS showed no significant difference with NHS. The most common immunoreactivity band is around the 47 KD region (38.5% and 48.1% in U2-OS and Saos-2 cell proteins), which implies that the targeting TAAs could be focused on the protein with molecular weight around 47KD.

Table 5: 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 antigens cellular protein 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: $P < 0.001$.

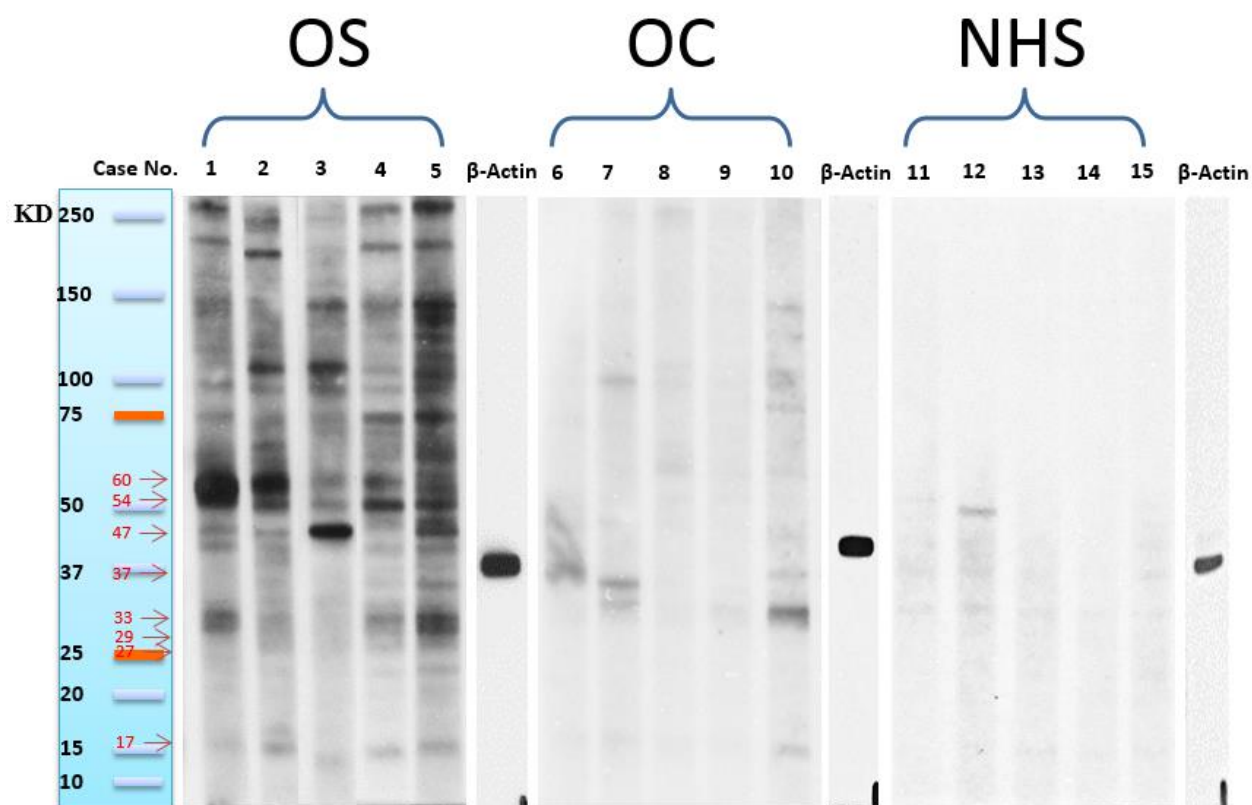
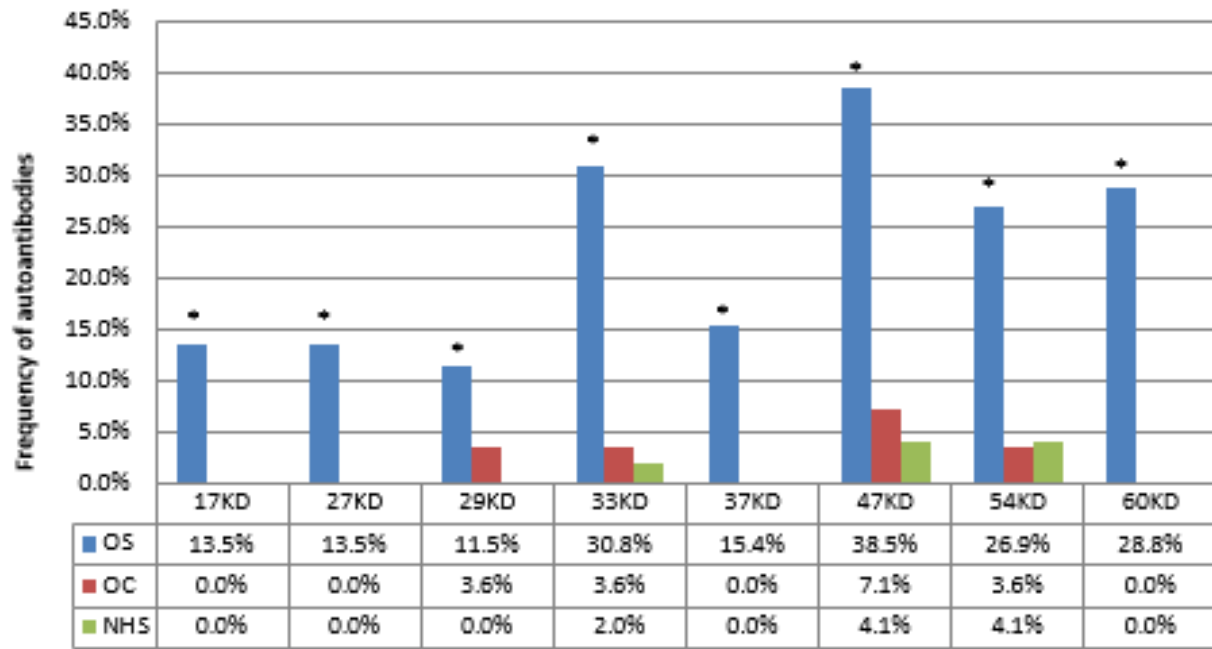


Figure 4: Detection of autoantibodies against cellular protein antigens extracted from U2-OS cell line in sera from patients with OS, OC and NHS. 1-D Western blotting shows that autoantibodies against the protein antigens extracted from U2-OS cell line detected after probing with sera OS# 16, 19, 21, 25, 26 (Lanes 1-5) were significantly higher than that in sera OC# 24, 25, 26, 27, 28 (Lanes 6-10) and normal human sera N# 28, 29, 30, 31, 32 (Lanes 11-15). OS: Osteosarcoma; OC: Osteochondroma; NHS: Normal Human Sera.



**P* value relative to NHS: *P* < 0.05.

Figure 5: Frequency of autoantibodies against the 47KD, 33KD, 60KD, 54KD, 37KD, 27KD, 17KD and 29KD autoantigens from U2-OS cell line in sera from patients with OS, OC and NHS.

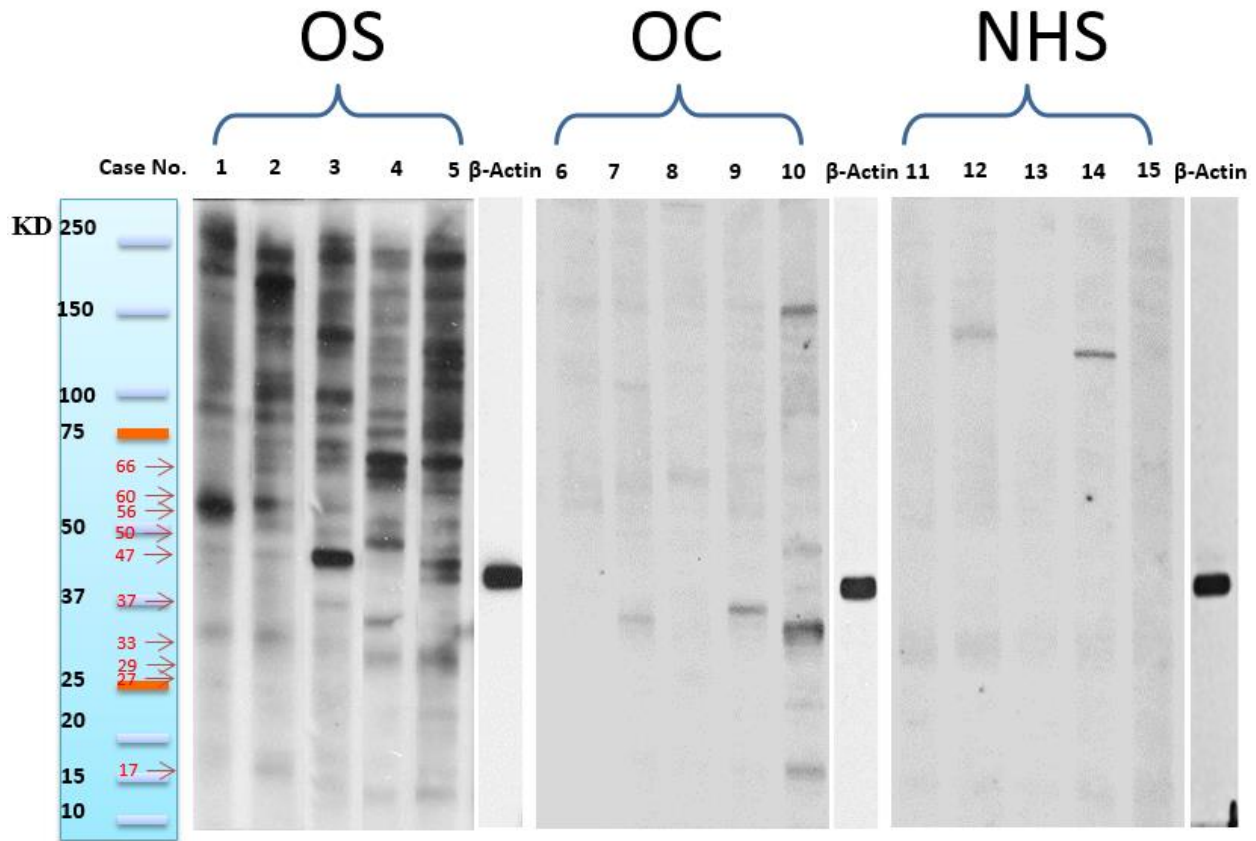
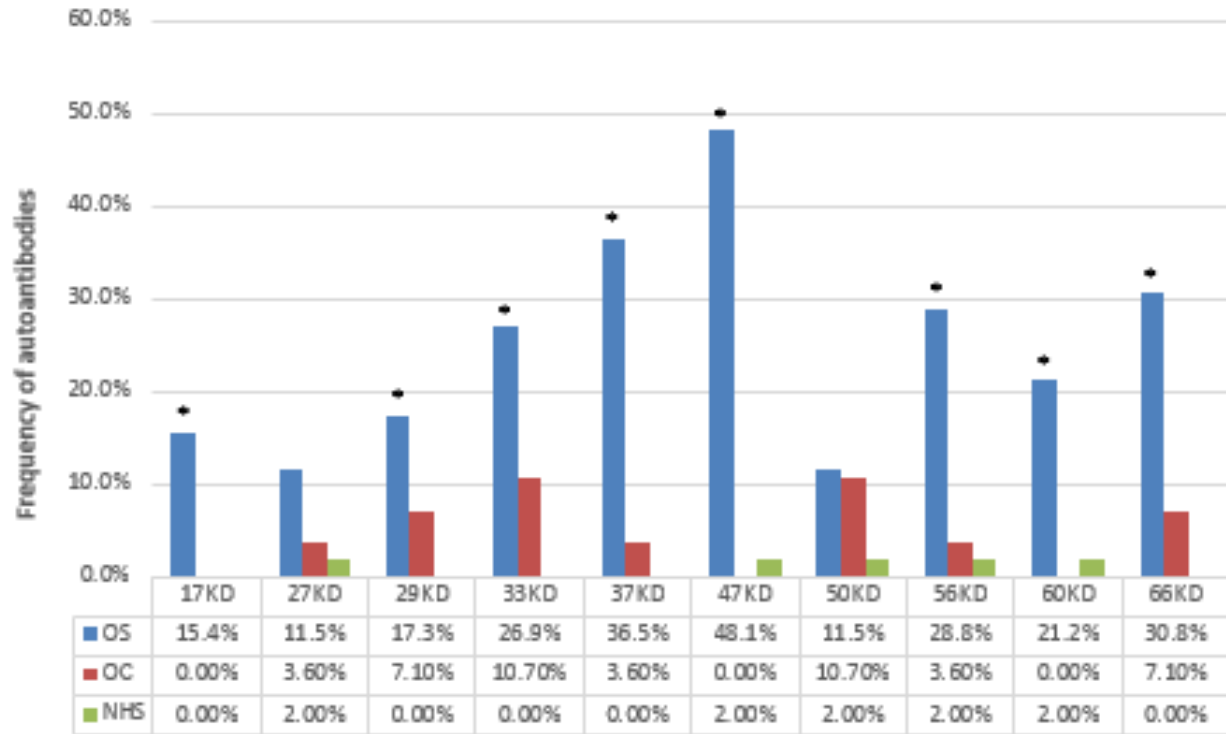


Figure 6: Detection of autoantibodies against cellular protein antigens extracted from Saos-2 cell line in sera from patients with OS, OC and NHS. 1-D Western blotting shows that autoantibodies against the protein antigens extracted from Saos-2 cell line detected in sera from patients with OS# 16, 19, 21, 25, 26 (Lanes 1-5) were significantly higher than that in sera OC# 24, 25, 26, 27, 28 (Lanes 6-10) and normal human sera N# 1, 2, 3, 4, 5 (Lances 11-15). OS: Osteosarcoma; OC: Osteochondroma; NHS: Normal Human Sera.



**P* value relative to NHS: *P* < 0.05.

Figure 7: Frequency of autoantibodies against the 47KD, 37KD, 66KD, 56KD, 33KD, 60KD, 29KD, 27KD, 17KD and 50KD autoantigens from Saos-2 cell line in sera from patients with OS, OC and NHS.

3.2 Indirect immunofluorescence (IIF) microscopy

Reactivity with cancer cells for all selected serum samples with strong immunoreactivity including those reacting with the top four common proteins band around 33KD, 47KD, 54KD and 60KD from U2-OS 1-D Western blotting analysis were further confirmed by indirect immunofluorescence (IIF) assay with commercially purchased HEp-2 antinuclear antigen tissue slides. Subcellular fractionations of HEp-2 cells were incubated with representative antigen (47KD, 54KD and 60KD protein antigens) enriched OS sera showing predominant cytoplasmic staining patterns with more intense staining in the perinuclear regions, whereas, the 33KD protein antigens shows nucleoplasm granular immunofluorescence staining pattern (Fig. 8).

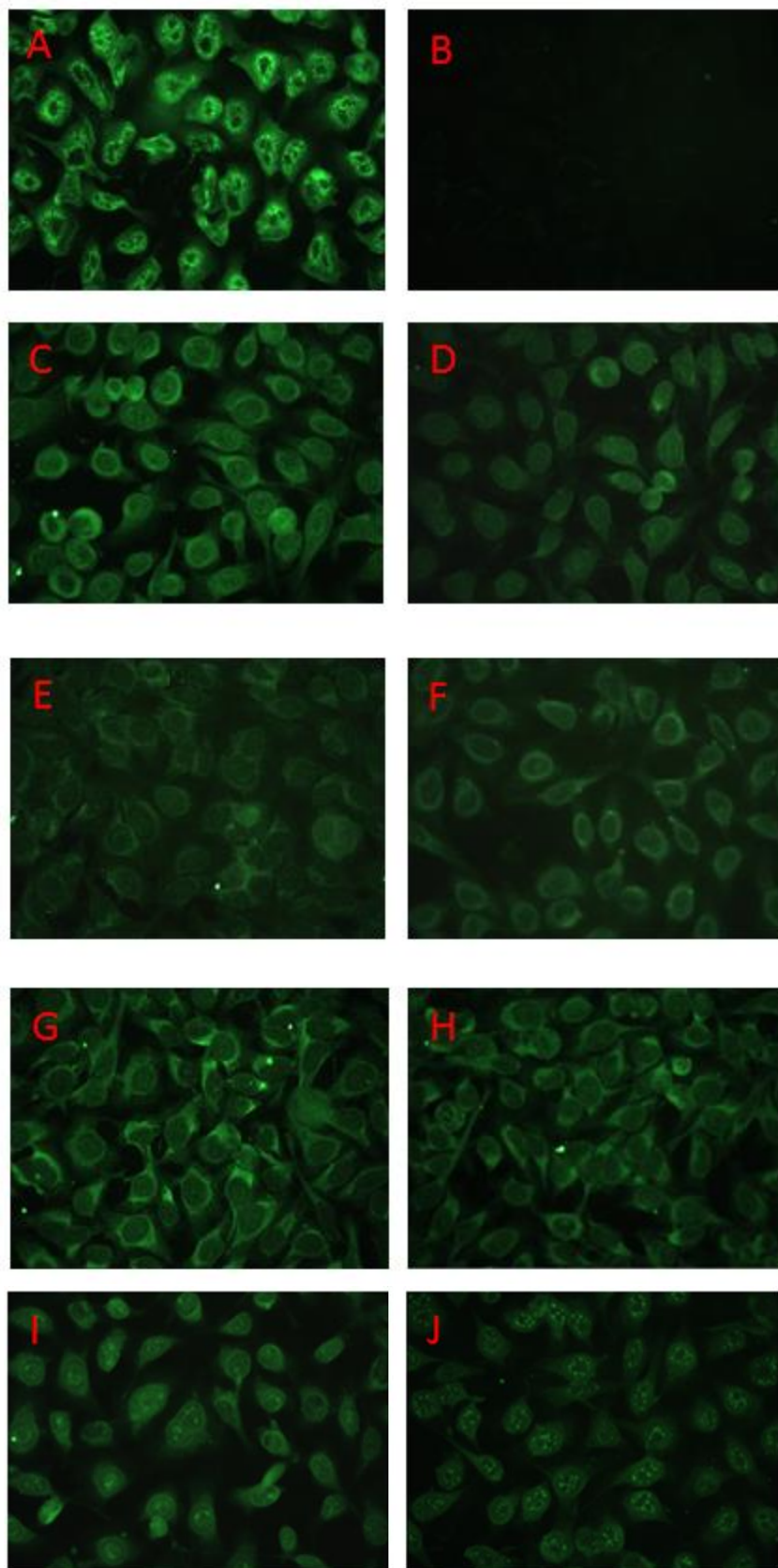


Figure 8: Representative immunofluorescence staining pattern of anti-33KD, 47KD, 54KD and 60KD autoantibodies positive in the OS serum. A, Sjogren's Syndrome A Control (SS-A) human sera which showed a nucleoplasm granular immunofluorescence staining pattern was used as positive control; B, PBS was used as blank control; C&D, two representative anti-60 KD autoantibody positive OS sera; E&F, two representative anti-54 KD autoantibody positive OS sera; G&H, two representative anti-47 KD autoantibody positive OS sera; I&J, two representative anti-33 KD autoantibody positive OS sera.

3.3 Identification of candidate TAAs by SERPA approach

In the present study, the SERPA approach was applied to identify the repertoire of immunoreactive potential TAAs as biomarkers in OS. Combined with advances in genomics and mass spectrometry, the SERPA approach has allowed the facile identification of immunoreactive proteins. A brief description of this approach is shown in Fig. 3. In order to obtain a global view of the proteins in U2-OS and Saos-2 cell lines, proteins extracted from US-OS and Saos-2 cells were analyzed by 2DE-PAGE using the broad-range pH 3-10 non-linear gradient gel strips for the isoelectricfocusing (IEF) strip. This broad range pH can provide the possibility for a view of most of the constituent proteins contained in cells and allow for the separation of protein with pI from 3 to 10. After electrophoresis, the gels were either stained with Coomassie blue or transferred onto nitrocellulose membrane. As shown in Fig. 9&10, a 15% SDS-PAGE was used in the second dimension gel to separate proteins with molecular weight from 10-250 KD. Subsequently, proteins were transferred to nitrocellulose membrane and probed with primary sera and conjugated secondary antibody, as per traditional Western blotting. We selected 11 representative sera from OS patients without any treatment and with common strong immunoreactivity in the 1-D Western blotting. The nitrocellulose membranes were incubated with these 11 representative OS sera and a pool of 5 NHS used as control. Images of immunoblots of 2-DE analysis gels exposed on films were overlaid and digitally matched with images of the corresponding Coomassie Blue-stained reference 2-DE gels (Fig. 11&12). Excising the identified immunoreactive proteins from a second protein stained 2DE-PAGE, and subsequent digestion with trypsin allows identification of proteins using mass spectrometry based techniques. Protein spots in stained 2D gels corresponding to the

immunoreactive spots 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. The filtering process for top scores of protein hits in the excised immunoreactive spots was based on molecular weight, isoelectric point, percent coverage, number of unique peptides, and total number of proteins identified for each spot.

Of interest, alpha-enolase (ENO1) was identified as the top hit from the 47 KD spot recognized by 10 of the 11 selected OS sera and without immunoreaction with NHS pool (Table 7). 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 (Table 6). As shown in Table 6, 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, TUBA1C) were identified successfully from both of two cell lines (Fig.13). 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 (Table 6). The representative peptide mass finger printing (PMF) and MS/MS spectra of two ENO1 peptides from the excised 47 KD spot from Saos-2 and U2-OS cell lines were shown in Fig.14. The other PMF detailed information was listed in supplemental file.

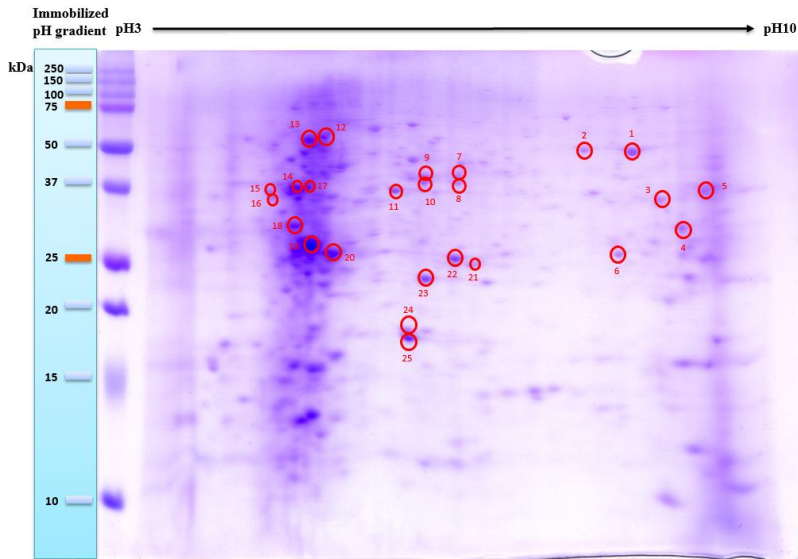


Figure 9: Total proteins from U2-OS cell analyzed by 2DE-gel. The U2-OS cell sample was lysed in IEF buffer and run on a pH3–10 Bio-Rad IPG IEF system. The second dimensional run was performed on 15 % gradient SDS-PAGE gels and the gels were subsequently processed with Coomassie blue staining.

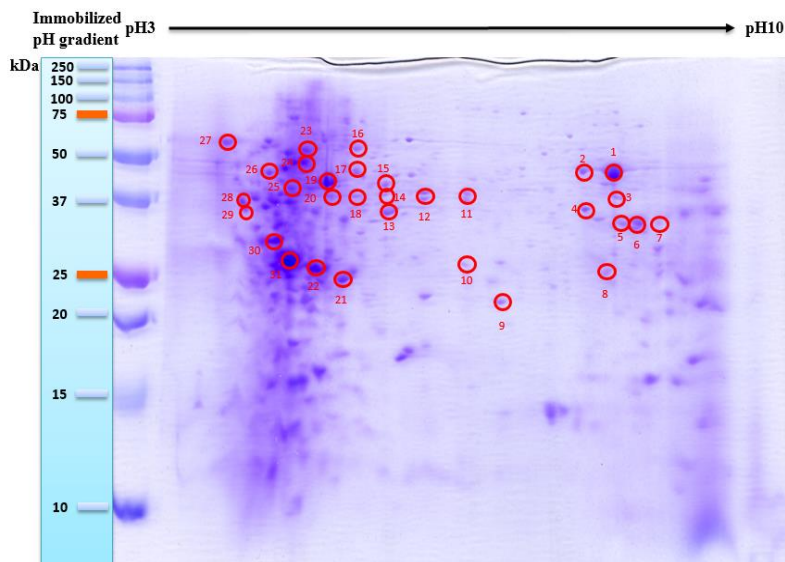


Figure 10: Total proteins from Saos-2 cell analyzed by 2DE-gel. The Saos-2 cell sample was lysed in IEF buffer and run on a pH3–10 Bio-Rad IPG IEF system. The second dimensional run was performed on 15 % gradient SDS-PAGE gels and the gels were subsequently processed with Coomassie blue staining.

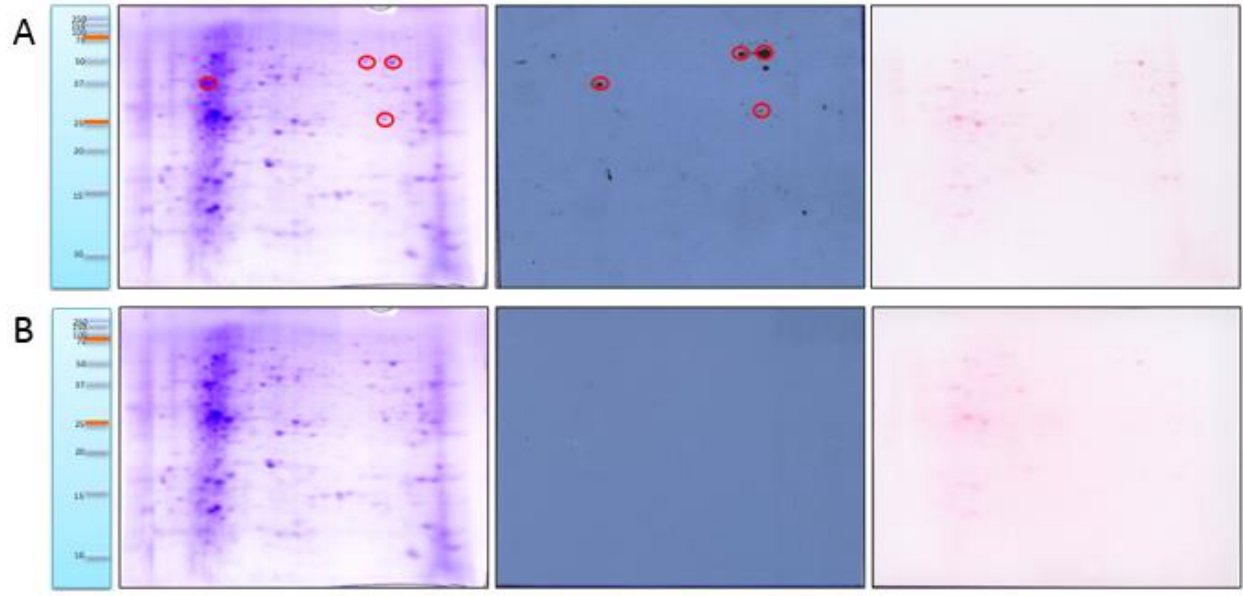


Figure 11: 2-DE Western blotting analysis of U2-OS cells was probed with one representative OS serum sample (# 26) and a pool of five 5 NHS (# 3, 8, 7, 1, 36) used as control. A, The 2-DE protein profile of U2-OS cells (left panel). Western blotting analysis of 2-DE gel was probed with one representative osteosarcoma serum sample OS (# 26) which contains antibodies to the 47KD protein (middle panel). The nitrocellulose membrane transferred from 2-DE gel was stained with Ponceasu S (right panel). B, The 2-DE protein profile of U2-OS cells (left panel). Western blotting analysis of 2-DE gel was probed with a pool of five 5 NHS (# 3, 8, 7, 1, 36) (middle panel). The nitrocellulose membrane transferred from 2D gel was stained with Ponceasu S (right panel).

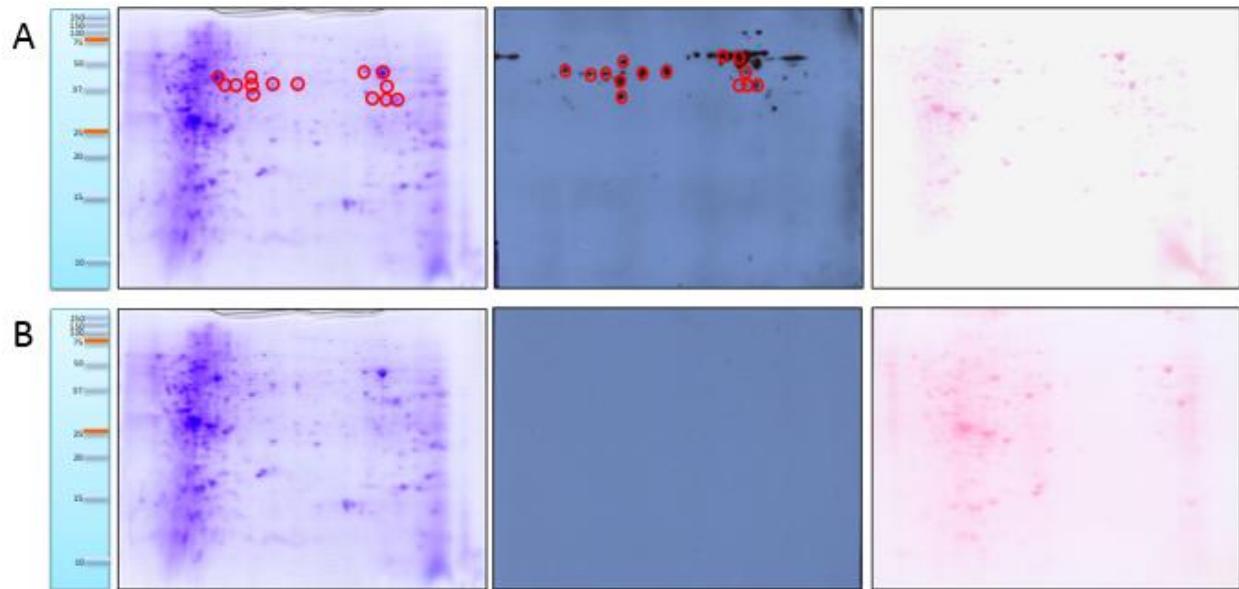


Figure 12: 2-DE Western blotting analysis of Saos-2 cells was probed with one representative OS serum sample (# 11) and a pool of five NHS (# 3, 8, 7, 1, 36) used as control. A, The 2-DE protein profile of Saos-2 cells (left panel). Western blotting analysis of 2-DE gel was probed with one representative osteosarcoma serum sample OS (# 11) which contains antibodies to the 47KD protein (middle panel). The nitrocellulose membrane transferred from 2-DE gel was stained with Ponceasu S (right panel). B, The 2-DE protein profile of Saos-2 cells (left panel). Western blot analysis of 2-DE gel was probed with a pool of five NHS (# 3, 8, 7, 1, 36) (middle panel). The nitrocellulose membrane transferred from 2D gel was stained with Ponceasu S (right panel).

Table 6: Top candidate OS autoantigens identified from autoantibodies profiling of multiple human OS sera

No.	No. in gel	Accession No.	Candidate TAAs	Acronym	Average # Unique Peptides	Score	Molecular function
1	S1	NP_001419.1	Alpha-enolase	ENO1	20	311	DNA-binding, Lyase, Repressor
	U1	3B97_C	Alpha-enolase	ENO1	16	284	
2	S4	NP_066272.1	PDZ and LIM domain protein 1	PDLIM1	18	155	Metal-binding, Zinc
3	S5	CAA37794.1	Uracil-DNA glycosylase	UNG	12	93	Hydrolase
4	S6	CAA25833.1	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	13	115	Oxidoreductase, Transferase
	U4	NP_001243728.1	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	13	86	
5	S8	4BR1_A	Triosephosphate isomerase	TPI1	18	328	Isomerase
	U6	AAH17917.1	Triosephosphate isomerase	TPI1	15	117	
6	S9	1PE0_A	Protein/nucleic acid deglycase DJ-1	PARK7	14	147	Chaperone, Hydrolase, Protease, RNA-binding
7	S11	ADK47995.1	C-myc promoter-binding protein	DENND4A	12	182	DNA-binding, Guanine-nucleotide releasing factor
	U7	ADK47995.1	C-myc promoter-binding protein	DENND4A	11	102	
8	S16	4PJ1_M	60 kDa heat shock protein, mitochondrial	HSPD1	20	128	Chaperone, Hydrolase
9	S17	NP_001814.2	Creatine kinase B-type	CKB	14	102	Kinase, Transferase
10	S19	5JLH_D	Actin, cytoplasmic 1	ACTB	17	437	Cell motility
11	S20	NP_001830.1	Calponin-3	CNN3	13	77	Actin-binding, Calmodulin-binding
12	S21	NP_004172.2	Ubiquitin carboxyl-terminal hydrolase isozyme L1	UCHL1	10	198	Hydrolase, Ligase, Protease, Thiol protease
13	S23	NP_116093.1	Tubulin alpha-1C chain	TUBA1C	18	169	GTP-binding, Nucleotide-binding
	U12	NP_116093.1	Tubulin alpha-1C chain	TUBA1C	14	142	
14	S24	NP_005733.1	Protein disulfide-isomerase A6	PDIA6	11	78	Chaperone, Isomerase
15	S24	ACA06103.1	Vimentin	VIM	27	192	Host-virus interaction
16	S29	3POS_C	Calreticulin	CALR	13	128	Chaperone
17	U13	NP_821133.1	Tubulin beta chain	TUBB	23	358	GTP-binding, Nucleotide-binding
18	U14	AAH16768.1	Nucleophosmin	NPM1	11	319	Chaperone, RNA-binding
19	U22	AAA62175.1	Heat shock protein beta-1	HSPB1	13	372	Chaperone
20	U25	NP_981946.1	Stathmin	STMN1	15	484	Developmental protein

Table 7: The appearance of identified proteins in 11 sera from patients with OS

Identified proteins	No. in gel	OS Sera No.										
		6	9	10	11	14	19	20	24	26	27	33
Alpha-enolase	S1&U1	X	X		X	X	X	X	X	X	X	X
PDZ and LIM domain protein 1	S4	X									X	X
Uracil-DNA glycosylase	S5		X		X	X					X	
Glyceraldehyde-3-phosphate dehydrogenase	S6& U4		X		X	X	X					X
Triosephosphate isomerase	S8&U6	X				X				X		X
Protein/nucleic acid deglycase DJ-1	S9											X
C-myc promoter-binding protein	S11&U7	X	X		X	X		X	X		X	X
60 KD heat shock protein, mitochondrial	S16						X		X			
Creatine kinase B-type	S17								X			
Actin, cytoplasmic 1	S19	X			X			X			X	
Calponin-3	S20	X			X		X					
Ubiquitin carboxyl-terminal hydrolase isozyme L1	S21	X					X					
Tubulin alpha-1C chain	S23&U12						X	X			X	
Protein disulfide-isomerase A6	S24	X										
Vimentin	S24	X										
Calreticulin	S29	X				X	X		X			
Tubulin beta chain	U13		X				X	X	X		X	
Nucleophosmin	U14			X						X	X	X
Heat shock protein beta-1	U22	X										
Stathmin	U25										X	

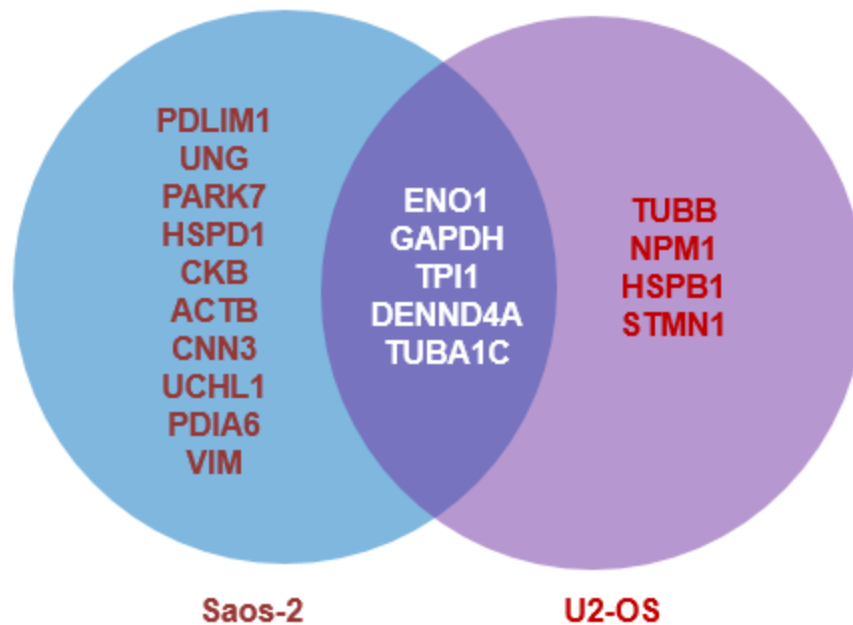


Figure 13: Comparison between proteins identified from U2-OS and Saos-2 cell lines. Five identified proteins (ENO1, GAPDH, TPI1, DENND4A, and TUBA1C) were overlapped between these two cell lines.

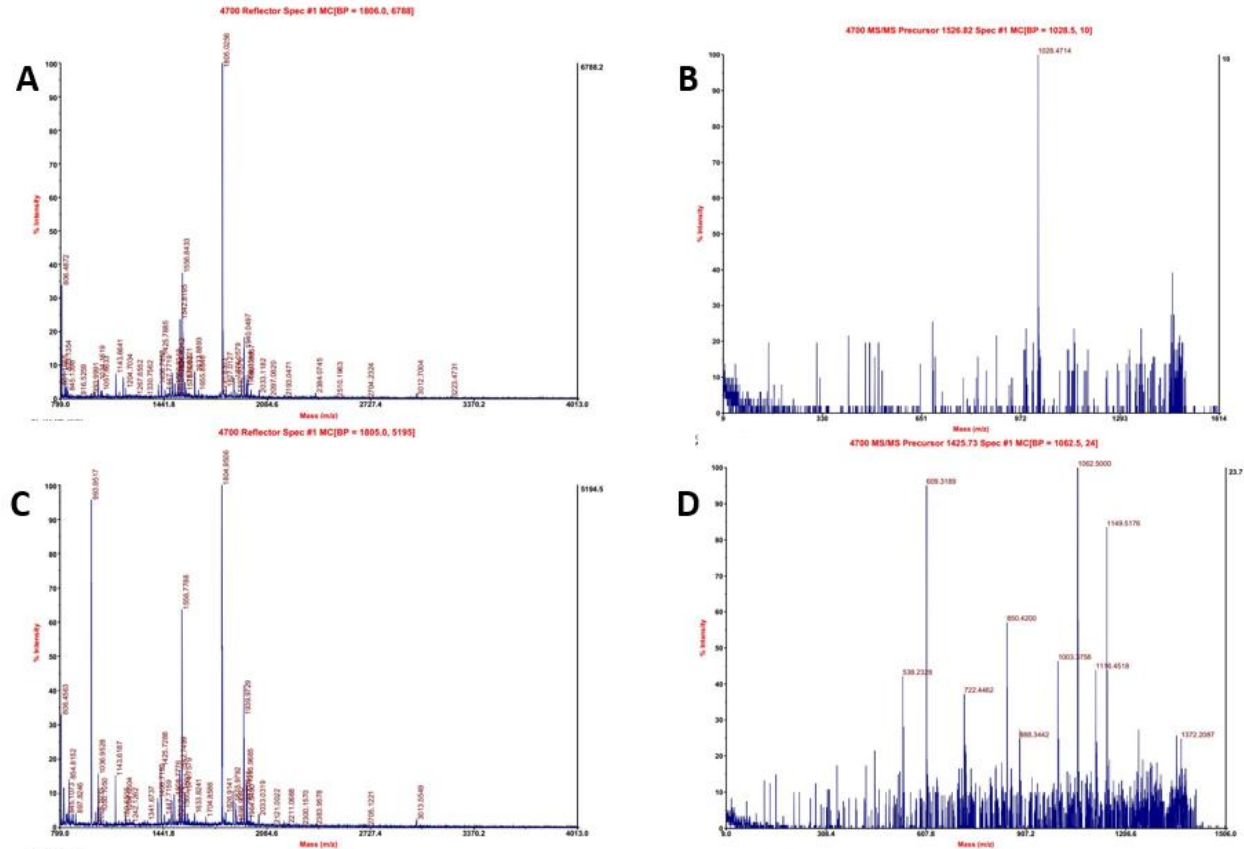


Figure 14: Peptide mass finger printing (PMF) and MS/MS spectra of representative ENO1 peptides identified from excised 2-DE gel protein spots from Saos-2 and U2-OS cell lines. A, PMF of Spot # 1 from 2-DE gel of Saos-2 cell line. B, MS/MS spectra of Spot # 1 from 2-DE gel of Saos-2 cell line. C, PMF of Spot # 1 from 2-DE gel of U2-OS cell line. B. MS/MS spectra of Spot # 1 from 2-DE gel of U2-OS cell line.

3.4 Functional categorization of identified proteins

Gene ontology (GO) annotation was performed to better understanding the biological functions of these differentially expressed proteins. The 20 identified proteins were categorized into three groups including molecular function, biological process, and cellular component. Proteins in each group were further categorized in different sub-groups. The results of GO annotation are presented Fig. 15. These differentially expressed proteins were predicted to be

involved in 48 different biological processes, to possibly have 10 kinds of molecular functions, and to involve 8 categories of cellular components.

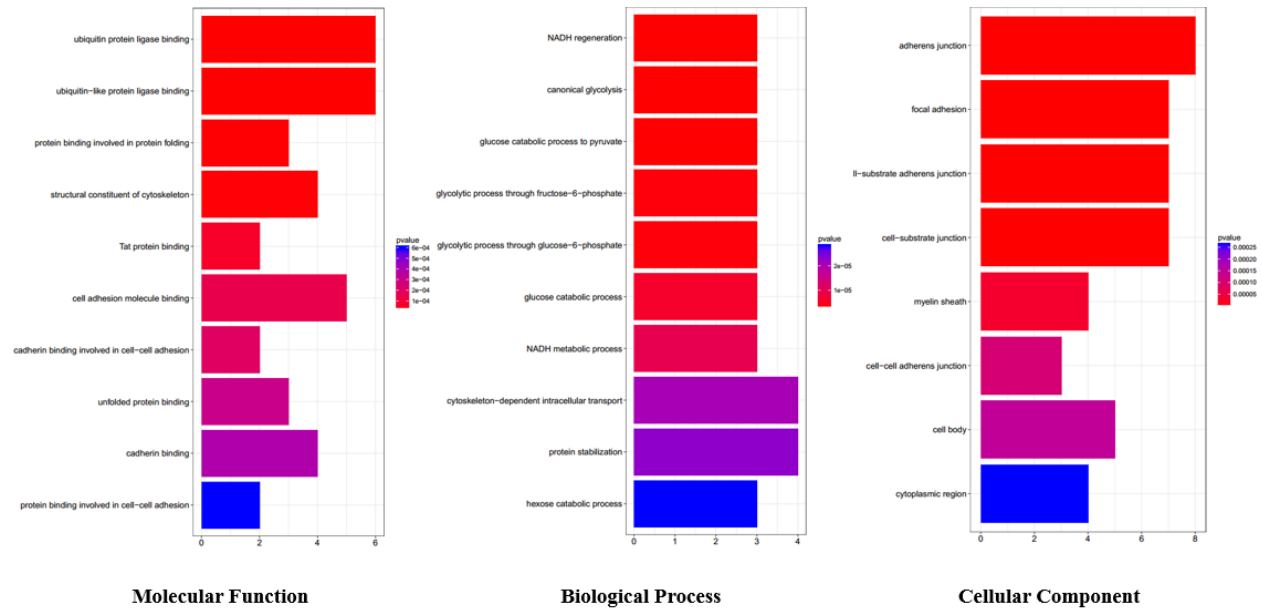


Figure 15: Functional categorization of twenty identified proteins. Twenty identified proteins were functionally categorized in three groups based on gene ontology (GO) annotation terms and searching sequences against the databases. Three functional groups include molecular function, biological process and cellular component. Proteins in each group were further categorized in different sub-groups.

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

After identifying that several sera in our initial OS patient test cohort contained autoantibodies to ENO1, we evaluated anti-ENO1 autoantibody levels in sera from patients with OS and controls, using the full-length recombinant ENO1 protein as coating antigen in ELISA^{97,99,100}, 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 ROC curves discriminated between OS and OC from NHS groups of anti-ENO1 autoantibody with AUCs of 0.853, $P < 0.001$ (OS vs NHS) and 0.758, $P < 0.001$ (OC vs NHS), respectively (Fig. 16). As shown

in Fig. 17, sera from OS patients showed a significantly higher frequency (12/52, 23.1%) and levels of autoantibodies to ENO1 compared to OC sera (1/28, 3.6%, $P<0.05$) and NHS (1/49, 2.0%, $P<0.01$), suggesting that they are OS-related. Western blotting analysis with representative positive sera against purified ENO1 showed that all of the 12 sera have reaction against purified ENO1 with different signal density. Nine of twelve sera reacted strongly against purified ENO1, but 3 weakly against purified ENO1 in Western blotting. By contrast, both of 10 randomly selected OC and NHS sera shows no immoreactivity or very weak reaction signal against purified ENO1 by Western blotting (Fig. 18).

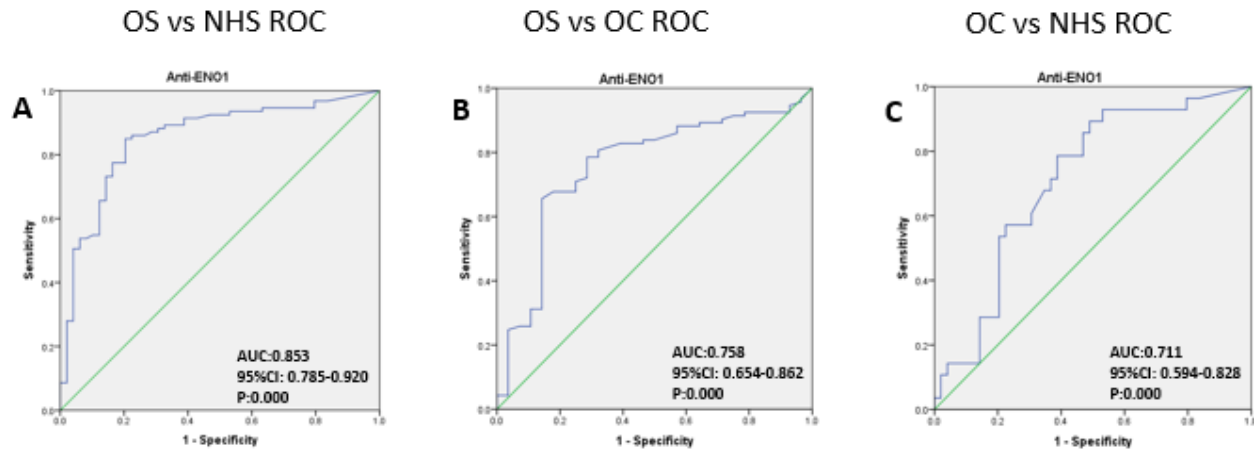


Figure 16: The ROC curves discriminate OS from OC and NHS groups of anti-ENO1 autoantibody. Receiver operating characteristic (ROC) curve analysis of ENO1 expression to discriminate the OS group from the OC and NHS groups. The area under the ROC curve (AUC) corresponding to the comparisons between pairs of these groups is indicated.

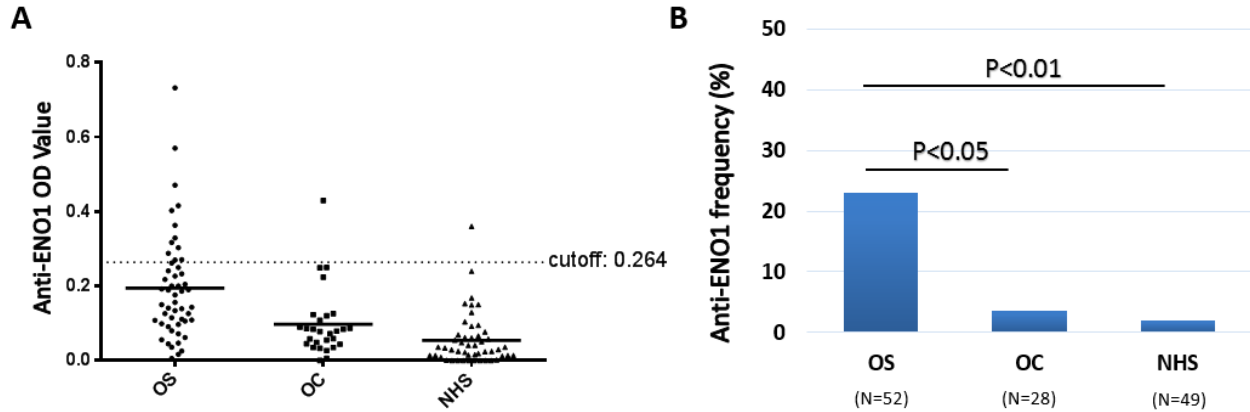


Figure 17: Frequency of serum anti-ENO1 autoantibody in sera from patients with OS, OC and normal human sera. A, ELISA scatter dot plot shows OD values with solid lines representing the mean of each cohort, OS, OC and NHS. The dotted line represent the mean value of the normal individuals' cohort plus three standard deviations to determine levels and frequency of autoantibody reactivity against ENO1. B, The graph shows higher frequency of anti-ENO1 antibodies in OS patient sera compared to OC and NHS sera.

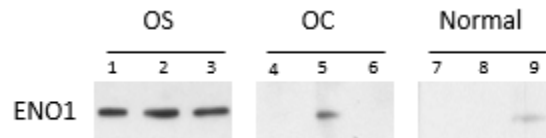


Figure 18: Western blotting analysis with representative positive sera in ELISA. Representative sera from patients with OS (lanes 1-3), OC patients (lanes 4-6) and normal human sera (lanes 7-9) probed against purified ENO1 showed differences in immunoreactivity by Western blotting. 9 of 12 (75.0%) sera with positive OD values were consistently positive in Western blotting.

3.6 Evaluation of anti-ENO1 autoantibody level in OS patients along the disease progression.

Since 67 serial serum samples from 24 OS patients were obtained at a wide range of time period (ranging 0 to 400 days after diagnosis), we questioned whether anti-ENO1 autoantibody levels might change along the disease progression. As shown in Fig. 19A, the presence of

autoantibodies to ENO1 was assessed by ELISA over time in the serially collected at three time points: diagnosis, before surgery and after surgery. The OD value of before and after surgery were 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). Unexpectedly, although there is no significant association of anti-ENO1 autoantibody titers at these three time points (Fig. 19A), descent of ENO1 immunoreactivity was observed in most patients along the disease progression (Fig. 19B&C&D), which may imply a potential association between changes in anti-ENO1 autoantibody titers and disease progression.

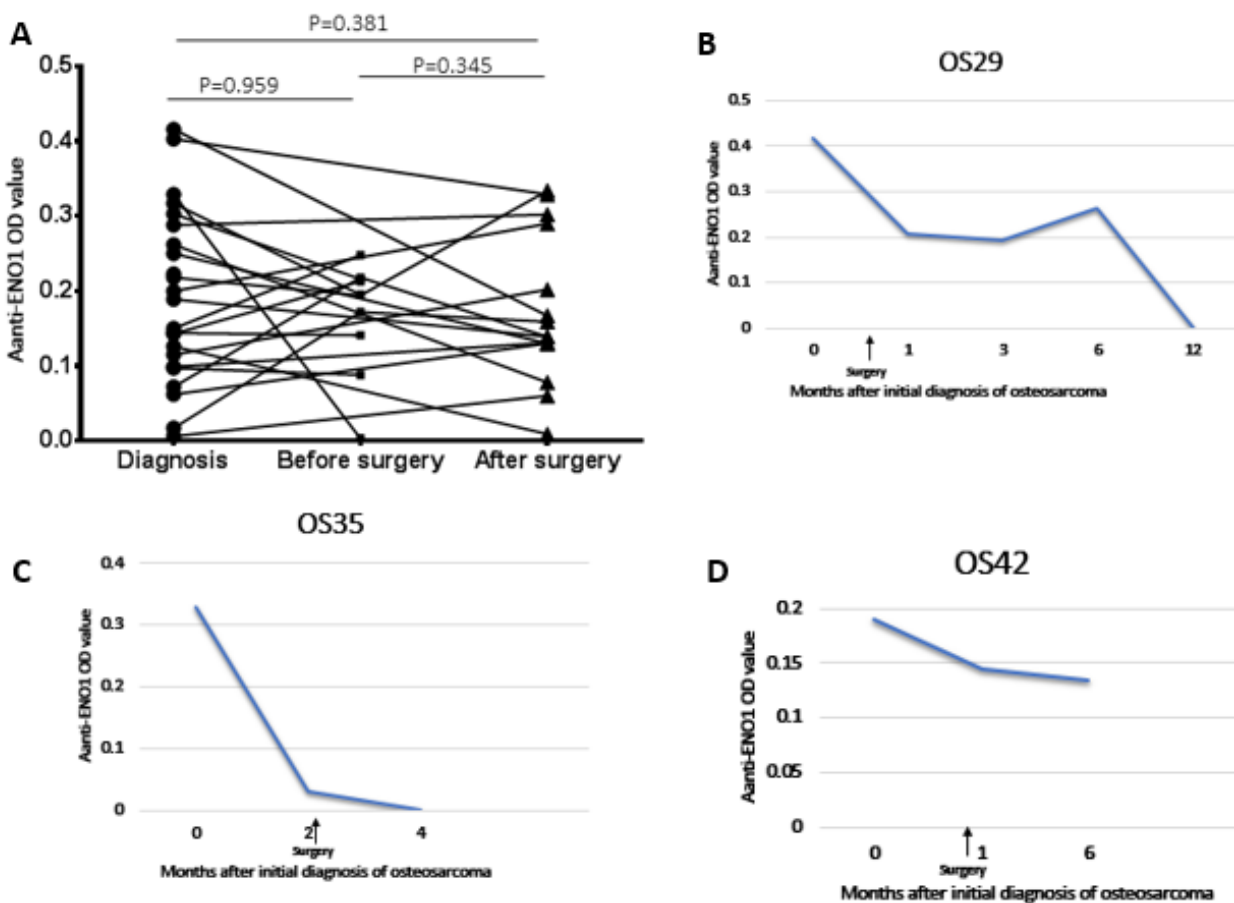


Figure 19: Serial assay of anti-ENO1 autoantibody by ELISA in 24 patients with OS along the disease progression. A, The OD values of anti-ENO1 autoantibody over time in the serially collected at three time points: diagnosis, before surgery and after surgery. B&C&D, Serial study of anti-ENO1 antibody levels (OD value) during a 1-year period in three representative OC patients.

3.7 Expression of ENO1 in OS tissues by immunohistochemistry with tissue microarray

To explore the possibility that ENO1 is overexpressed in OS, an expression analysis of this protein in OS tissue was performed by immunohistochemistry. Tissue microarray slides includes 51 cases of osteosarcoma (1 case was lost during performance of immunohistochemistry), 27 chondrosarcoma (4 cases was lost during performance of immunohistochemistry), plus 2 bone marrow tissue, and single core per case. And normal bone tissue microarray contains 11 cases of bone tissue, 1 osteosarcoma, and duplicate cores per case. As shown in Table 8, the results indicated that there was significant difference of the frequency of ENO1 overexpression in both osteosarcoma and chondrosarcoma tissues with 100% positive rate compared with normal bone tissue with 0% positive rate. However, the ability in distinguishing osteosarcoma from chondrosarcoma individuals was not found to have statistical significance. To investigate the possible relationship between theses histological characteristics and ENO1 expression, we analyzed the histological characteristics of 50 osteosarcoma specimens from the same tissue array that had information on TNM, pathology grade, and clinical stage. Eventually, there is no statistically significant difference between these groups. Fig. 20 shows representative normal with negative immunostaining for ENO1, osteosarcoma and chondrosarcoma tissues with positive immunostaining for ENO1.

Table 8: Expression profile of ENO1 in Osteosarcoma, Chondrosarcoma and normal bone tissue microarray

	Osteosarcoma	Chondrosarcoma	Normal
	n=50	n=23	n=22
Age			
Mean, SD	24.7, 13.7	37.8, 16.1	61.6, 11.0
Range	7-69	12-74	41-84
Gender (%)			
Male	30 (60.0)	15 (65.2)	20 (90.9)
Female	20 (40.0)	8 (34.8)	2 (9.1)
TNM (%)			
T1N0M0	7 (14.0)		
T2N0M0	43 (86.0)		
Grade (%)			
Grade1-2	6 (12.0)		
Grade 3-4	44 (88.0)		
Stage (%)			
<IIA	9 (18.0)		
>IIB	41 (82.0)		
ENO1 Expression (%)			
PR+SI \geq 4	50 (100)	23 (100)	0 (0)
PR+SI<4	0 (0)	0 (0)	22 (100)

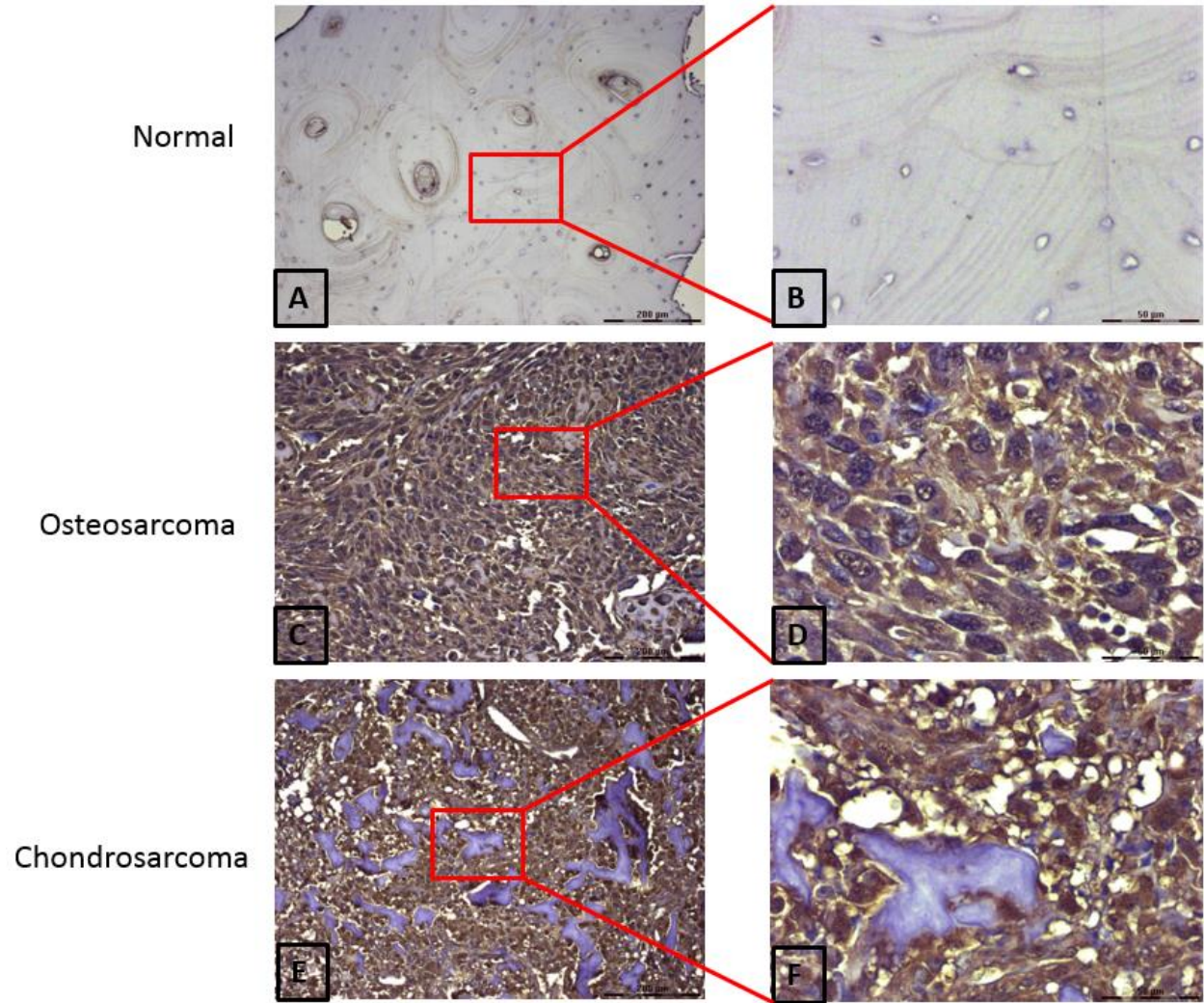
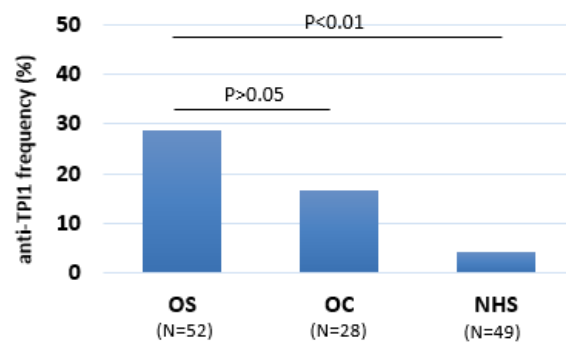
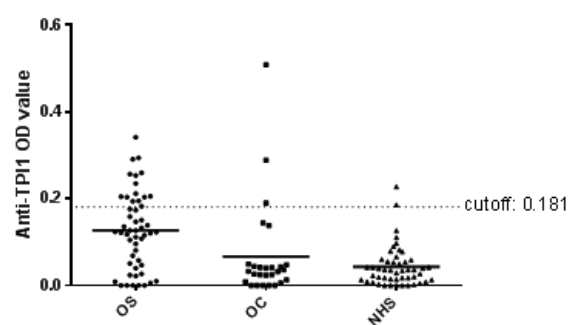
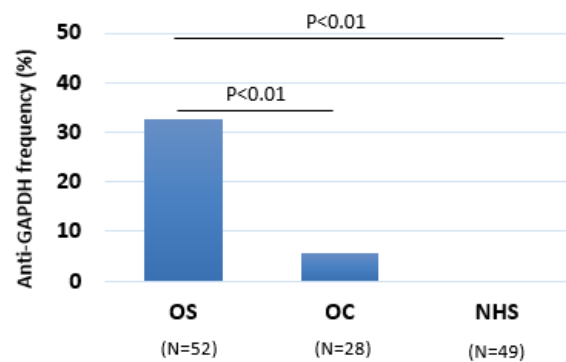
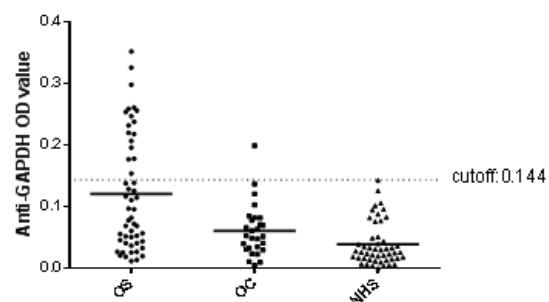
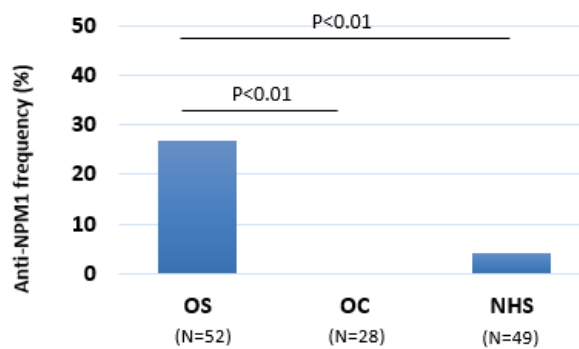
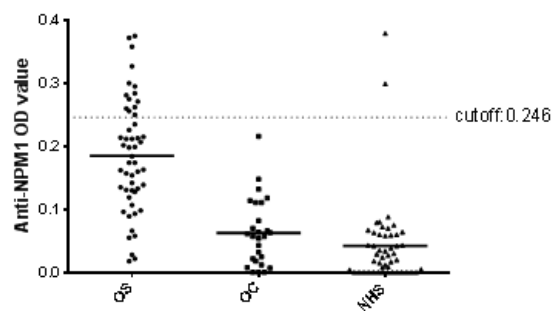


Figure 20: Evaluation of ENO1 protein expression in normal, osteosarcoma and chondrosarcoma tissues by immunohistochemistry. A&B, Negative staining of ENO1 expression in representative normal bone tissue at 100X and 400X magnification respectively; C&D, Positive staining of ENO1 expression in osteosarcoma tissue at 100X and 400X magnification respectively; E&F, Positive staining of ENO1 expression in chondrosarcoma tissue at 100X and 400X magnification respectively.

3.8 Autoantibodies levels to other 6-TAAs identified from SERPA in OS patients and control individuals

Six TAAs (NPM1, GAPDH, TPI1, HSP60, PDLIM1, STMN1) were selected from the 20 identified based on the findings of 1-D Western blotting and SERPA. Furthermore, they were considered to be oncogenes, tumor suppressor genes, growth factors, or cell cycle-related proteins. Many previous studies have demonstrated these serum antibodies to be autoimmunogenic, and detectable in serum from various cancer studies^{3,97,100,113-115}. We continuously evaluated autoantibodies against 6 TAAs using individual sera from 95 patients with OS, 28 sera from OC as well as 49 NHS. All of these 6 TAAs exhibited statistically significant increased autoantibody responses in patients with OS compared to NHS, while no differences were observed for the autoantibodies in OC and NHS groups (Fig. 21). However, the frequency of autoantibodies positive reactivity in OS sera against 4 of 6 TAAs (NPM1, GAPDH, PDLIM1, STMN1) was observed to be significant higher than that in OC group.



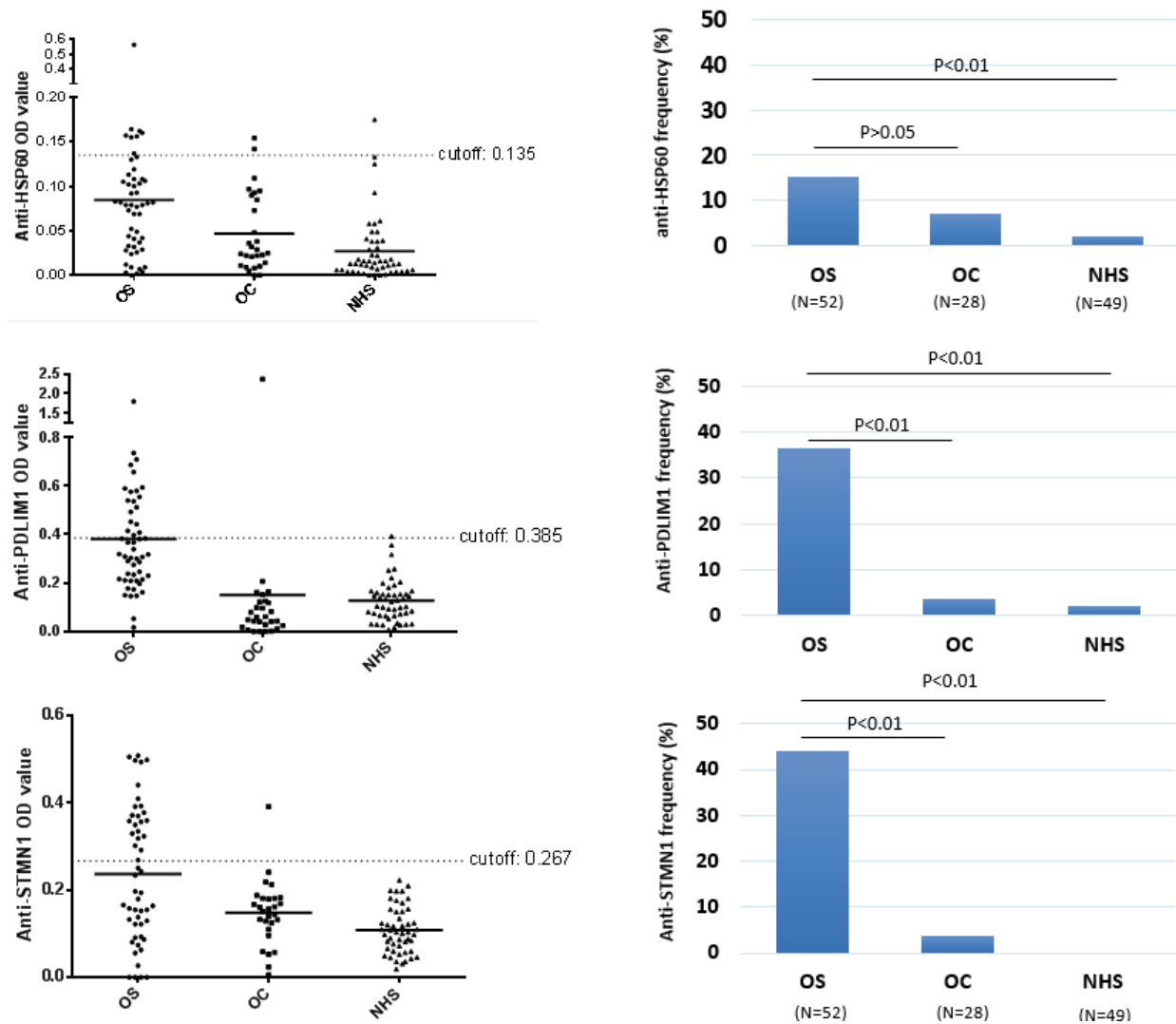


Figure 21: Frequency of serum anti-NPM1, anti-GAPDH, anti-TPI1, anti-HSP60, anti-PDLIM1, anti-STMN1 autoantibodies in sera from patients with OS, OC and normal human sera. ELISA scatter dot plot shows OD values of anti-6 TAAs respectively with solid line represents the mean of each cohort, OS, OC and NHS (Left Panel). The dotted line represents the mean value of the normal individuals' cohort plus three standard deviations to determine levels and frequency of autoantibody reactivity against these 6 TAAs. B, The graph shows higher frequency of anti-6 TAAs antibodies in OS patient sera compared to OC and NHS sera.

3.9 Design of TAA arrays for specific OS detection.

Our previous studies indicate that the combination of antibodies to multiple TAAs yield higher sensitivity for diagnosis of liver cancer¹⁰³, prostate cancer¹¹⁶, lung cancer¹¹⁷. Since the OS-related TAAs has been identified and validated, we further refined a miniarray to combine ENO1 with other 6 TAAs (NPM1, GAPDH, TPI1, HSP60, PDLIM1, STMN1) to enhance autoantibody detection and explore their utility in OS detection and diagnosis. Positive reactivity for 7-TAAs array was again defined as having an OD value above three standard deviations plus the mean OD in the 49 matched NHS. The frequencies of positive reactions for 7-TAAs array in OS, OC and NHS patients are shown in Fig. 22A. The 7-TAAs array showed that 90.4% of all OS patients were found to have levels above the OD cutoff value in one or more of these seven TAAs compared to 14.3% in the NHS ($P<0.01$) (Table 9). We generated a multiple-variable ROC curve by using the predictive probability variables of the 7-TAAs array. The analysis showed an AUC of 0.938 (95% CI: 0.886-0.990, $P=0.000$) within OS vs NHS (Fig. 22B) and an AUC of 0.911 (95% CI: 0.844-0.978, $P=0.000$) within OS vs OC (Fig. 22C).

Table 9: Evaluation of 7-TAAs array in the diagnosis of OS.

7-TAAs	OS	NHS	Total
Any +	47	7	54
All -	5	42	47
Total	52	49	101

Sensitivity= $47/52=90.4\%$

Specificity= $42/49=85.7\%$

False positive rate= $7/49=14.3\%$

False negative rate= $5/52=9.6\%$

Positive predictive value= $47/54=87.3\%$

Negative predictive value= $42/47=89.4\%$

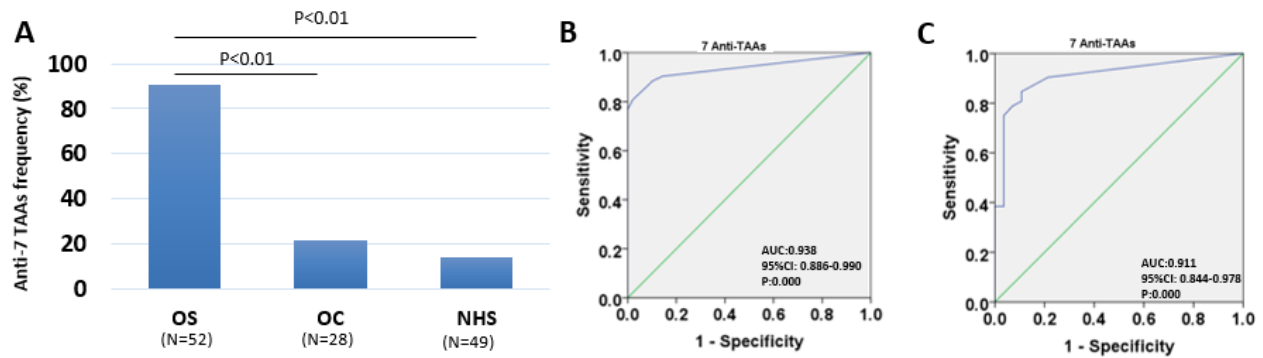


Figure 22: Frequency of autoantibodies against 7-TAAs (ENO1, NPM1, GAPDH, TPI1, HSP60, PDLIM1, STMN1) in sera from patients with OS, OC and normal human sera. A, The graph shows higher frequency of anti-7 TAAs autoantibodies in OS patient sera compared to OC and NHS sera; B, ROC curve of OS (n=52) vs NHS (n=49) for the panel of seven TAAs combination; C, ROC curve of OS (n=52) vs OC (n=28) for the panel of seven TAAs combination.

Chapter 4: Discussion

4.1 Screening of patients' sera with OS, OC and normal individuals for the presence of autoantibodies to potential TAAs.

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 the autoantibodies and 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 indirect immunofluorescence (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 is 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 (47KD, 33KD, 60KD, 37KD, 27KD, 17KD, 29KD) were observed from both of these two cell lines. Moreover, further analysis of the autoantibody positivity to 27KD and 50KD Saos-2 cellular antigens in OS shows 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 47KD 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 47KD.

HEp-2 cells have been shown to have greater sensitivity than tissue sections and yield sharper pattern recognition¹¹⁸, which is still considered the gold standard for screening of autoantibodies¹¹⁹. Reactivity with cancer cells for all selected serum samples with strong immunoreactivity including those reacting with the top four common proteins band around 33KD,

47KD, 54KD and 60KD from U2-OS 1-D Western blotting analysis were further confirmed by using IIF on HEp-2 antinuclear antigen tissue slides. Subcellular fractionations of Hep-2 cells were incubated with representative antigen (47KD, 54KD and 60KD protein antigens) enriched OS sera showing predominant cytoplasmic staining patterns with more intense staining in the perinuclear regions, whereas, the 33KD protein antigens showed nucleoplasm granular immunofluorescence staining pattern. Moreover, the same antigens enriched OS sera showed the similar staining pattern, which confirmed the results of immunoreactivity with 1-D Western blotting. Particularly, subcellular fractionation of HEp2 cells showed the unknown 47KD 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 studies¹⁰⁰, in U2-OS as well as MCF7 in other studies indicating mainly localization to plasma membrane & cytosol and in addition localized to the nucleoplasm (Fig. 23)¹⁰⁴.

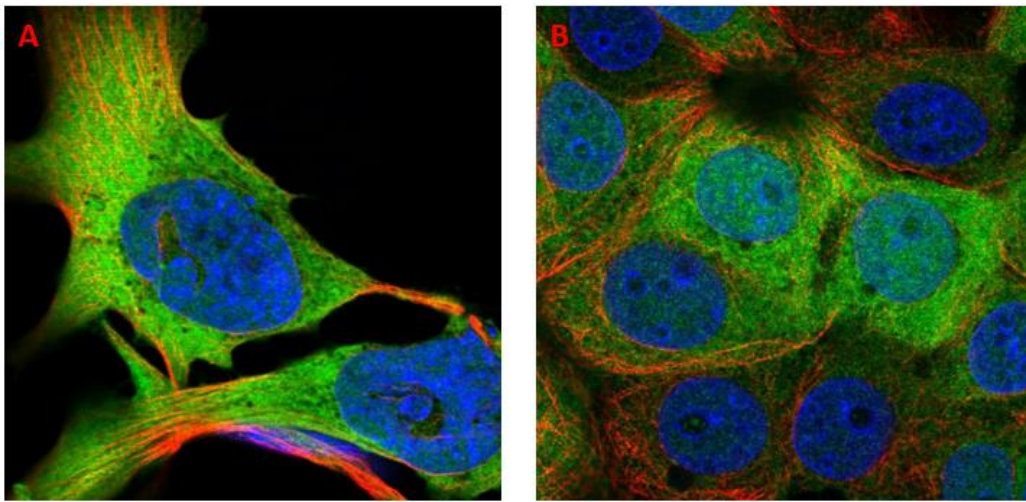


Figure 23: Immunofluorescent staining of human cell line U2-OS and MCF7 against ENO1. A, Immunofluorescent staining of human cell line U2-OS shows localization to nucleus, plasma membrane & cytosol. Antibody dilution: 1:66; B, Immunofluorescent staining of human cell line MCF7 shows localization to nucleoplasm, plasma membrane & cytosol. Antibody dilution 1:200.

4.2 Identify and characterize novel TAAs in OS using serological proteome analysis (SERPA) approach.

The rationale behind the SERPA approach is that intracellular proteins involved in carcinogenesis provoke autoantibody responses, and therefore autoantibodies can be used as probes to isolate, identify, and characterize potential oncoproteins. The SERPA approach has been recently widely implemented for identifying TAAs in cancer patients^{94,95,100}. While SEREX methodology has been used for over several decades, several limitations make it far from satisfactory to identified TAAs as potential markers in cancer. For instance, construction of cDNA expression library is essential for serological screening, however, this procedure is complex and time-consuming. Compared to SEREX, the SERPA allows individual screening of a large number of sera, as well as identification of a large number of candidate TAAs in a shorter time period. The proteome-based approach, which is generally named as immunoproteomics¹¹⁵, can also distinguish between antigen isoforms and detect the presence of autoantibodies directed against post-translational modifications of specific targets. Constantly evolving advances in SERPA offer great promise in the understanding of the molecular basis of cancer¹²⁰. This shed light on rapid advances in determining protein antigens for vaccine development¹²¹, immune correlates, and biomarkers for disease diagnosis and prognosis¹¹⁵. The need to reliably identify and validate protein biomarker expression and improve disease diagnosis is underscored by the increased survival of bone cancer patients who are diagnosed early¹²².

A SERPA approach was implemented in this project for the identification of TAAs that induce antibody responses in OS. The two-dimensional gel electrophoresis system (2-DE) has been set up, and the proteome-based technique has been used in our lab in some other project for identification of TAAs in hepatocellular carcinoma⁹⁴, esophageal squamous cell carcinoma⁹⁵, prostate cancer^{96,97}, lung cancer^{98,99} and liver fibrosis¹⁰⁰. Although though the SERPA approaches for cancer biomarkers has revealed many possible candidates, none have demonstrated a screening of sera from patients with OS and OC, particularly enough promise to be implemented clinically.

We selected 11 representative OS sera with common strong immunoreactivity from the results of 1-D Western blotting and a pool of 5 NHS used as control to examine them by SERPA. 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, alpha-enolase (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. 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.

Then we performed Gene ontology (GO) annotation study to better understanding the biological functions of these differentially expressed proteins. The 20 identified proteins were categorized in three groups including molecular function, biological process, physiological and cellular component. These differentially expressed proteins were predicted to be involved in 48 different biological processes, to possibly have 10 kinds of molecular functions, and to involve 8 categories of cellular components. For instance, in addition to ENO1, the protein hits included key chaperone such as Protein/nucleic acid deglycase DJ-1 (PARK7), 60 KD heat shock protein (HSPD1), Protein disulfide-isomerase A6 (PDIA6), Calreticulin (CALR), Nucleophosmin (NPM1), Heat shock protein beta-1 (HSPB1), Additional candidate TAAs identified by our SERPA approach have been associated with Metal-binding, Hydrolase, Transferase, Isomerase, DNA-binding, cell motility, GTP-binding and so on.

Cytoplasmic enzyme may also function in altering the tumor microenvironment^{123,124}. For example, a number of studies have demonstrated that glycolytic enzymes can relocate from the mitochondria to the plasma membrane during cytoskeletal remodeling, leading to influence on cell motility, extracellular matrix degradation, and plasminogen signaling¹²⁵⁻¹²⁷. ENO1 and GAPDH have been revealed to be “moonlight” as plasminogen receptors, contributing to the conversion of plasminogen to plasmin and promoting extracellular matrix remodeling with ensuing increased cancer cell migration and metastasis^{125,126,128}. In glioma tumors, ENO1 is overexpressed to

promote migration and tissue invasion¹²⁹, while its secretion from prostate cancer stromal cells was also found to improve cell migration¹³⁰. One group illustrated that a small molecular inhibitor of ENO1 reduced pancreatic cancer cell invasion and migration properties¹³¹, suggesting that this cytoplasmic enzyme plays 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 invasion¹³². This makes ENO1 and other cytoplasmic enzymes likely a contributor to plasminogen binding attractive candidates for anti-cancer immunotherapy.

4.3 Validate the potential value of newly identified TAA- Alpha-enolase (ENO1) as OS biomarkers.

As a potential TAA to be considered as a useful serological biomarker for cancer diagnosis or prognosis, it has to be preferentially recognized by sera from patients with the particular tumor type in which the antigen was identified, when compared to sera from normal controls, other bone tumors, or other diseases. Once a TAA is identified, the combined use of various immunoassays is required to enhance detection of the autoantibodies and determine more accurately the frequency of these autoantibodies in patient sera. The reason for this aim is that there is often an incomplete correlation in the detection of autoantibodies to TAAs between different immunoassays⁵⁹, probably due to differences in the sensitivity of the various assays or changes in antigen conformation from one assay platform to another. Therefore, it is essential to establish that the candidate TAA is indeed expressed in OS, and to correlate the presence of an anti-TAA autoantibody with TAA expression in autologous bone tumor tissue.

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 from one of the largest orthopedic specialty hospitals in the world- Henan Luoyang Orthopedic-Traumatological Hospital (HLOH) in China. 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. We chose 28 age-matched OC sera as a benign control because it is the most common benign bone tumor and these samples were readily available in our study. The 49 normal human sera used as control group includes both young and older patients, corresponding to the two age-specific peaks in frequency in OS. This analysis is essential to establish the sensitivity, specificity, and positive predictive value of individual antibody-antigen system as biomarkers of OS.

After demonstrating that most of sera in our initial 11 patients' test of SERPA contained autoantibodies to ENO1, we subsequently set up a validation by ELISA, using purified ENO1 as target TAA to determine the frequency of these autoantibodies in an expanded validation cohort of OS patients (n=95), as well as OC patients (n=28) and NHS (n=49). These cohorts contained all of the sera tested in our initial screening plus additional sera acquired at different time points along with the disease progression. The results of ROC curves analysis of anti-ENO1 autoantibody indicated it predominately discriminate the OS group from OC and NHS groups with AUCs of 0.853, $P<0.001$ (OS vs NHS) and 0.758, $P<0.001$ (OS vs OC). Furthermore, the ROC curves analysis also showed that anti-ENO1 can differentiate OC patients from normal individuals with AUC (95%CI) of 0.711 ($P<0.001$). Sera from OS patients showed a significantly higher frequency (12/52, 23.1%) and levels of autoantibodies to ENO1 compared to OC sera (1/28, 3.6%, $P<0.05$) and NHS (1/49, 2.0%, $P<0.01$), suggesting that they are OS-related.

Given these intriguing results, we then explored whether these selected positive sera that recognized ENO1 in cellular cells by 1-D WB would also recognize purified ENO1 by Western blotting, and whether the positive sera that reacted against ENO1 by ELISA would also recognize purified ENO1 by Western blotting. We then perform Western blotting with the selected 12 sera which have positive reaction with ENO1 in both 1-D WB and ELISA against purified ENO1.

Interestingly, all of the 12 sera have reaction against purified ENO1 with different signal density. Nine of twelve sera reacted strongly against purified ENO1, but 3 weakly against purified ENO1, which indicates 75.0% sera with positive OD values are consistently positive in Western blotting. By contrast, both of 10 randomly selected OC and NHS sera shows no immunoreactivity or very weak reaction signal against purified ENO1 by 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. Since 67 serial serum samples from 24 OS patients were available at a wide range of time period (ranging 0 to 400 days after diagnosis), we consequentially 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 in the serially collected at three time points: diagnosis, before surgery and after surgery. The OD value of before and after surgery were 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 signification association of ENO1 titers at these three time points, descant 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. As a result, different patients may produce autoantibodies against either non-denatured epitopes or denatured epitopes. Consequently, the across-the-board of different assays may enhance detection of specific autoantibodies in a particular cancer type. 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. To

further validate the identified TAA, we attempted to examine the ENO1 expression in OS tissue specimens by immunohistochemistry. For these experiments, we used commercially available human OS and chondrosarcoma tissue microarrays (TMA) which contain 51 paraffin-embedded OS and chondrosarcoma tissue specimens with clinical stage and TNM grading. We observed that, all of 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 examine for specificity of ENO1 expression with regards to cancer type, ENO1 expression level was compared with some other non-bone tumor tissues by analyzing the data from the Human Protein Atlas. The Human Protein Atlas demonstrates the feasibility of large-scale expression and purification of human antigens. Currently, the Human Protein Atlas hosts over 41,000 MS-verified human protein fragments, representing more than 18,000 human protein-coding genes¹⁰⁵. Most malignant cells exhibited moderate cytoplasmic and occasional nuclear positivity¹¹¹, which potentially implies the specificity of anti-ENO1 autoantibody might be higher in OS than other types of cancers. For instance, the Fig. 24 displays ENO1 protein expression in Prostate cancer (PCa) and Lung adenocarcinoma (LUAD) tissues by immunohistochemistry exhibiting moderate cytoplasmic positive staining.

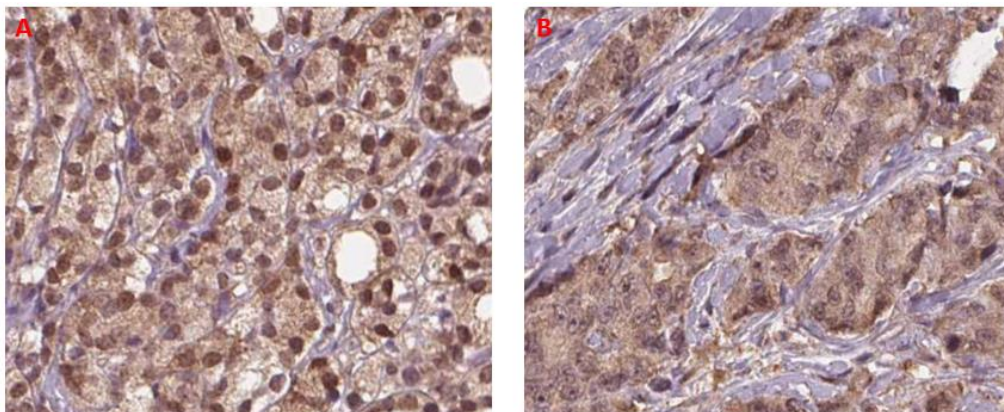


Figure 24: ENO1 protein expression in Prostate cancer (PCa) and Lung Adenocarcinoma (LUAD) tissues by immunohistochemistry. A, Moderate staining of ENO1 expression in PCa at 400X magnification shows cytoplasmic and occasional nuclear positivity; B, Moderate staining of ENO1 expression in LUAD at 400X magnification shows cytoplasmic and occasional nuclear positivity.

4.4 Design customized TAA mini-arrays that could be used to enhance autoantibody detection in OS.

Recent years have seen an enormous increase in efforts to exploit autoantigen arrays to profile serum autoantibody responses in human disease as a result of their potential value for enhancing early diagnosis, monitoring disease progression, identifying novel biomarkers, and guiding interventions for disease prevention or early treatment. Compared to autoimmune diseases, in which the presence of a particular autoantibody may have diagnostic value, anti-TAA autoantibodies may exhibit a relative little diagnostic value for several reasons if evaluated individually ²⁵. In general, the frequencies of autoantibodies specific for a particular TAA in a certain cancer population are often relatively low, approximately about 10%-40%^{48,56,133}. Secondly, in that certain TAAs are engaged at tumorigenesis in various cancer types, a specific autoantibody may present in different cancer types, which limits its application in clinical diagnosis due to its inadequacy in discriminating between different cancer types. Thirdly, the lack of specificity of some autoantibodies as a screening tool has also led to many possibly unnecessary

diagnostic (biopsies) and therapeutic procedures, as a results of its high degree of complexity in molecular events that are commonly associated with both cancer and other diseases associated with inflammation and aging. In addition, the TAA array specific for a certain cancer type may have a potential value for the development of cancer vaccines designed to boost the patient's immune system to fight the tumor. Moreover, certain TAA arrays could provide biosensor representing that a unique cellular signaling pathway is being preferentially targeted by the immune system in a given cancer type, indicating the highly association with the development of malignancy. This notion could be applied for identifying novel targets for therapeutic interventions or evaluating the effects of molecular targeting of components of that particular pathway in the sensitization of tumor cells to death induced by already established therapeutic agents such as antitumor drugs and radiation. Together, a critical unmet need in the diagnosis and management of OS is the development of a TAA array to acquire the highest sensitivity and specificity.

The OS-related TAAs identified and validated in Specific Aims 1 and 2 were further evaluated and customized in a TAA array for the specific detection of OS. Using ELISA multiplex as our initial approach, we undertook a comprehensive analysis and evaluation of different combinations of selected antibody-antigen systems to determine the optimal combination of TAAs that yields the highest sensitivity and specificity of anti-TAA antibody detection in OS sera. As controls, we used sera from individuals with normal human, patients with OC. Consequently, we expect that in the selection of OS-associated antigens, some may turn out to be unique to OS and others may not be specific. If an identified TAA can be validated as relatively specific for OS in terms of frequency of autoantibodies in OS sera and elevated expression in OS tissue, then priority will be given to that antigen for inclusion in the TAA mini-arrays for the immunodiagnosis of OS. Since 5 (ENO1, GAPDH, TPI1, DENND4A, TUBA1C) of 20 proteins were identified successfully from both of the two cell lines, we initially selected these five TAAs as candidate TAA array antigens. However, when combined with the results of 1-D Western blotting, DENND4A (209KD) and TUBA1C (50KD) were excluded from the list of candidate antigens for TAA array, because of their low immunoreactivities against corresponding autoantigens in OS sera. And then, in order

to get more widespread availability of TAA array to detect serum autoantibodies in OS patients, we continuously focused on the proteins corresponding to the seven identified protein bands (47KD, 33KD, 60KD, 37KD, 27KD, 17KD, 29KD), which were detected in both of these two cell lines. However, further analysis of the autoantibody positivity to 27 KD and 50KD of Saos-2 cellular antigens in OS shows no significant difference with NHS leading to weed out the corresponding proteins from the candidate list. Eventually, seven TAAs (ENO1, NPM1, GAPDH, TPI1, HSP60, PDLIM1, and STMN1) were selected from the 20 identified proteins, which were considered to be oncogenes, tumor suppressor genes, growth factors, or cell cycle-related proteins.

In addition to ENO1, we tested autoantibodies against the other 6 TAAs using individual sera from 95 patients with OS, 28 sera from OC as well as 49 NHS. All of these 6 TAAs were observed statistically significant increased autoantibody responses in patients with OS compared to NHS, as well as no differences for the autoantibodies in OC and NHS groups. However, the frequency of autoantibodies positive reactivity in OS sera against 4 of 6 TAAs (NPM1, GAPDH, PDLIM1, and STMN1) was obtained to be significant higher than that in OC group. Using the determined 7-TAAs array as a classifier, we were able to distinguish osteosarcoma from normal human with a respectable 90.4% sensitivity and 85.7% specificity. The multiple-variable ROC curve analysis by using the predictive probability variables of the 7-TAAs array also showed its possible ability for discrimination the OS from OC and NHS. It should be noted that this TAA array was relatively specific for cancer since the total autoantibody frequencies against the combined TAAs were less than 15% for sera from normal controls. Ultimately, we attained a highly specific and sensitive tailor-made TAA array (ENO1, NPM1, GAPDH, TPI1, HSP60, PDLIM1, STMN1) that discriminate OS from benign bone tumor and normal human individuals, when used for serological screening of antibody responses.

Chapter 5: Conclusion and Future Study

5.1 Autoantibody against ENO1 could be a potential diagnostic biomarker

Osteosarcoma is the most frequently diagnosed malignant bone tumor and remains one of the most harmful primary malignant tumors in childhood and adolescence, responsible for a high rate of amputation, disability and death. Although the 5-year survival probability has increased to almost 60% in recent years by combining neoadjuvant chemotherapy with surgical extirpation, the progression and prognosis for patients with advance disease has remained grim. A critical need in the diagnosis and management of OS is to determine a convincing combination of clinical biomarkers that could detect tumors early with high sensitivity/specificity and with limited invasiveness, and that could accurately predict which diagnosed patients will develop aggressive tumors requiring treatment. Recent researches on the immune response to tumors provide a great promise for developing new tools for the discovery of novel tumor biomarkers. These TAAs hold the key to offer a new understanding of the molecular mechanisms associated with the skeletal consequences of malignancy. Although data concerning the repertoire and function of TAA in OS is still limited, increasing evidence suggests that these tumors might express several diagnostic and/or therapeutic targets. The results of this study is eagerly anticipated.

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.

5.2 Seven-TAAs array can improve sensitivity in diagnosis of OS

Although anti-TAA autoantibodies in OS may have limited diagnostic and prognostic value when used individually, they have shown promise when profiled against carefully designed TAA arrays^{25,101,134}. These autoantibodies could also provide a platform for the development of novel antibody-based immunotherapies for OS. In this study, we were able to dramatically increase the frequency of positively reacting sera from 23.1% to 90.4% by adding 6 new candidate TAAs (NPM1, GAPDH, TPI1, HSP60, PDLIM1, and STMN1) to ENO1 developing a novel 7-TAAs array. The use of TAA arrays to profile the antitumor autoantibody response in a particular cancer patient could also provide key information on disease progression, which could guide physicians and patients in the making of important decisions regarding treatment options, combined with other available clinical information.

5.3 Future directions

This study focuses on the recent advances in proteomic technology that have thrust the skeletal cancer field into this exciting age of proteomics, and highlights the future work that is required to adapt this technology to specifically interrogate the skeletal consequences of malignancy. The findings represented in this study, while requiring further investigation in a large scale of samples, support the growing evidence for differences in the immunobiology of osteosarcoma compared with benign bone tumor and normal human. The present study has provided strong evidence that the selective 7-TAAs array provide a promising and powerful tool for enhancing cancer detection and treatment, but their utility in a clinical setting is currently in its infancy. Before this TAA array could be widely applied in screening programs for OS diagnosis or as a tool for monitoring tumor progression and guiding therapeutic interventions, it would be essential to enhance their sensitivity and specificity by identifying novel TAAs and defining systematically the optimal combination of TAAs. Different array platforms would also have to be evaluated to determine which one yields the highest sensitivity with minimal experimental

variation. Prospective studies in multiple clinical centers would then be required to ensure the reproducibility of these arrays.

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Abbreviations

1-D- One-dimensional gel electrophoresis

1D-IEF- First dimensional gel electrophoresis

2-DE- Two-dimensional gel electrophoresis

ABC- ATP-binding cassette

ACN- Acetonitrile

AJCC- American joint committee on cancer

AUC- Area under the curve

CHAPS- 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate

CI- Confidence interval

CV- Cut-off value

DAPI- 4',6'-diamidino-2-phenylindole

DTT- Dithiothreitol

ECL- Enhanced chemiluminescence

ELISA- Enzyme linked immunosorbent assay

ENO1- Alpha-enolase

ESCC- Esophageal squamous cell carcinoma

FA- Formic acid

FAP- Fibroblast activation protein

MAGE- Melanoma-associated antigen

GO- Gene ontology

HCC- Hepatocellular carcinoma

HDL- High-density lipoprotein

HLOH- Henan luoyang orthopedic-traumatological hospital

IEF- Isoelectric focusing

IHC- Immunohistochemistry

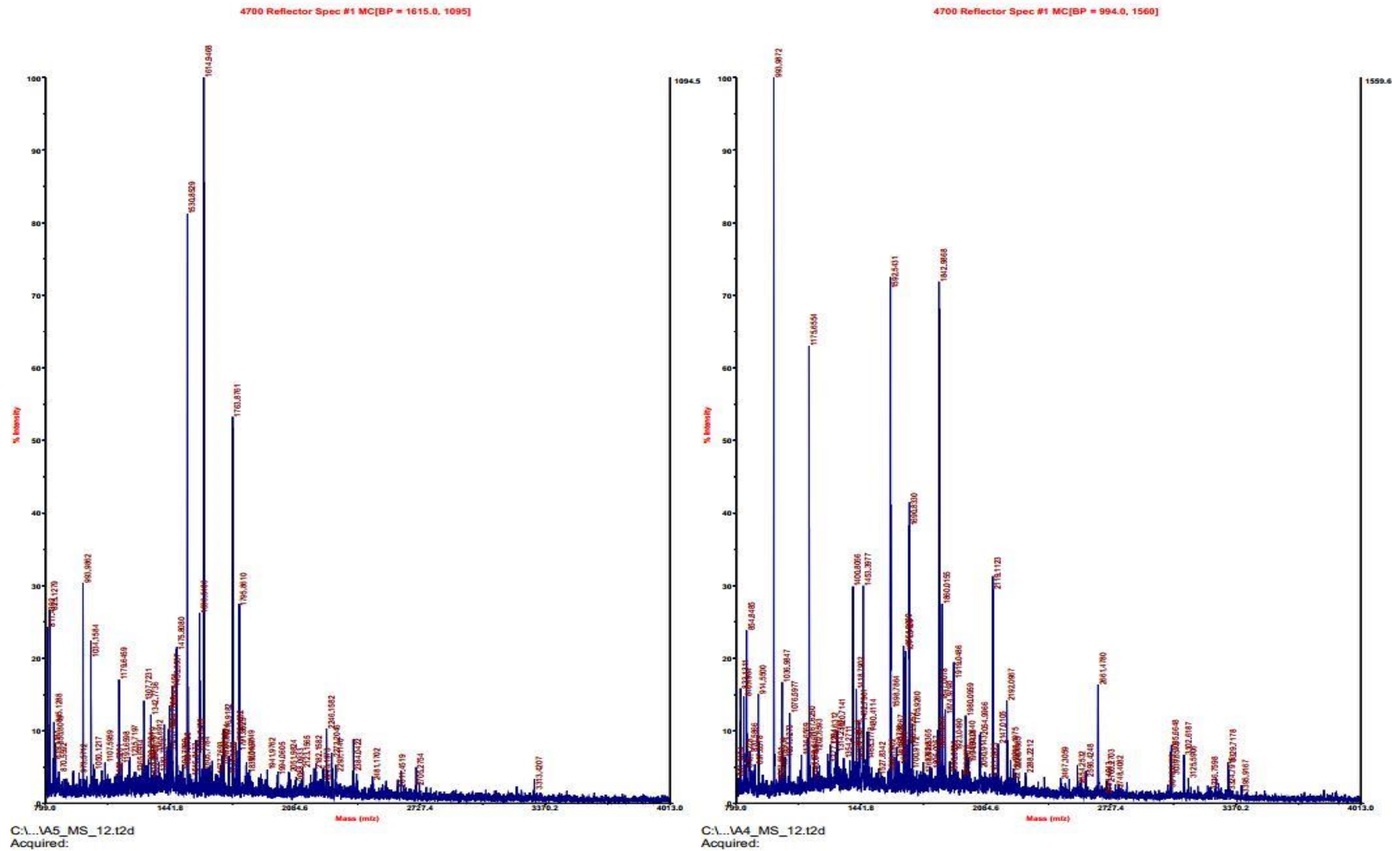
IIF- Indirect immunofluorescence
IL11R α - Interleukin 11 receptor alpha
LC- Lung cancer
LUAD- Lung adenocarcinoma
MCAM- Melanoma cell adhesion molecule
MS- Mass spectrometry
NH- Normal human
NHS- Normal human sera
NY-ESO-1- New York esophageal squamous cell carcinoma 1
OC- Osteochondroma
OD- Optical density
OS- Osteosarcoma
PBF- Papillomavirus binding factor
PBS- Phosphate buffered saline
PBST- Phosphate buffered saline containing 0.05% Tween-20
PCa- Prostate cancer
PMF- Peptide mass finger printing
PR- Positive rate
ROC- Receiver operating characteristic
RT- Room temperature
SDS-PAGE- SDS polyacrylamide
SEER- Surveillance, epidemiology, and end results
SEREX- Serological analysis of recombinant cDNA expression libraries
SERPA- Serological proteome analysis
SI- Staining intensity
SS-A- Sjogren's Syndrome A Control
TAAs- Tumor-associated antigens

TEM1- Endosialin/tumor endothelial marker 1

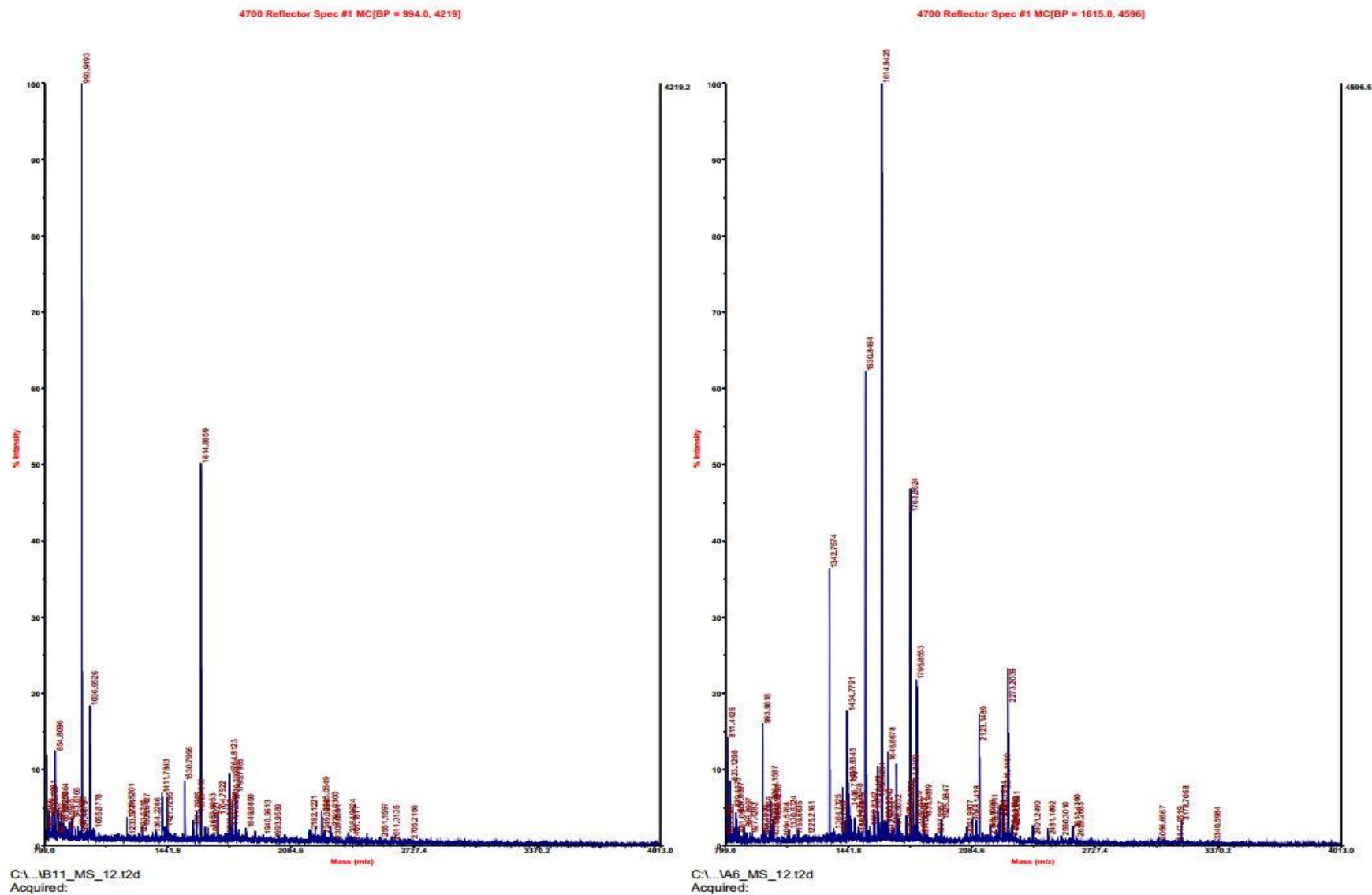
TMA- Tumor microarray

Supplemental File

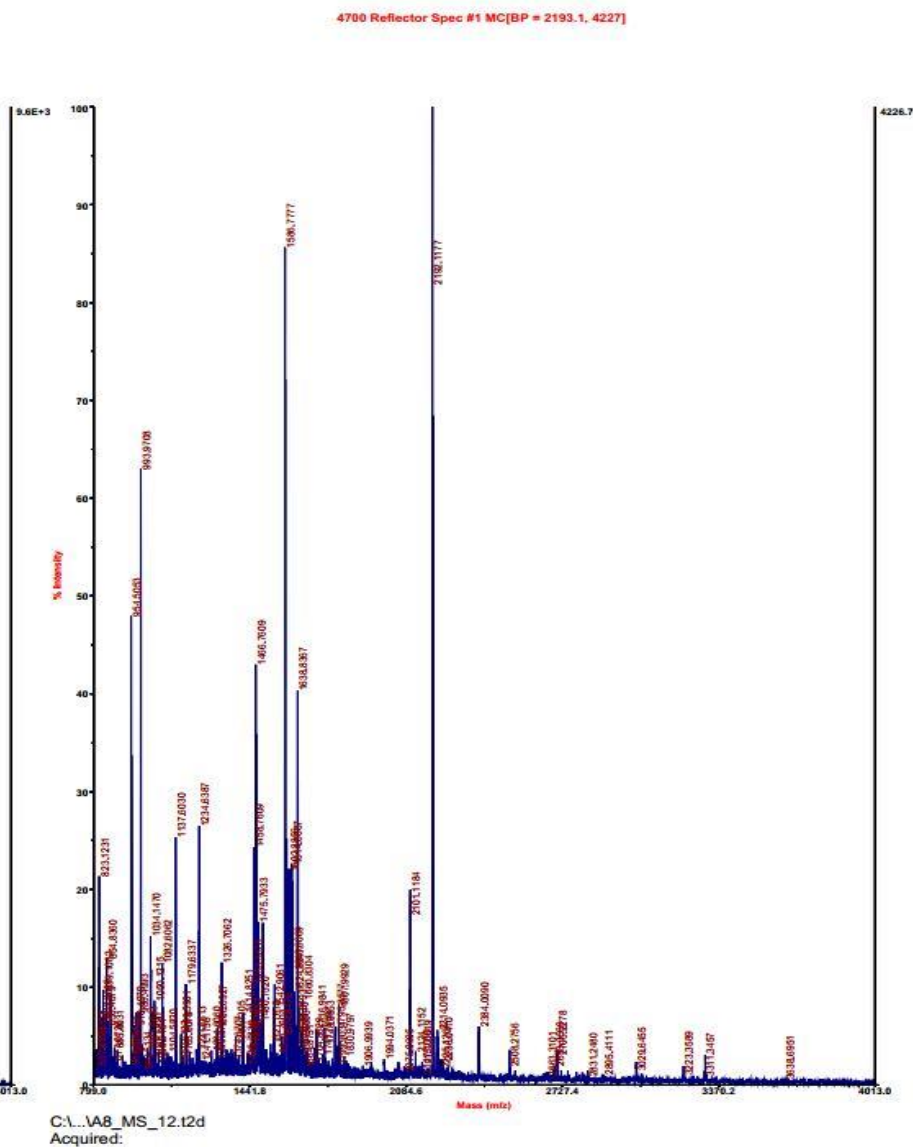
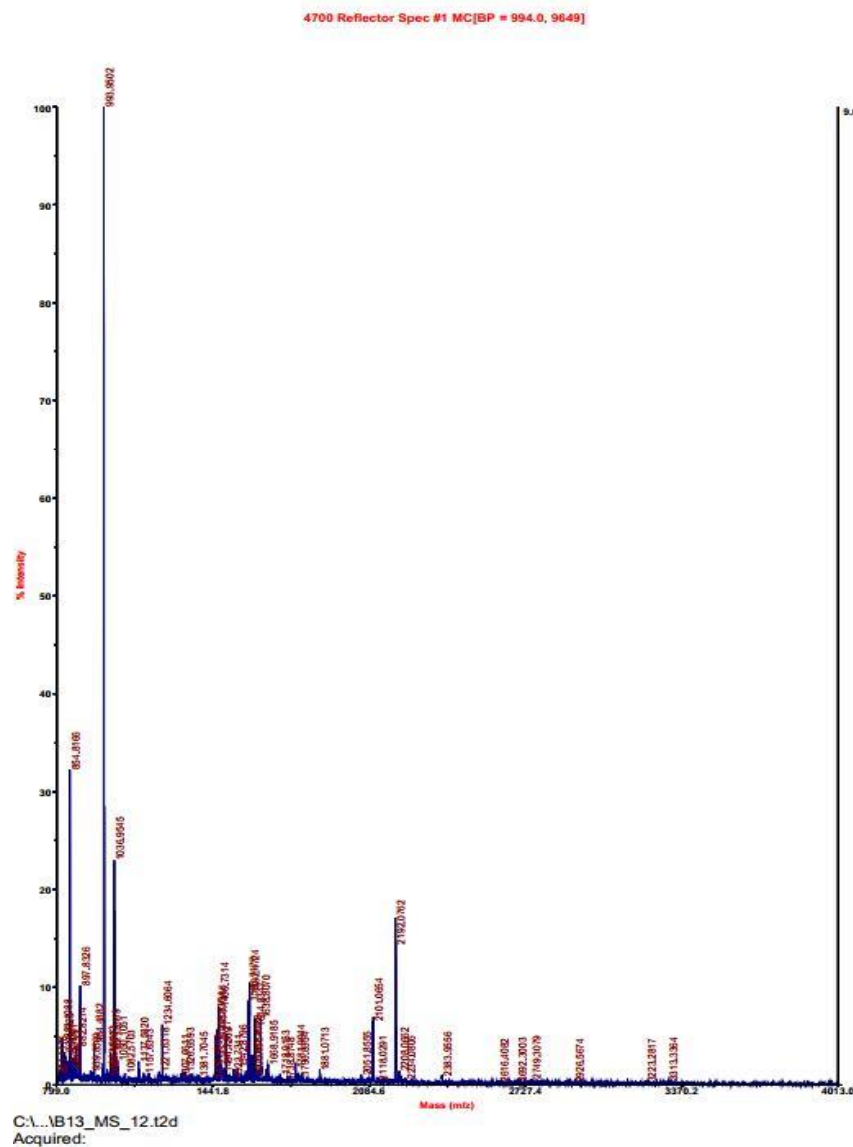
Supplementary Figure 1: Peptide mass finger printing for spots S5 and S4.



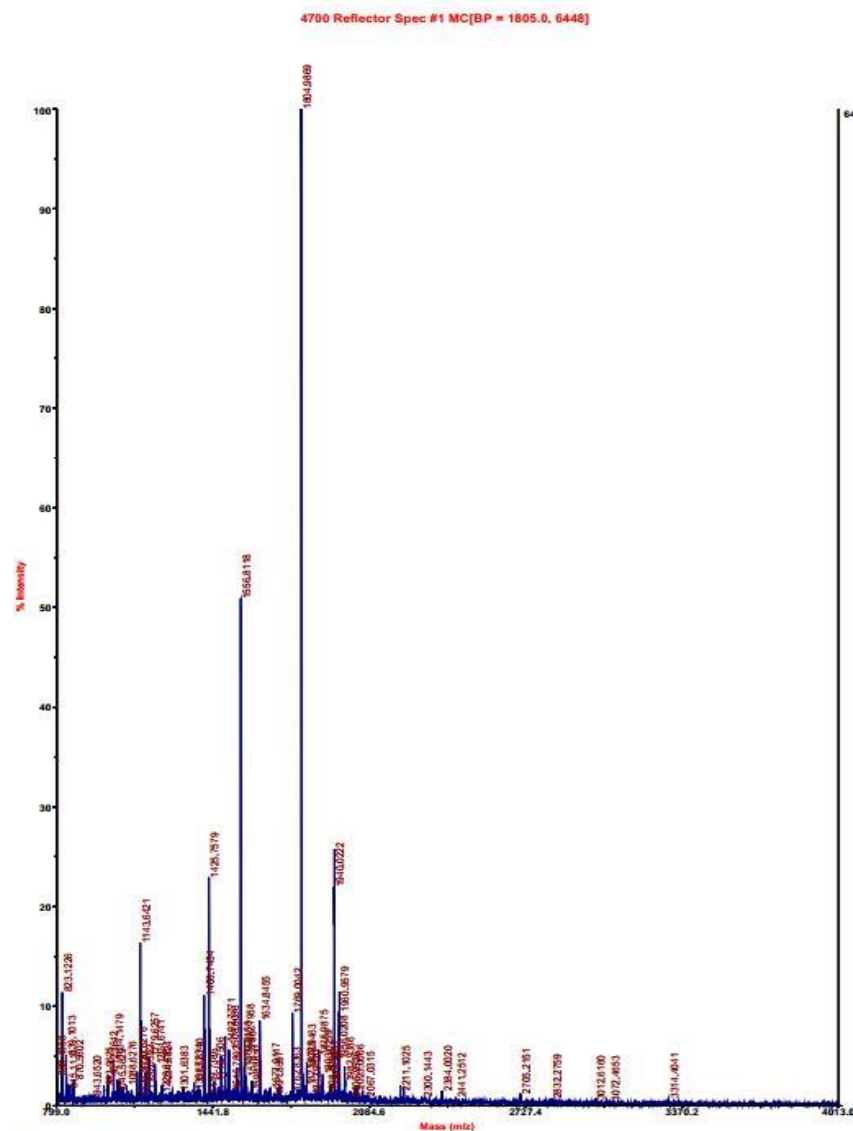
Supplementary Figure 2: Peptide mass finger printing for spots U4 and S6



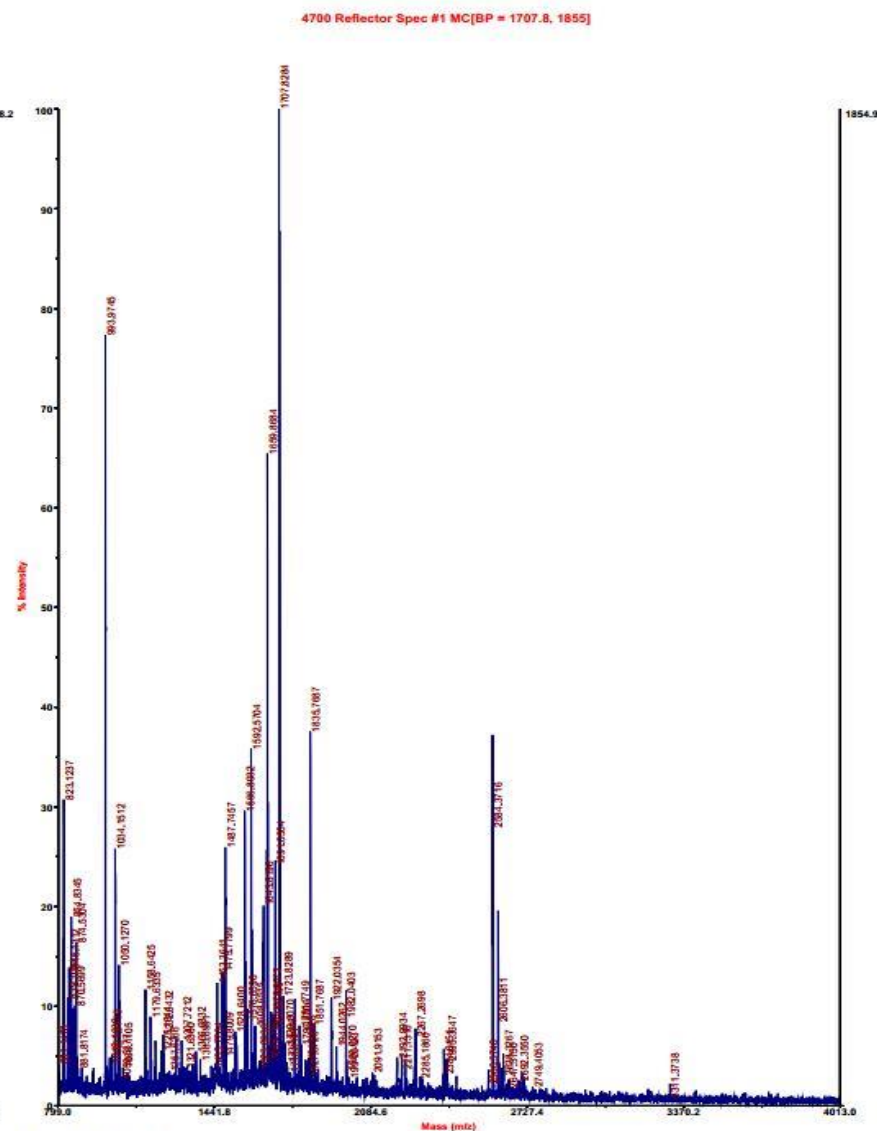
Supplementary Figure 3: Peptide mass finger printing for spots U6 and S8



Supplementary Figure 4: Peptide mass finger printing for spots S11 and S9

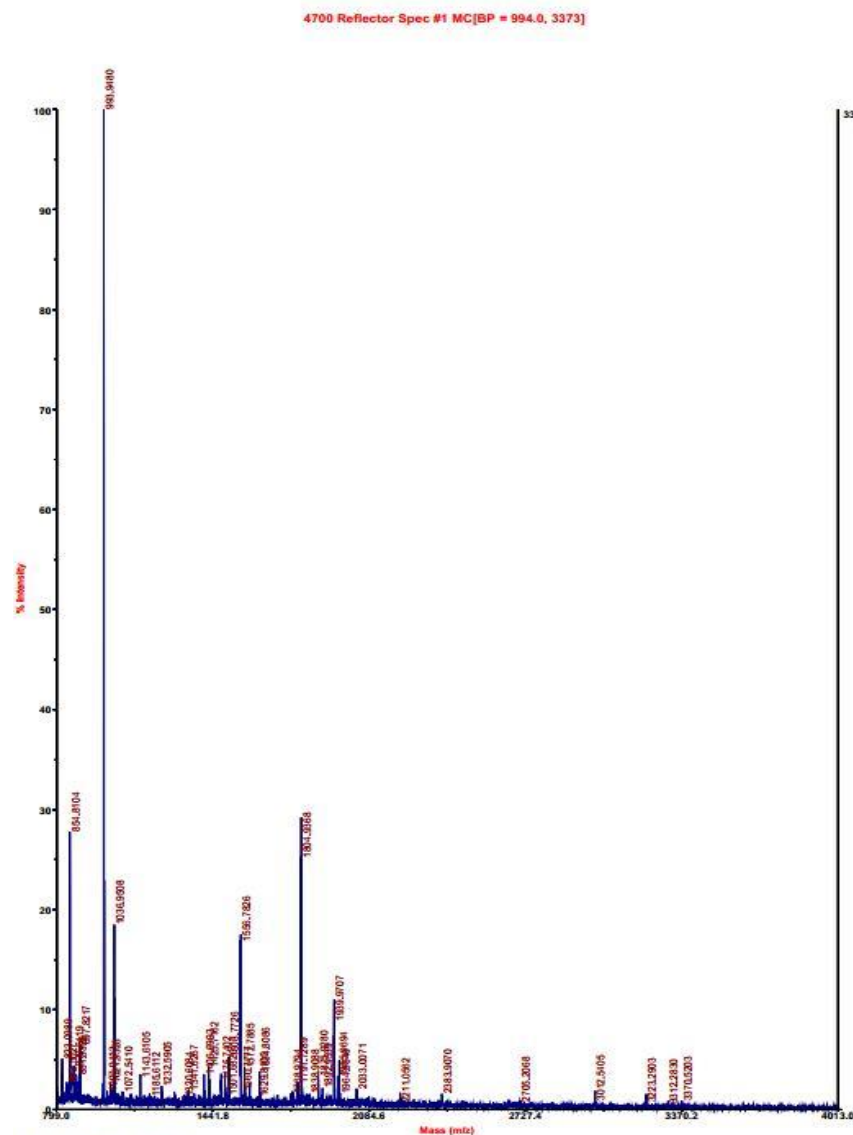


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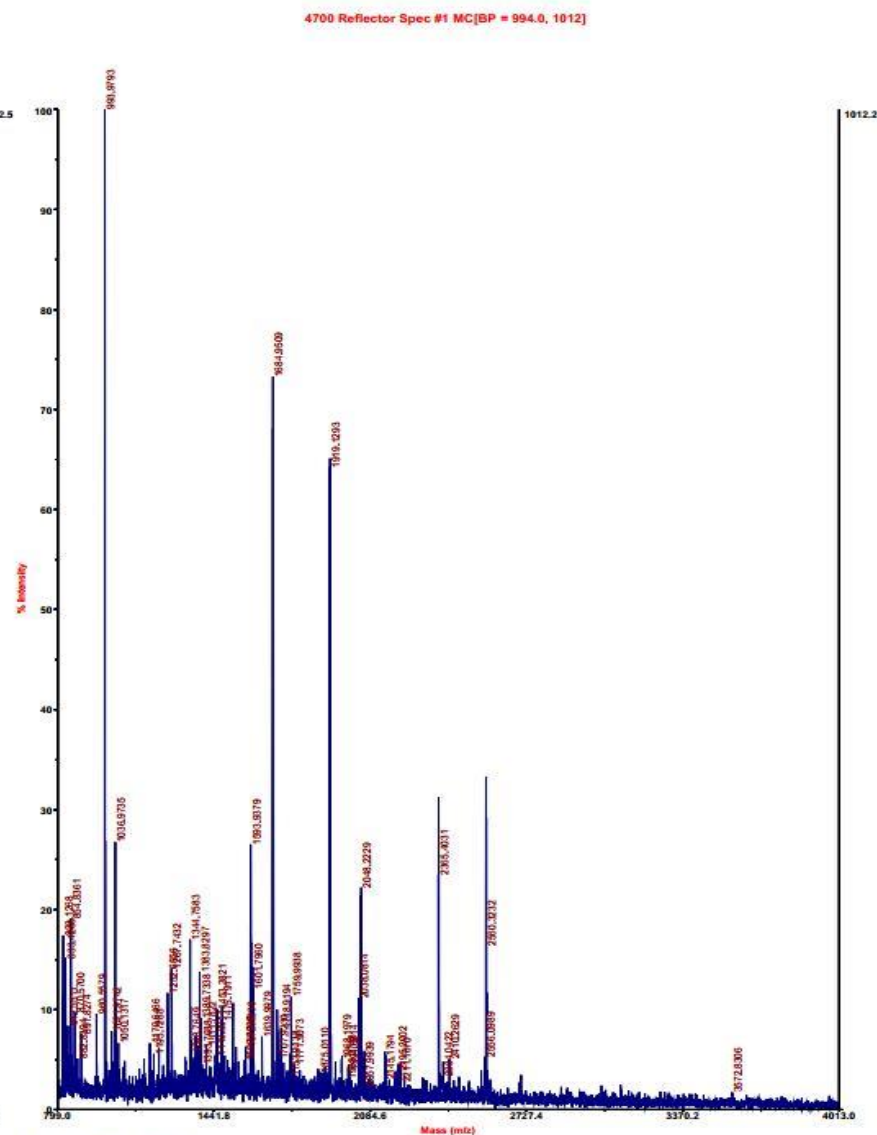


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Supplementary Figure 5: Peptide mass finger printing for spots U7 and S16

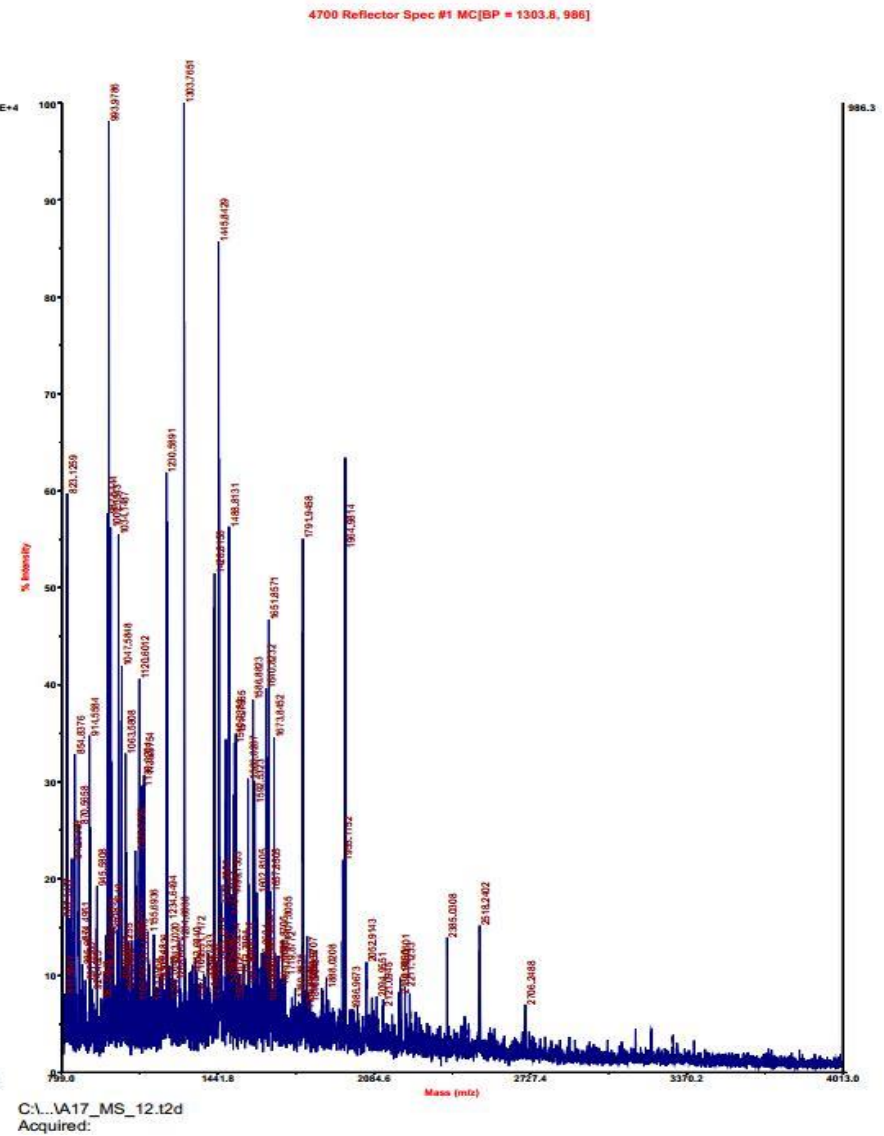
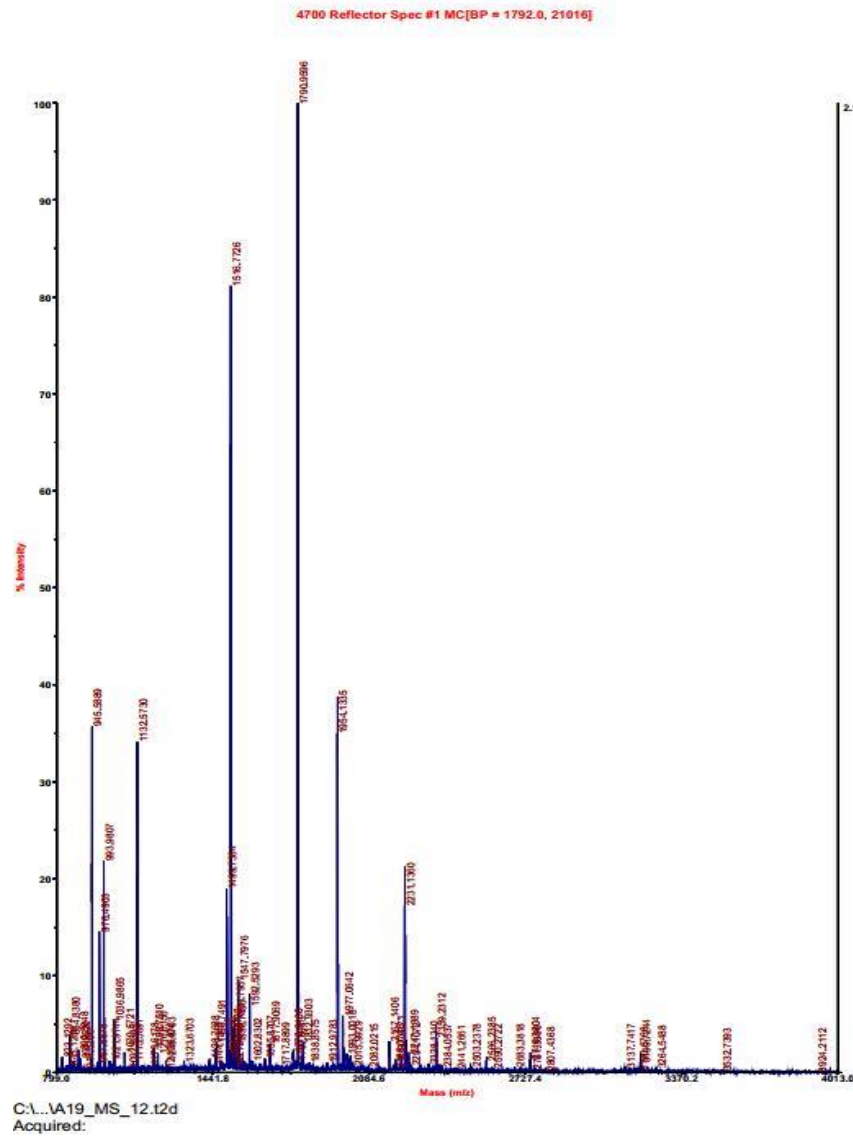


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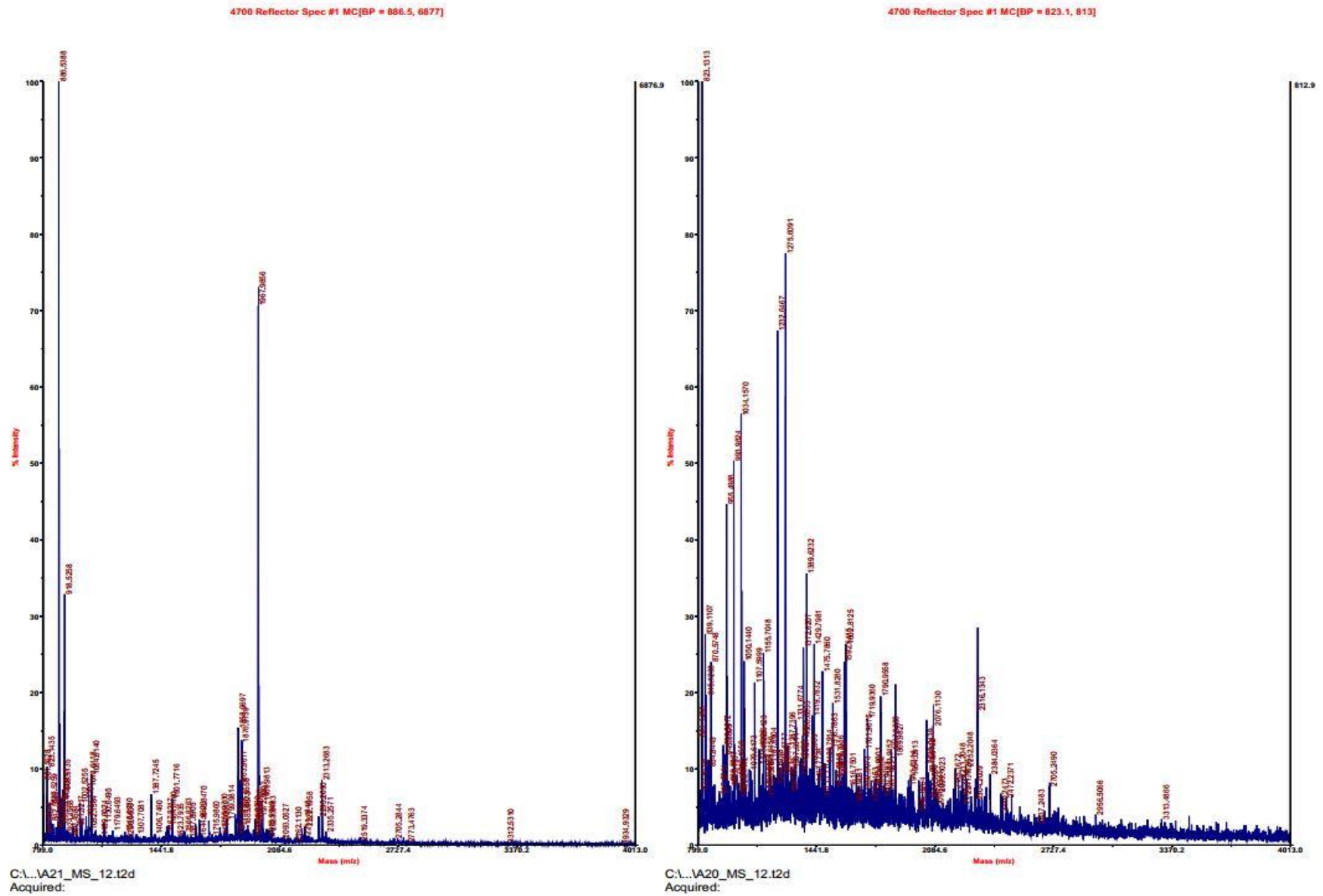


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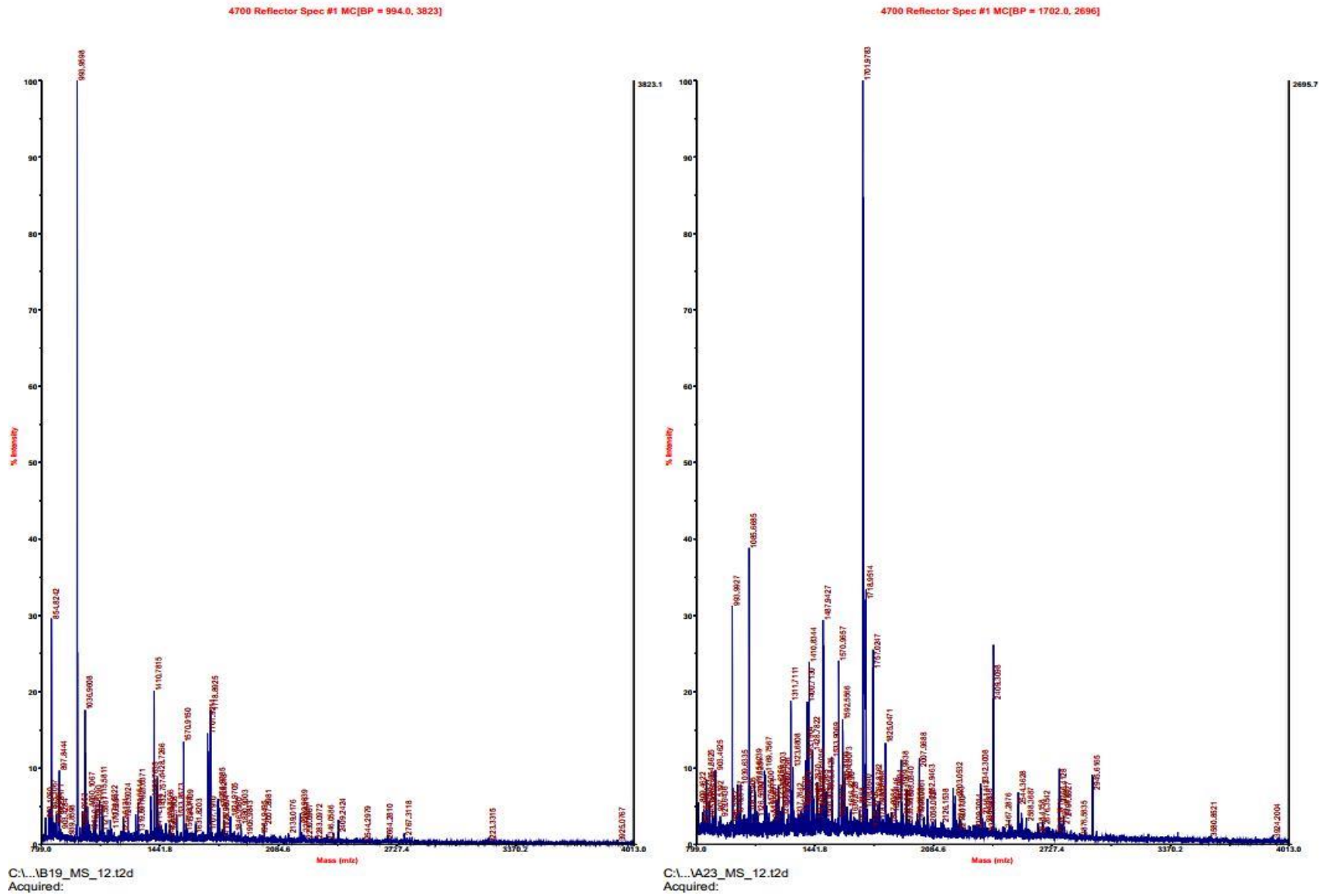
Supplementary Figure 6: Peptide mass finger printing for spots S19 and S17



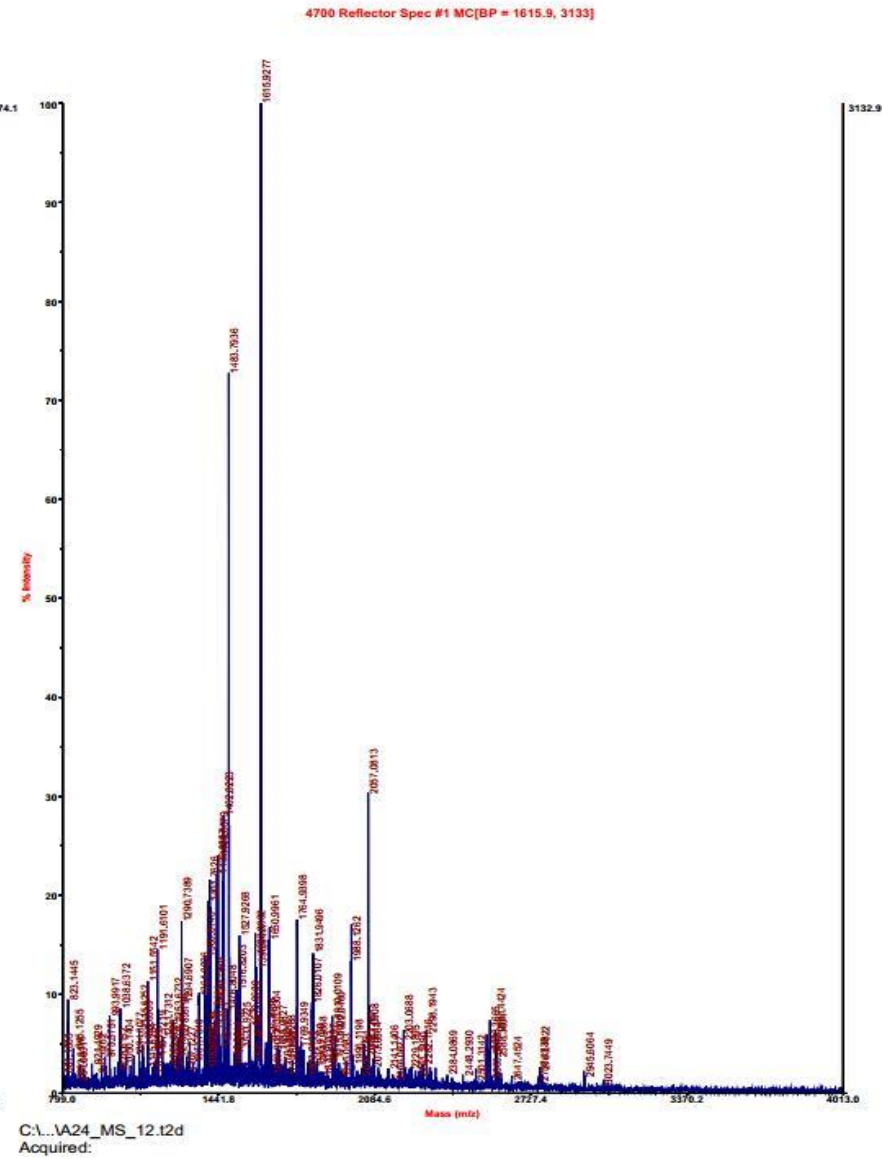
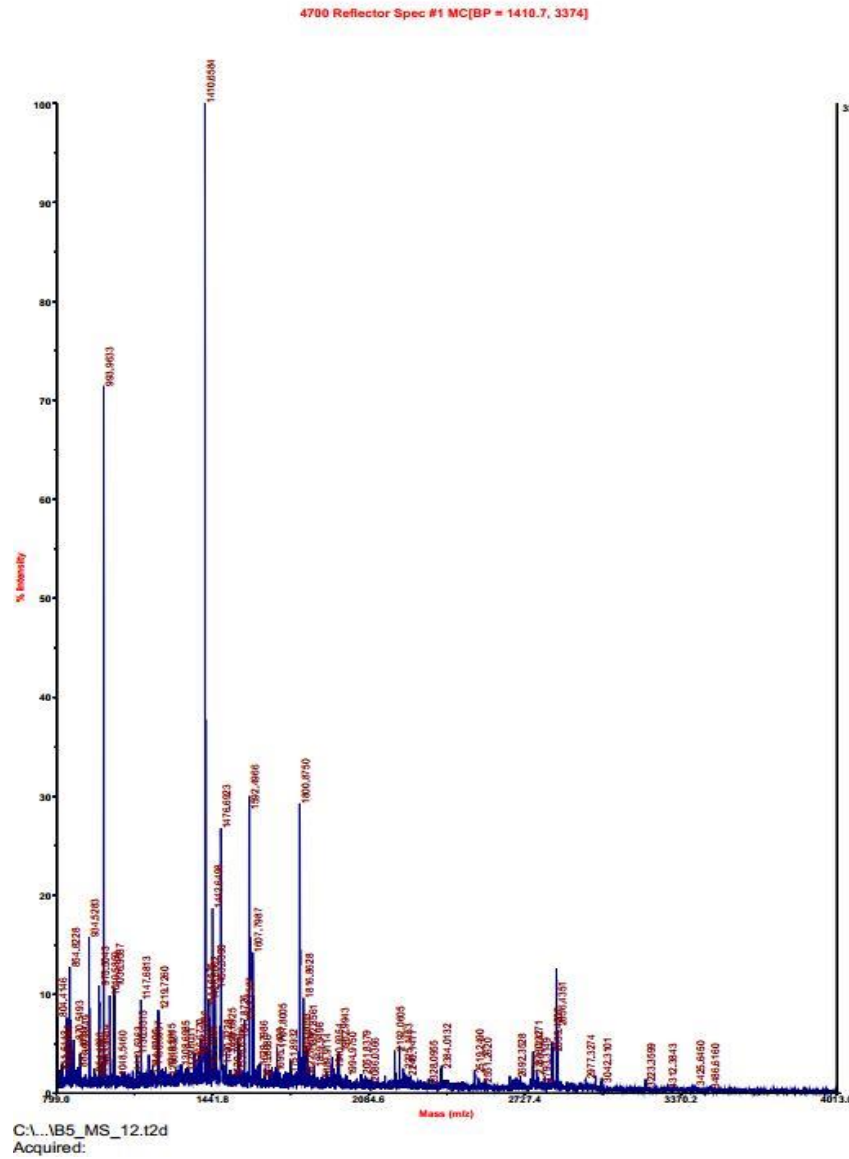
Supplementary Figure 7: Peptide mass finger printing for spots S21 and S20



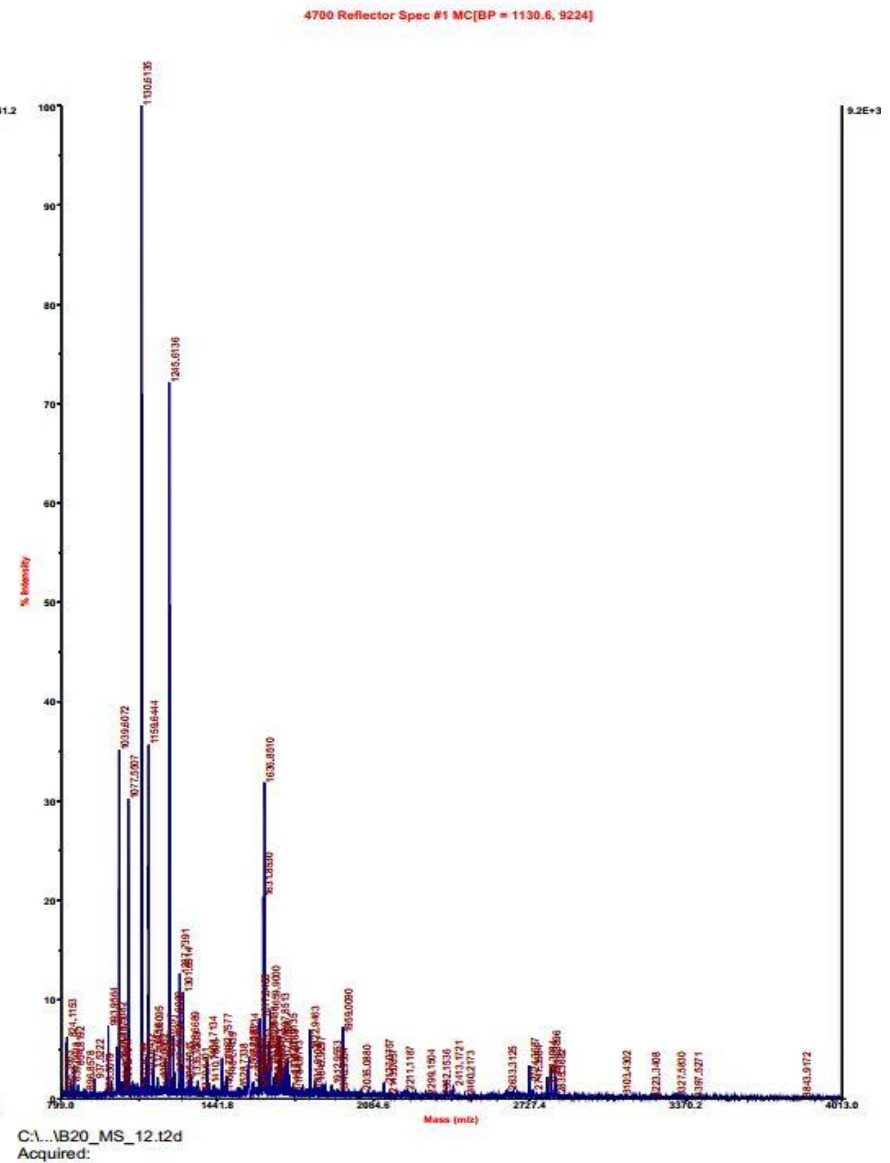
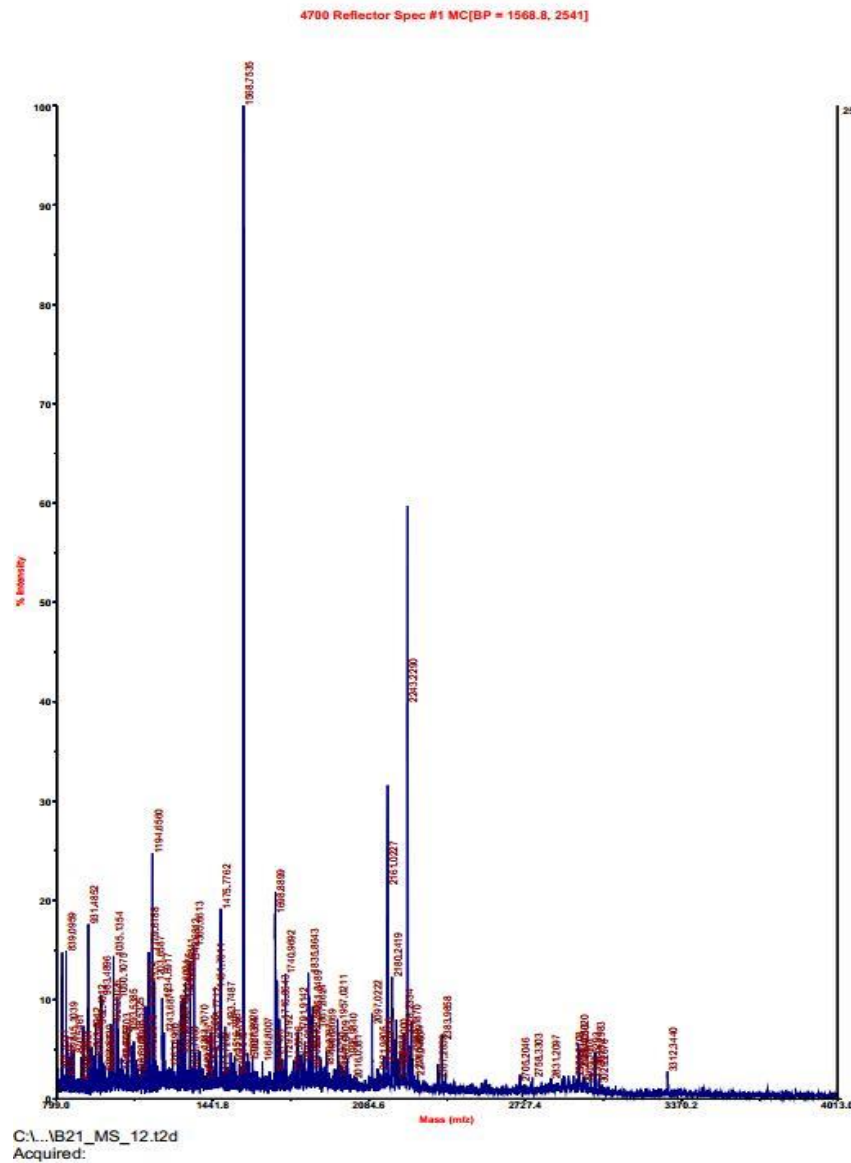
Supplementary Figure 8: Peptide mass finger printing for spots U12 and S23



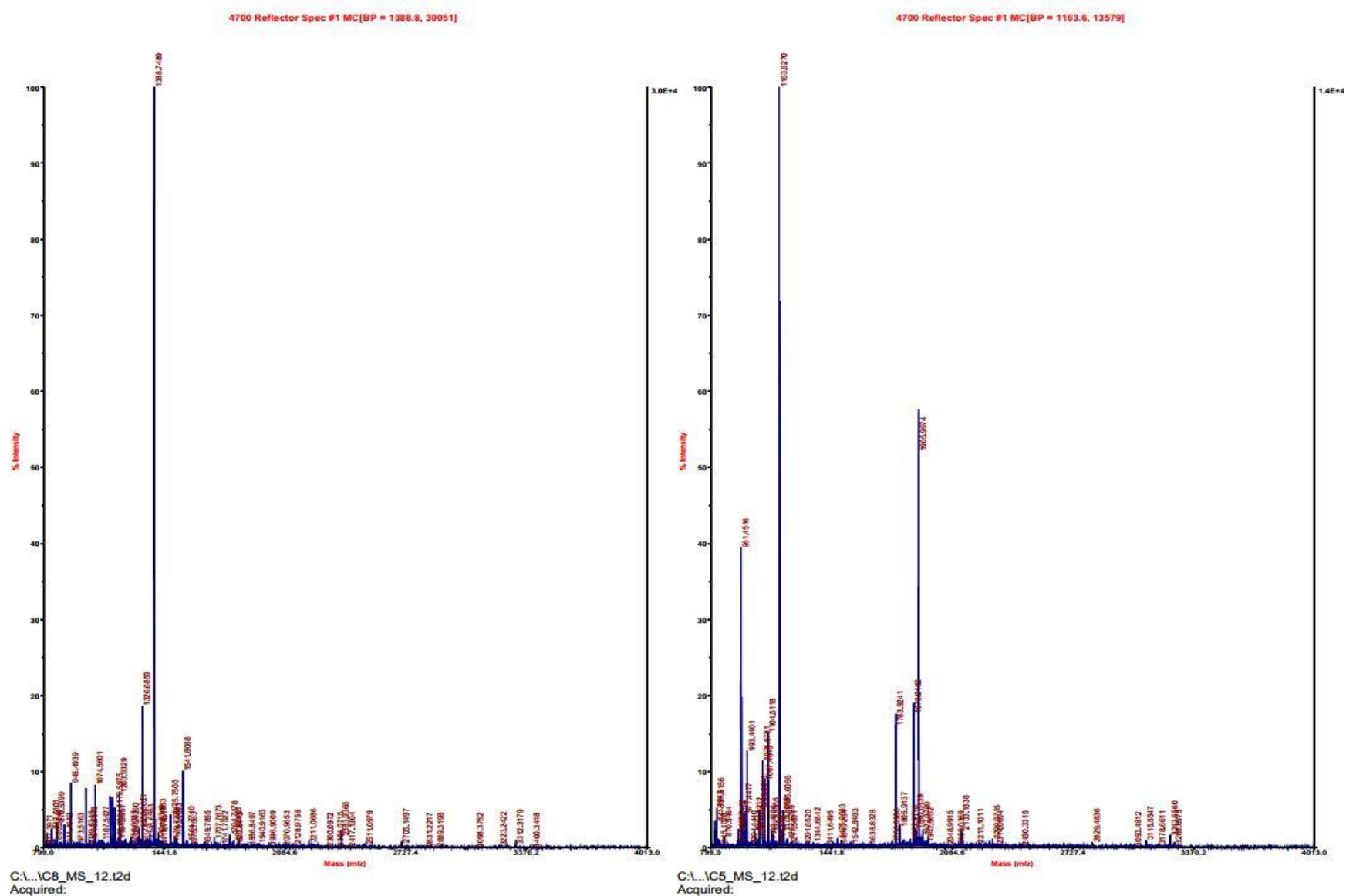
Supplementary Figure 9: Peptide mass finger printing for spots U29 and S24



Supplementary Figure 10: Peptide mass finger printing for spots U14 and U13



Supplementary Figure 11: Peptide mass finger printing for spots U25 and U2



Vita

Dr. Jitian Li earned his Bachelor Degree in Clinical Medicine (equivalent to M.D.) from Nanchang University School of Medicine in 2011. After his graduation from medical school in China, he came to The University of Arizona for his Master Degree in Public Health Policy and Management and received his Master of Public Health in 2012. From 2013, he joined the doctoral program in Biological Sciences at The University of Texas at El Paso.

Dr. Li was the recipient of numerous honors and awards including a Dr. Keelung Hong Fellowship, three Graduate Scholarships and four College Stipends. He was also awarded several grants including a Dodson Research Grant, a Wiemer Family Student Endowment for Excellence and two Graduate Travel Grant Awards. He published seven articles as first author as well as seven articles as co-authors. He presented his research in several national and regional meetings. For instance, he did five poster presentations on 2013 BMES, 2014-2017 AACR annual meetings respectively.

He worked hard in both of lab research and courses to maintain an excellent GPA (4.0/4.0). While pursuing his degree, Dr. Li worked as a research associate and assistant instructor for the Department of Biological Sciences. He served as the president of Chinese Students & Scholars Association (CSSA) at UTEP and as a grand award judge at the 2014 & 2016 Intel International Science and Engineering Fairs. He was also invited by several prestigious journals to act as a reviewer for other scientists' manuscripts and grant proposal.

Dr. Li's dissertation entitled, "Identification and characterization of tumor-associated antigens (TAAs) and anti-TAAs autoantibodies as biomarkers in immunodiagnosis of human osteosarcoma (OS) by serological proteome analysis" was under the mentorship of Dr. Jianying Zhang. Dr. Li will continue working as a post-doc researcher to combat cancer.