 doi:10.20944/preprints202201.0292.v1

Article

Frutalin Affinity Chromatography on Sepharose Gel as a Strategy for the Identification of Possible Tumor Markers in Myelodysplastic Syndromes

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Abstract: Myelodysplastic syndromes (MDS) are diseases that occur when blood-producing cells in the bone marrow are damaged; such damage can affect one or more types of blood cells. Common types of MDS are refractory anemia with ring sideroblasts and refractory anemia with excess blasts (MDS-RS and MDS-EB, respectively). This work analyzed the proteomics of the medullary plasma of 10 patients with MDS-RS and MDS-EB compared to healthy control people. Overexpressed proteins that may be potential candidates for biological markers for the evaluation, study, and diagnosis of these diseases have been identified. These samples were subjected to immunodepleting, concentrated, and digested for further analysis by mass spectrometry. The ratios between selected groups and healthy people were calculated. Seven overexpressed proteins in both syndromes were identified as potential biomarker candidates: vitronectin (VTN), (2) fibrinogen (FGA), (3) pregnancy zone protein (PZP), (4) kininogen (KNG1), (5) immunoglobulin lambda chain (IGLL1), (6) complement factor C4b, and (7) hemopexin (HPX). A modified affinity chromatographic column with lectin frutalin (FTL) was used for non-depleted samples. Immunoglobulin M (IgM) was expressed in the samples from both syndromes. Surprisingly, IgM from patients with syndromes was over retained on the frutalin (FTL) column when compared with the control group. We further hypothesized that over retention of this protein by the FTL is due to the presence of α-galactosidic residues in the IgM of MDS-RS and MDS-EB patients. Differential recognition of proteins on non-depleted samples from the use of FTL appears to be a powerful tool for proteomic analysis.

Keywords: Cancer biomarker; Proteomic analysis, Myelodysplastic syndromes, Frutalin, MDS-RS, MDS-EB

1. Introduction

In recent decades with the increase in life expectancy in some countries, the prevalence of cancer has increased. With its impact on public health, an interest in seeking alternatives to prevent or treat it early have evolved [1-4]. The Global Cancer Statistics (GLOBOCAN) foresees that in 2040, the number of people affected by some type of cancer will be able to reach 28.4 million cases worldwide[1] with about 12.0 million cancer deaths

 doi:10.20944/preprints202201.0292.v1

[1]. It is considered one of the main causes of death in the whole world representing a global area with economic consequences [5-8], both for developed and developing countries [1,9-11]. Myelodysplastic syndromes (MDS) to a large spectrum of clonal hematopoietic stem cell (HSC) disorders characterized by the presence of cytopenia(s) [12,13], normo/hypercellular bone marrow (BM) with dysplasia, and ineffective hematopoiesis. The incidence of MDS increases dramatically with age to 25 to 50 cases per 100,000 per year in populations aged 60 years, which makes MDS the most common bone marrow cancer in the western part of the world. The incidence and clinical characteristics of patients with MDS varies by geographical area, and these variations have been attributed to genetic, occupational, lifestyle, and environmental factors, none of which have been fully elucidated [12,14].

According to the World Health Organization (WHO) 2016, seven subtypes of MDS exist: (1) MDS with single lineage dysplasia, (2) MDS with ring sideroblasts, (3) MDS with multilineage dysplasia, (4) MDS with excess blasts-1, (5) MDS with excess blasts-2, (6) unclassifiable MDS, and (7) MDS with isolated 5q. MDS is a very heterogeneous disorder, and its clinical course is highly variable, ranging from stable disease over 10 or more years (for cases with ring sideroblasts and MDS with single lineage) to death within a few months due to leukemic transformation (for cases with excess blasts). The outcome of patients with MDS is one of the most critical points due to its very impressive clinical heterogeneity, making the search for possible biomarkers very important for predicting its course. The most used system of prognosis is the Revised International Prognostic Scoring System (R-IPSS), which is valuable, but sometimes imprecise [15].

Among the known post-translational modifications, phosphorylation, acetylation, alkylation, methylation, sulfation, iso-prenylation, glycosylation, ubiquitination, and formation of disulfide bridges have been described. Glycosylation is the most common form of enzyme-mediated post-translational modifications (MPT) in which oligosaccharide side chains are covalently attached to either side of the asparagine (N- or O-linked) serine/threonine chain. The first linkage is the most prevalent [16]. The protein-bound glycan fraction is often essential for cell recognition, signaling, and interactive events and may play an important role in protein folding and conformation [17-20].

The surfaces of mammalian cells contain carbohydrate complexes, collectively known as the glycocalyx, which is made up of simple monosaccharides in various oligosaccharide arrangements. Glycans have high complexity and diversity and mediate cell communication with the outside, a role of great importance in many biological processes, such as cell–cell adhesion, cell trafficking (red blood cells, polymorphonuclear neutrophil, lymphocytes), regulation of the catabolism pathway of glycosylated molecules, and maintenance of the specific conformation of membrane molecules or in solution in addition to acting as receptors for hormones and other biologically active molecules [21].

The biological significance of MPT has been well established. Among all types of MPTs, glycosylation is the most abundant, frequent, and complex. More than half of the serum proteins and a vast majority of membrane proteins are believed to be glycosylated [22]. Virtually all membrane and secreted proteins are glycosylated, and the presence of these carbohydrate chains improves their water solubility, contributes to the correct orientation of the molecule, protects them from proteases, and participates in intracellular transport. On the other hand, glycans affect protein recognition by specific receptors or can themselves be recognized by glycan binding proteins, the lectins. So, it is natural that glycosylation constitutes one of the most important modifications in newly formed proteins [23]. Changes in glycan structures can be associated with many physiological and pathological events, such as cell growth, migration, and differentiation. Consequently, the aberrant glycosylation that occurs in cancer cells can influence cell proliferation, adhesion, and motility in addition to angiogenesis and metastasis. Neoplastic transformation is often associated with the expression of antigens in cancer tissues rather than normal tissues [23,24]. Biological events, such as the onset of a disease, are often accompanied by changes not only in protein expression, but also by rapid and dynamic changes in their

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glycosylation patterns, thus making glycans qualitative biomarkers of health and disease [22].

Lectins (proteins of non-immune origin that specifically recognize carbohydrates) are capable of specifically binding to residues present in certain polypeptide segments [25]. Affinity chromatography with lectins immobilized on inert supports combined with protein identification by mass spectroscopy (MS) is a consistent, reproducible, and reliable method and is considered an excellent tool for comparative serum studies [26,27]. Its main advantage for separating proteins with different glycosylation patterns is its simplicity and low cost [28]. Frutalin (FTL) is a protein in the lectin family and is obtained from the seeds of *Artocarpus incisa*, which specifically binds to α-D-galactose [29]. FTL has been successfully used in immunobiology research for the recognition of cancer-associated oligosaccharides with their specificity of anomeric recognition for alpha galactosidic residues [17,18,29-33].

The aim of this report was to detect a panel of proteins that could explain the highly variable clinical course of MDS based on a comparison of proteomes from healthy people with those of patients with MDS. This comparison was made using results from the proteomic analysis of serum samples from bone marrow of patients with MDS (cases with ring sideroblasts and cases with excess blasts). On the other hand, affinity chromatography of undepleted samples was performed on FTL with the aim of detecting which proteins are prone to α-glycosylation for which visible results were found.

2. Results and Discussion

For identification and comparison of depleted fractions of the MDS-RS (n= 10) and MDS-EB (n= 10) pools compared with the control group (n= 10), 46,221 peptides were identified with 42,443 peptides having a mass error < 10 ppm. Thirty-one and 33 proteins for MDS-RS and MDS-EB were identified, respectively, (Figure A1 and A2) when compared with the control. Proteins that showed a significant increase in expression in one group when compared to the other were described as over expressed (ratio ≥ 1.5), proteins with a significant decrease in expression were described as under expressed (ratio ≤ 0.5), and all other proteins with non-significant expression changes (0.5 > ratio < 1.5) were described as a non-significant expression change. Some overexpressed proteins in MDS-EB were also overexpressed in MDS-RS and are summarized in Table 1.

Table 1. Overexpressed at least 1.5 times protein in myelodysplastic syndromes with ring sideroblasts (MDS-RS) or refractory anemia with excess blasts (MDS-EB) versus control.

Accession	Ratio MDS- RS	Ratio MDS-EB	Protein
A8K3E4_HUMAN	2,75	1,55	FGA
B4DPP8_HUMAN	3,35	2,01	KNG1
B4E1D8_HUMAN	1,82	2,14	C4b
HEMO_HUMAN	**6,75**	**4,22**	**HPX**
PZP_HUMAN	3,03	2,80	PZP
Q8N355_HUMAN	**12,06**	**9,49**	**IGLL1**

VTNC_HUMAN	1,88	1,6	VTN

FGA = Fibrinogen; KNG1 = Kininogen-1; C4b = Complement Component C4b; HPX = Hemopexin; PZP = Pregnancy zone protein; IGLL1 = Immunoglobulin lambda-like polypeptide; VTN = Vitronectin.

The overexpressed protein could be grouped into coagulation proteins [34] (vitronectin, fibrinogen, pregnancy zone protein, and kininogen [VTN, FGA, PZP, and KNG1, respectively]), proteins associated with defense functions [35] (immunoglobulin Lambda chain and complement factor C4b [IGLL1 and C4b, respectively]), and hemopexin (HPX). It seems that the overexpression of these proteins is not isolated biochemical events as the association between them appears to be correlated (Figure 1).

HPX has been suggested as an indicator of the status of the extracellular matrix and has been reported as a very important protein in healing processes [36]. Interactions between the domains of hemopexins form the basis for positive or negative interference with the formation of molecular complexes in the extracellular matrix and therefore, can be therapeutically exploited in cancer. In this work, the protein was overexpressed in two types of MDS possibly playing a useful role in tumor growth as it has the capacity to transform the entire molecular context of the extracellular matrix. The mutual correlation of overexpression with the presence of the MDS makes this protein a potential tumor biomarker.

Pro-coagulant proteins (VTN, FGA, PZP, KNG1) in MDS-RS [37] and -EB [38] play an essential role in hemostasis. Excessive expression of these compared to the control group was found, and it was suggested that they can be used as a biomarker for the disease. Similar behavior was observed in children with acute lymphoblastic leukemia; hence, the symptoms are more common and are only venous thromboembolism [39,40]. In addition, Rau et al. [40] detailed that the decrease or dysfunction of some of VTN, FGA, PZP, KNG1 factors can trigger bleeding or thrombotic states associated with cancer.

VTN induces cell differentiation in endoderm cells. In human plasma, malignant differentiation of lung and breast cancer has been reported [41,42] VTN mediates its effects predominantly through $\alpha V\beta 3$ integrin and as a result, causes a change in the β-catenin localization.[43] Primary lung cancer has been associated with overexpression of β-catenin protein in tissues. It is possible that overexpression of VTN protein in MDS-RS and MDS-EB contributed to tumor differentiation processes and disease progression.

An extensive discussion in the literature about the role of the PZP protein as a biomarker in cancer exists [43]. In light of reports by various investigators concerning PZP, a positive correlation between severe serum snows and breast tumors was found [44]. Furthermore, the tissue of the breast tumor seems to synthesize PZP, and serial doses of PZP plasma have been suggested to accompany the dissipation of non-cancerous micrometastatic breast tumors [45]. However, other reports have been incapable of reproducing these results, which suggests that PZP is useless as a tumor marker [46]. Also, recent reports on the preparation and immunological reactivity of PZP allow us to presume that many previous studies may have been influenced by non-specific antibodies and/or patterns of contaminated proteins [47]. However, PZP protein overexpression in patients with MDS-RS and MDS-EB suggests that it may be useful as a tumor marker for plasma-medullary studies.

Seraglia *et al.* [48] reported KNG1 as a potential new non-plasma marker for colorectal cancer. KNG1 is a multifunctional protein that plays an important pathophysiological role in many processes and has been described as a possible biomarker for fibrinolysis and oncogenesis [49,50]. In addition, Umemura and coworkers reported KNG1 protein as a possible tumor marker for colorectal cancer [51]. This current study reported a protein with super expression for the studied myeloblastic syndromes studied and also proposed it as interesting protein that could be part of a protein panel used to study these syndromes.

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Proteins of the complement system and immunoglobulins in MDS-RS and MDS-EB were identified as overexpressed proteins associated with the complement system (C4b). The activation mechanisms of the complement system are already known and have been described in various studies [52,53]. The complement system is an important component of the innate and adaptive immune system and is composed of more than 40 plasma and membrane proteins. This finding is more important in the complement system or in reconstitution of pathogenic agents in which the activation of the chemotaxis of leukocytes and the induction of cell lysis by formation of the membrane attack complex (MAC). Due to the large number of genetic and epigenetic alterations associated with carcinogenesis, neoplastic transformation can increase the capacity of the malignant cells to activate complement [54,55], a fact that can explain the overexpression of C4b, which also agrees with other works that report or increase expression of complement proteins in lung cells in cancer [56].

Traditionally, the only source of Igs was believed to be mature B lymphocytes. Although some researchers have reported that Igs could also be detected in carcinoma cells, which are derived from epithelium. Recently, a study demonstrated that human cancer cell lines express the IgG variable region of mRNA [57]. Immature B lymphocytes generate diversity in the antibody repertoire and lead to reorganization of the variable chain of Ig. Some important proteins for genetic recombination that generate variability in Igs, such as recombination-activating gene (RAG) proteins, have been found, a finding that allows us to think that they may be present in cancer that could hypothetically generate Ig. Qiu *et al.* [57] found gene expression of RAG1 and 2 in three epithelial cancer cell lines. In this sense and with subsequent studies, it was found that other types of Ig could also be produced by cancer cells. They reported that IgM was expressed in some tumor cell lines although at a much lower level than IgG. In addition, Chen *et al.* [58] reported expression of the heavy chain of IgM and IgA in non-lymphoid lineage and neoplastic cells. We report over expression of IgLL1 and expression of IgM in both MDS-RS and MDS-EB. However, these results place this protein as a very interesting biomarker candidate due to the importance and previously demonstrated activity of cancer cells to produce immunoglobulins [59-63].

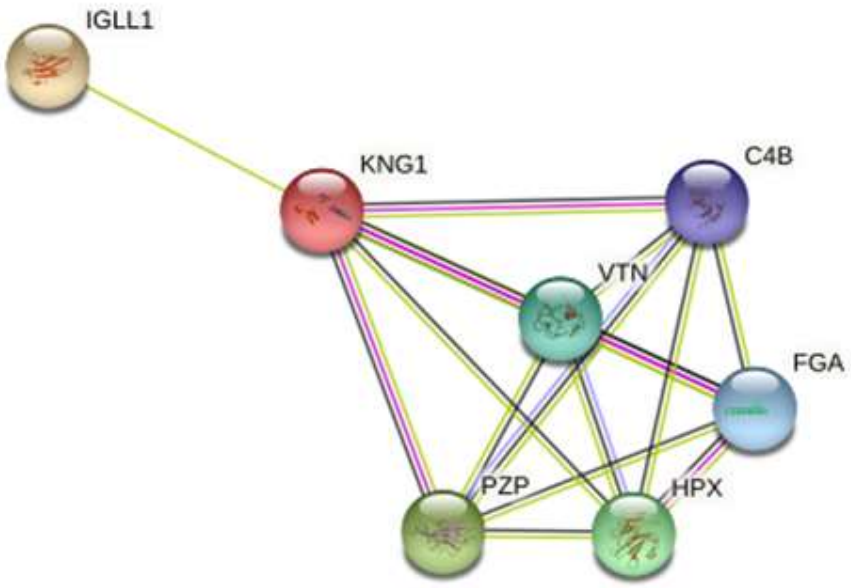

Figure 1. Association between the overexpressed protein in myelodysplastic syndromes with ring sideroblasts (MDS-RS) or refractory anemia with excess blasts (MDS-EB) versus the control group. Uncharacterized proteins were excluded from protein association. The protein association was

 doi:10.20944/preprints202201.0292.v1

carried out by Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) 10.0 – 2022, Required score was 1.5.

A chromatographic affinity column was made using peanut agglutinin (PNA) and FTL lectin proteins that were individually immobilized on Sepharose 4B. The FTL column was assembled using an elution solution containing an extract of the seeds of *A. incisa.* Two peaks were observed after sample elution (Figure A3). Peak (I) represents the fraction not retained on the column, while peak (II) represents the retained fraction. Samples were eluted using Buffers A and B. Lectin proteins were used to cause a decrease in the number of observed peptides and to expand the detection capacity of less abundant proteins in plasma samples from patients with MDS-RS and MDS-EB.

PNA is a lectin that has been widely described as a tool for the procuring of galactose β1-3-N-acetylgalactosamine (Tf) antigen [64]. However, it was not possible to observe any retained fraction for the column that had immobilized PNA. For the FTL sepharose column, a retained fraction was observed. It was possible to observe and identify 15 proteins for the MDS- RS versus the control and 10 proteins for the MDS-EB versus the control (Figure 2). The observed proteins were analyzed via mass spectrometry. The proteins that were over retained that presented a ratio > 1 are summarized in Table 2.

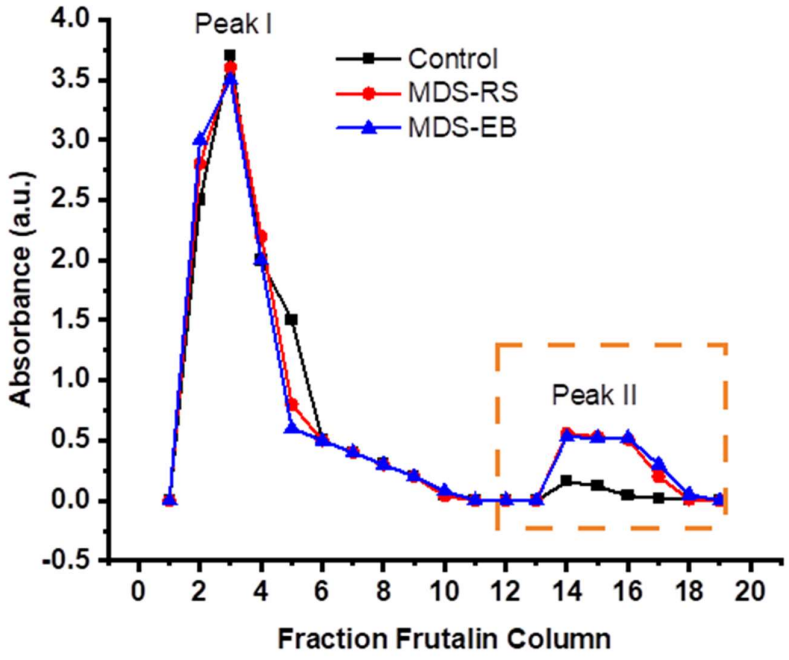

Figure 2. Chromatographic elution profiles of pooled protein of MDS-RS (Figure A4) and MDS-EB (Figure A5) samples in a FTL chromatographic column eluted with Buffer A (Peak I was eluted with 20 mM Tris in 150 mM NaCl pH 7.4.) and Buffer B (Peak II elution was performed later with 20 Mm Gly pH: 2.6). Absorbance at was followed at 220 nm in each sample of 1 mL.

In comparison of the chromatographic profiles, the retained fractions of the samples derived from MDS-RS and -EB are shown and are greater than the one obtained for the control group. It is surprising that the alterations in the glycosylation profiles of proteomes derived from MDS generated a fraction by mainly retaining an increase in the appearance of alpha galactosidic residues in glycoproteins. The results are in full agreement with those from other studies using FTL that reported a majority of retained fractions in

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cancer samples on affinity chromatography compared with samples from the control group.

The retention capacity of the FTL affinity chromatographic columns allows studies of undepleted samples with the aim of searching for protein interactions. This lectin specifically binds α-D-galactose [29]. FTL has been used successfully in immunobiology research for the recognition of cancer-associated oligosaccharides, taking into account its anomeric recognition specificity for alpha galactosidic residues [17,18]. In oncogenesis, glycosylation is affected by alteration in the transferred glycosyl in such a way that residues that commonly should be associated with the proteins, such as beta-galactosidic residues, are manifested as alpha residues [17,18]. The Galβ1-GlcNAc groups and Galβ1-GlcNAc repeats are further elongated by the processes of fucosylation, sialylation, galactose, glucuronylation, and/or sulfation. A GalNAcβ1 residue is added to some GlcNAc residues. Variations in the type of binding from beta to alpha or exposure to galactose associated with the Tn antigen are indicators of cell malignancy. Figure 3 shows the interaction between FTL and α-D-galactose.

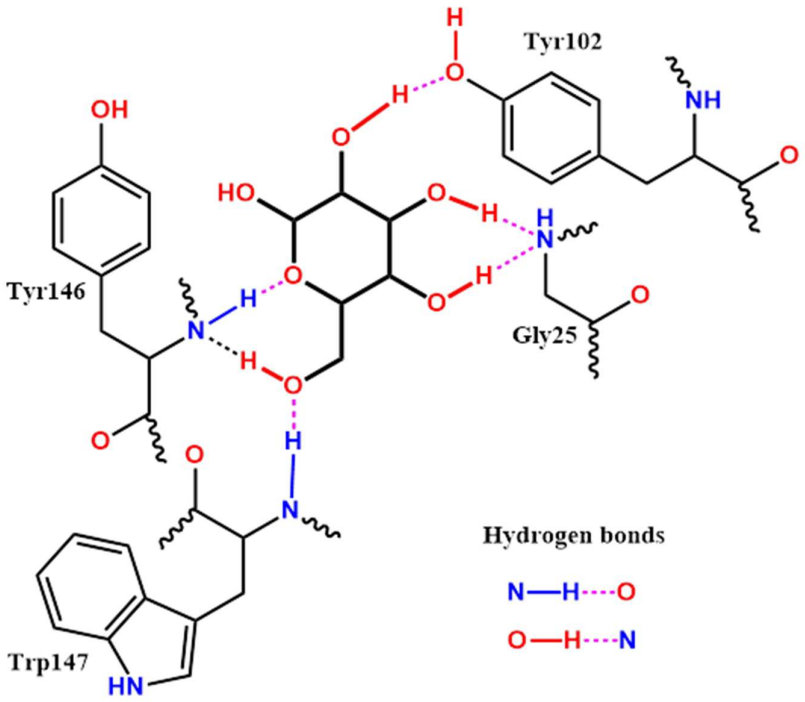

Figure 3. Simplified representation for the binding site of a-D-Galactose to the frutalin (FTL) protein. Scheme based on crystalline structure reported by Neto *et al.* [29].

Frutalin has been used successfully in immunobiology research for the recognition of cancer-associated oligosaccharides [17,18]. However, the molecular basis by which FTL promotes these specific activities remains poorly understood. The interactions between frutalin and α-D-galactose was previously characterized by X-ray crystallography [29] Lectin exhibits a post-translational cleavage that produces α- and β-chains (133 and 20 amino acids, respectively). The carbohydrate binding site (CBS) involved in the N-terminus of the α chain contains four key residues: (1) Gly25, (2) Tyr146, (3) Trp147, and (4) Asp149. Molecular dynamic simulations suggest that cleavage of the Thr-Ser-Ser-Asn (TSSN) peptide leads to a reduction in the stiffness of FTL CBS, thus increasing the number of interactions with ligands and resulting in multiple binding sites and anomeric recognition of α-D-galactose.

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Table 2. Peptides founded after elution in frutalin (FTL) affinity chromatography column.

MDS-RS peptide	Description	Ratio
IGHM_HUMAN	**Ig mu chain C region OS Homo sapiens GN IGHM PE 1 SV 3**	**4.00**
Q6GMX6_HUMAN	IGH protein OS Homo sapiens GN IGH PE 1 SV 1	1.42
Q9NPP6_HUMAN	Immunoglobulin heavy chain variant Fragment OS Homo sapiens PE 2 SV 1	1.30
A2NJV5_HUMAN	Kappa light chain variable region Fragment OS Homo sapiens GN IGKV A18 PE 4 SV 1	1.26
MDS-EB peptide	**Description**	**Ratio**
IGHM_HUMAN	**Ig mu chain C region OS Homo sapiens GN IGHM PE 1 SV 3**	**1.72**
Q9NPP6_HUMAN	Immunoglobulin heavy chain variant Fragment OS Homo sapiens PE 2 SV 1	1.31
A8K3E4_HUMAN	cDNA FLJ78367 highly similar to Homo sapiens fibrinogen A alpha polypeptide FGA transcriptvaria	1.18

Overexpression of IgM in MDS-EB and subexpression of IgM in MDS-RS was observed on mass spectrometry when compared to profiles of healthy people. However, a higher retention of IgM was demonstrated in the fraction retained on FTL chromatography for MDS samples compared to the control. The affinity of FTL on the chromatography column for α-glycoprotein was increased in the expressed proteins in MDS-RS and MDS-EB. This effect could be due to the conformational exchange of α-IgM exposed glycosylation profiles.

IgM plays an important role in primary defense mechanisms [65]. Studies have reported that IgM is involved in early recognition of external invaders, such as bacteria and viruses and cellular and self-modified debris in addition to recognition and elimination of precancerous and cancerous lesions [65]. The membrane-attached IgM is found in most normal lymphocytes B together with IgD [66]. Membrane-bound IgM induces the phosphorylation of CD79a and CD79b by the Src family of protein tyrosine kinases, which can induce cell death by apoptosis. IgM has also been found in soluble form and represents around 30% of the total number of Igs in plasma in which it is found exclusively as a homopolymer [67]. Once the antigen binds to the B lymphocyte receptor, the secreted form is released in large amounts [68]. Tisch et al. described the recognition of IgM with a diversity of tumor antigens [69], hence, the increase in IgM was shown to be associated with different types of cancer.

3. Conclusions

From the 31 proteins overexpressed in MDS-RS and the 33 overexpressed in MDS-EB, a panel of seven overexpressed proteins (VTN, FGA, PZP, KNG1, IGLL1, C4b, and

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HPX) in both myeloid syndromes were generated, which could be used as biomarkers for MDS-RS and MDS-EB. The seven proteins presented strong associations between them, which suggests that studies should be carried out to understand mechanisms of expression and self-regulation. It was possible to fractionate the proteins using Sepharose chromatography columns modified with lectin proteins; hence, FTL, distinct from PNP, presented a retention capacity that contained α-galactosidic residues. Due to the direct relationship between Igs with altered expression and glycosylation processes in cancer, proteomic studies that use FTL columns make it possible to fractionate non-depleted samples, which makes it a powerful tool for cancer studies. Surprisingly, IgM was strongly retained by the FTL column due to the α-galactosidic residues present in it. This finding suggests that the glycosylation of IgM in its synthesis process could be modified to factors associated with the MDS-RS and MDS-EB.

4. Materials and Methods

4.1. Chemicals

RapiGest SF Surfactant Waters was purchased from Milford, USA and Trypsin Gold, MA. Spectrometry grade extreme resistance to autolytic digestion kit was purchased from Promega, Madison, USA. Dithiothreitol DTT, (±)-threo-1,4-dimercapto-2,3-butanediol solution, Tris base (2-amino-2-(hydroxymethyl)-1,3-propanediol), HCl 36%, *Saccharomyces cerevisiae*, alcohol dehydrogenase (ADH - access code P00330 in SwissProt), acetonitrile anhydrous 99.8%, Sepharose 4BCross-linked, galactose European Pharmacopoeia (EP) Reference Standard, NaCl ACS reagent ≥ 99.0%, Glycine ReagentPlus® ≥ 99% (HPLC), acid formic p.a., ACS reagent Ph. Eur. ≥ 98%, and ammonium bicarbonate BioUltra, ≥ 99.5% (T) were purchased from Sigma Aldrich.

5.2. Patients and Samples

All samples were obtained by bone marrow aspiration after obtaining informed consent at diagnosis. All patients were diagnosed according to the guidelines [70] from WHO 2016 and to the Revised International Prognostic Scoring System (R-IPSS) [71]. A total of 30 marrow serum samples were obtained: 10 patients diagnosed with MDS with ring sideroblasts (MDS-RS), 10 patients with MDS with excess blasts (MDS-EB-1 and -2), and 10 as control group. The study was approved by the Ethics Committee of the NPDM Federal University of Ceará (CAAE 69366217.6.0000.5054). All samples were stored at –80 °C until used, and concentrations were determined using the Nanovue PlusTM instrument (GE Healthcare, Uppsala, Sweden).

5.3. Protein management

The protocol of Lobo and collaborators was used with some modifications [17]. Three groups were formed with the different samples: (1) Group 1 - Control (healthy marrow plasma donors), (2) Group 2 - Samples from people with MDS-RS, and (3) Group 3 - people with MDS-EB-1 and MDS-EB-2. Initially, all samples were centrifuged at 3000 x g after thawing, the resulting supernatant was then saved, individual samples were frozen at –30 °C, and the pellet was discarded. Subsequently, pools were grouped, ensuring that the protein chastity contribution of each sample was the same (10.6 mg) for each of the pools, resulting in a pool of 1.6 mL (6.625 mg/mL). These pools were used to perform all assessments and ensured the same amount of protein for each procedure. Each sample containing 50 µg of protein, which was denatured with 0.2% RapiGestTM SF (Waters, Milford, USA), reduced (10 mM dithiothreitol), alkylated (10 mM iodoacetamide), and enzymatically digested with trypsin (Promega, Madison, WI, USA). At the end of this process, the

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samples were centrifuged, and the supernatant was transferred to a vial to which 5 µL of internal standard, alcohol dehydrogenase (ADH, 50 fmol, access code P00330 on SwissProt) and 85 µL of 3% acetonitrile with acid were added in 0.1% formic acid. The final concentrations of glycoproteins and ADH were estimated at 250 ng/µL and 25 fmol/µL, respectively, and the final volume in the microtube was 200 µL. Quantitative and qualitative experiments using nano ultrapure liquid chromatography (nanoUPLC) and tandem nano electrospray ionization mass spectrometry (ESI/MSE) of the digested samples were performed using reverse phase chromatography of peptides with 3% to 40% (v/v) of acetonitrile containing 0.1% formic acid for 90 min. A flow rate of 600 nL /min was maintained for 100 min on a nanoACQUITY UPLC core system. A 1.7 µm, 100 µm × 10 cm nanoACQUITY C18 UPLC BEH reverse phase column was used in conjunction with the SCX 5 µm, 180 µm x 23 mm precolumn.

5.4. Protein Samples Depletion

The protocol of Lobo and collaborators was used.[17] Human serum albumin (HSA) and Immunoglobulin G (IgG) were removed from marrow plasma by affinity chromatography performed on a HiTrap Albumin IgG depletion™ column (GE Healthcare) coupled to the ÄKTApurifier 10 automated fast protein liquid chromatography (FPLC) system (GE Healthcare). Initially, marrow serum plasma samples were filtered on a 0.22 µM membrane (Vertipure™ PVDF syringe filters, Veritical) after which the samples were manually and individually injected into the FPLC system. The matrix was subjected to a constant flow of 1 mL/min of 5 mL of buffer A (20mM Tris-HCl pH 7.4 in 0.15 M NaCl) until the injection of 150 µL of sample when the matrix was washed with 8 mL of buffer A followed by 7 mL of buffer B (0.1 M Glycine-HCl pH 2.6 in 0.15 M NaCl) under the same conditions with protein elution measured at absorbances of 280 and 220 nm.

5.5. Mass Spectrometry

The fractions were digested (50 ug) and tryptic peptides were separated using a nanoACQUITY UPLC system (Waters) equipped with an HSS T3 C18 reverse-phase column (1.8 µm, 75 µm × 20 mm) for 110 min using a 0%–40% gradient for 90 min and 40%–85% gradient for 5 min after which the column was re-equilibrated for 15 min at 35 °C. The flow rate was 0.35 µl/min, and mobile phases A and B contained 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively.

A data-independent analysis (MSE) of tryptic peptides was performed using a Synapt HDMS mass spectrometer (nanoESI-Qq-oaTOF; Waters, Manchester, UK). All measurements on the mass spectrometer were done in the "V" mode with a resolving power of at least 12,000 times. All analyses were performed using nanoESI (+). The collection channel of the analyzed sample was closed every 30 sec for the passage of the reference ion (Glu–Fib (Glu1) derived from fibrinopeptide B human ([M + 2H]2+ = 785.2486). The exact mass retention times of nanoLC-MSE data were collected using alternative lower (3 eV) and elevated collision ramp energies (12–40 eV) applied to the argon collision cell with argon gas using a scan time of 1.5 s with a 0.2-s interscan delay for each MS scan from m/z 50 to 2,000. The radio frequency RF offset (MS profiles) was adjusted such that the LC/MS data were effectively acquired from m/z 300 to 2,000, which ensured that any mass observed in the LC/MSE data < m/z 300 arose from dissociations in the collision cell.

5.6. Mass spectra interpretation

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LC/MSE data were processed, and proteins were identified using ProteinLynxGlobalServer v.2.4 software (PLGS) with the UniProtKB/Swiss-Prot 57.1 and UniProtKB/TrEMBL 40.1 Homo sapiens annotated database.

The selected databases were randomized during database queries and appended to the original database to assess the false-positive identification rates. The identified proteins were organized by Protein Lynx Global Server (PLGs) into a list that corresponded to a single protein for both conditions (study or control group) and a logarithmic ratio between the different groups was plotted on a scatter plot to visualize differences between the groups. Proteins only in points of presence and confidence greater than 99% (3x3 assays) were considered for accepting searches in the database, and when the same protein was identified for different MS/MS ion fragmentations, those that presented the highest scores were considered for comparisons and data presentation.

For searching spectra and the database, we used the default parameters of PLGS followed by a maximum of one missed trypsin-cleavage and fixed carbamidomethyl and variable oxidation modifications [72, 73]. The absolute quantification of each run was calculated according to the three most intense peptides (label-free Hi3 method) using ADH peptides as internal standards [73].

The average quantitative values of all samples were calculated, and the p value ($p < 0.05$) calculated using ExpressionE software to refer to the differences between biological replicates.

5.7. Protein interaction network analysis

The protocol of Cavalcante and collaborators was used [18]. The proteins identified in this work as having differential expression were used for an interaction analysis. The Search Tool for the Retrieval Interaction of Genes/Proteins (STRING) program version 9.05 was used, and the different proteins were identified with the accession number that identifies them in the database. Swiss-Prot protein data, the analysis parameters were Homo sapiens species, significance level between 0.400 and 0.900 with parameter prediction methods enabled. Protein lists were then filtered to show only those present in all three repeat injections of each sample after which the PLGs created an output table. This table presented the names and access codes in addition to the expression levels of the proteins based on the ratio (ratio) of the proteins among the selected groups. The probability of the ratio demonstrated the significance of the protein over-expression (up-regulated) ratio ≥ 2, under-expression (down-regulated) ratio ≤ 0.5, and others without significant changes between groups (unchanged) $0.5 < \text{ratio} < 2$.

5.8. Affinity Chromatographic Column (Frutalin as stationary phase)

The marrow plasma samples were thawed, centrifuged at 12,000 x g for 15 min at 8 °C and filtered through a 0.22 μM membrane (Vertipure™ PVDF syringe filters, Veritical) to prevent obstruction of the chromatography column. Affinity chromatographs with plasma samples (150 uL) were performed on a matrix with the isolated lectins coupled (Frutalin and PNA) on an activated Sepharose 4B column as described in the manufacturer's protocol (Sigma). Peak I was eluted with 20 mM Tris in 150 mM NaCl pH 7.4. Non-retained fractions with an absorbance ≥ 0.100 at wavelengths of 280 and/or 220 nm were stored in a pool, and peak II elution was performed later with 20 Mm Gly buffer, pH of 2.6. The retained and eluted fractions with absorbances ≥ 0.050 occurring before mentioned wavelengths were concentrated and dialyzed with ammonium bicarbonate 50 mM Nanosep 3 kDa 10000 x g at 20 °C and later stored at –30 °C for further proteomic analysis as indicated Cavalcante *et al*. [18].

5.9. Frutalin extraction

Purification of the lectin lectin D-galactose-ligand from the seeds of *Artocarpus incisa* (frutalin). During the purification process of the D-galactose-binding lectin of the seeds of frutalin, a chromatographic process was carried out on a column of D-Galactose/Agarose (PIERCE) previously balanced with saline solution (150 mM NaCl). All of the proteins that did not interact with the matrix (non-retained fraction, Peak I) were eluted with the equilibrium solution and followed at an absorbance of 280 nm. The retained and eluted fraction with 0.2 M D-Galactose solution in 150 mM NaCl (Fraction retained, Peak II), contained the fructyl that had interacted with the matrix. The exposed galactosidic residue was fixed, and when eluted with a solution with D-Galactose, the chemical balance was displaced to favor the affinity on the galactose binding proteins coupled to the galactose in suspension.

Author Contributions: Conceptualization, C.T. and R.M; methodology, A.M., M.L., M.C. and R.P; formal analysis, C.T., A.R., M.C. A.M, R.P. and R.M; data curation, C.T., A.R. and M.L. writing—original draft preparation, C.T. and A.R.; writing—review and editing, C.T. and A.R.**;** funding acquisition, Y.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The data in this work are available from the corresponding author upon reasonable request.

Acknowledgments: This work was supported by research grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). Additionally, this work was supported by research grants from Centro de Innovación y Productividad (CIP) Dosquebradas of Universidad Nacional Abierta y a Distancia (Colombia) to finance and allow the development of the project PIE 09-08-2016 that allowed the interdisciplinary analysis of the reported data. Additionally, specially thankfull to Joaquin Rodriguez-Lopez for the support during the research. A. I. B. Romo thanks to ANID (#72170429) from Chile.

Conflicts of Interest: The authors declare that there are no conflict of interest.

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Appendix

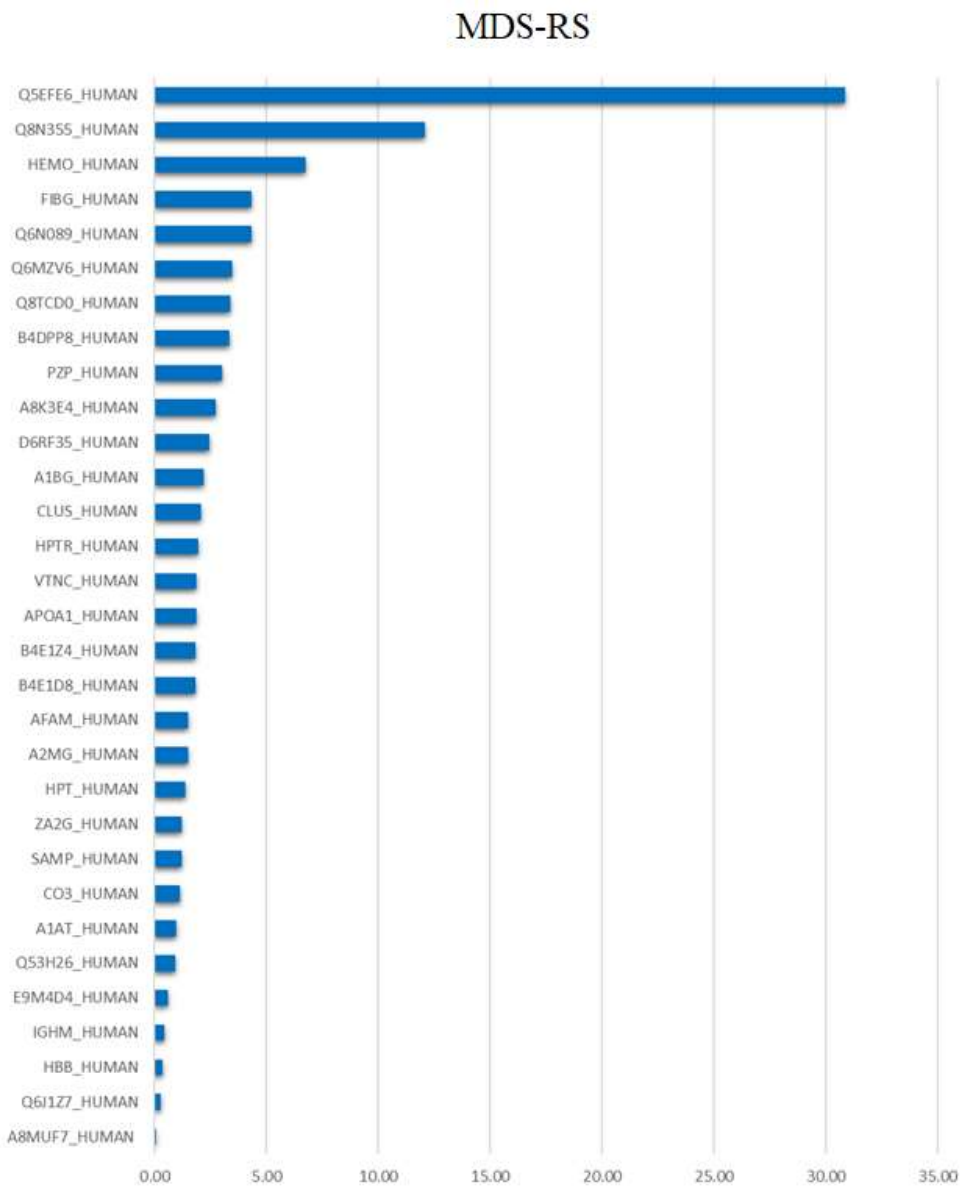

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Figure A1. Histogram of protein expressed in ratio of MDS-RS/Control.

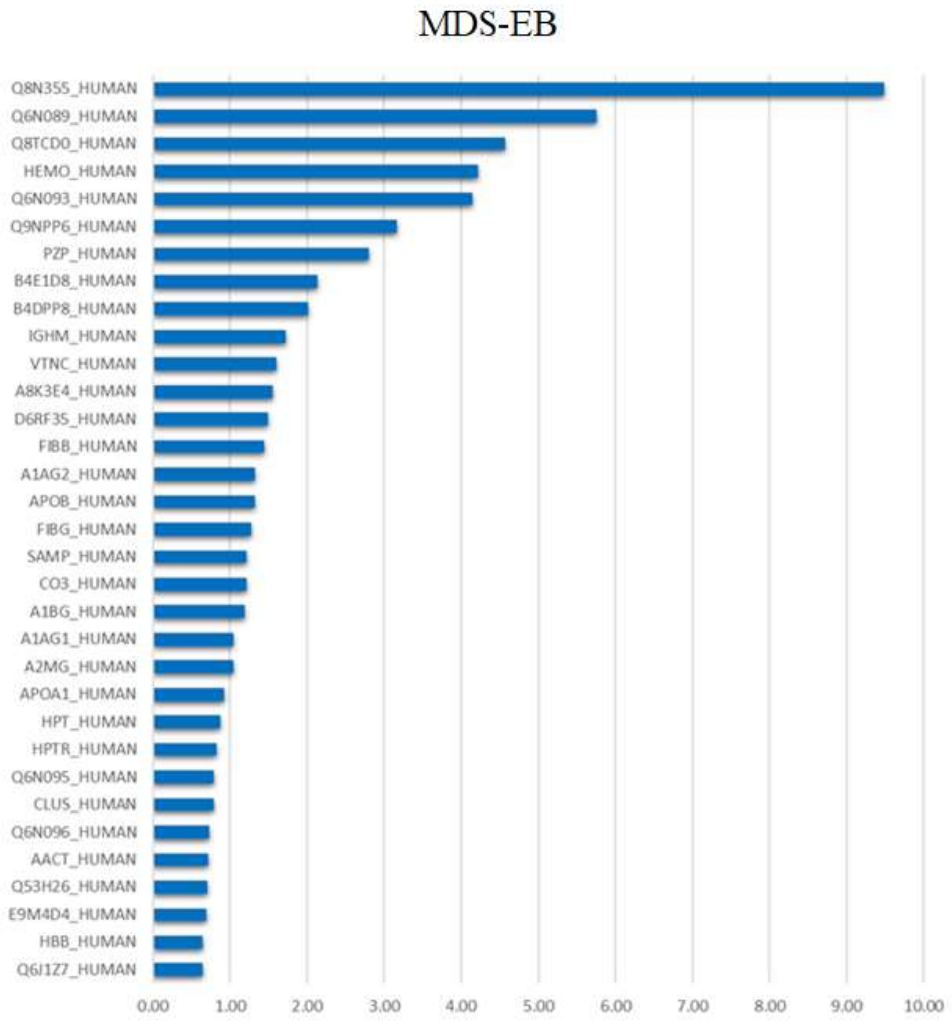

Figure A2. Histogram of protein expressed in ratio of MDS-EB/Control.

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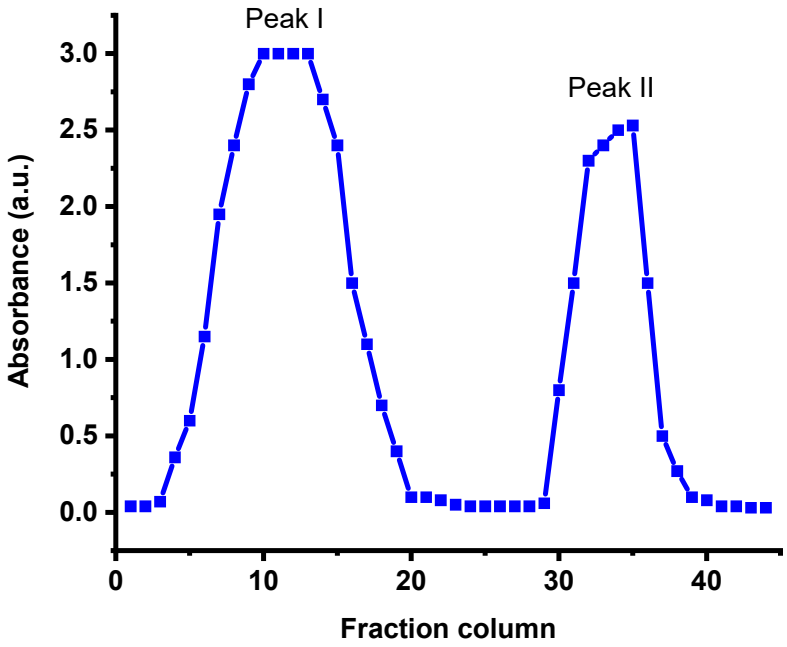

Figure A3. Chromatographics perfil elution of Frutalin purification by retention in a D-Galactose – Agarose column. Absorbance in 280 nm was follow in each samples of 1 mL.

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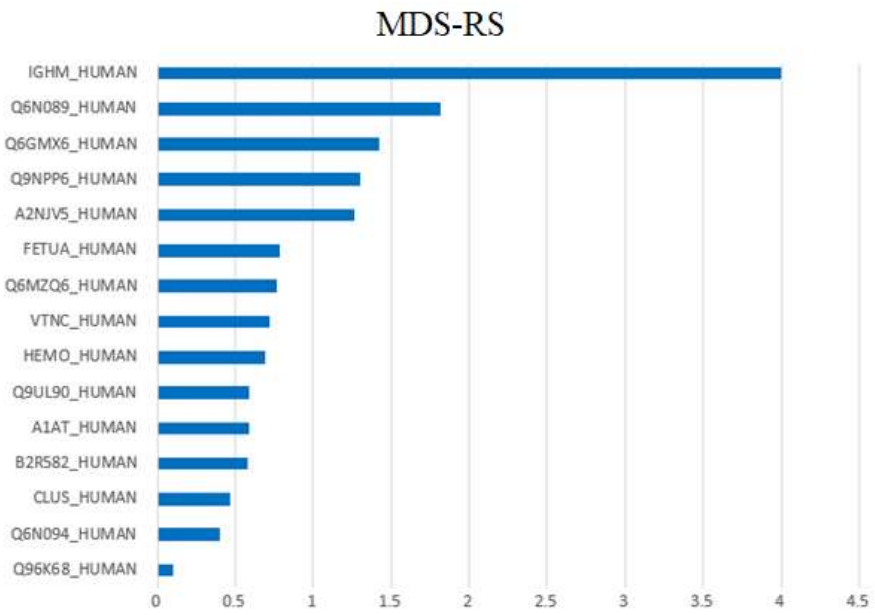

Figure A4. Histogram of protein expressed in ratio of MDS-RS/Control after elution in frutalin modified chromatographic column.

 doi:10.20944/preprints202201.0292.v1

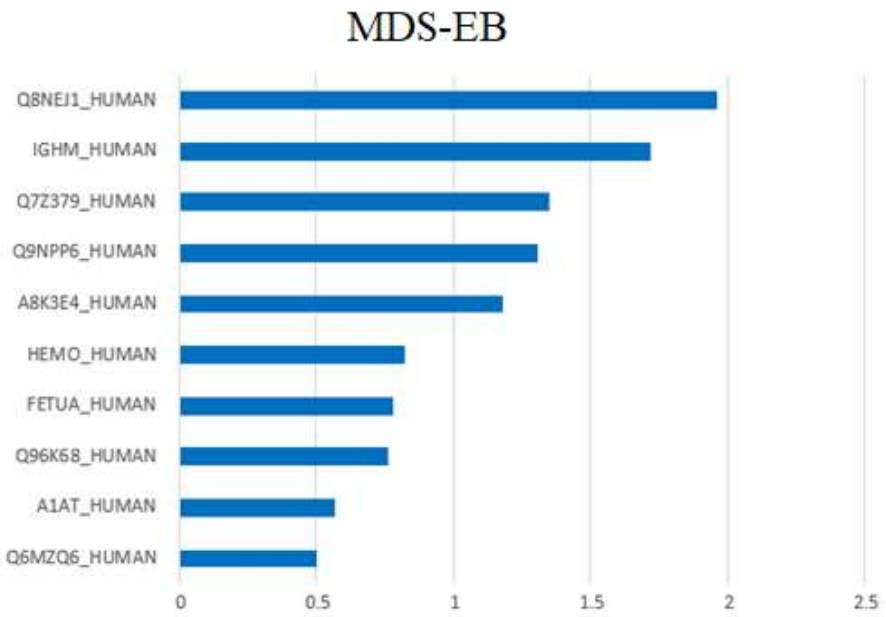

Figure A5. Histogram of protein expressed in ratio of MDS-EB syndrome/Control after elution in frutalin modified chromatographic column.

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