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RESEARCH ARTICLE

SUPEROXIDE DISMUTASE 2 PROTEIN LEVELS IN BLOOD MAY ACT AS A PROGNOSTIC MARKER FOR HIGH-RISK NEUROBLASTOMA PATIENTS

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ABSTRACT

Purpose: Determination of proteomic differences plays an important role in biomarker investigations. Due to its heterogenic molecular background, identification of certain biomarkers is still a demand both for diagnosis and prognosis of neuroblastoma. In this study, it is aimed to identify marker proteins/mechanisms that may play role in neuroblastoma prognosis.
Material and Methods: Real-time PCR analyses were performed for 2p24.3, 11q23, 1p36 and 17q25 status from tumor samples of the patients to determine the risk groups. A proteomic approach was used for different risk groups of the disease by using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF/TOF) approach. Mononuclear cell pools from blood samples of patients for different risk groups were constructed and protein expression changes for different groups were identified.
Results: Manganese-superoxide dismutase (SOD2) protein was found to significantly increase in high-risk group of neuroblastoma patients.
Conclusion: Our results showed that SOD2 may play an important role in neuroblastoma progression and be a candidate prognostic peripheral blood marker for neuroblastoma patients.

Keywords: Neuroblastoma, SOD2, risk-classification, proteomics, MALDI-TOF/TOF

INTRODUCTION

Neuroblastoma (NB), which is the most common extra cranial solid tumor of childhood, arises from neural crest cells of sympathetic nervous system (1,2,3). Although developments in multimodal therapies, survival rates are low in advanced stage disease (4). Neuroblastoma shows clinical heterogeneity from spontaneous regression to aggressive disease. N-myc amplification is seen in aggressive and proliferative tumors and is considered as high-risk criteria (5). However, N-myc amplification is not the only criteria in risk classification of this disease. Some chromosomal changes such as 1p36 LOH, 11q23 loss and 17q25 gain, also play role in NB risk classification (6-10). Along with these genetic changes, DNA index is also important in risk classification.

Schimada and his friends (1984) created the first NB classification through clinical behavior and histopathological properties of the tumor (11). The international Neuroblastoma Stratification System (INSS) uses radiographic and surgical assessments

of the NB patients in addition to clinical behavior (12,13), while the International Neuroblastoma Risk Group Stratification System (INRGSS) is based on tumor stage, age, MYCN status, chromosomal changes and tumor cell ploidy of the patient along with Shimada histology (14). Aggressive surgical procedures could change the INSS stage. Therefore, an alternative approach was developed according to imaging findings and clinical evaluation. Nowadays, International Neuroblastoma Risk Group (INRG) consensus pretreatment classification schema, including INSS and INRG, are being used for risk classification.

Reactive oxygen species (ROS), including hydrogen peroxide, superoxide anion and hydroxyl radical are produced by partial reduction of oxygen molecule. ROS can be generated both endogenously in the process of mitochondrial oxidative phosphorylation and exogenously by interactions with several exogenous sources. When ROS levels exceed the levels that a cell can overcome by cellular antioxidant defense mechanisms, oxidative stress takes place, which is known to play role in pathology of several diseases including neurodegenerative disorders such as Alzheimer's, Parkinson's disease and aging (15-17). Among the intrinsic antioxidant defense mechanisms are mainly glutathione peroxides, thioredoxins, catalase and superoxide dismutases (SOD1 and SOD2). Mitochondria are particularly vulnerable to oxidative damage as being the main site of ROS generation; therefore, contain antioxidant enzymes to eliminate ROS within the site of generation. Manganese-superoxide dismutase-SOD2 is one of those mitochondrial antioxidant proteins and Manganese (Mn) is an essential co-factor for SOD2.

Proteomics is a post-genomic approach that is used for identification, separation and quantification of proteins generally by using high technologies. Proteomic studies in pediatrics is of great importance. Proteomic studies in pediatrics mainly focus on neuroblastoma and acute lymphoblastic leukemia. In one study, dedifferentiation assessment was performed in SK-N-BE2 NB cells by using mass spectrometry and 239 proteins found to play role in spheroid dedifferentiation (18). In another study, Tropomyosin receptor kinase A (TrkA)-dependent target proteins were determined in SK-N-MC neuroblastoma cells by using a proteomic approach (19). In a study by Kim et al., component C3 was proposed as a candidate plasma biomarker in TH-MYCN+/+ mouse model by using liquid tandem mass spectrometry (20). Receptor tyrosine kinases RET, insulin growth factor 1 like- 1 receptor/1K and fibroblast growth factor receptor-1 were shown to play an important role in NB upon comparison of protein profiles of different neuroblastoma cell lines by using shotgun Liquid Chromatography/Mass Spectrometry (LC/MS) (21).

Although several studies exist, to our knowledge there is limited data about proteomic changes in neuroblastoma patients. Presence of a blood protein marker that can be used for risk classification would be useful since it may function as a non-interventional analysis, which is quite valuable due to the young age of the NB patients. Therefore, we asked if there exist any proteins in blood that are differentially expressed in different risk groups and aimed to identify the differences in protein profiles in neuroblastoma patients of different risk groups. Our hypothesis was that there exist proteins that may be useful in discriminating low-risk and high-risk groups from blood samples at different clinical stages. In order to identify the possible protein changes, we constructed protein pools from the peripheric blood mononuclear cell samples of different patients in different risk groups and performed protein analysis by MALDI-TOF/TOF. Our results revealed that Manganese-superoxide dismutase (SOD2) levels are significantly increased in blood samples of high-risk neuroblastoma patients.

MATERIAL AND METHODS

Patients and Tissue Samples

Fresh tumor tissue samples of NB patients were chosen from oncology centers of several universities from all over Turkey, within the scope of "Turkish Pediatric Oncology Group-Neuroblastoma-2009 (TPOG-NB-2009) Protocol", which is based on INRGSS. Real-time PCR analysis were performed for N-MYC, 11q, 1p and 17q status of these patients and risk groups were determined according to INRGSS. Along with the tissue samples, peripheral blood samples were collected form these patients as well and, mononuclear cells from peripheral blood samples of 42 neuroblastoma patients between 1-5 years old were used. Among 42 patients, 19, 12 and 11patients were in high-risk, middle-risk and low-risk group, respectively. As a control group, mononuclear cells from blood samples of 10 healthy children between 1-5 years old who are not diagnosed with neuroblastoma were used. Control group samples

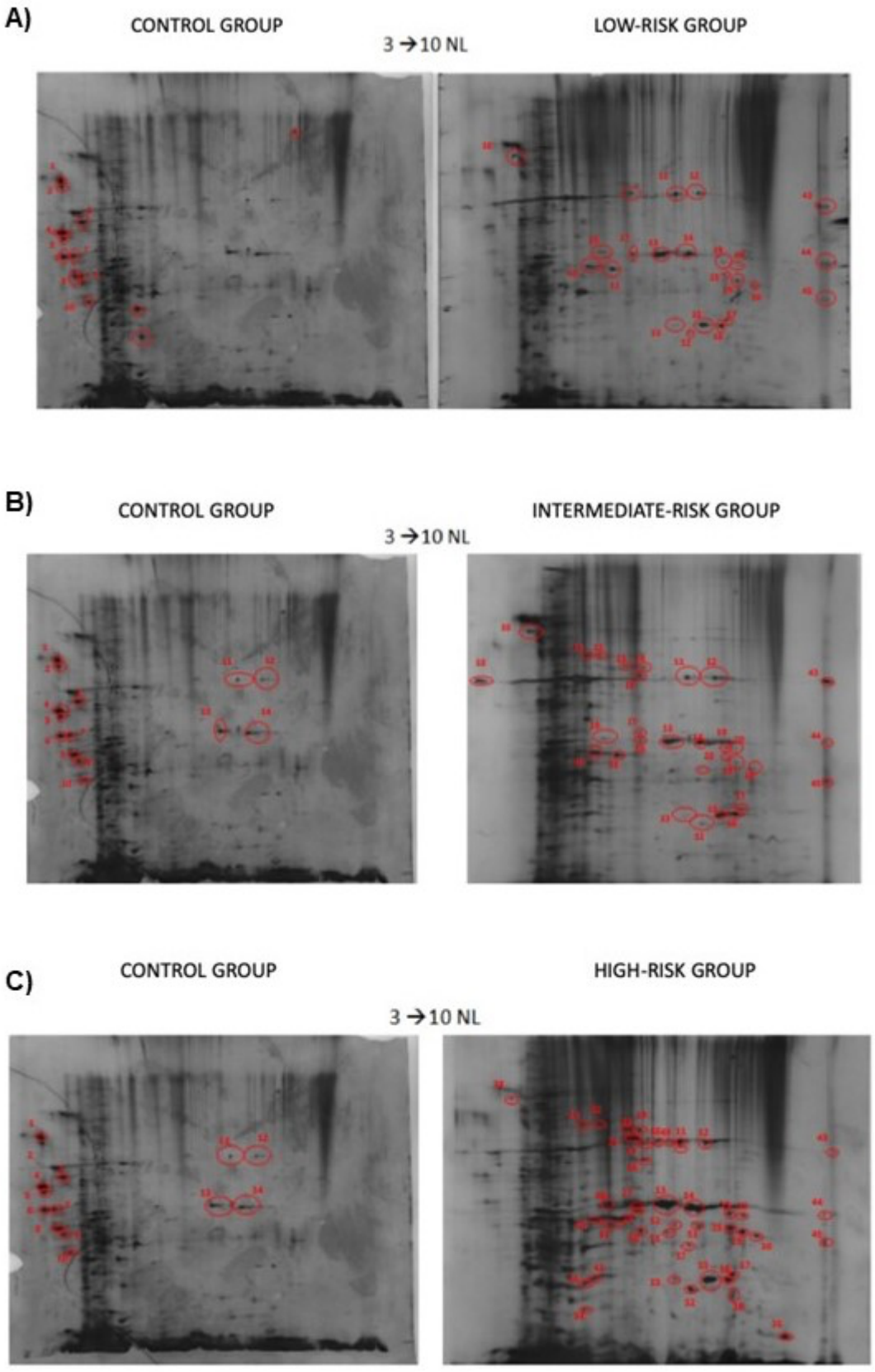


Figure 1. Comparison of 2D gel electrophoresis results of **A)** Low-risk group **B)** Intermediate-risk group and **C)** High-risk group to the control group

The Ethical approval for the study was taken from Izmir Dr. Behcet Uz Hospital Non-Drug Clinical Research Ethics Committee with an archive number 2013/44 which was approved on 25.09.2013.

Mononuclear Cell Isolation

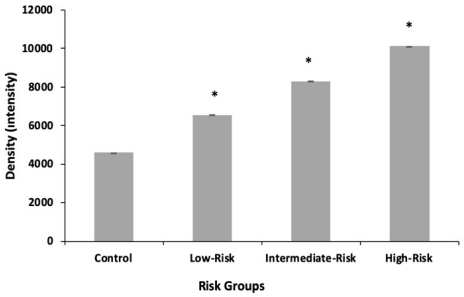
Mononuclear cells were isolated from blood samples by using Ficoll. Briefly, blood samples and phosphate buffer saline (PBS) were mixed as 1:1 ratio and this mixture added on Ficoll solution gently with a 45o angle. The samples were centrifuged at 800xg for 20 minutes and mononuclear cells, were transferred into a new falcon tube. These cells were washed with PBS and the isolated mononuclear cells of each patient were kept at -80oC until analysis.

Protein Isolation

Protein pools for each risk group were constructed by mixing the mononuclear cells of patients from similar risk groups. These mononuclear cell pools for each group were washed with dH2O for 2 times. The cells were suspended in lysis solution (7M Urea, 2M Thiourea, 1% dichloro diphenyl trichloroethane (DTT)(w/v), 2% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)(w/v) and were lyzed on ice (22). Cell lysates were centrifuged at 4oC, 25000g for 30 minutes and the supernatants were obtained. Protein concentrations were determined with Bradford method (Coomassie Plus (Bradford) Assay kit) (Thermo) at 595nm.

Two-Dimensional (2D) Gel Electrophoresis

Isoelectric point separation of protein samples was performed by using Protean i12 IEF system (BioRad). 350µg protein were mixed with rehydration buffer and loaded on pH3-10 non-linear IPG strips (BioRad). Passive rehydration was performed for 2 hours and after addition of 2 ml mineral oil, isoelectric focusing (IEF) program was run (12 hours active rehydration; 500V for 10 minutes; 4000V linear for 1,5 hours; 8000V fast for 25000Vh). IEF strips were kept at -80oC until analysis. IPG strips were incubated in Equilibration buffer I (6M urea, 0,375M Tris-HCl, pH 8.8, 2% (w/v) sodium dodecyl sulfate (SDS), 20% (v/v) glycerol, 2% (w/v) Dithiothreitol (DTT) and Equilibration buffer II (6M urea, 0.375M Tris-HCl, pH 8.8, 2% (w/v) sodium dodecyl sulfate (SDS), 20% (v/v) glycerol, 2.5% (w/v) iodoacetamide), respectively for 15 minutes each. Proteins were isolated according to their weights in 12% sodium dodecyl sulfate (SDS) (v/v) gel at 16mA for 1 hour and 8 hours at 180V by using Protean II XI Cell system. 2D Gel electrophoresis were performed for 3 times.



Group Name	Density (intensity)	Fold increase compared to Control Group	P-value
Control	4589,939	-	
Low-risk	6567,177	1,43	4,45973E-10
Middle-risk	8307,78	1,81	2,3436E-11
High risk	10111,43	2,20	3,6342E-12

Figure 2. SOD2 spot density results analyzed by Quantity One v.4.6.5 program and fold increase changes of SOD2 in risk groups compared to control group. * indicates p-value <0.005.

Silver Staining

2D gels were stained with mass compatible silver staining protocol (23,24). Briefly, gels were incubated in fixation solution (50% (v/v) ethanol, 16% (v/v) acetic acid) for 1 hour and washed with wash solution (25% (v/v) ethanol) and ultra-pure water, respectively for 15 minutes each. Gels were further incubated in sensitization solution (1,2mM sodium thiosulfate pentahydrate) for 1, 5 minutes, in ultra-pure water for 20 seconds (2 times), in silver stain solution (12mM silver nitrate) for 30 minutes and washed with ultra-pure water for 20 seconds (2 times). Gels were subsequently incubated in development solution (0,2M potassium carbonate, 0,06mM sodium thiosulfate pentahydrate ,37% (v/v) formaldehyde) (3-5 minutes) until the appearance of the protein spots. Once the protein spots appeared clearly, incubating gels with stopping solution (0,025% (v/v) acetic acid, 0,4M Tris base) for 2 minutes inhibited the reaction. After the gels were washed 2 times with ultra-pure water for 10 minutes, gel visualization was performed by using VersaDoc visualization system (Bio Rad). The gels were kept at 4oC in ultra-pure water until analysis.

The gels for each risk group were compared with control group gels. Differentially expressed protein spots were identified separately by three different people, compared and confirmed. These protein

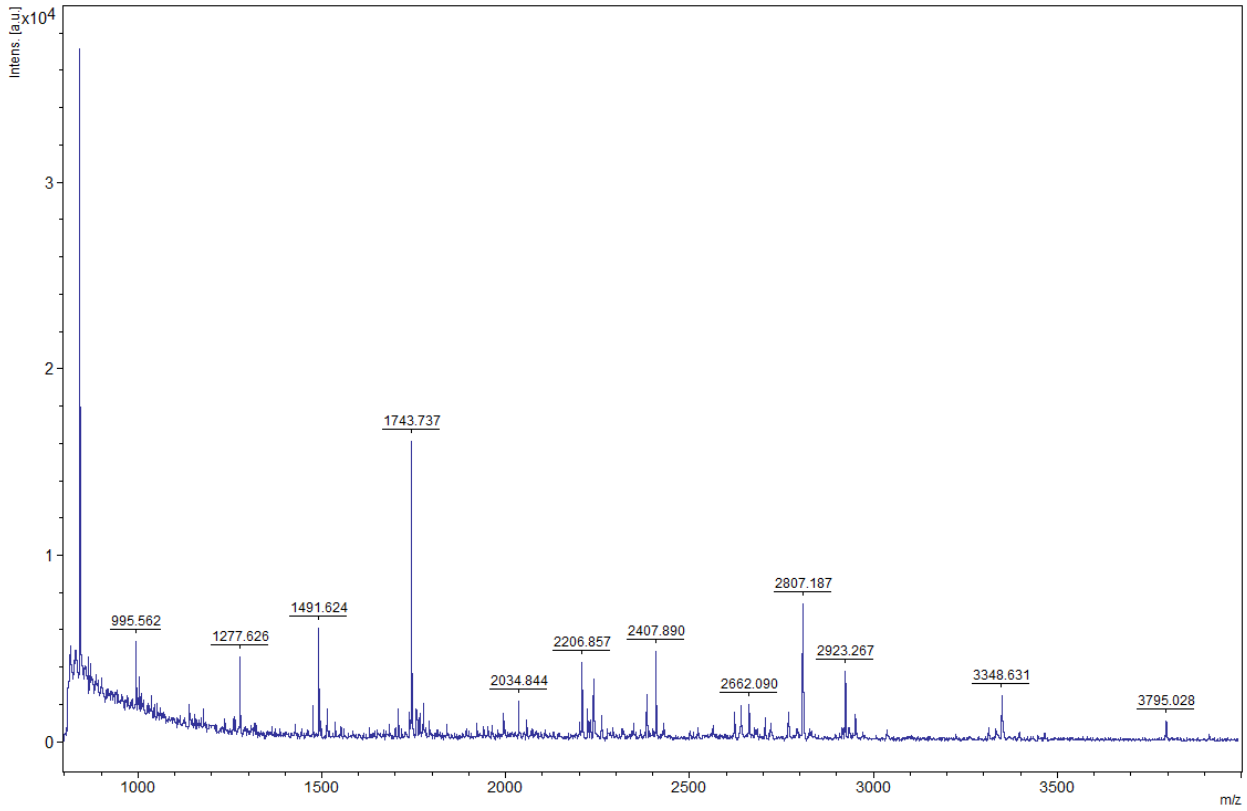


Figure 3. MS spectrum of spot 16 that gave a match with Superoxide Dismutase 2 (SOD2) protein.

spots were cut as 2-3mm in diameter and 1mm in thickness. The cut gel pieces were transferred into low-protein binding micro centrifuge tubes (Protein LoBind Tube Eppendorf).

Protein In-Gel Digestion

Silver stain from differentially expressed protein spots was removed by using a destaining protocol (25). Briefly, gel spots were incubated with spot destaining solution (30mM potassium ferricyanide) for 5-10 minutes at room temperature. The solution is then removed and gel pieces were washed 5 times with ultra-pure water for 5 minutes. 200mM ammonium bicarbonate was added on gels and incubated at room temperature for 20 minutes. The solution is then removed and gel pieces were washed 5 times with ultra-pure water for 5 minutes. After the removal of water, gel spots were dehydrated with acetonitrile solution and dried with vacuum-centrifuge for 5 minutes at room temperature. The spots were incubated in 10mM DTT, 100mM iodoacetamide and acetonitrile, respectively and rehydrated in ammonium bicarbonate. After dehydration with acetonitrile, samples were dried and rehydrated in 20nM trypsin (Sigma-Aldrich) solution prepared in 50mM ammonium bicarbonate. Trypsin digestion was performed overnight at 37oC. 50mM ammonium bicarbonate was added on digested samples and vortexed. After centrifugation, supernatants were transferred into new microcentrifuge tubes. This step was repeated twice. After collection of the supernatants, the samples were vacuum-centrifuged until 10µl of sample is left in the tubes and then kept at -80oC until analysis.

Sample Desalting by Solid-Phase Extraction

Prior to mass spectrometry analysis, ZipTipTM (Millipore) protocol was performed to samples according to manufacturer's protocol. Briefly, protein extracts were transferred into new microcentrifuge tubes and pipetting was performed with trifluoroacetic acid. C18 ZipTipsTM were moisten with 50% (v/v) acetonitrile and equilibrated with 0.1% (v/v) trifluoroacetic acid by pipetting procedure. In order to bind acidified peptides to ZipTipsTM, peptide mixes were pipetted with ZipTipsTM for 10 times and ZipTipsTM were washed with 0.1% (v/v) trifluoroacetic acid. 50% (v/v) acetonitrile was added into new microcentrifuge tubes and pipetting was performed by ZipTipsTM in order to obtain peptides within acetonitrile solution.

Table 1. MASCOT search engine results of significant differentially expressed protein spots

Spot Number	NCBI MASCOT SEARCH	Zone	Score	SwissProt MASCOT SEARCH	Zone	Score
11	β -fibrinogen	White	59	β-fibrinogen	White	59
15	Fibrinogen-gamma	White	68	Fibrinogen-gamma	Green	17
16	Mn-Superoxide Dismutase	White	38	Mn-Superoxide Dismutase	White	38
17	Fibrinogen-gamma	White	70	Fibrinogen gamma	White	70
18	Mutant- β actin/ actin- β	White	47	Putative β actin like protein	White	31
20	Mutant- β actin/ actin- β	White	47	Fibrinogen gamma	White	68
22	Type-I Keratin	White	79	Keratin	White	91
31	Fibrinogen-gamma	White	55	Fibrinogen-gamma	White	31
32	Fibrinogen-gamma	White	30	Fibrinogen-gamma	Green	0

Mass Spectrometry Analysis

MALDI TOF/TOF analyses were performed by using Autoflex III MALDI TOF/TOF MS system (Bruker Daltonics, Bremen, Germany) (3). For each spectrum, average of 1000 laser hits with 100Hz laser frequency was considered and system was calibrated by using peptide calibration mix (Bruker Daltonics, Peptide Calibration mixture). α-Cyano-4-hydroxycinnamic acid(α-CHCA) matrix was mixed with the samples, dropped on a gold-covered MALDI target plate and dried at room temperature. MS/MS analyses were performed and the results were analyzed with MASCOT search engine (v.2.2).

Real Time-PCR Analysis

DNA isolation was performed as described by the manufacturer (High Pure PCR Template Preparation Kit/Roche, Mannheim, Germany) from tissue samples (23). 2p24.3 (*MYCN*) amplification, 1p36 (*GNB1*) LOH, 11q23 (*ARCN1*) deletion and 17q25 (*SURVIVIN*) gain status of DNA samples were assessed by TaqMan real-time PCR (RT-PCR) (Roche LightCycler Nano Real-Time PCR Instrument, Germany) with custom designed TaqMan labelled primers for each corresponding gene region. The reference genes for each genetic region were again selected from the corresponding chromosomes. Briefly, 2p13.3 (*NAGK*) was used as a reference gene for N-MYC while 1p13.1 (*NGFB*), 11p11.2 (*MYBPC3*) and 17p13.1 (*TP53*) were used as reference genes for 1p36, 11q23 and 17q25 regions, respectively.

Statistics

Statistical analysis for the comparison of protein densities of different risk groups for spot 16 was performed by using student's t-test, while statistical analysis was performed automatically by the system during MASCOT search. According to the MASCOT analysis, data with the score >56 or the peaks within the white region (trustable region) of the results table are considered as statistically significant ($p<0.05$). Ions score is -10*Log(P), where P represents the probability of random match. Individual ion scores >37 indicate identity or extensive significant homology. Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

RESULTS

Outcome Data

In this study, we aimed to identify a marker protein that can be useful to distinguish different risk groups of NB. In order to eliminate individual differences, mononuclear cell pools of different NB risk groups were generated and protein profiles of these groups were compared. In total, 53 protein spots were found to be differentially expressed in different risk groups compared to the control group, of which nine protein spots resulted in a protein match in MASCOT search. Among those 9 proteins most of the protein signals

were further masked with fibrinogen-gamma or keratin-I-type, while one of the spots which gave a match with Superoxide Dismutase 2 and proposed to have a significant role in NB progression since its protein expression showed direct correlation with the risk classifications.

Main Results

The risk groups of 42 patients were determined according to the International Neuroblastoma Risk Group Stratification System (INRGSS). Mononuclear cells of 19 patients from high-risk NB group, 12 patients from intermediate-risk NB group, 11 patients from low-risk NB group and 10 normal children from control group (Supplementary Table 1) were pooled separately to construct the corresponding mononuclear cell pools of patients from different risk groups. Protein isolations were performed from these pools and protein concentrations were found to be 1762µg/ml, 2047µg/ml, 2051µg/ml and 2049µg/ml for control, low-risk, intermediate-risk and high-risk group, respectively.

2-D Gel electrophoresis performed in triplicates with equal amounts of protein for each group. Mass compatible silver staining, which can detect 5-10ng amounts of protein, was used for staining since less proteins detected with Coomassie blue staining. Mass compatible silver stained gels of different risk groups were compared with control group gels and the differentially expressed proteins were labeled. In total, 53 protein spots were found to be differentially expressed (up-regulated/down-regulated) in different risk groups compared to the control group. (Fig.1) These differentially expressed protein spots were common in all triplicate experiments and although most of them were common among different risk groups, some were unique to a single risk group (Fig. 1A, 1B, 1C).

Protein spots labeled from 1-10 were seen to be lost/downregulated in all risk groups compared to control groups. Protein spots 11-17 and 19-20 were found to be upregulated gradually in risk groups. Protein spot 18 found to be only upregulated in intermediate-risk group. Protein spots 21-24 increased in intermediate-risk and high-risk groups only while 25 found to be increased in all risk groups. Protein spot 26 is a newly appeared spot in intermediate-risk and high-risk groups while protein spots 27-33, 37-40 and 43-45 are new protein spots appeared in all risk groups. Protein spots 34-36, 41-42 and 46-53 newly appeared in high-risk group (Fig. 1C).

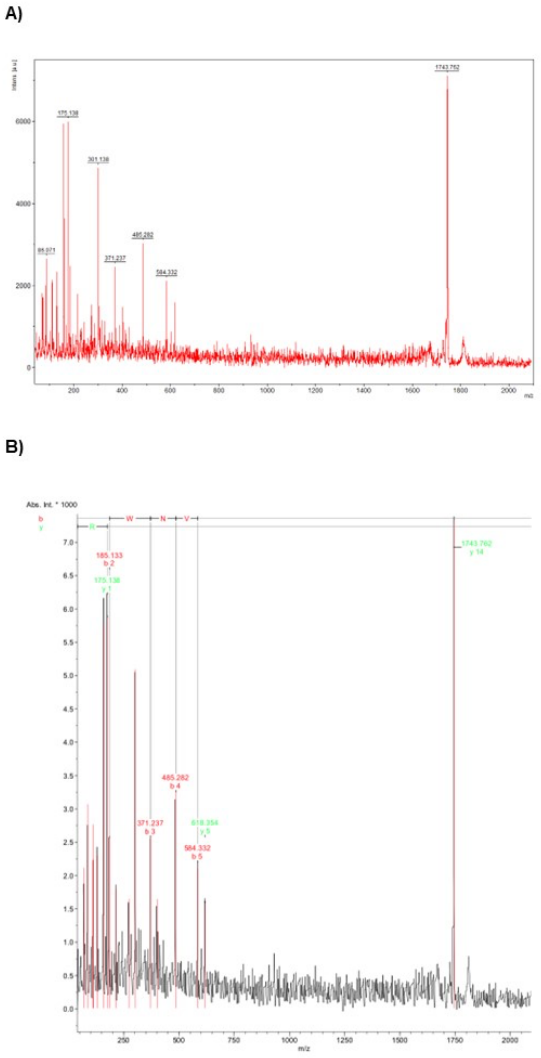


Figure 4. A) The MS/MS spectrum and **B)** the MS/MS spectrum analysis for the parental mass 1743.733 of spot 16

Among 53 protein spots, the analyzed proteins were mostly found to be within the green region of the MASCOT search result tables, which is a statistically insignificant area. Therefore, the results of these proteins are not shown since significant protein matches could not be observed. MASCOT search that is performed by protein and peptide BLAST method was performed by using two different protein databases (NCBI and Swiss Prot). Among 53 protein spots, 9 were found to give a significant protein matches in MASCOT search and 8 of these belong to either fibrinogen-gamma, keratin or beta-actin protein (Table 1, Supplementary Fig. 1-8). Since proteins such as fibrinogen and keratin are highly abundant in

Table 2. A) The peak list and **B)** the calculated masses for the MS/MS analysis of the parent mass 1743.733 of spot 16

A)

Peak	Mass	Intensity	Peak	Mass	Intensity
1	70.038	2030.008	2	86.071	2951.437
3	110.078	2656.938	4	112.082	2149.899
5	130.091	2205.526	6	159.111	5542.646
7	175.138	5648.861	8	185.133	2454.581
9	214.112	1725.337	10	273.134	1582.139
11	301.138	4904.570	12	371.237	2497.305
13	400.231	1576.346	14	485.282	3156.264
15	584.332	2024.274	16	618.354	1516.713
17	1743.762	7061.820			

B)

Calculated Masses:
AIWNVINWENVTER

N-Term.	Ion	a	b	y	dL	dI	i	wL	wI	a-NH3
1	A	44.049	72.044	**175.119**	2.003	16.018	44.049	116.045	**130.061**	27.023
2	I	157.134	**185.128**	304.162	115.087	129.102	**86.096**	245.088	259.104	140.107
3	W	343.213	**371.208**	405.209	**301.166**	315.182	**159.091**	346.136	360.151	326.186
4	N	457.256	**485.251**	504.278	415.209	429.224	87.055	445.204	459.220	440.229
5	V	556.324	**584.319**	**618.321**	514.277	528.293	72.080	559.247	573.263	539.298
6	I	669.408	697.403	747.363	627.361	641.377	**86.096**	688.290	702.305	652.382
7	N	783.451	811.446	933.442	741.404	755.420	87.055	874.369	888.385	766.425
8	W	969.530	997.525	1047.485	927.484	941.499	**159.091**	988.412	1002.428	952.504
9	E	1098.573	1126.568	1160.569	1056.526	1070.542	102.054	1101.496	1115.512	1081.547
10	N	1212.616	1240.611	1259.638	1170.569	1184.585	87.055	1200.564	1214.580	1195.589
11	V	1311.684	1339.679	1373.681	1269.637	1283.653	72.080	1314.607	1328.623	1294.658
12	T	1412.732	1440.727	1559.760	1370.685	1384.701	74.059	1500.687	1514.702	1395.706
13	E	1541.775	1569.770	1672.844	1499.728	1513.743	102.054	1613.771	1627.786	1524.748
14	R	1697.876	1725.871	**1743.881**	1655.829	1669.845	129.113	1684.808	1698.823	1680.849

blood, the signals from these proteins masked the ions of the original protein spots which limited the number of proteins to be detected.

The only spot that revealed a possible candidate marker protein was spot 16. Protein densities of this protein spot in different risk groups were analyzed and according to these results, *SOD2* expression showed 1.43, 1.81 and 2.20-folds increase in low-risk, intermediate-risk and high-risk group, respectively, which all found to be statistically significant ($p<0.005$) (Fig. 2). Further MS and MS/MS analysis of this spot revealed a significant match with Manganese-superoxide dismutase (SOD2) (Fig. 3 and 4). The MASCOT search results for this spot showed expected mass as 1742.7296, calculated mass as 1742.8740 with -82.82 ppm and no mis-cleavages, Both the MS spectrum (Fig. 3) and the MS/MS spectrum for the parental mass 1743.733 (Fig. 4) matched with Sod2 protein. Although only one peak gave a significant match with Sod2 in MS/MS analysis, since both MS and MS/MS analysis resulted in the same match, the results are considered as acceptable. The detailed peak list and calculated masses for the MS/MS analysis of the parent mass 1743.733 for spot 16 was given in Table 2 and Supplementary Table 2.

DISCUSSION

Solid tumors are known to exhibit hypoxia which may trigger the increase in oxidative environment (27). An imbalance in oxidation/antioxidation status was previously shown to occur in blood of children with neoplastic diseases including solid tumors (28). However, no studies exist so far that defines oxidation related parameters as indicators of severity or progression of NB.

In our study, expression of Sod2 protein showed a proportional increase in all risk-groups compared to control group. Several studies found SOD2 to be increased in several cancer types; however, its role in cancer is not clear yet. In some cases, such as breast cancer, high Sod2 levels were found to increase the risk of cancer development (29,30). In colorectal cancer, four folds of increase in *SOD2* expression was found in tumor cells compared to normal mucosa (31) as well as a link between high levels of sod2 and colorectal lymph node metastasis and low rates of 5-years of survival were shown (32). Our results are

also in correlation with these findings by showing increased sod2 levels in neuroblastoma patients especially in high-risk group of patients. Increase in sod2 levels but not Sod1 was found in SH-SY5Y NB cell lines upon induction of neurotoxicity with carbamates (33). Similarly, dopamine induced toxic effects in human SH-SY5Y NB cell lines were rescued with superoxide dismutase sod1 and sod2 (34). In SK-N-BE2 neuroblastoma cells, neuroglobin (NGB) was found to be co-localized in mitochondria with Sod2 during its function of neuroprotection through mitochondrial lipid raft-associated complexes (35) and reactive oxygen species induced cell death was shown to be inhibited by overexpression of *SOD2* in SH-SY5Y NB cells with a tumor stem cell-like phenotype (36). *SOD2* expression was also found to rescue the mitochondrial dysfunction in neuropathy patients (37). Therefore, increase in *SOD2* expression in correlation with increase in NB risk groups, may also be a rescue response of the cells with increased oxidative stress conditions. SOD2 signaling was shown to play an important role in neuronal oxidative stress induced by several agents (38) and its increased expression in stem cell-like phenotypes (36) is supportive of our data since the cellular phenotypes resemble more to stem cell-like phenotypes as the tumor progress in higher risk groups.

There are also contradictory findings about *SOD2* expression levels in cancer cells (39). In a study performed with plasma and mononuclear cells of patients with different malignancies, low levels of superoxide dismutase were found in some patients (40). In the same study, three neuroblastoma patients were assessed and similarly low levels of Sod2 were shown in these patients. In another study that is performed on 99 neuroblastoma patients, comparison of cancer and healthy cases revealed no significant difference in superoxide dismutase and glutathione peroxides levels (41). In SK-N-BE cells, oxidative metabolism that increase during differentiation was shown to lead increased Sod2 levels (42). In the same study, less detoxification enzyme levels were found in malign tumor cells compared to normal cells.

Dynamic epigenetic regulation of *SOD2* transcription may contribute to both decreased *SOD2* expression in cancer development and increased *SOD2* levels in tumor progression (43-45). NF-kB is also known to be an intronic enhancer of *SOD2* and increased Sod2 levels may be due to increased NF-kB activity as well. NF-kB signaling was shown to play a central role in neuroblastoma. Its activation was shown to be a potential mechanism in which cell metastasis is fostered (46). Therefore, increased SOD2 levels in blood may be due to increased activation of NF-kB in neuroblastoma as well.

In our study, we aimed to decrease the individual differences by constructing a sample pool for each risk group and to determine the protein expression differences among risk groups more generally. Construction of sample pools were previously used in some studies to eliminate the personal differences within a group (19) and pooling approach is considered as valid and potentially valuable (47). MS analysis of differentially expressed protein spots and MS/MS analysis of at least 3 peptides for each protein spot were performed by MALDI. Results of our analysis were limited by the presence of high amounts of fibrinogen protein in our samples, which suppressed the signals of other proteins. Small amounts of different peripheral blood proteins, which are difficult to be detected with Coomassie staining, resulted in low signals and made analysis difficult. Also, some protein spots can be subunits of the same protein and protein spots 11-14 are an example for this. These protein spots were found to belong to fibrinogen-beta and fibrinogen-gamma subunits. Presence of excess fibrinogen may cause contaminations to other protein spots; however, there is no way to eliminate fibrinogen from the total protein pool in this kind of methodologies. Due to the dominant presence of actin and fibrinogen proteins and presence of small amount of other proteins, resulted in determination of fibrinogen and actin in most of the protein spots.

Although in the presence of dominant proteins, our results revealed that SOD2 levels increased 2,2 folds in high risk group ($p<0.001$) compared to control group. High *SOD2* expression is most commonly associated with transcriptional up regulation in response to intra- and extra-cellular stimuli, including those associated with redox stress.

There are three major limitations of this study. Firstly, the number of patients is low, which is restricted due to the study population being pediatric patients. Secondly, although pooling process is a methodology that is used by several studies for the comparison of different groups, it may reduce the power of the analysis. Mainly the protein expressions from a pool sample is close to the mean expression of the individuals that make up the pool for most proteins

and the biological variance among individuals and among groups is found not to differ so much (47). However, further confirmation of the results at the individual level would be required for more exact data. Thirdly, ion masking was one of the most restricting limitations in protein identification during mass analysis. Since the samples studied were the peripheral mononuclear cells of the patients, proteins that are abundant in blood, such as fibrinogen and keratin, masked the identification of the main protein spots. Although many more proteins could be obtained, we ended up with a single protein due to this restriction. Using other mass spectrum analysis than MALDI, can be helpful to decrease such unwanted results. Individual assessment of patients is required, with a larger number of patients to further confirm the results. These are among our future plans for this study.

CONCLUSION

Increase in Sod2 levels in blood, either as an oxidative rescue or as a resemblance to stem cell-like phenotype, may lead to new diagnostic or prognostic approaches in neuroblastoma. However, more studies should be performed to clarify the molecular mechanisms that play role in *SOD2* expression in neuroblastoma. Conducting comparative studies of *SOD2* expression in NB on individual patients from different risk groups as well as understanding the molecular mechanisms behind its differential expression are among the future studies of our group.

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Author contribution: The project was constructed by Z.A., A.B.D., S.A. and N.O. A.B.D., Z.A., S.A. and N.O. designed, performed and analysed experiments. A.B.D and Z.A. wrote the manuscript, and S.A. and N.O. edited the manuscript.

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REFERENCES

1. Maris JM, Matthay KK. Molecular biology of neuroblastoma. J Clin Oncol 1999;17:22642279.
2. Olgun N, Kansoy S, Aksoylar S, Cetingul N, Vergin C, Oniz H, et al. Experience of the Izmir Pediatric Oncology Group on Neuroblastoma: IPOG-NBL-92 Protocol. Pediatr Hematol Oncol. 2003; 20(3):211-18.
3. Li C, Wu Z, Liu M, Pazgier M, Lu W. Chemically synthesized human surviving does not inhibit caspase-3. Protein Sci. 2008; 17(9):1624-29.
4. Berthold F. Neuroblastoma-90 (NB-90) protocol. German Pediatric Oncology Group (GPOG-NBL),1990.
5. Matthey KK, Perez C, Seeger RC, Brodeur GM, Shimada H, Atkinson JB, et al. Successful treatment of stage III neuroblastoma based on prospective biologic staging: A Children's Cancer Group study. J. Clin Oncol. 1998;16:1256.
6. Krona C, Ejeskär K, Abel F, Kogner P, Bjelke J, Björk E, et al. Screening for gene mutations in a 500 kb neuroblastoma tumor suppressor candidate region in chromosome 1p; mutation and stage-specific expression in UBE4B/UFD2. Oncogene. 2003;2343-51.
7. De Preter K, Vandesompele J, Menten B, Carr P, Fiegler H, Edsjö A, et al. Positional and functional mapping of a neuroblastoma differentiation gene on chromosome 11. BMC Genomics. 2005;6:97.
8. Wang Q, Diskin S, Rappaport E, Attiyeh E, Mosse Y, Shue D, et al. Integrative genomics identifies distinct molecular classes of neuroblastoma and shows that multiple genes are targeted by regional alterations in DNA copy number. Cancer Res. 2006;66(12):6050-6062.
9. Souzaki R, Tajiri T, Higashi M, Kinoshita Y, Tanaka S, Kohashi K et al. Clinical implications of a slight increase in the gene dosage of MYCN in neuroblastoma determined using quantitative PCR. Pediart Surg Int. 2008;24:1095-1100.
10. Spitz R, Hero B, Ernestus K, Berthold F. FISH analyses for alterations in chromosomes 1,2,3, and 11 define high-risk groups in neuroblastoma, Med Pediatr Oncol. 2003;41:30-35.
11. Shimada H, Chatten J, Newton WA Jr, Sachs N, Hamoudi AB, Chiba T, et al. Histopathologic prognostic factors in neuroblastic tumors: definition of subtypes of ganglioneuroblastoma and an age-linked classification of

neuroblastomas. J Natl Cancer Inst. 1984; 73(2):405-416.
12. Brodeur GM, Pritchard J, Berthold F, Carlsen NL, Castel V, Castelberry RP et al. Revisions of the international criteria for neuroblastoma diagnosis, staging and response to treatment. J Clin Oncol. 1993;11(8):1466-1477.
13. Brodeur GM, Seeger RC, Barrett A, Castleberry RP, D'Angio G, De Bernardi B, et al. International criteria for diagnosis, staging, and response to treatment in patients with neuroblastoma. J. Clin Oncol.1988;6(12):1874-1881.
14. Monclair T, Brodeur GM, Ambros PF, Brisse HJ, Cecchetto G, Holmes K et al. The International Neuroblastoma Risk Group (INRG) staging system: an INRG Task Force report. J Clin Oncol. 2009;27(2):298-303.
15. Demir AB, Aktas S, Altun Z, Ercetin P, Aktas TC, Olgun N. Questioning How to Define the "Ultra-High-Risk" Subgroup of Neuroblastoma Patients. Folia Biol (Praha). 2021; 67(1):1-9.
16. Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat Rev Drug Discov. 2009;8:579–591.
17. Andersen JK. Oxidative stress in neurodegeneration: cause or consequence? Nat Med. 2004; 10(Suppl): S18–S25.
18. Saini RK, Attarha S, da Silva Santos C, Kolakowska J, Funa K, Souchelnytskyi S. Proteomics of dedifferentiation of SK-N-BE2 neuroblastoma cells. BBRC. 2014;454:202-209.
19. Jung EJ, Park HC, Chung KH, Kim CW. Proteomic analysis of SP600125-controlled TrkA-dependent targets in SK-N-MC neuroblastoma cells: Inhibition of TrkA activity by SP600125.Proteomics. 2014;14:202-215.
20. Kim PY, Tan O, Diakiw SM, Carter D, Sekerye EO, Wasinger VC, et al. Identification of plasma Complement C3 as a potential biomarker for neuroblastoma using a quantitative proteomic approach. Journal of Proteomics. 2014;96:1-12.
21. DeNardo BD, Holloway MP, Ji Q, Nguyen KT, Cheng Y, Valentine MB, et al. Quantitative phosphoproteomic analysis identifies activation of the ret and igf-1r/ir signaling pathways in neuroblastoma. Plos One. 2013;8(12): e82513.
22. Fuchs D, Vafeiadou K, Hall WL, Daniel H, Williams CM, Schroot JH, Wenzel U. Proteomic biomarkers of peripheral blood mononuclear cells obtained from postmenopausal women undergoing an intervention with soy isoflavones. Am J Clin Nutr. 2007;86:1369-75.
23. Mass Spectrometry Compatible Silver Staining. Center for Biotechnology, Turku Bioscience, University of Turku, Findland. 2009.
24. O'Connell KL, Stults JT. Identification of mouse liver proteins on two-dimensional electrophoresis gels by matrix-assisted laser desorption/ionization mass spectrometry of in situ enzymatic digests. Electrophoresis. 1997;18(3-4):349-59.
25. Gharahdaghi F, Weinberg CR, Meagher DA, Imai BS, Mische SM. Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity. Electrophoresis. 1999;20(3):601-605.
26. Haigis MC, Yankner BA. The aging stress response. Mol Cell. 2010;40:333–344.
27. Di Rita A, Strappazzon F. Neuroblastoma and oxidative stress: From pathogenesis to in vitro models of neurodegeneration. Oxidative stress and Dietary Antioxidants in Neurological Diseases. Academic Press, Elsevier, 2020;67-79.
28. Drabko K, Kowalczyk J. Imbalance between pro-oxidative and anti-oxidative processes in children with neoplastic disease. Med Wieku Rozwoj. 2004;8:217-23.
29. Bica CG. de Moura da Silva LL, Toscani NV, da Cruz IB, Sá G, Graudenz MS, Zettler CG. MnSOD gene polymorphism association with steroid-dependent cancer. Pathol. Oncol. Res. 2009;15:19-24.
30. Alateyah N, Gupta I, Rusyniak R.S, Ouhtit A. SOD2, a potential transcriptional target underpinning CD44-promoted breast cancer progression. Molecules. 2022;27:811.
31. Janssen AM, Bosman CB, Kruidenier L, Griffioen G, Lamers CB, van Krieken JH, et al. Superoxide dismutases in the human colorectal cancer sequence. J. Cancer Res. Clin. Oncol. 1999;125:327-335.
32. Nozoe T. Honda M, Inutsuka S, Yasuda M, Korenaga D. Significance of immunohistochemical expression of manganese superoxide dismutase as a marker of malignant potential in colorectal carcinoma. Oncol Rep.2003;10:39-43.
33. Khan Z, Akhtar Ali S. Increased mitochondrial superoxide dismutase 2 level but not cytosolic

Sod1 is associated with oxidative stress of SH-SY5Y neuroblastoma cells induced by carbamates pesticide. Eur J Mol Clin Med. 2020;7(11):6774-6778.
34. Biosa A, De Lazzari F, Masato A, Filograna R, Plotegher N, Beltramini M, et al. Superoxide dismutases SOD1 and SOD2 rescue the toxic effect of dopamine-derived products in human SH*SY5Y neuroblastoma cells. Neurotoxicity Res. 2019;36:746-755.
35. Garofalo T, Ferri A, Sorice M, Azmoon P, Grasso M, Mattei V, et al. Neuroglobin overexpression plays a pivotal role in neuroprotection through mitochondrial raft-like microdomains in neuroblastoma SK-N-BE2 cells. Mol and Cel Neurosci. 2018;88:167-176.
36. Ruggeri P, Farine A.R, Di Ianni N, Cappabianca L, Ragone M, Ianni G, et al. The TrkAIII oncoprotein inhibits mitochondrial free radical ROS-induced death of SH-SY5Y neuroblastoma cells by augmenting SOD2 expression and activity at the mitochondria, within the context of a tumour stem cell-like phenotype. Plos One. 2014; 9(4):e94568.
37. Zhou Q, Yao S, Yang M, Guo Q, Li Y, Li Lei, et al. Superoxide dismutase 2 ameliorates mitochondrial dysfunction in skin fibroblasts of Leber's hereditary optic neuropathy patients. Front Neurosci. 2022;16:917348.
38. Liu Z, Xu S, Ji Z, Xu H,Zhao W, Xia Z, et al. Mechanistic study mtROS-JNK-SOD2 signaling in bupivacaine-induced neuron oxidative stress. Aging.2020;12(13):13463-13476.
39. Azzolin VF, Cadoná FC, Machado AK, Berto MD, Barbisan F, Dornelles EB, et al. Superoxide-hydrogen peroxide imbalance interferes with colorectal cancer cells viability, proliferation and oxaliplatin response. Toxicology in Vitro. 2016;32:8-15.
40. Abdel-Aziz A.F, El-Naggar M.M. Superoxide dismutase activities in serum and white blood cells of patients with some malignancies. Cancer Letters.1997;113:61-64.
41. Stachowicz-Stencel T, Synakiewicz A, Owczarzak A, Aleksandrowicz-Wrona E, Sliwinska A, Lysiak-Szydlowska W, Balcerska A. The antioxidant status and response to therapy in children with soft tissue sarcomas and neuroblastoma. Pediatr. Blood Cancer. 2011;57:561-568.
42. Steinkühler C, Mavelli I, Melino G, Piacentini M, Rossi L, Weser U, Rotilio G. Antioxygenic enzyme activitites in differentiating human neuroblastoma cells. Annals. New York Academy of Sciences.1988;551:137-140.
43. Hitchler MJ, Oberley LW, Domann FE. Epigenetic silencing of SOD2 by histone modifications in human breast cancer cells. Free Radic. Biol. Med. 2008;45:1573–1580.
44. Cyr AR, Hitchler MJ, Domann FE. Regulation of SOD2 in cancer by histone modifications and CpG methylation: Closing the loop between redox biology and epigenetics. Antioxid. Redox Signal. 2013; 18:1946–1955.
45. He C, Danes J.M, Hart P.C, Zhu Y, Huang T,Luelsdorf de Abreu A, et al.SOD2 acetylation on lysine 68 promotes stem cell reprogramming in vreast cancer. PNAS. 2019;116(47):23534-23541.
46. Zhi Y, Lu H, Duan Y, Sun W, Guan G, Dong Q, Yang C. Involvement of the nuclear factor-κB signaling pathway in the regulation of CXC chemokine receptor-4 expression in neuroblastoma cells induced by tumor necrosis factor-α. International Journal of Molecular Medicine. 2015;35:349-357.
47. Diz AP, Truebano M, Skibinski DOF. The consequences of sample pooling in proteomics: An empirical study. Electrophoresis. 2009;30:2967-2975.

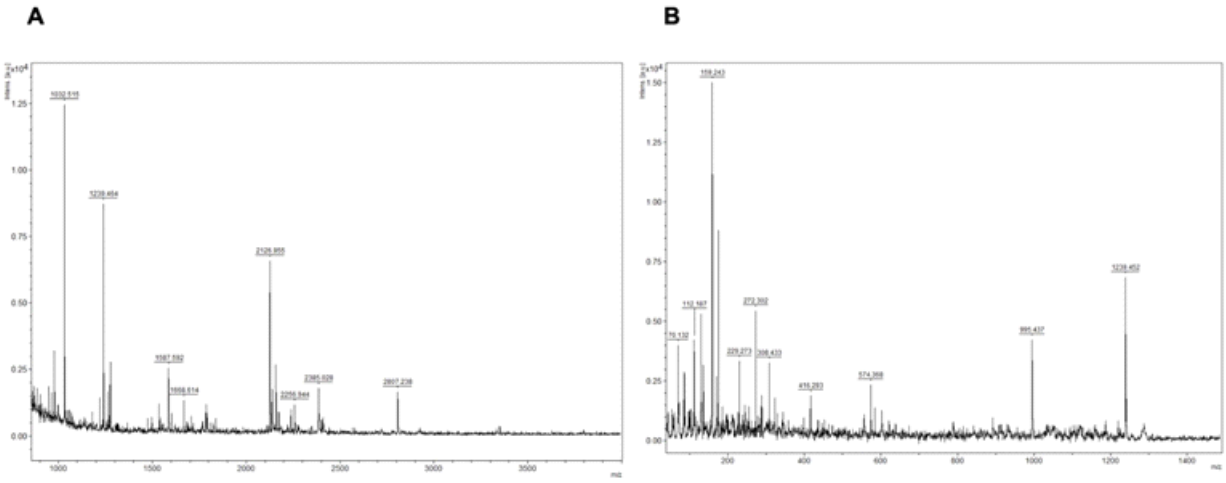
Supplementary Table 1. Detailed data of the patients used in this study.

Patient ID	Source University of the Sample	Age (months)	Gender	Risk Group
NB110	Kanuni Sultan Suleyman Training and Research Hospital	21	Female	High
NB193	Dr. Sami Ulus Training and Research Hospital	48	Male	High
NB245	-	30	Male	High
NB275	Celal Bayar University Hospital	18	Male	High
NB347	Erciyes University Hospital	30	Male	High
NB369	Hacettepe University Hospital	10	Female	High
NB379	Akdeniz University Hospital	56	Female	High
NB438	Ankara University Hospital	36	Female	High
NB442	Ok Meydani Training and Research Hospital	54	Male	High
NB446	Hacettepe University Hospital	28	Female	High
NB127	Ege University Hospital	36	Male	High
NB246	Ankara University Hospital	40	Female	High
NB351	Dokuz Eylul University Hospital	12	Male	High
NB371	Ege University Hospital	40	Female	High
NB352	Hacettepe University Hospital	49	Female	High
NB388	Ege University Hospital	60	Male	High
NB456	Sisli Hamidiye Etfal Training and Research Hospital	54	Male	High
NB402	Uludag University Hospital	48	Male	High
NB439	Akdeniz University Hospital	22	Female	High
NB225	Marmara University hospital	1,5	Female	Intermediate
NB320	Medical Park Bahcelievler Hospital	7 (days)	Male	Intermediate
NB321	Erciyes University Hospital	6	Female	Intermediate
NB228	Hacettepe University Hospital	26	Male	Intermediate
NB376	Hacettepe University Hospital	5	Male	Intermediate
NB432	Hacettepe University Hospital	7	Male	Intermediate
NB354	Ankara Pediatric Hematology Oncology Hospital	24	Female	Intermediate
NB392	Erciyes University Hospital	1,5	Male	Intermediate
NB403	Goztepe Training and Research Hospital	11	Male	Intermediate
NB410	Erciyes University Hospital	15	Female	Intermediate
NB412	Göztepe Training and Research Hospital	8	Male	Intermediate
NB462	Ok Meydanı Training and Research Hospital	4	Female	Intermediate
NB224	Dr. Sami Ulus Training and Research Hospital	13	Female	Low
NB358	Medical Park Bahcelievler Hospital	-	Female	Low
NB363	Uludag University Hospital	4	Male	Low
NB413	Ankara University Hospital	24	Female	Low
NB448	Hacettepe University Hospital	8	Male	Low
NB264	Ege University Hospital	48	Male	Low
NB319	Dr. Sami Ulus Training and Research Hospital	7	Female	Low
NB409	Erciyes University Hospital	42	Male	Low
NB390	Ege University Hospital	-	Male	Low

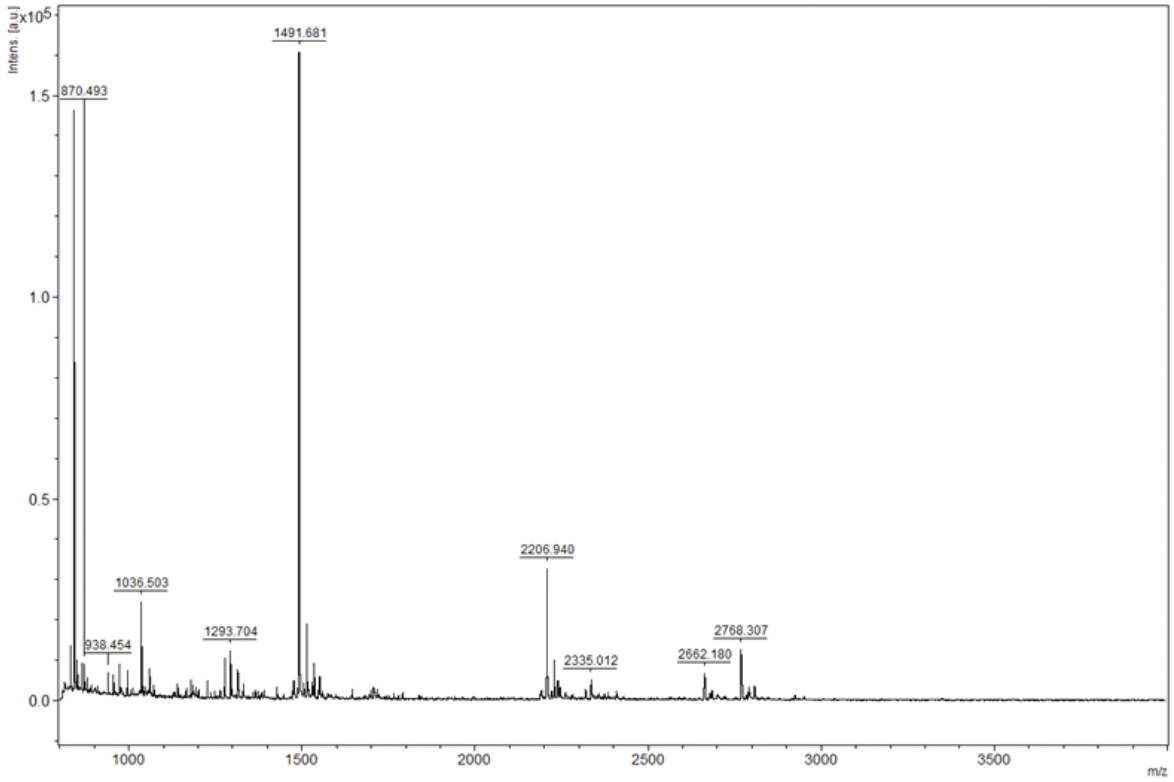
Supplementary Table 1continue

NB461	Uludag University Hospital	24	Female	Low
NB384	Dr. Sami Ulus Training and Research Hospital	11	Female	Low
CNTRL1	Dokuz Eylul University Hospital	48	Female	N/A (Control)
CNTRL2	Dokuz Eylul University Hospital	36	Female	N/A (Control)
CNTRL3	Dokuz Eylul University Hospital	60	Female	N/A (Control)
CNTRL4	Dokuz Eylul University Hospital	48	Female	N/A (Control)
CNTRL5	Dokuz Eylul University Hospital	36	Female	N/A (Control)
CNTRL6	Dokuz Eylul University Hospital	48	Male	N/A (Control)
CNTRL7	Dokuz Eylul University Hospital	24	Male	N/A (Control)
CNTRL8	Dokuz Eylul University Hospital	48	Male	N/A (Control)
CNTRL9	Dokuz Eylul University Hospital	60	Male	N/A (Control)
CNTRL10	Dokuz Eylul University Hospital	24	Male	N/A (Control)

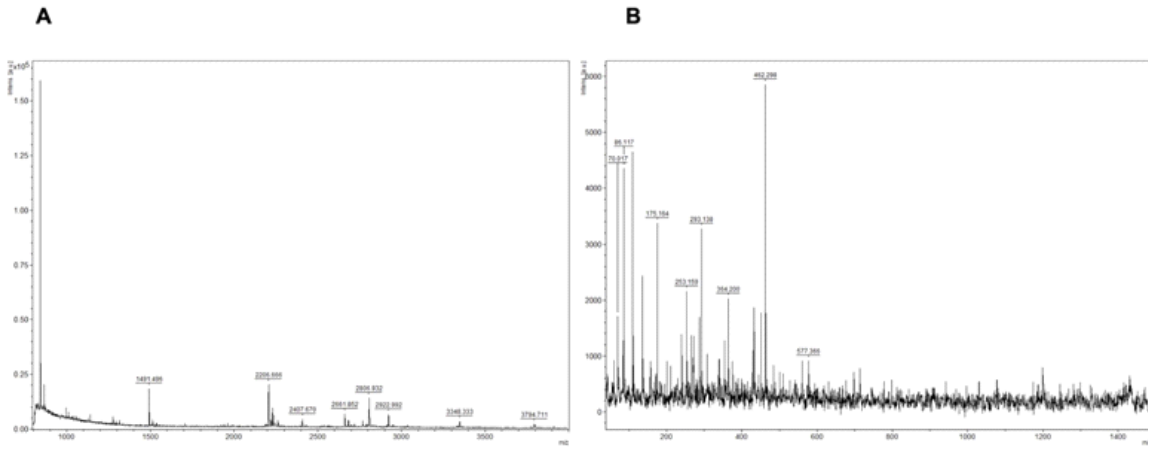
Supplementary Figures



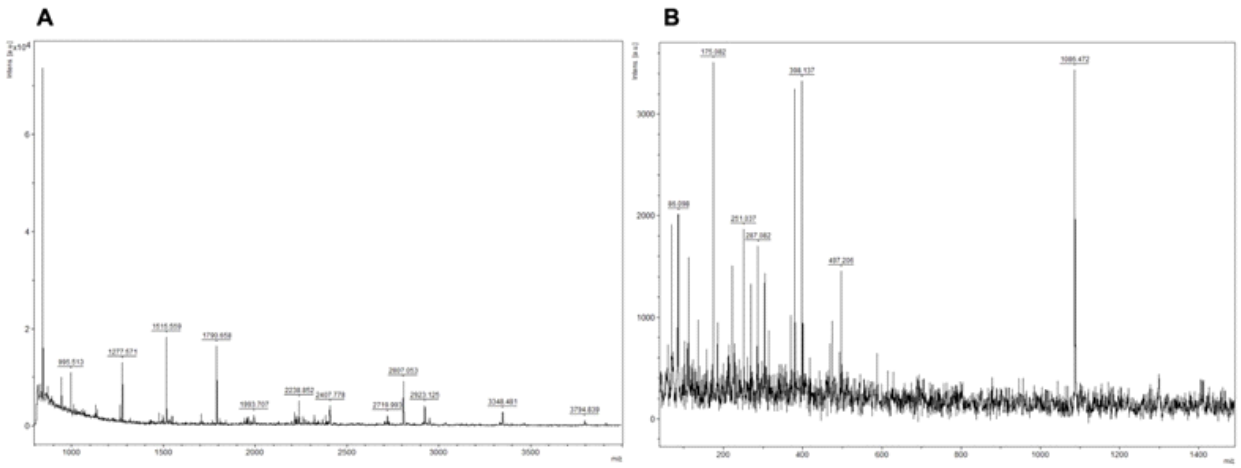
Supplementary Figure 1. A) The MS spectrum and **B)** the MS/MS spectrum for the parental mass 1239 of spot 11 that gave a match with β -fibrinogen



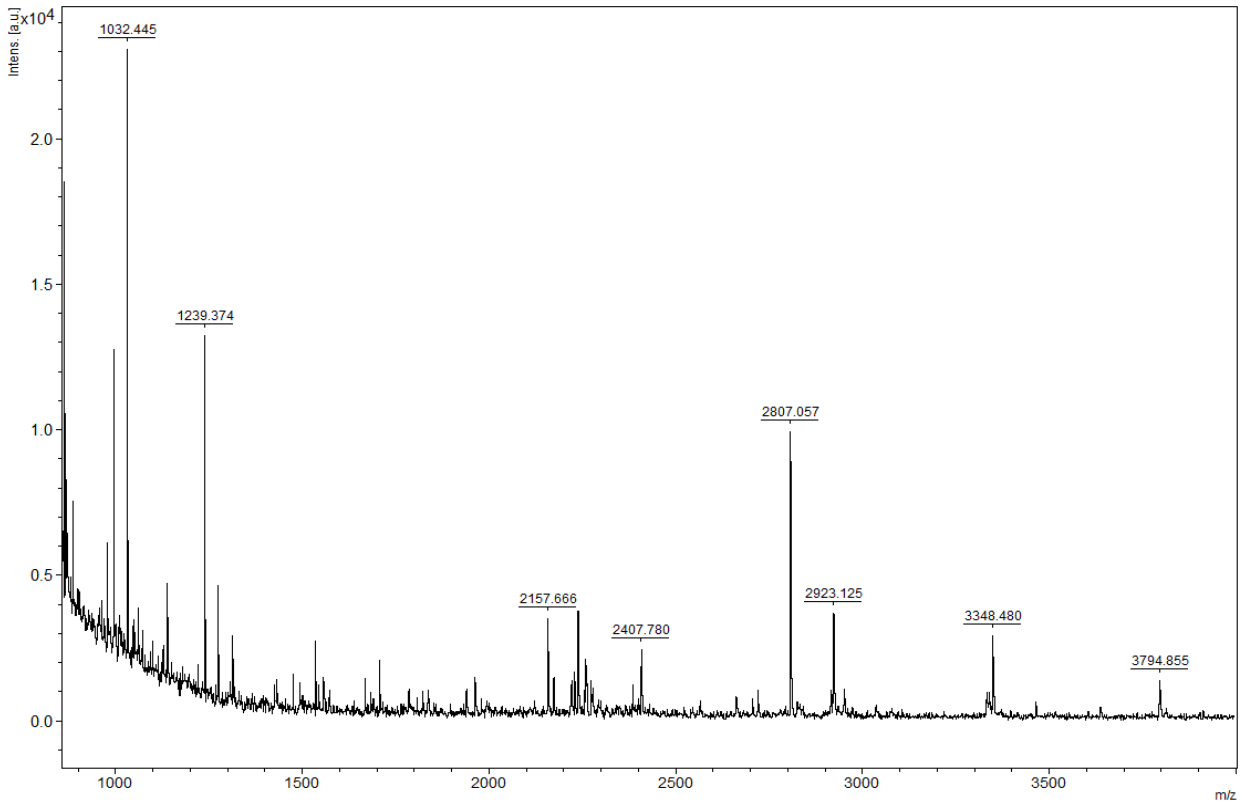
Supplementary Figure 2. MS spectrum of spot 15 that gave a match with Fibrinogen-gamma



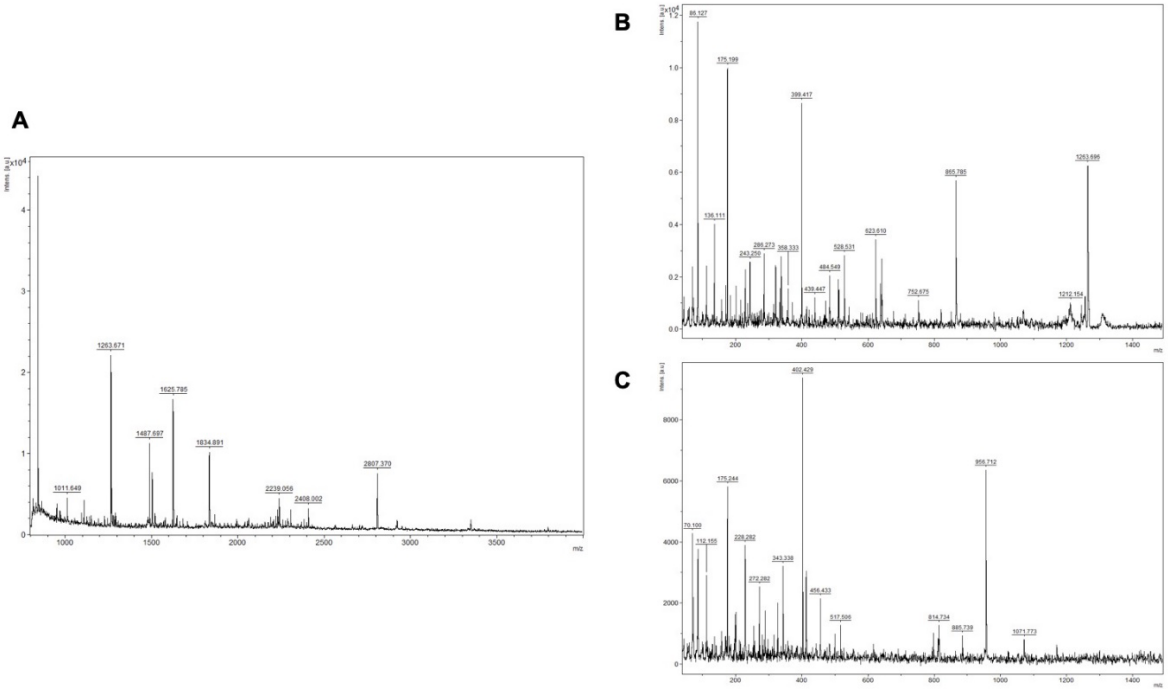
Supplementary Figure 3. A) The MS spectrum and **B)** the MS/MS spectrum for the parental mass 1491 of spot 17 that gave a match with Fibrinogen-gamma.



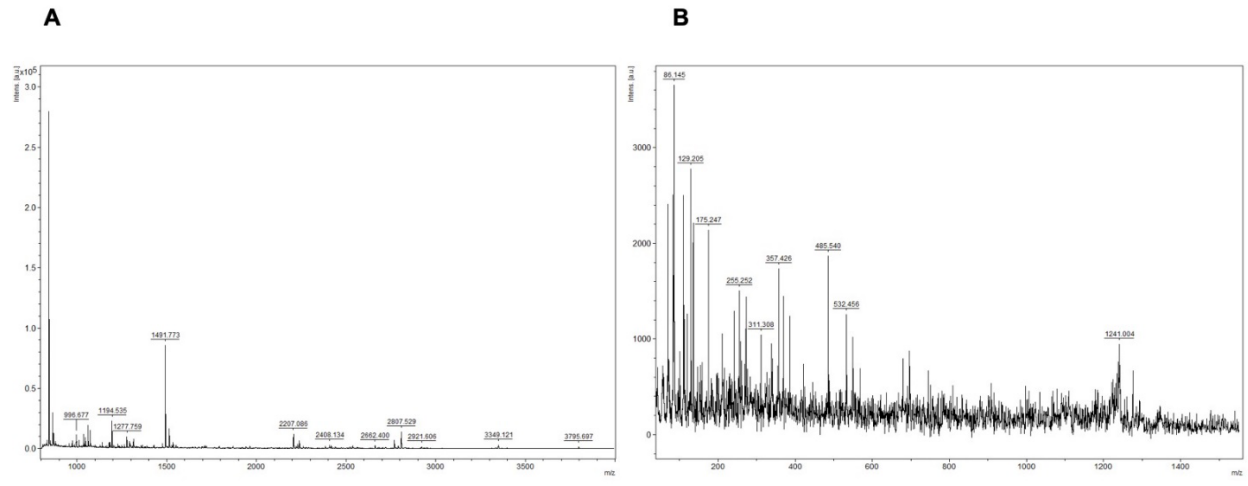
Supplementary Figure 4. A) The MS spectrum and **B)** the MS/MS spectrum for the parental mass 1790 of spot 18 that gave a match with mutant- β actin/ actin- β



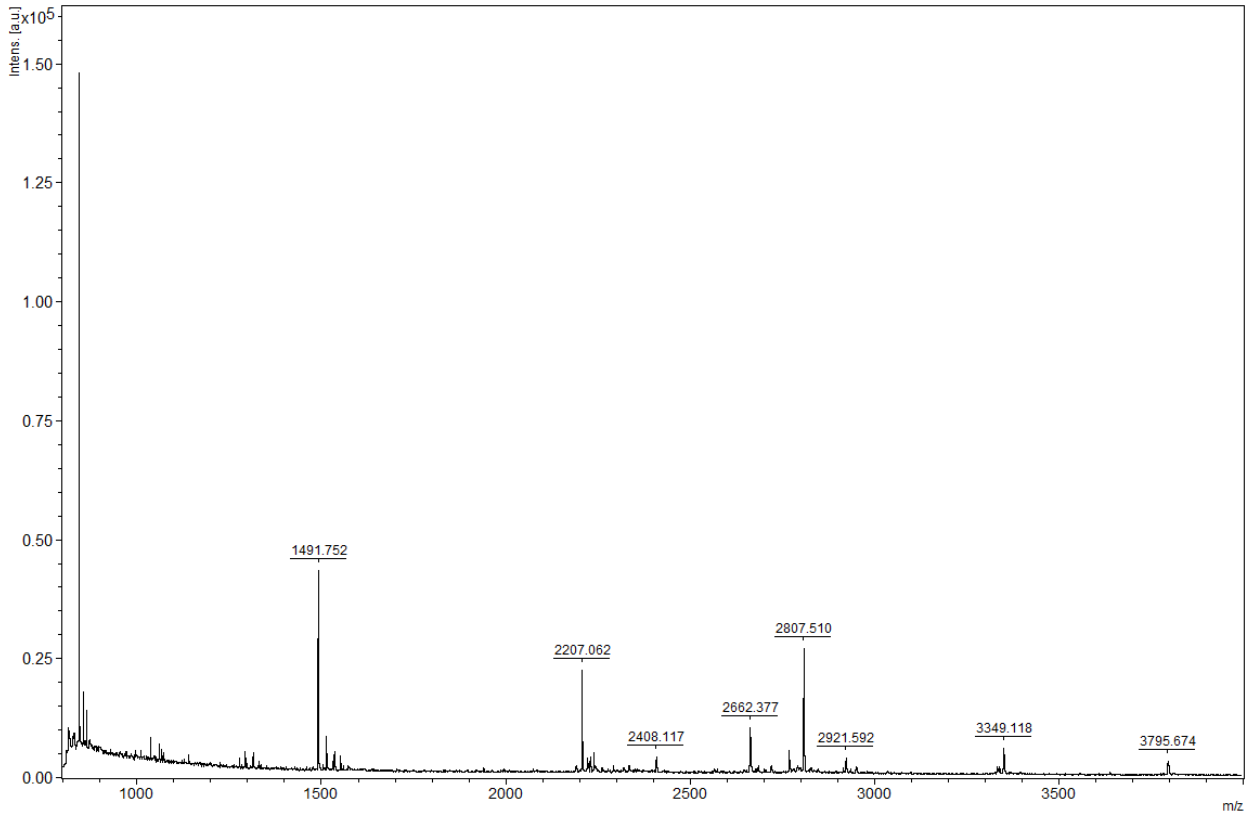
Supplementary Figure 5. MS spectrum of spot 20 that gave a match with mutant- β actin/ actin- β



Supplementary Figure 6. A) The MS spectrum of spot 22 and the MS/MS spectrum for the parental masses **B)** 1263 and **C)** 1625 that gave a match with Type-I-Keratin



Supplementary Figure 7. A) The MS spectrum and **B)** the MS/MS spectrum for the parental mass 1293 of spot 31 that gave a match with Fibrinogen-gamma



Supplementary Figure 8. MS spectrum of spot 32 that gave a match with Fibrinogen-gamma