ORIGINAL ARTICLE

Plasma and Cell Lysate Proteins Associated With Treatment Outcome in Acute Myeloid Leukaemia

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ABSTRACT

Introduction: Drug-resistance is a major hindrance to successful treatment of AML. Current predictive biomarkers are mainly genetic aberrations and insufficient in foretelling treatment outcome in all acute myeloid leukaemia (AML) due to its heterogeneous and aggressive nature. Proteins are stable and reliable. Secreted proteins in AML may have predictive or prognostic values for early intervention. Proteomic studies on AML are few and further investigations will benefit in selection of best markers. The aim of the study was to identify differentially expressed plasma proteins in AML with different treatment outcome. **Methods:** Two-dimensional electrophoresis (2-DE) technique was utilised to identify proteins differentially expressed in chemo-sensitive/chemo-resistant AML. Plasma and peripheral blood mononuclear cell (PBMC) lysate proteome analysis were performed on six chemo-resistant, four chemo-sensitive and six healthy controls and seven chemo-resistant, three chemo-sensitive and six healthy controls, respectively. Each experiment was conducted in duplicate or triplicate. Images were captured and protein spots detected by software. Differentially expressed protein spots were excised from gel and proteins were identified using LC/MS/MS. Proteins spots that were also detected in healthy controls were excluded. **Results:** Comparing mean % volume of each spot demonstrated significantly enhanced expression of apoliprotein-E (APO-E) and haptoglobin (HP) ($p<0.05$) in plasma and HNRNP H1 ($p=0.049$) in cell lysate of chemo-sensitive group. Serotransferrin (STF) from plasma and DNA-PK from cell lysate ($p=0.01$) were associated with chemo-resistance. **Conclusion:** This preliminary study identified several potential predictive biomarkers associated with chemo-resistance/chemo-sensitivity to treatment in AML. Further studies with a larger number of samples are required to validate the results.

Keywords: Proteomics, Acute myeloid leukaemia, Two-dimensional electrophoresis; Mass spectrometry; Chemo-resistance.

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INTRODUCTION

Acute myeloid leukaemia (AML) arises from the aberrant proliferation of hematopoietic myeloid progenitors which also demonstrates a partial block in cell maturation (1). Immature leukaemic blasts originate from the bone marrow and accumulate in the peripheral blood.

Intensive chemotherapy is a standard treatment and the aim of induction chemotherapy is to achieve complete remission (CR) (2). Stem cell transplantation has further improved cure rates, however, this has limited application. Initial respond to treatment is more favourable in younger AML patients as the majority achieve CR (65-75%) but is less so among the elderly patients (40-50%) (1). The rest are drug resistant. Most patients relapse within three years after initial CR and many become resistant to further chemotherapy (3, 4).

Several chromosomal translocations and gene mutations are recommended by the World Health Organisation (WHO) as prognostic markers in classification of AML (5), however, these are unavailable to a substantial number, for example, those who are normal for cytogenetics or gene mutations.

Screening for potential biomarkers in diagnosis, prognosis, monitoring and therapy of cancers including AML has been actively investigated and with the use

of high throughput technologies has generated vast amount of data. However, due to the heterogeneous as well as the aggressive nature of AMLs which requires close monitoring, pathophysiology of the complete disease is only beginning to be clear. Development of personalized medicine is expected to greatly improve long-term survival of chemo-resistant patients but is not expected to be without challenges [reviewed in (6)]. Genetic aberrations such as translocations involving MLL-AML, CBF, point mutations affecting FLT-3 and NPM1 are routinely tested. Nevertheless, random translocations/mutations involving other genes are frequently observed in AML. Abnormally up-regulated mediators of signalling pathways in JAK/STAT, Raf/MEK/ERK and PI3K/AKT (7,8) as well as leukaemia stem cells have been implicated in promoting survival and resisting chemotherapy in AML (9,10). Epigenetic changes due to hyper/hypo methylation, associated non-coding RNAs and proteomics contributions to AML prognosis are still under study [reviewed in (11)]. Bai et al. (12) profiled AML peptidomes to identify serum candidates in minimal residual disease samples to predict impending relapse and identified 47 potential biomarkers including increased UBA1, and decreased isoform 1 of fibrinogen alpha chain precursor and platelet factor 4(PF4) in newly diagnosed and relapsed+refractory AML. Nevertheless, biomarkers of refractory/chemo-resistance in newly diagnosed cases may differ.

Proteins are the functional components of a cell. Its stability permitted many of the long established, reliable protein-based diagnostic markers critical in clinical settings. Test methods range from easy to use, rapid results to full automation. Due to gene regulation at the transcriptional and post-transcriptional level, protein abundance and RNA expression levels rarely exhibit a linear correlation (13).

Powerful technologies in microarrays and proteomic approaches including mass spectrometry have made it possible to discover and identify new markers. Novel tools in protein detection with aptamers, peptoids and small molecules will super advance diagnostics, as it did before with antibodies (14).

Investigation on protein biomarkers in AML is still inconclusive. The aim of this study was to identify proteins associated with outcome from chemotherapy in AML at initial diagnosis from plasma and peripheral blood mononuclear cells (PBMC) using proteomic approach.

MATERIALS AND METHODS

Patients

Peripheral blood samples were collected from newly diagnosed AML patients admitted to Hospital Ampang between July 2009 and July 2011. Included were patients of all ages, both sexes and Malaysians. Excluded were samples from chemotherapy treated patients, patients without complete induction therapy, and insufficient samples. Samples were collected after informed consent from patients. This study was approved by the Medical Research Ethics Committees at Institutional and Ministry of Health, Malaysia. All procedures were in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). AML treatment was according to the Berlin-Frankfurt-Munich (BFM)-83 protocol with modifications. All clinical and pathological data were obtained from hospital records. Diagnosis of AML patients was based on presence of more than 20% of myeloblast defined by morphology, cytochemistry and immunophenotyping according to guidelines from the 2016 revision of World Health Organisation classification of myeloid neoplasms and acute leukaemia (5). Criteria for chemotherapy resistance status were based on bone marrow examination, showing > 5 % blast cells after recovery from induction chemotherapy. The patients were categorized as chemotherapy resistant or sensitive/responder, where chemotherapy sensitive/responder patients achieved complete remission (i.e. <5% blasts in bone marrow) as diagnosed in bone marrow report, one month after induction therapy. For proteomic study of PBMC, 10 AML patients (chemo-resistant = 7, chemo-sensitive = 3) and 6 normal controls (3 males, 3 females) were included while for plasma analysis, 10 AML patients (chemo-resistant = 6, chemo-sensitive = 4) and 6 normal controls (3 males, 3 females) were included in this study.

PBMC and plasma samples preparation

Peripheral blood was separated by gradient density centrifugation on Ficoll-Hypaque Plus (GE Healthcare, Sweden) to isolate peripheral blood mononuclear cells (PBMC). Washed pellet was resuspended in cryopreservation medium consisting of 10% DMSO (Ameresco, USA) and 40% FBS (Gibco®, Invitrogen, South America) in RPMI 1640 (Gibco®, Invitrogen, South America) and stored over liquid nitrogen. Plasma layer was transferred into tubes and stored at -80°C until further analysis.

To perform 2-DE, frozen tubes were quick thawed and PBMC was washed. Pellets containing 10^7 cells were solubilised in 200 µl lysis buffer. For plasma samples, 20 µl was added to 100 µl lysis buffer. Lysis buffer consisted of 7M urea (Bio-Rad, USA), 2M thiourea (Sigma, USA), 4% (w/v) CHAPS (Amresco, USA), 2% IPG (Immobilized pH Gradient) buffer pH 3-10 nonlinear (GE Healthcare-Sweden), 0.5 % Triton X-100 (Amresco, USA), 1% (w/v) DTT (Amresco, USA), 1% nuclease mix (GE Healthcare, Sweden) and 1% protease inhibitor mix (GE Healthcare, Sweden).

Following lysis, PBMC samples were centrifuged at 16,000 xg at 4°C for 45 minutes. The supernatant was cleaned with 2D-CleanUp Kit (GE Healthcare, Sweden) to reduce salt and other contamination. For plasma

samples, proteins were subjected to immunodepletion of albumin and IgG with the albumin IgG removal kit (GE, Healthcare, Sweden) and acetone precipitated to enrich for low abundant proteins and increase resolution. For acetone precipitation, 10 µl was mixed with four volumes of ice-cold acetone and left at -20°C overnight. Protein pellet was then obtained by centrifugation at 16,000 xg for 10 min at 4°C. Acetone was decanted and pellet air dried for 5 minutes at room temperature.

Precipitated proteins were resuspended in the same lysis buffer and protein concentration was determined with a Bio-Rad Protein Assay kit (Bio-Rad, USA) based on Bradford protein test. Lysate samples were aliquoted and stored at -80°C.

Two dimensional electrophoresis (2-DE)

For isoelectric focusing of PBMC samples, 7 cm IPG strips, pH 3-10 nonlinear (GE Healthcare, Sweden) were used while for plasma samples, isoelectric focusing was conducted on 17 cm IPG strips, pH3-10, nonlinear (GE Healthcare, Sweden) to increase protein loading. Passive rehydration with 30 µg of protein lysate took place in rehydration buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.2% IPG buffer (pH 3-10 NL, GE Healthcare) and 0.002% bromophenol blue for 18 hrs. Isoelectric focusing was performed at 20°C using PROTEAN IEF cell (Bio-Rad, USA). Standard focusing program, recommended by manufacturer was applied with some modifications as follows: 250 V in a linear gradient over 40 minutes, a linear gradient to 4000V over 2 hours, 4000 V until 10000 V/h in a ramping mode.

For equilibration of focused proteins, strips were incubated in equilibration buffer (0.05 M Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 0.001% bromophenol blue) containing 2% DTT for 15 min followed by further incubation in the same equilibration buffer but with 2.5% iodoacetamide instead of DTT for an additional period of 15 min.

For second dimension electrophoresis, proteins were separated on 12.5% SDS-polyacrylamide gel (Mini-PROTEAN Tetra Cell electrophoresis system, Bio-Rad). The voltage was set initially at 50 V for 30 minute followed by 100 V until the tracking bromophenol blue dye reached the end of the gel. Gels were stained with silver stain plus kit (Bio-Rad, USA) according to manufacturer's instruction. Each sample was run in triplicate to reduce technical variation.

Scanning and image analysis

Images of two dimensional electrophoresis gels were captured using Image Scanner (GE Healthcare, Sweden) with 300 dots per square inch (dpi). Protein spots were detected automatically using the ImageMaster 2D Platinum software, version 7.0 (GE Healthcare, Sweden). To analyse protein expression levels, percentage of volume contribution (% vol) was used. All values are presented as mean ± S.E.M (standard error of the mean).

Protein identification by liquid chromatography/mass spectrometry/mass spectrometry (LS/MS/MS)

Selected protein spots were manually excised and outsourced for LC/MS/MS analysis at Proteomics International Pty Ltd (Australia). Mascot sequence matching software (Matrix Science) with Ludwig NR database was used. Proteins were identified based on statistically significant molecular weight search (MOWSE) score ($p < 0.05$).

Statistical analysis

All values were expressed in mean ± SEM. Non-parametric Mann-Whitney U test was performed to compare median percentage of spots volume between two groups. A p value < 0.05 was considered significant.

RESULTS

Clinical data and diagnosis

Clinical details of AML patients included in this study are listed in Table I. Mean age of patients was 38 years (range19-66 years). There were more female (58.3%) than male patients. Majority of the samples were used for both peripheral blood mononuclear cell (PBMC) and plasma analysis.

Table I: Clinical details of AML samples used in this study

Response to chemo-therapy	No	Code	[a]FAB Subtype	Gen-der	Treat-ment[b]	Age
	1	51H	M1	F	D+A	33
	2	54H	M1/M2	F	D+A	34
	3	56H	M4	M	M+A	64
	4	32H	M4	M	M+A	66
Resistant	5	52H	M1	F	D+A	51
	6	14H	M1	F	D+A	23
	7	45H*	ND	F	D+A	29
	8	35H#	M2	M	I+A	32
	9	33H	M5b	M	D+A	36
Responsive	10	43H	M2	F	D+A	47
	11	53H	ND	M	I+A	19
	12	49H*	ND	F	D+A	57

[a]FAB : French-American-British classification system. ND= Not determined
[b]Treatment: A= Ara-C, D= Daunorubicine, I= Idarubicin, M=Mitoxantrone
*Only plasma sample #Only PBMC sample

2-DE gel analysis

Three separate experiments were performed for all samples except two where the amount of protein was insufficient. Similar protein spot patterns were obtained for all groups. Representative 2-DE gel images for each group are shown in Fig. 1 and Fig. 2. Approximately 1070 protein spots were detected in PBMC samples in silver stained gels. Scanned images were firstly analysed

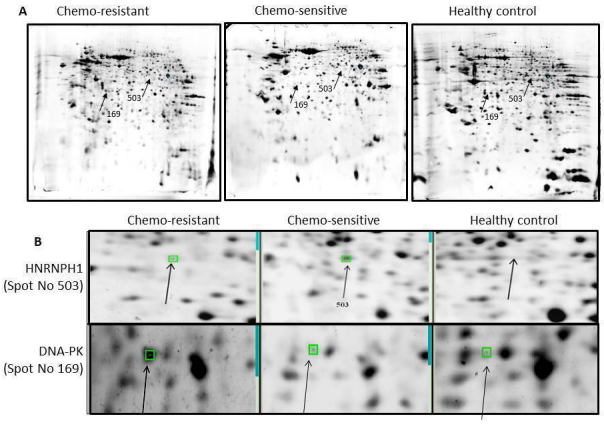

Figure 1: A) Representative full 2-DE gel images of PBMC samples. B) Close-up 2-DE gel images of differentially expressed protein spots in chemo-resistant, chemo-sensitive and healthy controls from PBMC samples.

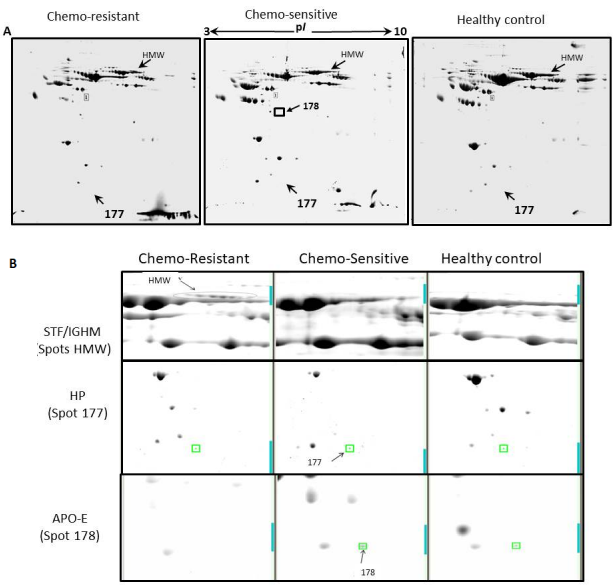

Figure 2: A) Representative full 2-DE gel images of plasma samples. B) Close-up 2-DE gel images of differentially expressed protein spots in chemo-resistant, chemo-sensitive and healthy controls from plasma samples.

using the ImageMaster software then checked manually. Spots >4 fold changes were chosen as differential spots between resistant and sensitive groups. Two spots expressed significantly different in the two patient groups were selected, excised and sent for mass spectrometry (MS) analysis. Spot No. 169 (p=0.01) was overexpressed in resistant group (Fig. 1) while Spot No. 503 (p=0.049) was up-regulated in the chemo-sensitive group.

In plasma samples approximately 560 spots were detected. Among them, a total of eight proteins were significantly different in plasma of resistant and sensitive patients. Five high molecular weight (HMW, MW~70 kDa) spots were expressed in 3/6 chemo-resistant samples and absent from the other groups. Of these 3 were selected for mass spectrometry analysis. Spots No. 177 (<20 kDa) and 178 (~36 kDa) were significantly overexpressed in chemo-sensitive group (P<0.05).

Protein identification by LC/MS/MS

Representatives of full 2-DE gel images of PBMC and plasma samples are shown in Fig. 1 and Fig. 2, respectively. Proteins spots differentially expressed in chemo-resistant and chemo-sensitive AML PBMC and plasma are shown in Fig. 1B and Fig. 2B, respectively. Characteristics of the individual protein spots identified by LC/MS/MS analysis in PBMC and plasma samples are shown in Table II and Table III, respectively.

For PBMC proteins, Spot No.169 was successfully identified as heterogeneous nuclear ribonucleoprotein H1 (HNRNP H1). Identification of Spot No. 503 was not successful and reported as a putative uncharacterized protein of Streptococcus downei. However, by blasting the peptide sequence with NCBI database against *Homo sapiens*, protein DNA-PK (DNA-dependent protein kinase) was found as a hit (Table II).

For plasma proteins, two spots (Spot No. 177 and 178) and a set of high molecular weight (HMW) proteins of similar molecular weight were differentially expressed in the AML groups and absent in normal controls (Fig. 2). LC/MS/MS identified Spot. No. 177 as haptoglobin (HP). Spot. No. 178 was identified as apoliprotein-E (APO-E). All three HMW spots selected (HMW 1, 2 and 3) aligned to basically two proteins, serotransferrin (STF) or in short for transferrin (TF) and Ig mu chain C region (IGHM) (Fig. 2, Table III). TF and IGHM have high molecular weights at 80 kDa and 65 kDa, respectively. However, TF is closer to the observed molecular weight for these spots and thus, is likely the differentially expressed protein. Annotation and function of proteins were retrieved from protein databases such as UniProt and NCBI websites.

Table II: Characteristics proteins from PBMC lysate identified by LC/MS/MS differentially expressed in chemo-resistant and chemo-sensitive AML

Spot No.	NCBI Accession No.	Sequence coverage	Protein name	MW (Da)	PI	Score	Peptides (Unique)
169	NP_005511.1	4%	Heterogeneous nuclear ribonucleoprotein (HNRNP H1) complexes	47379	5.93	64	2
503	E3CKV6 (In Uniprot)	2%	Putative uncharacterized protein (Streptococcus downei) Or DNA-PK	45221	8.28	52	1

Table III: Characteristics of plasma proteins identified by LC/MS/MS differentially expressed in chemo-resistant and chemo-sensitive AML

Spot No	Accession No	Sequence Coverage	Protein Name	MW (Da)	PI	Score	Peptides (Unique)
178	NP_000032.1	31%	Apolipoprotein E precursor	36123	5.65	557	11
HMW 1	B4E1B2	19%	cDNA FLJ53691, highly similar to Serotransferrin	74783	6.81	689	25
	P01871	26%	IGHM Ig mu chain C region	49276	6.35	616	21
	B2R8I2	17%	cDNA, FLJ93914, highly similar to Homo sapiens histidine-rich glycoprotein (HRG), mRNA	59475	7.01	362	11
HMW 2	B4E1B2	24%	cDNA FLJ53691, highly similar to Serotransferrin	74783	6.81	651	24
	P01871	27%	IGHM Ig mu chain C region	49276	6.35	632	23
HMW 3	B4E1B2	8%	cDNA FLJ53691, highly similar to Serotransferrin	74783	6.81	170	7
	P01871	13%	IGHM Ig mu chain C region	49276	6.35	268	7
177	H3BMJ7	16%	Haptoglobin	10147	4.88	379	13

DISCUSSION

In the present study 2-DE identified HNRNP H1 from PBMC lysate, APO-E and haptoglobin from plasma were significantly up-regulated in chemo-sensitive patients. On the other hand, DNA-PK from PBMC lysate and serotransferrin from plasma were significantly increased in chemo-resistant patients.

HNRNP H1 belongs to a subfamily of RNA-binding proteins that possess three repeats of quasi-RRM domains and has a key role in RNA processing and metabolism. HNRNP H1/H2 is involved in splicing of pre-thymidine phosphorylase (TP) mRNA. It was demonstrated that abnormal splicing of TP pre-mRNA caused loss of expression and function of TP gene in a tumour cell line, therefore, resulting in resistance to fluoropyrimidine-based anti-cancer drugs (15). A unique glycosylated HNRNP H1 was found to be associated with 11q23 translocation (16) but not other AML cytogenetic risk groups. An analysis of 148 AML patients showed presence of 11q23 did not alter overall survival (OS) but improved OS and relapse-free survival in allogeneic stem cell transplantation patients (17). HNRNP H1 is a putative ZEB2 interaction partner in fusion proteins in T-cell acute lymphoblastic leukaemia, T-ALL (18) and recognised as a main novel splicing regulator in murine AML model among novel tumour suppressors and oncogenes (19). Strong HNRNP H expression was associated with better survival in colorectal cancer (20). The identification of HNRNP H1 protein here supports its importance as a potential predictive biomarker in leukaemia.

ApoE is a 299 residue protein and key regulator of plasma lipid levels which binds to lipid and low density lipoprotein receptor inducing lipolytic processing and endocytosis of triglyceride-rich lipoprotein. It is secreted by cells in the liver as well as the brain. Three ApoE isoforms are recognised, E2, E3 and E4 with different clinical significance. E3 is the dominant form with normal functional abilities (21). Spot 178 was identified as apoliprotein E isoform b precursor (NP_000032.1). ApoE3 is identified as an Identical Protein Group (https://www.ncbi.nlm.nih.gov/ipg/NP_000032.1). These proteins are apparently not differentiated at the protein level. Braoudaki et al. (22) analysed serum samples from acute lymphoblastic leukaemia (ALL) and identified APO-E at similar position in 2-DE proteome as this study but found it to be associated with high risk ALL. This is in contrast to increased expression in chemo-sensitive cases here. However, it is increased in ovarian cancers and expression in nuclei was associated with better survival (23). Women with APOE4 genotype survived significantly longer than non-APOE4 chronic lymphocytic leukaemia (CLL) patients (24). A study on serum from AML patients found it to be absent in contrast to normal controls (25). Thus, importance of APO-E in cancer and leukaemia may be subjected to its various isoforms.

Haptoglobin (HP) is a serum protein essential in acute-phase response during inflammation with an important inhibitory function (26). It combines with plasma haemoglobin for recycling of heme iron. This complex is picked up by macrophage CD163 scavenger receptor and degraded through the endocytic lysosomal pathway in liver Kupffer cells. Haptoglobin was identified on three regions on 2-DE with nonlinear IPG strip on the pH range 3-10 (27) where the smallest molecular weight corresponded to truncated alpha-chain proteins of haptoglobin as identified here. Increased HP1 in leukaemias has been reported since the 1960s (28). In AML, HP was significantly elevated compared to controls (29). Mitchell et al. (28) examined HP phenotypes in AML and showed frequency of HP1 was increased while HP2 was decreased compared to controls. HP2-1 and HP0 phenotypes were also identified. A more recent study showed HP2 was elevated in AML in contrast to normal control (25). Haptoglobin-1 precursor was significantly increased in all grades of ovarian cancer

suggesting suitability as an early diagnosis biomarker (30). Our results support increased haptoglobin protein in AML particularly among chemo-sensitive cases.

DNA-dependent protein kinase (DNA-PK) catalyses the repair of DNA double-strand breaks via involvement of the non-homologous end-joining pathway. Cancer cells that are able to repair DNA damage controls their sensitivity to radio- or chemo-therapy. It is widely accepted that increase in DNA-PK activity enhances resistance of cancer cells to chemotherapies since their DNA repair function unavoidably lead to protection of the cancer genome and survival of tumour cells (31). DNA-PK stimulates angiogenesis, migration and invasion in the development of metastasis (32). In AML, increased DNA-PK activity correlated with chemo-resistance against various anti-tumour agents (33). Our result on DNA-PK supports these earlier studies.

Transferrin (TF) is a serum glycoprotein for the delivery of iron and is a test routinely used in diagnosis of diseases. Higher transferrin saturation was associated with increased risk of non-skin cancer and cancer death (34). CD71 (transferrin receptor, TFR) is a preferred marker for study and was not found to be expressed on leukaemia cells in AML cases (35). A proportion of AML blasts expressing TFR was related to proliferative potential and could be stimulated to proliferate (36). AML expressing high levels of TFR (>60%) was 30.8%, however, did not appear to impact survival time (37). The availability of this biomarker in routine test makes it all the more suitable as it can be easily incorporated into current practise in management of AML.

CONCLUSION

In conclusion, proteins selected from this study support findings of earlier studies in having identified the same proteins with potential as biomarkers in AML. However, its precise role to predict treatment outcome varied possibly due to the heterogeneity in cancers as well as complexity of proteins. Future work should include a larger number of samples to validate the predictive potential of these markers.

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