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Identification of Target Antigens in AML:

Profiling the Surfaceome

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Erklärung

Diese Dissertation wurde im Sinne von § 7 der Promotionsordnung vom 28. November 2011 von Frau Prof. Dr. Marion Subklewe betreut und von Herrn Prof. Dr. Roland Beckmann von der Fakultät für Chemie und Pharmazie vertreten.

Eidesstattliche Versicherung

Diese Dissertation wurde eigenständig und ohne unerlaubte Hilfe erarbeitet.

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Xilong Liu

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1. Gutachter: Prof. Dr. Roland Beckmann

2. Gutachter: Prof. Dr. Marion Subklewe

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I dedicate this work to my family!

路漫漫其修远兮，吾将上下而求索

－屈原《离骚》

The journey is long, I'll search up and down.

-Qu yuan 《Li Sao》

Translated by Su Man-shu

List of Abbreviations

5-FU	5-fluorouracil
ALK	Anaplastic lymphoma kinase
ALL	Acute lymphoblastic leukemia
ALCL	Anaplastic large cell lymphoma
AML	Acute myeloid leukemia
AML-MRC	AML with myelodysplasia-related changes
APC	Antigen-presenting cell
APL	Acute promyelocytic leukemia
ATO	Arsenic trioxide
ATRA	All-trans-retinoic acid
BCL2	B-cell lymphoma 2
BCR-ABL	Breakpoint cluster region protein-Abelson murine leukemia viral oncogene homolog
BiTE®	Bispecific T cell engager
BHES	2,2‘-Thiodiethanol
BM	Bone Marrow
BSA	Bovine serum albumin
BTD	Breakthrough therapy designation
BTK	Bruton's tyrosine kinase
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen
cHL	classical hodgkin lymphoma
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor cell
CR	Complete remission
CRh	Complete response with partial hematologic recovery
CSC	Cell Surface Capturing
CTCL	Cutaneous T-cell lymphoma
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
DFS	Disease-free survival
DFSP	Dermatofibrosarcoma protuberans
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
DPBS	Dulbecco`s Phosphate Buffered Salt Solution
DTH	Delayed-type hypersensitivity
DTT	DL-Dithiothreitol
early HPC_BM	Hematopoietic progenitor cells from bone marrow
EDTA	Ethylenediaminetetraacetic acid
EGFR	Endothelial growth factor receptor
E:T	Effector-to-target
ET/NET	Endocrine/neuroendocrine tumor
ELN	European LeukemiaNet
FAB	French–American–British classification system
FBS	Fetal bovine serum
FDA	Food and Drug Administration (U.S.)
FGFR	Fibroblast growth factor receptor
FIH	First-in-human
FL	Follicular lymphoma
FLT3	Fms-like tyrosine kinase 3
FOLFOX4	Oxaliplatin/5-FU/leucovorin
GCTB	Giant cell tumor of the bone
GIST	Gastrointestinal stromal tumor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Granulocyte monocyte progenitors
GO	Gemtuzumab ozogamicin
GVAX	Vaccine comprised of cancer cells genetically modified to secrete granulocyte-macrophage colony-stimulating factor

HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor 2
HNC	Head and neck cancer
HL	Hodgkin lymphoma
HSC_BM	Hematopoietic stem cells from bone marrow
HSCs	Hematopoietic stem cells
HSP	Heat shock protein
iBAQ	Intensity based absolute quantification
IDH2	Isocitrate dehydrogenase-2
IFN	Interferon
IL	Interleukin
ITD	Internal tandem duplication
i.v.	Intravenous injection
LAA	Leukemia-associated antigen
LC-MS	Liquid chromatography coupled to tandem mass spectrometry
LSA	Leukemia-specific antigen
LSC	Leukemic stem cell
M	mol/l
MCC	Merkel cell carcinoma
MCD	Multicentric Castleman disease
MCL	Mantle cell lymphoma
mDC	$CD11c^+$ myeloid dendritic cells
MD/MPDs	Myelodysplastic/myeloproliferative disorders
MEP	Megakaryocyte-erythroid progenitor cell
MES	2-(N-Morpholino)ethanesulfonic acid hydrate
mg	Milligram
ml	Milliliter
mM	Milli mol/l
MM	Multiple myeloma
MRC	Medical Research Council
MRD	Minimal residual disease
MS	Mass spectrometry
MY_BM	Myelocyte from bone marrow
NA	Not available
NB	Neuroblastoma
NCI	National Cancer Institute
NHL	Non-hodgkin's lymphoma
NIH	National Institutes of Health
NK cells	$CD56^+$ natural killer cells
NSCLC	Non-small cell lung cancer
NPM1	Nucleophosmin-1
OEC/FTC/PPC	Ovarian epithelial/fallopian tube/primary peritoneal cancers
OS	Overall survival
PB	Peripheral blood
PBD	Pyrrolobenzodiazepine
pcALCL	Primary cutaneous anaplastic large cell lymphoma
PCNSL	Primary central nervous system lymphoma
pDC	$CD123^+$ plasmacytoid dendritic cells
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PDX	Patient-derived xenograft
Pen-Stre-Glu	Penicillin-Streptomycin-Glutamine
PDGFR	Platelet-derived growth factor receptor
Ph+	Philadelphia chromosome-positive
Ph-	Philadelphia chromosome-negative
PI3K-δ	Phosphatidylinositol-3-kinase- delta
PM_BM	Promyelocyte from bone marrow
PMN_BM	Polymorphonuclear cells from bone marrow
PMN_PB	Polymorphonuclear cells from peripheral blood
PP	Primary patient
PRAME	Preferentially expressed antigen in melanoma
PTM	Post-translational modification
PTCL	Peripheral T-cell Lymphoma

rh	Recombinant Human
RARs	retinoic acid receptors
RIL	Relapsed indolent lymphoma
RPKM	Reads Per Kilobase of transcript per Million mapped reads
RXRs	Retinoid X receptors
R/R	Relapsed or refractory
sALCL	systemic anaplastic large-cell lymphoma
SCT	Stem cell transplantation
SEER	Surveillance, Epidemiology, and End Results Program
SILAC	Stable-isotope labeling with amino acids in cell culture
SLAMF7	Signaling lymphocytic activation molecule F7
SLL	Small lymphocytic lymphoma
SM	Systemic mastocytosis
STS	Soft tissue sarcoma
sulfo-NHS-SS-biotin	sulfosuccinimidyl-2-(biotinamido)-ethyl-1,3'-dithiopropionate
t-AML	Therapy-related AML
TCEP	Tris (2-carboxyethyl) phosphine
TIM-3	T-cell immunoglobulin domain and mucin domain 3
TLR	Toll-like receptor
TMs	Transmembrane domains
T_{regs}	regulatory T cells
VEGFR	Vascular endothelial growth factor receptor
WHO	World Health Organization
WT	Wildtype
WT1	Wilm's tumor protein 1

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Summary

Acute myeloid leukemia (AML) is a heterogenous hematopoietic disorder which includes a number of categories and many subtypes. The incidence rate correlates with age showing higher incidence rate for individuals above the age of 65. Patients with AML have worse 5-year survival rates compared to other subtypes of leukemia, such as acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML) and chronic lymphocytic leukemia (CLL).

Despite many considerable advances in the field of immunotherapy, such as chimeric antigen receptor (CAR) T cells, checkpoint inhibitors, dendritic cell vaccination, T cell-recruiting antibody constructs and antibody-drug conjugates, clinical immunotherapy of AML to date has failed to show the same efficacy seen in other types of leukemia. In addition, on-target off-leukemia toxicity remains a challenge. Thus, identification of more suitable target antigens for immunotherapy might improve the therapeutic efficacy of immunotherapy in AML and ultimately improve over outcome for this disease.

The original „Cell Surface Capturing“(CSC) technology (Glyco-CSC) and it‘s variants (Cys-Glyco-CSC and Lys-CSC) technology are Mass spectrometry-based technologies which offer the advantage of multiplexed and unbiased detection of cell surface proteins independently from existing antibody collections. They provide the possibility to identify the entirety of surface proteins expressed on living cell populations. However, direct processing of primary leukemia samples has been limited by the substantial number of viable cells needed for the CSC-workflow. We therefore addressed this issue by improving the technique including the use of our *ex vivo* culture system. This allowed for an unbiased, direct assessment of primary AML

patient samples and thus enabled us to interrogate the AML surfaceome in clinically relevant samples. Primary cells from patients with newly diagnosed or relapsed AML were cultured in our *ex vivo* co-culture system as previously described (Krupka et al. 2014) using MS-5 feeder cells. After 3 days, non-adherent cells were harvested and immediately subjected to the CSC-workflow. Glyco-CSC and its variants Cys-Glyco-CSC and Lys-CSC were initially performed as described by Wollscheid and colleagues (Wollscheid et al. 2009 and Bausch-Fluck et al. 2012) and subsequently modified to improve the yield on AML samples. CSC samples were analyzed by tandem mass spectrometry on an Orbitrap Elite instrument and modified peptides were identified using MaxQuant software.

To enhance the number of successfully identified surface proteins, we also adapted the original CSC protocol. These modifications doubled the yield in identified proteins from AML cell lines from initially 125 to 252. More importantly, the modifications increased the specificity of the assay significantly. In the original Glyco-CSC experiments, only 54% of all identified peptides displayed a mass shift (of 0.984 Da) associated with successful N-glycosylation and had a transmembrane domain or a signal peptide annotated in UniProt. After modification of the protocol, 80.4% of all peptides fulfilled these criteria. The modified protocol was therefore used for all primary samples. 5 representative primary patient samples from initial diagnosis and 2 samples from relapsed disease were analyzed. All samples yielded sufficient viable cell numbers after *ex vivo* culture and could successfully be subjected to the CSC workflow. We identified a total of 719 surface proteins fulfilling all filter rules. 22.9% of these proteins had CD annotations. Next, we only considered proteins that were detected by CSC in at least half of the primary patient samples tested. In addition, proteins were filtered to eliminate targets that are abundantly

expressed on normal human hematopoietic stem and progenitor cells as well as relevant healthy tissue using publicly available transcriptome databases. 85 proteins were selected as potential candidates for manual screening. Of note, the expression of several antigens currently under investigation for AML immunotherapy (i.e. CD33, CD123, CD135, CLL-1) were detected by the method. We selected 5 promising novel candidate markers previously not described as relevant targets in AML. These were assessed by FACS analysis in independent patient samples. 4/5 of our novel targets showed uniform expression in all independent primary AML samples tested (defined as MFI ratio >1.5).

In conclusion, improvements in the CSC-Workflow combined with our *ex vivo* culture system allowed for the successful identification of the AML surfaceome from primary patient samples without the necessity of xeno-amplification. We identified 5 novel targets, 4/5 were found to be uniformly expressed in independent primary AML samples. These candidates are now being evaluated further as potential targets for antibody and CAR based immunotherapy in AML.

* A majority of this "Summary" part was submit to ASH, Blood 2017 130:3968, which I am the second author. Slightly changes have been made after more clinical data was carried out from AML patient samples.

Zusammenfassung

Akute myeloische Leukämie (AML) ist eine genetisch und klinisch heterogene Erkrankung. Die Inzidenz der Erkrankung korreliert mit dem Alter und ist deutlich erhöht in Individuen über dem Alter von 65 Jahren. Verglichen mit anderen Leukämien – akute lymphatische Leukämie (ALL), chronische myeloische Leukämie (CML) und chronisch lymphatische Leukämie (CLL) – verbleibt die Prognose für Patienten mit AML deutlich schlechter. Trotz deutlicher Fortschritte auf dem Feld der Immuntherapie und entsprechenden Anstrengungen, chimäre antigen-rezeptor T Zellen, Checkpoint-Inhibitoren, dendritische Zell-Vakzinierungen, T Zell rekrutierende Antikörperkonstrukte sowie Antikörper-toxin-Konjugate gegen AML einzusetzen, ist der klinische Erfolg für Patienten mit AML bislang begrenzt. Ein möglicher Grund ist das die bislang verwendeten Zielantigene auf AML-Zellen entweder keine effiziente Eliminierung der malignen Zellen ermöglichen oder eine erhöhte Toxizität gegenüber gesunden Zellen aufweisen. Daher besteht ein erhebliches Interesse an der Identifikation von geeigneteren Antigenen zur Entwicklung von immuntherapeutischen Strategien.

Die „Cell Surface Capture“ (CSC) Technologie erlaubt die Massenzytometrie-basierte Identifikation der Gesamtheit der Zell-Oberflächenproteine (das sog. „Surfaceome“), unabhängig von bestehenden Antikörperkollektionen. Bislang war eine direkte Anwendung dieser Technologie auf primäre Leukämieproben von Limitationen bezüglich der notwendigen Zellzahlen begrenzt. Wir haben diesen Aspekt durch die Verwendung unseres ex vivo Kultursystems (Krupka et al., 2014) adressiert. Primäre Patientenproben von Patienten mit neu diagnostizierter oder rezidivierter AML wurden mit MS-5 Stromazellen kokultiviert und nach drei Tagen konnten viable, nicht-adhärante

Zellen der CSC Technologie zugeführt werden. Die CSC Proben wurden mittels Tandem Massenspektrometrie auf einem Orbitrap Elite Instrument detektiert und mittels MaxQuant software ausgewertet.

Um die Anzahl der erfolgreich identifizierten Proteine zu erhöhen, haben wir zudem das vorhandene CSC-Protokoll (Wollscheid et al. 2009 and Bausch-Fluck et al. 2012) modifiziert. Durch diese Modifikationen konnten wir die Anzahl der identifizierten Proteine auf AML Zelllinien von initial 125 auf 252 erhöhen. In diesem Kontext ist jedoch noch relevanter, dass die Modifikationen auch zu einer Erhöhung der Spezifität der Technologie führen konnte. So fand sich initial die mit einem für die korrekte N-Glycosylation charakteristische Massenverschiebung (0.984 Da) in nur 54% aller identifizierten Peptide. Nach Einführung der Protokoll-Modifikationen erfüllten 80.4% aller identifizierten Peptide dieses Kriterium. Das modifizierte Protokoll wurde daher bei allen primären AML Proben eingesetzt.

5 repräsentative Patienproben zum Zeitpunkt der Primärdiagnose sowie 2 Proben von Rezidivfällen wurden analysiert. Von allen Proben konnten ausreichende Zellzahlen der CSC-Technologie zugeführt werden.

Wir konnten in Summe 719 Oberflächenproteine identifizieren. 22.9% dieser Proteine waren mit Cluster of Differentiation (CD) Annotationen versehen. In einem nächsten Schritt wurden diejenigen Antigene Eliminiert, die entweder auf weniger als der Hälfte der analysierten Proben nachweisbar waren oder eine hohe Expression auf gesunden hämatopoetischen Stamm- und Vorläuferzellen sowie relevante Organe des nicht-hämatopoetischen Systems zeigten.

84 Proteine wurden als potentielle Kandidaten für ein manuelles Screening identifiziert. Interessanter Weise wurde durch unsere Methode mehrere Antigene, die derzeit als potentielle Zielantigene für immuntherapeutische Strategien

untersucht weden (CD33, CD123, CD135, CLL-1). Wir wählten 6 vielversprechende, neue Antigene, die bislang nicht als relevante Zielantigene in der AML beschrieben worden sind, aus. Für 4/5 Antigene konnte eine relevante Expression auf unabhängigen AML Patientenproben mittels FACS (definiert als MFI ratio >1.5) nachgewiesen werden.

Zusammenfassend, konnten wir durch Verbesserungen des CSC-Protokolls gemeinsam mit der Verwendung unseres ex-vivo Kultursystems erfolgreich das Surfaceome in primären AML Proben identizieren. Wir konnten 5 potentiell relevante Zielantigene identifizieren, 4/5 ließen sich zudem mit einer relevanten Expression auf unabhängigen AML Patientenproben nachweisen. Diese Kandidatenantigene werden nun weiter bezüglich Ihrer Eignung als Zielstrukturen für Antikörper- oder CAR-T Zell-basierte Immuntherapie evaluiert

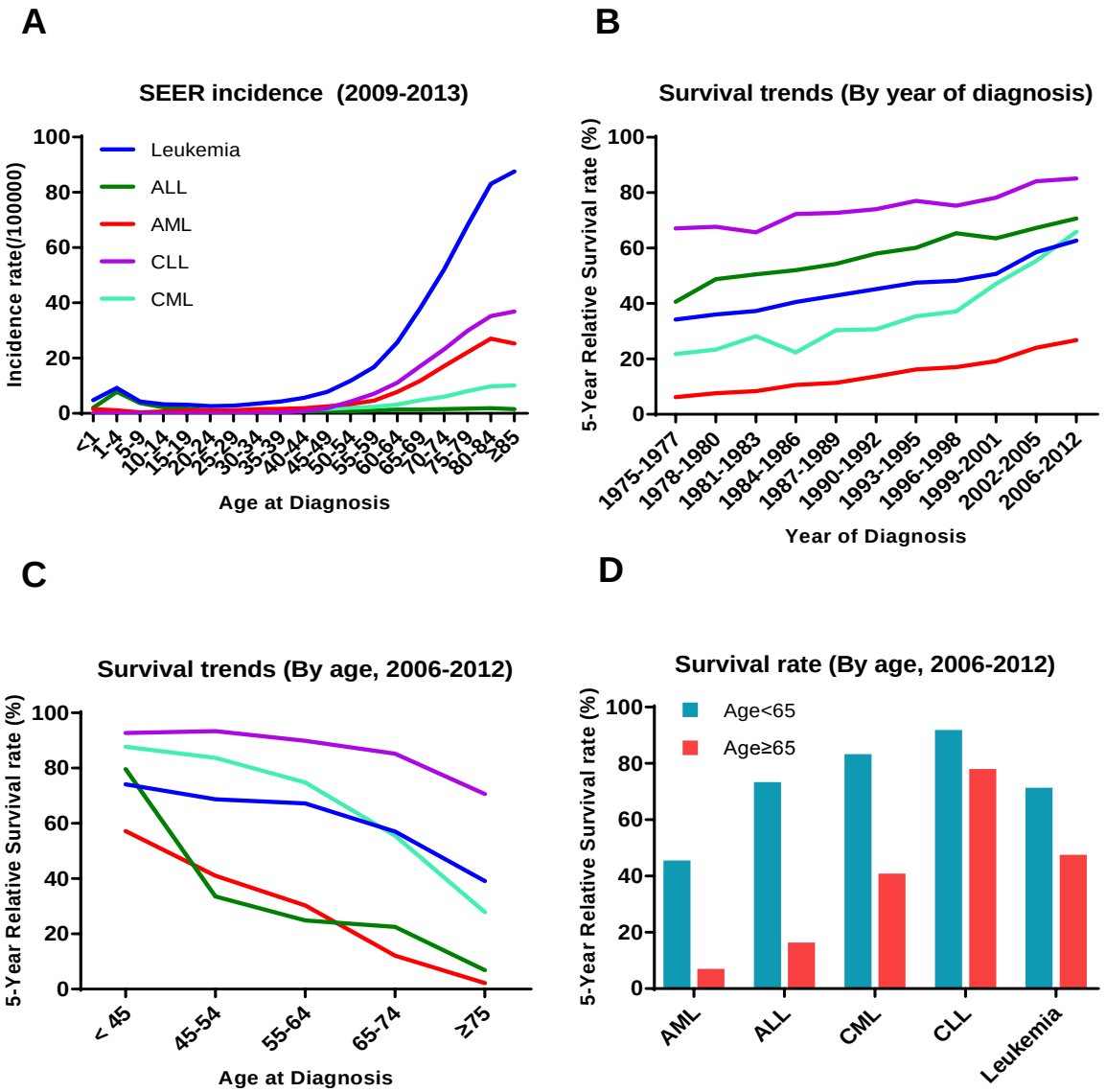
1. Introduction

1.1 Leukemia and acute myeloid leukemia

Leukemia is a group of cancers caused by malignant clonal expansion of hematopoietic stem- and precursor cells. Alfred-Armand-Louis-Marie Velpeau, an anatomist and surgeon, described leukemia for the first time in 1827[1,2], though it is possible that leukemia had already been seen as early as 1811[1,3]. Leukemia may occur in every stage of people's whole life, most often in older adults, but it is the most common cancers in young children. The incidence and death rate are low before age 60, 16.8 and 5.8 (rate is per 100,000 per year), respectively. But there is a rapid increase from age 65 to 85+, with incidence rates increasing from 38.3 to 87.5 and death rates increasing from 17.7 to 84.0[4].

Leukemia can be divided into 4 main types: acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL) and chronic myeloid leukemia (CML)[5,6]. ALL is the most common childhood leukemia, about 75% of all ALLs were accounted in children (Figure 1-1 A). By contrast, AML, CLL and CML occur mainly in adults. According to data statistics from National Cancer Institute (NCI, at the National Institutes of Health (NIH)), AML is the most common acute leukemia in adults with a incidence of 4.1/100,000 cases (year 2009-2013)[4] in the US. Incidence dramatically increases with age, resulting in 19.1/100,000 cases in the age group 65+ (Figure 1-1 A)[4]. Despite some improvement since 1975, 5-year relative survival rate for AML patients remain low for now (26.8%) (Figure 1-1 B). In addition, survival rates correlate with age, resulting in a 5-year relative survival rate of 6.3% in patients aged above 65 while younger patients display 5-year survival rates of 44.8% (Figure 1-1 C and D). One significant reason is that in spite of remission rates of up to 80% after intensive induction chemotherapy, patients with AML have a

high risk of disease recurrence. Therefore, more research is needed to improve survival rates or achieve long-term cure for the majority of AML patients in the future.



*Data source: https://seer.cancer.gov/csr/1975_2013/sections.html, NCI SEER, all races, both sexes, 1975-2013.

Figure 1-1. Updated data of leukemia. (A) SEER incidence of leukemias show that leukemia occurs in every age group. ALL patients are mainly younger children (age<14) while AML, CLL and CML are mainly found in adult patients, especially those age 55+. (B) Great improvements have been made since 1975. Leukemia patients have a much higher 5-year relative survival rate now (62.7%) compare to 34.2% in 1975. AML patients have the poorest 5-year relative survival rate (26.8%) while CLL patients display a more favorable prognosis (85.1%). (C) With increased age, survival rates decrease: AML patients aged 65+ have the lowest survival rate, only 6.3%, compare to the other 3 types (D).

Most patients eligible for intensive therapy, excluding acute promyelocytic leukemia, are treated with standard "7+3" intensive induction therapy. After continuous

intravenous (i.v.) infusion of cytarabine (7 days) and anthracycline (3 days, mostly daunorubicin, alternatively mitoxantrone or idarubicin), 60-80% of younger adults and 40-60% of older adults (65+) will achieve a complete remission (CR)[7-10].

Despite some improvements, especially for patients below 60 years of age, 50-70% of patients achieving first CR are expect to relapse within 3 years[11,12]. Relapses in patients with a continued CR of >5 years are rare (1.16% of all relapses) [13]. However, prognosis for relapsed or refractory AML (R/R-AML) is especially poor[14-16]. Many factors might contribute to the poor outcomes at relapse, such as cytogenetics, unidentified mutations, relapse after hematopoietic stem cell transplant (HSCT), older age, and duration of first CR less than 12 months[14,17-20].

The major reason considered to cause relapse in leukemia is a small number of leukemic cells still detectable in the patients during or after treatment when a CR achieved. These cells have been termed “minimal residual disease” (MRD) or “minimal measurable disease”. MRD assays have been established and are commonly performed in ALL patients[21-24]. In AML, MRD assessment is more complex due to the genetic as well as phenotypic heterogeneity of the disease. One potential target for MRD assessment in AML is are nucleophosmin (NPM1) gene mutations, which occur in about 30% patients, which represent the most frequent genetic alteration in AML[25]. More than 50 NPM1 mutations have been found to date. Type A, B and D are the three most common variants found in 90% of all mutated cases[26-32]. Fms-like tyrosine kinase 3 (FLT3) internal tandem duplications (ITD) is a second commonly affected gene mutation and can be identified in about 25% of all AML patients. However, heterogeneity and instability limits its suitability as a MRD marker[28-38]. These examples highlight the fact that AML is a biologically heterogeneous disease.

Traditional chemotherapy-based treatment of AML patients are unlikely to improve outcomes. Various novel immunotherapy strategies are being evaluated in pre-clinical and clinical trials, such as chimeric antigen receptor (CARs) T cells[39-45], dendritic cells (DC) vaccination[46-48] and antibody based immunotherapy[49-56]. Clinical results from these functional cells or antibody drugs are expected to further improve survival interval and prevent relapse of AML patients.

1.2 Advanced cancer therapies

Figure 1-2. Current strategies to treat cancers and hematopoiesis disorders. 8 treatment strategies were developed to fight against cancer and hematopoiesis diseases to date. Surgery, radiation therapy, and chemotherapy are still important choices for many types of cancer, especially solid tumors. Immunotherapy and targeted therapy are two most potential strategies for all types of cancers, with the development of new/novel therapeutic markers and relevant drugs. Hormone therapy is applied to treat gender-special cancer, like breast cancer; while stem cell transplant (SCT) is specific for numbers of hematopoiesis diseases, like leukemias. Precision medicine aims to supply personalise treatment strategy to the patient based on the rapid development of gene sequencing technology and big data, i.e. genome, proteome, transcriptome and metabolome.

The National Cancer Institute (NCI) lists 8 main types of cancer treatment (Figure 1-2). Besides the 3 conventional strategies (surgery, radiation therapy and chemotherapy), 5 more recent strategies are also involved, which are immunotherapy, targeted therapy, hormone therapy, precision medicine, and stem cell transplant

(SCT). Currently, cancer patients commonly receive a combination of treatments, including surgery (especially for solid tumors such as breast cancer, lung cancer and prostate cancer), radiation therapy, chemotherapy, immunotherapy and SCT (especially for hematological cancers). Immunotherapy utilizes the host immune responses to produce potent cancer destruction; whereas, targeted therapies commonly aim to block essential molecular pathways crucial for proliferation and maintenance of cancer. But NCI claims that immunotherapy is one type of targeted therapies, which possibly indicates that there is obscure boundary between immunotherapy and targeted therapy. Since they both are focusing on suitable "targets" on tumor cells, immunotherapy and targeted therapy play very important roles in the development of new/novel anticancer drug.

1.2.1 Cancer immunotherapy

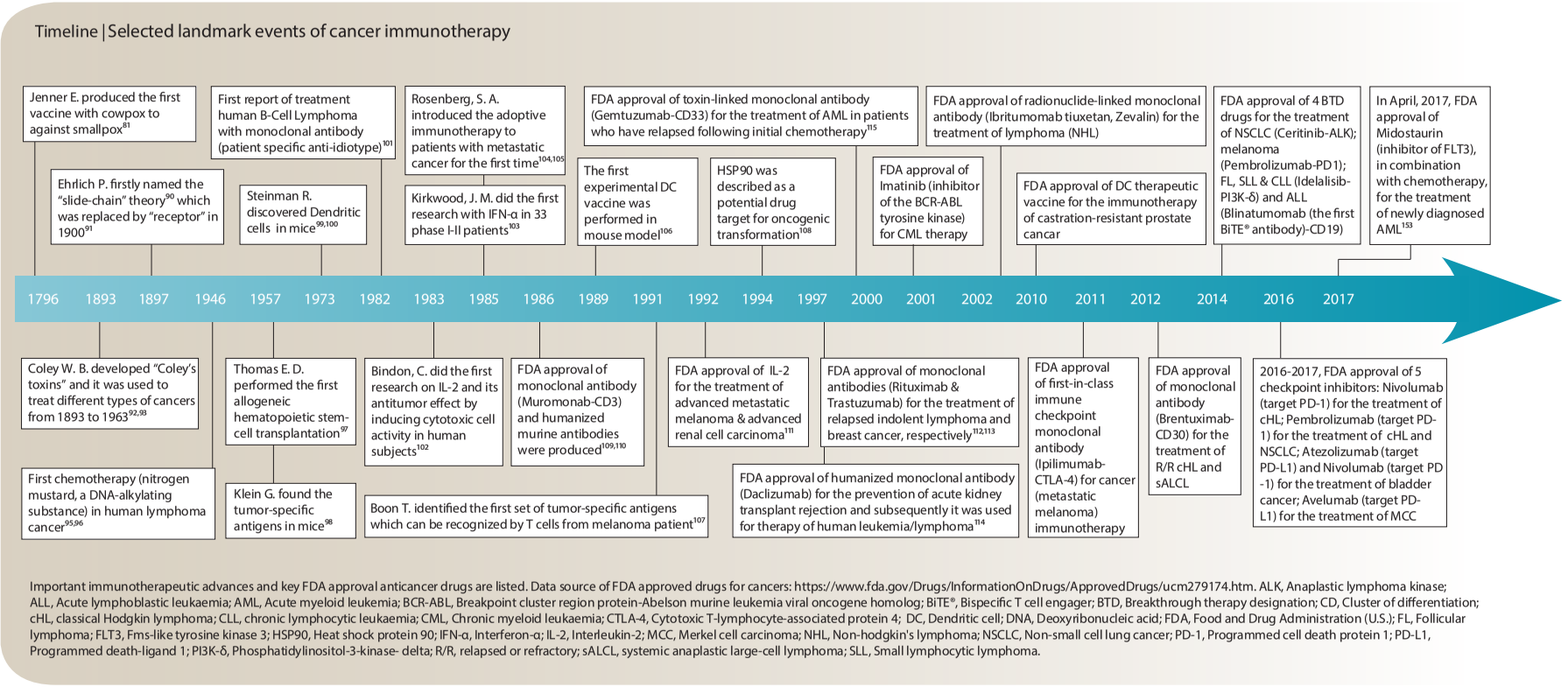
Briefly, the aim of cancer immunotherapy is to utilize a patient's own immune system to treat cancer. Four main types strategies are accounted for cancer therapy: 1) antibody therapy; 2) cellular immunotherapy (i.e. DC and CAR T cells); 3) cytokine therapy (interleukin and interferon); 4) Therapeutic vaccines (Figure 1-2). Cancer immunotherapy has a long history in its development (Figure 1-3)[57,58] and now is given rise to new options for cancer treatment[57,59-65].

In 1796, Edward Jenner produced the first vaccine with cowpox to against smallpox[57]. About 100 years later, Paul Ehrlich firstly named the "slide-chain" theory on 1897[66], but it was replaced by "receptor" in 1900[67] and it was accepted by doctors and scientific researchers and still in use now. Meanwhile, Coley's toxins (also called Coley's treatment) was developed by William Coley, a bone surgeon, to treat with

different types of cancer from 1893 to 1963[68,69]. But it did not show more promising results in several small clinical trials. Chemotherapy and HSCT were developed from 1940s to 1960s[70-72]. More and more important discoveries came up after the tumor-specific antigen was found in mice[73], including the discovery of DC[74,75], first report of treatment lymphoma with monoclonal antibody[76], research on IL-2 and IFN-α on human[77,78]. In the late 20th century, Steven Rosenberg *et al*. had introduced immune cell therapy for cancer. They received a low tumor regression rate (2.6-3.3%) in 1205 metastatic cancer patients[79,80]. The first experimental DC vaccine[81] and the first set of tumor-specific antigens from melanoma patient[82] were studied in the 1990s.

FDA approved drugs, especially for immunotherapy and targeted therapy, promoted the improvement of cancer therapy since the 1980s[83-88]. With the coming of the 21st century, totally 395 new therapeutic drugs had been proved by FDA between 2001 and 2015, among which 112 (28.4%) were used for treatment of hematologic, oncologic, or immune-modulating disease[89,90]. Figure 1-3 listed some selected FDA approval drugs from 2000 to 2017 and information of those drugs can be found online (FDA Approved drugs: Hematology/Oncology (Cancer) Approvals & Safety Notifications):

https://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm279174.htm.



Important immunotherapeutic advances and key FDA approval anticancer drugs are listed. Data source of FDA approved drugs for cancers: https://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm279174.htm. ALK, Anaplastic lymphoma kinase; ALL, Acute lymphoblastic leukaemia; AML, Acute myeloid leukemia; BCR-ABL, Breakpoint cluster region protein-Abelson murine leukemia viral oncogene homolog; BiTE®, Bispecific T cell engager; BTD, Breakthrough therapy designation; CD, Cluster of differentiation; cHL, classical Hodgkin lymphoma; CLL, chronic lymphocytic leukaemia; CML, Chronic myeloid leukaemia; CTLA-4, Cytotoxic T-lymphocyte-associated protein 4; DC, Dendritic cell; DNA, Deoxyribonucleic acid; FDA, Food and Drug Administration (U.S.); FL, Follicular lymphoma; FLT3, Fms-like tyrosine kinase 3; HSP90, Heat shock protein 90; IFN-α, Interferon-α; IL-2, Interleukin-2; MCC, Merkel cell carcinoma; NHL, Non-hodgkin's lymphoma; NSCLC, Non-small cell lung cancer; PD-1, Programmed cell death protein 1; PD-L1, Programmed death-ligand 1; PI3K-δ, Phosphatidylinositol-3-kinase- delta; R/R, relapsed or refractory; sALCL, systemic anaplastic large-cell lymphoma; SLL, Small lymphocytic lymphoma.

Figure 1-3. History timeline of selected milestones in the development of cancer immunotherapy

1.2.2 Targeted cancer therapy

4 different types of cancer therapy are commonly involved in the current treatment with anticancer drugs: chemotherapy, hormonal therapy, targeted therapy and immunotherapy (Figure 1-2)[91]. After the first tumor-specific antigens were identified and isolated from melanoma patient[82], the race was on to target them as therapeutic vaccines. Technically, targeted cancer therapy is considered as a special type of chemotherapy. However, different from conventional chemotherapy, targeted cancer therapy makes combination with specific proteins involved in tumorigenesis and show cytostatic instead of cytotoxic[92,93]. 3 mainly types of drugs are developed in targeted cancer therapies: 1) monoclonal antibodies, 2) small molecule inhibitors and 3) immunotoxins (Figure 1-2)[93].

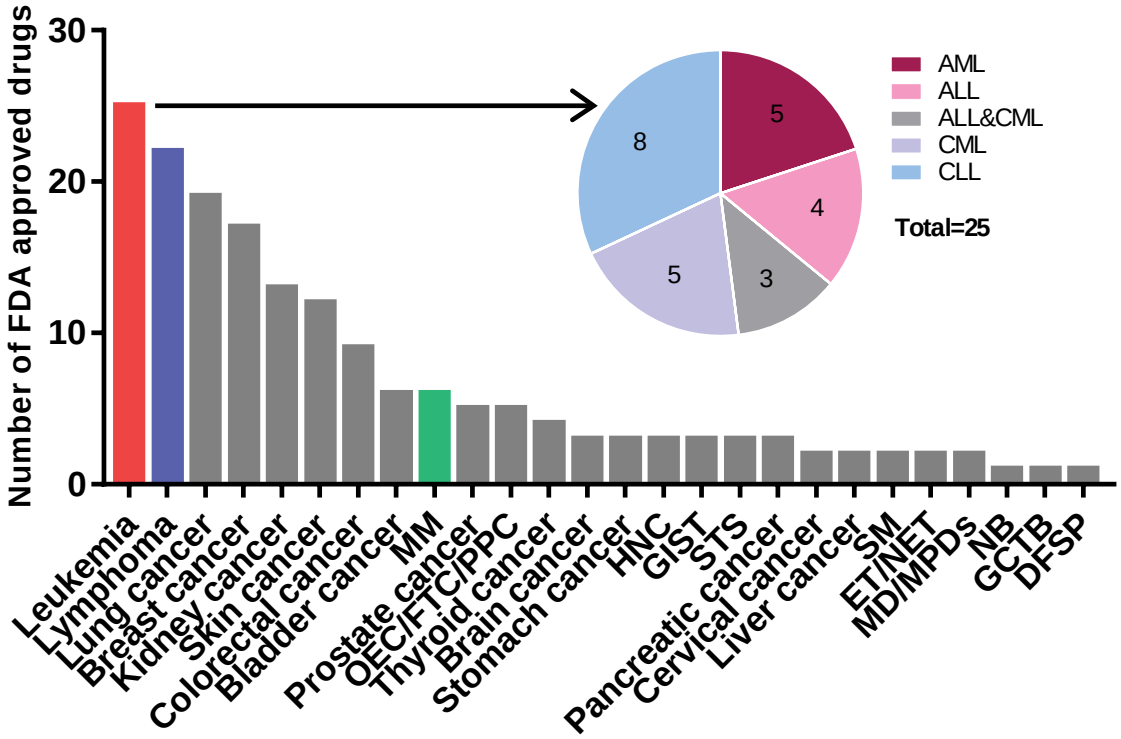
NCI summed up 7 types of targeted therapies for cancer treatment, including hormone therapies, signal transduction inhibitors, gene expression modulators, apoptosis inducers, angiogenesis inhibitors, immunotherapies, and toxin delivery molecules. Sometimes, cancer vaccine and gene therapy are also considered as targeted therapies since they attack and interfere with the growth of cancer cells. Taking cancer vaccine as an example, in 1993, Glenn Dranoff developed the first therapeutic vaccine comprised of cancer cells genetically modified to secrete granulocyte-macrophage colony-stimulating factor, shorted as GVAX[94]. GVAX and CRS-207, a supplemental vaccine, were granted by FDA in 2015 as 2 vaccines in patients with metastatic pancreatic adenocarcinoma[95]. GVAX can be an option to treat many other cancers, such as melanoma, ovarian, NSCLC and prostate cancer[96-99]. GVAX also showed effective roles in post-transplantation AML and CLL studies[100,101].

Targeted therapies had been expected less toxic than traditional chemotherapy since they commonly target with cancer cells rather than normal cells. But scientists and doctors did find side effects during the research and treatment. Moreover, there are still limitations of targeted cancer therapies for cancer treatment, such as drug resistant; not all cancers respond to targeted therapy; the drugs are expensive or not yet developed for specific types of cancer. Mutation of target and new pathway discovered for cancer survival would cause the resistant, which makes targeted cancer therapies most often used in combination with more other targets[102], or other strategies, such as immunotherapy[103,104], surgery, chemotherapy and/or radiation therapy.

New anti-cancer drugs are urgent need to treat those patients. FDA had approved 169 drugs for target cancer therapy since December 31, 2017. Among them, 53 drugs (31.4%) were approved to treat with hematologic cancers, 19 for leukemia, 17 for lymphoma and 6 for multiple myeloma (MM), (Figure 1-4, Table 1-1). Some drugs shared the same target and some of them have been approved for more than one type of cancer[105]. Nivolumab and pembrolizumab are two widely used checkpoint inhibitors that targeted programmed cell death protein 1 (PD-1) to block a signal which prevent active T cells from eliminating cancer cells. The FDA has approved these to treat several specific types of cancer, including head and neck cancer (HNC), melanoma, lymphoma and lung cancers. Nivolumab also had been approved for the treatment of advanced form of kidney cancer in 2015 and more recently for urothelial carcinoma, in February, 2017. Anti-cluster of differentiation antigen (CD) 20 antibody, rituximab, was approved for the treatment of both previously untreated and previously treated CLL and certain types of B-cell non-Hodgkin lymphoma (NHL). Another anti-CD20 antibody, obinutuzumab, have been approved to treat previously untreated

CLL and R/R Follicular Lymphoma (FL). Idelalisib, acts as a phosphatidylinositol-3-kinase-delta (PI3Kδ) inhibitor, has been approved for the treatment of recurred CLL and NHL patients (Table 1-1).

Side effects, like skin problems and blood pressure, still exist though most researchers had expected that targeted cancer therapies would show less cytotoxic than conventional chemotherapy. But some side effects have been noticed that they were linked to better outcomes[106,107]. Children and adults patients may have different side effects to specific target therapy[108]. Many mutations and uncertain changes may involve in the development of cancer makes it a multifactorial disease. Different types of cancer may own different critical targets. Therefore, targeting one specific molecular target or pathway may not be the final option. Many targeted therapies have been studied in combination with established chemotherapies or more than 2 critical targets. Bevacizumab is the first angiogenesis inhibitor drug approved to treat numerous types of cancer. It didn't improve patient survival rate as a monotherapy for metastatic colorectal cancer, however, it provided a 2.5 months benefit in progression-free survival and overall survival when combination with FOLFOX4 (oxaliplatin/5-FU/leucovorin)[109]. In addition, blockade of co-inhibitory pathways, like PD-1, PD-L1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), also provided an effective and durable cancer immunotherapy in several types of cancer[110-112]. More recently, FDA granted accelerated approval to pembrolizumab (in combination with pemetrexed and carboplatin) for the treatment of previously untreated NSCLC patients. There is still a long way ahead for all cancer treatments besides the achievements researchers have now. Side effects, therapeutic index and myriad possibilities are challenges of combination therapies, which would be overcame and improved by scientific researchers and clinical doctors in the future.



*Data sources:
https://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm279174.htm
https://www.cancer.gov/about-cancer/treatment/types/targeted-therapies/targeted-therapies-fact-sheet

Figure 1-4. FDA approved drugs for targeted cancer therapy. Totally 169 drugs (or combined drugs) had been approved by FDA for targeted cancer therapy (some of them have been proved for more than one specific type of cancer). 53 drugs (31.4%) were approved for hematologic malignancies. *(Reviewed: December 31, 2017)*

Abbreviations: DFSP, Dermatofibrosarcoma protuberans; ET/NET, Endocrine/neuroendocrine tumors; GCTB, Giant cell tumor of the bone; HNC, Head and neck cancer; GIST, Gastrointestinal stromal tumor; MD/MPDs, Myelodysplastic/ myeloproliferative disorders; MM, Multiple myeloma; NB, Neuroblastoma; OEC/FTC/PPC, Ovarian epithelial/fallopian tube/primary peritoneal cancers; SM, Systemic mastocytosis; STS, Soft tissue sarcoma.

Table 1-1. FDA approved targeted therapy drugs for hematologic malignancies. *(Reviewed: December 31, 2017)*

Drugs	FDA approved indication(s)	Target(s)
Leukemia		
Tretinoin/ATRA (Vesanoid®)	APL, AML	RARs
Imatinib Mesylate (Gleevec®)	ALL (Ph^+), CML (Ph^+)	ABL, KIT, PDGFR
Dasatinib (Sprycel®)	ALL (Ph^+), CML (Ph^+)	ABL
Nilotinib (Tasigna®)	CML (Ph^+)	ABL
Bosutinib (Bosulif®)	CML (Ph^+)	ABL
Rituximab (Rituxan®)	CLL	CD20
Alemtuzumab (Campath®)	CLL	CD52
Ofatumumab (Arzerra®)	CLL	CD20
Obinutuzumab (Gazyva®)	CLL	CD20
Ibrutinib (Imbruvica®)	CLL	BTK
Idelalisib (Zydelig®)	CLL	PI3Kδ
Blinatumomab (Blincyto®)	ALL (Ph^-)	CD3, CD19

Venetoclax (Venclexta™)	CLL (del[17p])	BCL2
Ponatinib Hydrochloride (Iclusig®)	CML (Ph^+)T315I mutation ALL (Ph^+)T315I mutation	FLT3, VEGFR2, ABL, FGFR1-3
Midostaurin (Rydapt®)	AML ($FLT3^+$)	FLT3
Combination of rituximab and hyaluronidase human	CLL and diffuse large B-cell lymphoma	CD20
Blinatumomab (Blincyto®)	ALL (Ph^+)	CD3, CD19
Enasidenib (Idhifa®)	AML (IDH2 mutation)	IDH2
Liposome-encapsulated combination of daunorubicin and cytarabine	t-AML or AML-MRC	DNA/RNA
Inotuzumab ozogamicin (Besponsa®)	ALL	CD22
Tisagenlecleucel (Kymriah®)	ALL	CAR T cell therapy
Gemtuzumab ozogamicin (Mylotarg®)	AML	CD33
Dasatinib (Sprycel®)	CML (Ph^+), in the chronic phase	ABL
Bosutinib (Bosulif®)	CML (Ph^+), newly-diagnosed chronic phase	ABL
Nilotinib (Tasigna®)	CML (Ph^+), updated	ABL
Lymphoma		
Ibritumomab Tiuxetan (Zevalin®)	NHL	CD20
Denileukin Diftitox (Ontak®)	CTCL	CD25
Brentuximab Vedotin (Adcetris®)	ALCL, HL	CD30
Rituximab (Rituxan®)	NHL	CD20
Vorinostat (Zolinza®)	CTCL	HDAC
Romidepsin (Istodax®)	CTCL	HDAC
Bexarotene (Targretin®)	Skin problems caused by CTCL	RXRs
Bortezomib (Velcade®)	MCL	Proteasome
Pralatrexate (Folotyn®)	PTCL	DHFR
Ibrutinib (Imbruvica®)	MCL, NHL	BTK
Siltuximab (Sylvant®)	MCD	IL-6
Idelalisib (Zydelig®)	FL, SLL	PI3Kδ
Belinostat (Beleodaq®)	PTCL	HDAC
Obinutuzumab (Gazyva®)	FL	CD20
Nivolumab (Opdivo®)	cHL	PD-1
Pembrolizumab (Keytruda®)	cHL	PD-1
Combination of rituximab and hyaluronidase human	FL	CD20
Copanlisib (Aliqopa®)	FL	PI3K-α and PI3K-δ
Axicabtagene ciloleucel (Yescarta™)	R/R large B-cell lymphoma	CAR T cell therapy
Brentuximab Vedotin (Adcetris®)	pcALCL	CD30
Obinutuzumab (Gazyva®)	FL	CD20
Acalabrutinib (Calquence®)	MCL	BTK
Multiple myeloma (MM)		
Bortezomib (Velcade®)	MM	Proteasome
Carfilzomib (Kyprolis®)	MM	Proteasome
Ixazomib citrate (Ninlaro®)	MM	Proteasome
Panobinostat (Farydak®)	MM	HDAC
Elotuzumab (Empliciti™)	MM	SLAMF7
Daratumumab (Darzalex™)	MM	CD38

*Data sources:
https://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm279174.htm
https://www.cancer.gov/about-cancer/treatment/types/targeted-therapies/targeted-therapies-fact-sheet

1.2.3 AML therapies

A number of immunotherapeutic strategies have been developed[113,114]. Immune checkpoint inhibitors, which were previously studied in various solid tumors[115], have shown considerable promise in the treatment of AML and other types of hematologic malignancies[116,117]. Hereby, we mainly discuss three types of treatment: antibody-based immunotherapy, Immune checkpoint pathways and Immune cells (DCs and CAR T cells)-based strategies.

1.2.3.1 Antibody-based immunotherapy

Antibody-based immunotherapy demonstrated efficacy by targeting surface antigens expressed on tumor or immune cells. Monoclonal antibodies (mAbs) were identified as unique tools in clinical diagnosis and basic research investigation after its first description in 1975[118] and now hare considered as a standard component of cancer treatment. CD33 is a cell surface therapeutic target which has been found to be broadly expression on AML blasts[52]. FDA granted accelerated approval to Gemtuzumab ozogamicin (GO), an anti-CD33 antibody conjugate, for treatment of AML patients who have relapsed following initial chemotherapy in 2000[119]. Subsequently, it was voluntarily withdrawn from the US market in June 2010 since a randomized phase 3 study found addition of GO showed no improvement in CR, disease-free survival (DFS), or overall survival (OS)[120]. There still are several clinical trials focus on GO (or combined with other drugs) for the treatment of AML patients (Table 1-2). SGN-CD33A is another immune-conjugated anti-CD33 antibody that is being evaluated in several clinical trials (Table 1-2). SGN-CD33A showed improved efficacy compared to GO in xenograft mice models[121]. Additionally, other antibody based therapies are being evaluated in clinical trials, including antibodies targeting

CD37, CD45, CD47 and CD123. AMG 330 is a novel bispecific T-cell-engaging antibody (BiTE), which belongs to a novel class of cytotoxic drugs derived from antibody-based immunotherapy. By targeting both CD33 and CD3, AMG 330 would recruit T cells and show effective antibody-mediated cytolysis in *ex vivo*[52] and *in vivo*[122] experiments at low effector-to-target (E:T) ratio. AMG 330-regulated cytotoxicity and immune responses were significantly enhanced after blocking the PD-1/PD-L1 pathway[53]. CD33 antigen universally expressed on AML blast cells from individual patients which indicated CD33 as an useful target for debulking $CD33^+$ AML cells. But it is not an ideal target to eradicate all types of AML cells since it was still not crystal clear whether, or to what degree, this antigen was displayed on AML progenitor and stem cells[123], though some data from cDNA microarray showed CD33 lower expressed on normal hematopoietic stem cells (HSCs) than on AML leukemic stem cells (LSCs)[124]. JNJ-63709178, another BiTE antibody targets CD123 and CD3, was considered to be a candidate drug to eliminate AML stem cells since CD123 was presumed widely expressed on those cells. The first-in-human (FIH) phase 1 clinical trial was carrying out by *Amgen* and *Janssen Research & Development, LLC*, focus on the research of AMG 330 and JNJ-63709178, respectively (Table 1-2). As no AML-specific antigens were identified and BiTE antibodies could be highly effective at low target antigen level, target antigens should be selected prudently and their expression on regenerative and normal tissues need to be assessed carefully.

1.2.3.2 Immune checkpoint pathways

Immune checkpoint pathways, particularly the CTLA-4 and PD-1/PD-L1, have become promising therapy strategies in both solid and blood cancers[110,116,125-127].

Several phase 1 or 2 clinical trials start learning on the effect of nivolumab, pidilizumab, atezolizumab and ipilimumab for the treatment of AML patients (Table 1-2). FLT3, also known as CD135, is a surface antigen expressed on many hematopoietic progenitor cells. FLT3-ITD is the most common mutation associated with AML patients which indicates worse prognosis[33]. On April 28th 2017, FDA approved midostaurin (RYDAPT, *Novartis Pharmaceuticals Corp.*), in combination with standard cytarabine and daunorubicin induction and cytarabine consolidation, for the treatment of adult patients with newly diagnosed (AML) who are FLT3 mutation-positive ($FLT3^+$) (Table 1-1). This is the first FDA approved multi-targeted protein kinase inhibitor drug for AML therapy since 1990. The efficacy and safety of midostaurin-combined treatments were studied in a randomized phase 3 clinical trial of 717 AML patients. The trial showed significant improvement in OS for midostaurin received patients compared to placebo treated patients, median months from 16.3 (placebo) to 20.9 (midostaurin) (HR=0.77, p=0.016)[128]. AGS62P1 is another novel site-specific antibody drug conjugate target FLT3. Its preclinical assessment of anti-leukemic activity had been tested with AML and ALL cell lines, *in vitro* and i*n vivo*[129,130]. *Agensys, Inc.* sponsored a phase 1 clinical trial to evaluate the safety, tolerability, pharmacokinetics and the anti-leukemic activity of AGS62P1 in AML patients' age of year 18 and older (Table 1-2).

More recently, FDA approved enasidenib, a small molecule inhibitor of isocitrate dehydrogenase 2 (IDH2), for the treatment of adult R/R AML patients with an IDH2 mutation on August 1, 2017 (Table 1-1). With orally treatment with 100 mg of enasidenib, 23% of 199 adult AML patients experienced CR or complete response with partial hematologic recovery (CRh) lasting a median of 8.2 months (NCT01915498). This is the first FDA approval for R/R AML specifically with an IDH2

mutation and FDA concurrently approved a companion diagnostic, the RealTime IDH2 Assay, used to detect the IDH2 mutation.

B-cell lymphoma 2 (BCL2) was identified as an apoptosis regulator in 1986 and was continuous researched on how its functional roles in cancer biology for more than 20 years[131]. Recently, a novel BCL2 inhibitor drug, venetoclax, was reported with a 79% overall response rate for all 116 R/R CLL or small lymphocytic lymphoma (SLL) patients[132]. FDA approved venetoclax as the first drug targets BCL2 for the treatment of CLL patients who have a chromosomal abnormality called 17p deletion (del[17p]) and who have been treated with at least one prior therapy in April 2016 (Table 1-1). *AbbVie*, which developed the drug in collaboration with *Roche*, also claimed a phase 2 breakthrough designation that combination of venetoclax with hypomethylating agents in naïve AML patients. S 055746 is another candidate BCL2 inhibitor drug and now is performing phase 1 trials with AML patients (Table 1-2).

1.2.3.3 Immune cells (DCs and CAR T cells)-based strategies

DCs are professional antigen-presenting cells (APCs), and thus usually are considered as “nature's adjuvants” for antigen delivery in cancer immunotherapy. DC-based vaccines[133-136] and immunotherapies[136-138] are essential therapy approches to induce immune responses through the patients’ own immune system. Numerous of leukemia-associated antigens (LAAs), such as FLT3, preferentially expressed antigen in melanoma (PRAME) and Wilm’s tumor protein 1 (WT1), were identified with the development of the tumor biology and biochemistry. Clinical phase 1/2 trials on those peptide vaccines also have showed exciting results for AML

patients. DCs, however, retain the potential to be more successful than peptide vaccines since its antigen delivery properties in cellular and humoral immunity. The first clinical study of autologous monocyte-derived DCs on patients was carried out to treat AML relapse after autologous SCT[139]. More recently, 17 of in-remission but at high risk with relapse AML patients were administrated with WT1 mRNA-electroporated DCs that differentiated from $CD14^+$ monocytes. In this phase 1/2 study, 8 patients reached clinical and molecular remission. Median of OS was from 6 months (non-responders) to 52 months (responders), p=0.0007; and median of relapse-free survival was 3 months (non-responders) compared to 47 months (responders), p<0.0001. Excitingly, two out of three patients, who only got partial remission with chemotherapy-refractory disease, achieved CR after 4 vaccinations with this cellular product[140,141]. Other clinical studies also have shown that DC-based immunotherapies were safe and improved the survival rate by inducing anti-tumor immune responses[142].

Normally, DCs can be generated from hematopoietic precursor cells, monocytes, embryonic stem cells and peripheral blood stem cells[143,144] with a standard 7-day protocol. An improved 3-day protocol developed in our lab to obtain mature DCs from monocytes which stimulated by cytokines, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 for 48 hours, and subsequently, incubated with toll-like receptor (TLR)7/8 agonist-containing maturation cocktail for another 24 hours[48]. A phase 1/2 study (NCT01734304) held by our lab had revealed the feasibility and safety of the next-generation DCs for post-remission therapy of AML patients[145]. Delayed-type hypersensitivity (DTH) responses were achieved in 6/6 patients, and 9/9 patients in updated data at December 2016[146], accompanied by slight erythema and indurations at the injection site, but no grade III/IV toxicities.

Autologous leukemic apoptotic corpse pulsed DCs also showed significantly improvement of OS in elderly AML patients (NCT01146262). In this study, most patients (19/21) received non-intensive chemotherapy. After 5 vaccines administrated, the median of OS was extended from 4.75 months to 13 months, p=0.009[47]. However, another literature showed that 71% (12/17) of patients, the median age was 63 years, remain alive without recurrence at a median follow-up of 57 months after treated with autologous DCs[147]. Current DC-based therapy had demonstrated promising results in solid, skin and blood cancers[114,142,148]. Further deeply and widely researches are clearly needed with more sophisticated strategies developed, like identification of individual patient antigens or mutations.

CAR T cells are another potent candidate strategy to treat AML and several of other types of cancer[39,43,45,149,150]. CAR T cells were usually engineered by grafting an arbitrary specificity onto T cells to increase the specificity of those T cells which could be adoptively transferred to cancer patients. Many CD (CD19, CD20, CD22, CD33 and CD123) and non-CD (IL-13Rα, endothelial growth factor receptor (EGFR), carcinoembryonales antigen (CEA) and human epidermal growth factor receptor 2 (HER2)) targets for CAR T cells were submitted for clinical trials[39,149]. Many factors in the whole immune system, like the regulatory T cells, checkpoints and tumor microenvironment, would influence the function of CAR T cells[151-153].

T cell-based immunotherapies, including antibody bases immunotherapy, DC vaccines and CAR T cells, together with checkpoint-targeted drugs and standard established chemotherapy, mainly shaped the advanced AML therapy[114]. Some approaches might have achieved milestone success, e.g. APL therapy[154], but not with broadly feasible for treatment with other cancers. The high risk of relapse in AML patients, the poor survival rate after chemotherapy and SCT and tolerance ability for

individual treatment of patients with SCT prompt researchers to look for more LAAs or even the leukemic specific antigens (LSAs). New therapeutic drug or checkpoint targets are urgently needed for more promising preclinical and clinical trials, with the final purpose to completely eliminate both leukemic cells and leukemic stem cells (LSCs).

High relapse rates and poor prognosis remain challenging in AML, especially compared to other subtypes of leukemia (Figure 1-1 B). Current immunotherapeutic approaches for AML therapy have had only shown limited success in clinical trials. Thus, novel, more specific targets are needed for AML immunotherapy. “Cell Surface Capturing” (CSC) technology provide the possibility to identify the whole leukemia surfaceome (cell surface proteome) of AML blasts or cell lines. In this study, we employed this technology and its variants to discover new targets for AML immunotherapy. In addition, the identification of more specific surface markers is also likely to improve MRD detection.

Table 1-2. Ongoing clinical trials of antibody/immune checkpoint-based treatments for AML patients.

Drug(s)	Target(s)	NCT trial number	Phase
AMG 330	CD33/CD3	NCT02520427	1
SGN-CD33A	CD33	NCT02326584	1
SGN-CD33A	CD33	NCT01902329	1
SGN-CD33A	CD33	NCT02785900	3
IMGN779	CD33	NCT02674763	1
GO (combination)	CD33	NCT02473146	2/3
GO (combination)	CD33	NCT02724163	3
AGS67E	CD37	NCT02610062	1
Yttrium Y 90	CD45	NCT01300572	1
Hu5F9-G4	CD47	NCT02678338	1
SGN-CD123A	CD123	NCT02848248	1
KHK2823	CD123	NCT02181699	1
Talacotuzumab	CD123	NCT02472145	3
JNJ-63709178	CD123/CD3	NCT02715011	1
AGS62P1	FLT3	NCT02864290	1
Nivolumab	PD-1	NCT02532231	2
Nivolumab	PD-1	NCT02275533	2
Pidilizumab	PD-1	NCT01096602	2
Atezolizumab	PD-L1	NCT02935361	1/2

Nivolumab/Ipilimumab	PD-1/CTLA-4	NCT02397720	2
Ipilimumab	CTLA-4	NCT01757639	1
Ipilimumab	CTLA-4	NCT02890329	1
S 055746	BCL2	NCT02920541	1

*Data source : https://clinicaltrials.gov/

1.3 AML surfaceome and LC-MS-based CSC technology

Surfaceome, also referred to as the cell surface sub-proteome, are proteins that are expressed on the cell surface. These proteins represent important cellular functions in cell-cell interaction, cell signaling and microenvironment. Cell surface proteins, offer great potential as therapeutic targets[105]. A comprehensive analysis of the surface proteome promises to be useful for the identification and validation of novel targets, which may be considered for the development of antibody-, DC vaccines-, and CAR T cell-based AML therapy. However, until recently, a comprehensive mapping of the surface proteins was technologically limited.

Mass spectrometry (MS)-based technologies offer the advantage of multiplexed and unbiased detection of proteins independent of existing antibody collections. The original “Cell Surface Capturing” (CSC) technology (Glyco-CSC) and its variants (Cys-Glyco-CSC and Lys-CSC) technology provide the ability to identify all surface proteins of any cell population[155-157]. These three strategies were developed based on the following features of membrane proteins: 1), most cell surface proteins are known or predicted to be glycosylated[158,159] (for original CSC); 2), the glycosylated peptides often contain cysteine residues within disulfide bridges[160,161] (for Cys-Glyco-CSC); 3), lysine-contained proteins can be identified by interaction with biotin[162-164] (for Lys-CSC). Taking advantages of these features, membrane peptides can be detected and the associated specific proteins identified. The procedure encompasses six steps which are depecited in Figure 1-4. Compared to other

strategies, such as lectin-based methods, cell surface shaving, antibody-mediated membrane enrichment, silica bead coating method, cell surface biotinylation, reversed phase capillary liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)-based CSC technologies provide increased specificity (95%) and thus allow for the mapping of a more representative surfaceome[155,165,166].

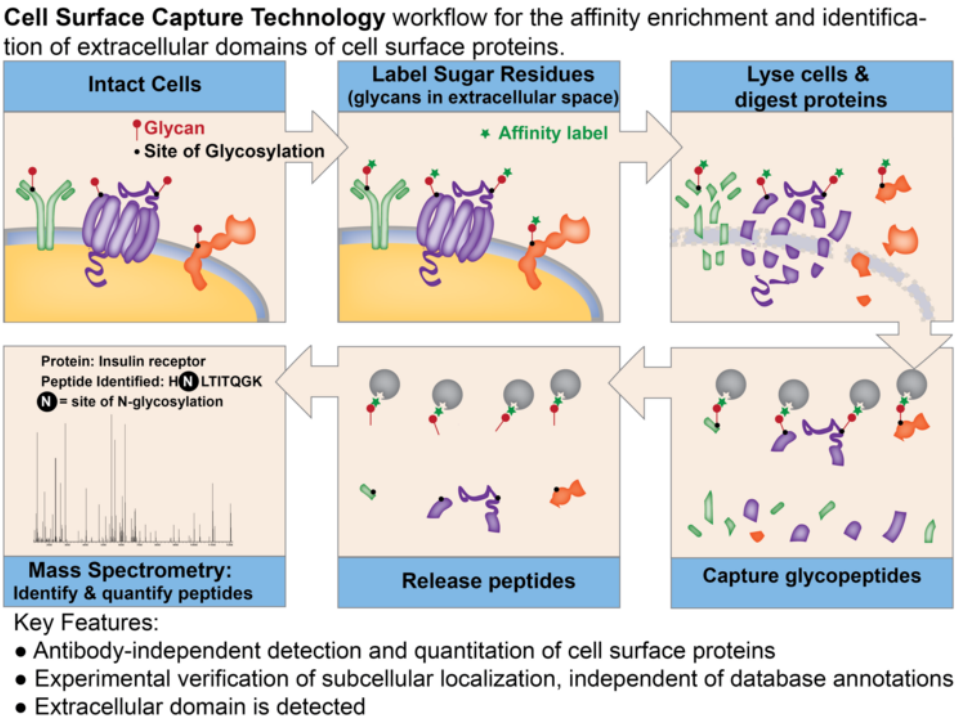


*Figure source : http://www.gundrylab.com/research/#/cellsurfaceproteins/

Figure 1-4. Overview of the CSC workflow. Six mainly steps involved in CSC and it's variant technologies: (1) oxidation of reactive protein groups, (2) biocytin hydrazide or NHS-SS-biotin labelling, (3) cell lysis, membrane protein collected and digested, (4) affinity purification of glycopeptides, (5) enzymatic or reduction release peptides and (6) analysis and identification of peptides.

In a previous study using two AML cell lines, more than 500 membranes were identified, including 137 CD antigens[160]. Another group identified 823 proteins with an EZ-link sulfo-NHS-LC-biotin-based protocol; however, only 320 proteins could be annotated to the cell surface membrane. Membrane specificity was not high, with a percentage of putative membrane proteins in all six samples ranging between 44 and 53%[166]. The hematopoietic cell surface marker CD45, the myeloid marker CD33 and

granulocyte differentiation markers such as CD11b and CD35 were identified in both studies. However, no comprehensive mapping of the surfaceome has been published using primary AML primary patient samples[167-169].

1.4 Objectives

The objectives of this work were:

(1): To establish the use of CSC and it's variants technologies on AML cells from primary patient samples.

(2): To identify new biomarkers to measure MRD and target antigens from primary AML patient samples.

2. Materials and methods

2.1 Materials

2.1.1 Chemicals and Kits

Table 2-1. Chemicals and Kits

Name	Product No.	Company
Sodium (met)periodate ($NaIO_4$)	30323-100 g	Sigma-Aldrich
Biocytin hydrazide	ABD-3086	Biomol
Tris base	93350-100 g	Sigma-Aldrich
Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	13152-1 kg	Sigma-Aldrich
MES hydrate	M8250-250 g	Sigma-Aldrich
Sodium chloride (NaCl)	S1679-500 g	Sigma-Aldrich
Sucrose	16104-1 kg	Sigma-Aldrich
Ammonium bicarbonate (NH_4HCO_3)	11213-1 kg-R	Sigma-Aldrich
RapiGest	186001860-1 mg	Waters
Tris (2-carboxyethyl)phosphine hydrochloride	C4706-2 g	Sigma-Aldrich
Iodoacetamide	I1149-5 g	Sigma-Aldrich
2,2'-Thiodiethanol	166782-100 g	Sigma-Aldrich
Sequence grade modified trypsin	V5111-5X20 µg	Promega
Glycerol free PNGase F	P0705 S-15000 units	NEB
Pierce™ Streptavidin Plus UltraLink™ Resin	53117-5 ml	ThermoFisher
EZ-Link™ Sulfo-NHS-SS-Biotin	21331-100 mg	ThermoFisher
Sodium bicarbonate($NaHCO_3$)	6885.2-500 g	Carl Roth
Sodium carbonate (Na_2CO_3)	A135.1-500g	Carl Roth
DL-Dithiothreitol	43815-1 g	Sigma-Aldrich
HPLC Grade Water	W/0110/PB17-2.5 L	ThermoFisher
Formic acid (HCOOH)	56302-10 X 1 ml	Sigma-Aldrich
Acetonitrile (CH_3CN)	34967-250 ml	Sigma-Aldrich
LC-MS grade water	39253-1 l-R	Sigma-Aldrich
Ultrapure Water	Cay400000-4 L	Biomol
Glycerol	G5516-500 ml	Sigma-Aldrich
Triton™ X-100	T9284-500 ml	Sigma-Aldrich
Fetal Bovine Serum	10270-106-500 ml	ThermoFisher
Horse Serum	H1270-500 ml	Sigma-Aldrich
Recombinant Human G-CSF (rh-G-CSF)	300-23-1 mg	PeproTech
Recombinant Human IL-3 (rh-IL-3)	200-03-1 mg	PeproTech
Recombinant Human TPO (rh-TPO)	300-18-1 mg	PeproTech
2-Mercaptoethanol (14.3 M)	M6250-100 ml	Sigma-Aldrich
Penicillin-Streptomycin-Glutamine (100X)	10378016-100 ml	ThermoFisher
Pierce™ Bovine Serum Albumin Standard	23209-10 x 1 ml	ThermoFisher
Pierce™ BCA Protein Assay Kit	23227-500 ml	ThermoFisher
Pierce™ C18 Spin Columns	89870-25 columns	ThermoFisher

2.1.2 Solutions, Mediums and Buffers (Commercial)

Table 2-2. Solutions, Mediums and Buffers (Commercial)

Name	Product No.	Company
Phosphoric Acid	438081-500 ml	Sigma-Aldrich
Sodium hydroxide solution (4 mol/l)	T198.1-1 L	Carl Roth
Hydrochloric acid (6 mol/l)	0281.1-1 L	Carl Roth
UltraPure™ 0.5M EDTA, pH 8.0	15575-038-100 ml	ThermoFisher
HEPES buffer (1M)	15630-080-100 ml	ThermoFisher
DPBS	P04-36500-500 ml	PAN-Biotech
RPMI 1640	P04-16500-500 ml	PAN-Biotech
Alpha MEM Eagle	P04-21500-500 ml	PAN-Biotech

2.1.3 Solutions, Mediums and Buffers (Handmade)

Table 2-3. Solutions, Mediums and Buffers (Handmade)

Name	Preparation method
R10 medium (10% FBS, 1% HEPES, 1% Penicillin-Streptomycin-Glutamine in RPMI 1640)	Add 50 ml FBS, 5 ml of HEPES and 5 ml of Penicillin-Streptomycin-Glutamine into 500 ml RPMI 1640 medium. Store at 4 °C.
Blasts Base medium (12.5% FBS ,12.5% horse serum ,1% Penicillin-Streptomycin-Glutamine in ALPHA MEM Eagle Medium)	Add 62.5 ml FBS, 62.5 ml of horse serum and 5 ml of Penicillin-Streptomycin-Glutamine into 500 ml ALPHA MEM Eagle Medium. Store at 4 °C.
Long-Term culture medium (20 ng/ml of rh-G-CSF, rh-IL-3 and rh-TPO, 57.2 µM β-mercaptoethanol in Blasts Base medium)	Add 2 µl (0.1 mg/ml) of rh-G-CSF, rh-IL-3 and rh-TPO to 10 ml Blasts Base medium. Add 4 µl (1:100 dillution) of β-mercaptoethanol. Mix and store at 4 °C.
FACS buffer	500 ml DPBS, 1% bovine serum albumin (BSA), 0.1% sodium azide
Glyco labeling buffer (PBS, pH=6.5)	500 ml DPBS, adjust the pH to 6.5 with 85% (w/v) Phosphoric Acid. Add 0.5 ml fetal bovine serum.
$NaIO_4$ stock solution (160 mM)	Add 34 mg $NaIO_4$ to 0.9 ml labeling buffer and make up to 1 ml with labeling buffer. (Keep in dark)
Biocytin hydrazide solution (5.4 mM)	Add 25 mg biocytin hydrazide to 10 ml labeling buffer, mix and make up to 14 ml with labeling buffer. (Prepare before use)
Iodoacetamide stock solution (500 mM)	Add 92.48 mg iodoacetamide to 800 µl water, mix, and make up to 1 ml with water. Aliquot and store at −20°C. (Keep in dark)
Lysine labeling buffer (2 mM sulfo-NHS-SS-biotin and 4 mM iodoacetamide)	Add 5.55 mg sulfo-NHS-SS-biotin and 40 µl of iodoacetamide stock solution to 4.5 ml PBS, mix and make up to 5 ml with PBS. (Prepare before use)
Hypotonic lysis buffer (Glyco-CSC) (10 mM Tris pH=7.5, 0.5 mM $MgCl_2$)	Add 0.6 g Tris Base and 50.5 mg $MgCl_2 \cdot 6H_2O$ to 45 ml water. Mix and adjust the pH to 7.5 with 1M HCl. Make up to 50 ml with water. Store at 4 °C.
Hypotonic lysis buffer (Lys-CSC) (10 mM Tris pH=7.5, 0.5 mM $MgCl_2$,10 mM iodoacetamide)	Add 0.6 g Tris Base, 92.48 mg iodoacetamide and 50.5 mg $MgCl_2 \cdot 6H_2O$ to 45 ml water. Mix and adjust the pH to 7.5 with 1M HCl. Make up to 50 ml with water. Store at 4 °C. (Keep in dark)
MES stock solution (500 mM, pH=6)	Add 9.76 g MES hydrate to 80 ml water. Mix and adjust the pH to 6 with 1 M NaOH. Make up to 100 ml with water.

Membrane preparation buffer (50 mM MES pH=6, 10 mM $MgCl_2$, 450 mM NaCl, 280 mM sucrose)	Add 2.03 g $MgCl_2 \cdot 6H_2O$, 26.30 g NaCl, 95.84 g sucrose and 100 ml of the 500 mM MES stock solution pH=6 to 700 ml water. Mix and make up to 1 L. Aliquot in 50 ml tubes and store at −20°C.
Membrane wash buffer (25 mM Na_2CO_3)	Add 0.265 g Na_2CO_3 to 90 ml water. Mix and make up to 100 ml with water.
Sucrose buffer (35% sucrose in membrane preparation buffer and water)	Add 15.08 g sucrose to 20 ml water. Mix and make up to 25 ml with water. Add 25 ml of Membrane preparation buffer. Store at −20°C.
NH_4HCO_3 buffer (100 mM)	Add 0.395 g NH_4HCO_3 to 45 ml water. Mix and make up to 50 ml with water. (Prepare before use)
2,2-Thiodiethanol stock solution (100 mM BHES)	Add 12.22 mg BHES to 900 µl water. Mix and make up to 1 ml. Store at −20°C
RapiGest stock solution (1% (w/v) RapiGest surfactant)	Add 1 mg RapiGest to 10 µl water. Mix and store at 4 °C.
Tris (2-carboxyethyl) phosphine stock solution (100 mM TCEP)	Add 28.66 mg TCEP to 900 µl water. Mix and make up to 1 ml. Aliquot and store at −20°C.
Digestion buffer (Glyco-CSC, Cys-Glyco-CSC) (100 mM NH_4HCO_3 , 1 mM iodoacetamide, 1 mM BHES)	Add 100 µl of BHES stock solution and 200 µl of iodoacetamide stock solution to 9 ml NH_4HCO_3. Make up to 10 ml with NH_4HCO_3.
Digestion buffer (Lys-CSC) (100 mM NH_4HCO_3, 1.25 mM iodoacetamide, 1.25 mM BHES, 2 mg/ml RapiGest.)	Add 5 µl of BHES stock solution and 1 µl of iodoacetamide stock solution to 320 µl NH_4HCO_3. Mix and add 80 µl of RapiGest stock solution. (Keep in dark)
NaCl washing buffer (5 M NaCl)	Add 14.6 g NaCl to 30 ml water, mix and make up to 50 ml with water.
Detergent buffer (137 mM NaCl, 50 mM Tris pH=7.8, 10% glycerol, 0.5 mM EDTA pH 8, 0.1% Triton X-100)	Add 0.606 g Tris base to 70 ml water and adjust pH to 7.8 with 1 M HCl. Then, add 0.8 g NaCl, 10 ml glycerol, 0.1 ml EDTA stock solution, and 1 ml Triton X-100 and make up to 100 ml with water.
$NaHCO_3$ washing buffer (100 mM $NaHCO_3$, pH=11)	Add 0.84 g $NaHCO_3$ to 90 ml water. Adjust pH with 1 M NaOH and make up to 100 ml with water. (Prepare before use)
Dithiothreitol (DTT) stock solution (100 mM DTT)	Add 15.4 mg DTT to 800 µl water. Mix and make up to 1 ml. Aliquot and store at −20°C.
Elution buffer (100 mM NH_4HCO_3 , 10 mM TCEP, 1 mM DTT)	Add 100 µl DTT stock solution and 200 µl TCEP stock solution to 9 ml NH_4HCO_3. Mix and make up to 10 ml with 100 mM NH_4HCO_3 buffer.
10% formic acid (10% (v/v) formic acid)	Add 1 ml formic acid to 9 ml water.
50% acetonitrile (LC-MS grade water, 50% (v/v) acetonitrile, 0.1% (v/v) formic acid)	Add 5 ml acetonitrile and 10 µl formic acid to 4.5 ml LC-MS grade water. Mix and make up to 10 ml with LC-MS grade water.
80% acetonitrile (LC-MS grade water, 80% (v/v) acetonitrile, 0.1% (v/v) formic acid)	Add 8 ml acetonitrile and 10 µl formic acid to 1.5 ml LC-MS grade water. Mix and make up to 10 ml with LC-MS grade water.
Sample buffer (LC-MS grade water, 5% (v/v) acetonitrile, 0.1% (v/v) formic acid)	Add 0.5 ml acetonitrile and 10 µl formic acid to 9 ml LC-MS grade water. Mix and make up to 10 ml with LC-MS grade water.

2.1.4 Instruments and Equipments

Table 2-4. Instruments and Equipments

Name	Company
5 ml, 15 ml and 50 ml Tubes	BD
FACS tubes	BD

1.5 ml and 2 ml Tubes	Eppendorf
Ultracentrifuge Tubes (SW40)	Seton Scientific
pH Meter (MP225)	METTLER TOLEDO
Vortexer	Heidolph Instruments
Mobicol "classic" column-M1002	Mobitec
Filter (large) 35 µm pore size-M523515	Mobitec
Countess™ Automated Cell Counter	Invitrogen
Glass 7 mL Dounce Tissue Grinder-357542	Wheaton
Centrifuge-5920R	Eppendorf
Centrifuge-5417R	Eppendorf
Thermomixer-Comfort (1.5 ml)	Eppendorf
Ultracentrifuge-L70	Beckman
Rotary mixer-LD-76	Labinco-BV
Roll mixer-SRT6D	Stuart
Vacuum Concentrator Centrifuge (SpeedVac)-UNIVAPO 150 H	Uniequip
Bioruptor	Diagenode
LC-MS (Elite-velos-pro)	ThermoScientific
FACS (10-color Navios flow cytometer)	Beckman Coulter

2.1.5 Antibodies for FACS

Table 2-5. Antibodies for FACS

Name	Product No.	Company
PE-CD172b	Cat # 323906	BioLegend
PE-CD148	Cat # 328708	BioLegend
Brilliant Violet 421-CD49d	Cat # 304321	BioLegend
FITC-PLXA1	Cat #: abx107198	Abbexa
PE-ITB7	Cat #: 321204	BioLegend

2.2 Methods

2.2.1 Cell culture

The AML cell line OCI-AML3 was maintained in RPMI1640 medium, supplemented with 10% fetal calf serum, 1% penicillin/streptomycin/glutamine and 0.005 mM 2-mercaptoethanol. The cell suspension was kept in the incubator at 37°C, 5% CO_2.

2.2.2 Patient-derived xenograft (PDX) cells

In cooperation with Prof. Dr. med. Irmela Jeremias (Group Leader of Apoptosis Group in Helmholtz Zentrum München), we collected 2 patient-derived xenograft (PDX) samples. The PDX cells were obtained from mice spleen as previously described[170]. Briefly, patient cells were transplanted i.v. into NSG mice. After 6 to 20 weeks, when mice showed clinical signs of illness, mice were sacrificed and PDX cells were isolated from bone marrow and spleen. PDX cells were either directly retransplated or frozen for later analyses or retransplantation.To amplify transgenic PDX cells, they were retransplanted into mice. Fresh PDX cells were incubated with hypotonic lysis buffer (V_{cell}:V_{buffer}=1:2) for 10 minutes at room temperature to lysis the blood and spleen cells. Then, the sample was centrifuged at 500×g for 5 minutes. The pellet was resuspended in 50 ml labeling buffer. Around 10^8 cells were harvested, with a cell viability of 89% and 91% for PDX1 and PDX2, respectively. Fresh PDX cells were immediately performed with experiments following the improved CSC protocol. Patient characteristics were collected in Table 2-6.

2.2.3 Primary patient (PP) cells

Peripheral blood (PB) and/or bone marrow (BM) samples were collected from primary diagnosis or relapsed AML patients after signed informed consent in accordance with the Declaration of Helsinki and approval by the Institutional Review Board of the Ludwig-Maximilians-University (Munich, Germany). Primary AML patient cells were isolated by gradient centrifugation and then stored in -80°C, -150°C or liquid nitrogen until further experiments were performed. Primary patient (PP) samples for CSC tests were subjected to the long-term cell culture workflow as reported previously with slight modifications[52]. Briefly, irradiated MS-5 cells were resuspended in blast medium and 2.5 x 10^5 cells /well were seeded on 6-well plates. The plates were incubated at 37°C overnight to allow the cells to adhere to the plate. The primary patient cells were thawed and washed with pre-heated blasts basal medium (550 g, 5 min). Cells were counted and washed one more time with blast medium. Then the cell pellet was resuspended in blasts basal medium, and 20 ng / ml of IL-3, TPO and G-CSF + 57.2 µM β-mercaptoethanol were added to the medium. Then, the medium in the 6-well plate was carefully removed without scratching the feeder layer. 1-1.5 x 10^7 cells/well were added into the plate. The cells were incubated at 37°C for 3 days and then were collected, counted and subjected to the Glyco-CSC protocol. Patient characteristics are summarized in Table 2-6.

Table 2-6. Patient characteristics for CSC experiments.

Sample	Sex	Age (Y)	FAB	Disease Stage	Cytogenetics	NPM 1	FLT3 -ITD	ELN	BM/ PB
PDX 1	F	47	M4	Relapse after SCT	46,XX, ins(10;11) (p12;q23q23)	WT	WT	Adv	BM
PDX 2	M	57	M4	Primary Diagnosis	46,XY,t(6;11) (q27;q23)	WT	WT	Adv	BM
PP 01	F	35	M1	Primary Diagnosis	46,XX	WT	WT	Fav	BM

PP 03	F	28	M5	Primary Diagnosis	46,XX,t(11;19)(q23;p13.3)	WT	WT	Adv	BM
PP 04	M	55	NA	Relapse	46,XY	WT	WT	NA	PB
PP 05	F	53	NA	Primary Diagnosis	46,XX	Mut	Mut	NA	BM
PP 06	F	31	NA	Primary Diagnosis	46,XX,der(8)(8;21)(q22;q22),der(22)t(8;22)(q22;q1?3)[21]	WT	WT	NA	BM

PDX (patient-derived xenograft); PP (Primary Patient); F (female); M (male); FAB (French–American–British classification system); SCT (stem cell transplantation); NPM1 (nucleophosmin-1); WT (wildtype); Adv (adverse); Mut (mutated); FLT3 (Fms-related tyrosine kinase 3); ITD (internal tandem duplication); ELN (European LeukemiaNet classification system); PB (peripheral blood); BM (bone marrow); NA (not available)

2.2.4 CSC technology

2.2.4.1 Original protocol

Original standard Glyco-CSC and it's variants (Cys-Glyco-CSC and Lys-CSC) technology were performed as previously described[155,156]. Briefly, about 10^8 cells were labeled with biocytin hydrazide (Glyco-CSC and Cys-Glyco-CSC, 10 ml, 6.5 mM) after the oxidation with $NaIO_4$ (50 ml ,1.6 mM), or NHS-SS-biotin (Lys-CSC, 5 ml, 2 mM). Afterwards, cells were lysis with Dounce homogenizer or bioruptor. Subsequently, surface proteins were collected by ultracentrifugation in a 1:1 ratio with membrane preparation buffer and hypotonic lysis buffer (Glyco-CSC and Cys-Glyco-CSC, 100,000 × g, 60 minutes) or in a sucrose gradient (Lys-CSC, 150,000 × g, 60 minutes, collect the phase border containing plasma membranes into a new ultracentrifuge tube and fill the tube with hypotonic lysis buffer and then centrifuge at 200,000 × g, 20 hours). Membrane protein pellets from Glyco-CSC and Cys-Glyco-CSC were incubated with 400 µl membrane wash buffer and then filled with hypotonic lysis buffer and centrifuged at 100,000 × g, 60 minutes. Membrane protein pellets were completely collected into a new 2 ml tube and added 340 µl of

100 mM NH_4HCO_3 buffer and 40 µl of 1% RapiGest stock solution (Glyco-CSC and Cys-Glyco-CSC) or 320 µl of digestion buffer and 80 µl of 1% RapiGest stock solution (Lys-CSC), then were completely dissolved by indirect sonication. Protein concentrations were determined by BCA kit. For Glyco-CSC samples, membrane proteins were reduced by TCEP (8 µl, 100 mM, 30 minutes) and then alkylated by iodoacetamide (12 µl, 500mM, 30 minutes). While for Lys-CSC and optionally for Cys-Glyco-CSC samples, 1 µl PNGase F were added to solubilize and de-glycosylate the membrane proteins. The pre-treated membrane proteins were then digested overnight with trypsin at a ratio of trypsin: protein=1:50. The membrane proteins in the digestion were captured by affinity streptavidin beads and then released by 1 µl of glycerol-free PNGase F (Glyco-CSC and Cys-Glyco-CSC) or by reduction agents (cysteine contained peptides in Cys-Glyco-CSC and Lys-CSC). Those peptides were then desalted and washed in C18 columns and concentrated in a SpeedVac concentrator. The complete dried peptides were resuspended in 20 µl of sample buffer and stored at -20°C for further LC-MS detection.

In summary, the differences between Cys-Glyco-CSC and Glyco-CSC are that the Cys-Glyco-CSC skips the protein reduction and alkylation step and instead releases cysteine peptides by chemical reduction from the solid phase. Lys-CSC is also differing from Glyco-CSC in biotin labeling, ultracentrifugation and peptides release steps. The systematic workflow of CSC and it’s variants (Cys-Glyco-CSC and Lys-CSC) technology are illustrated in Figure 2-1.

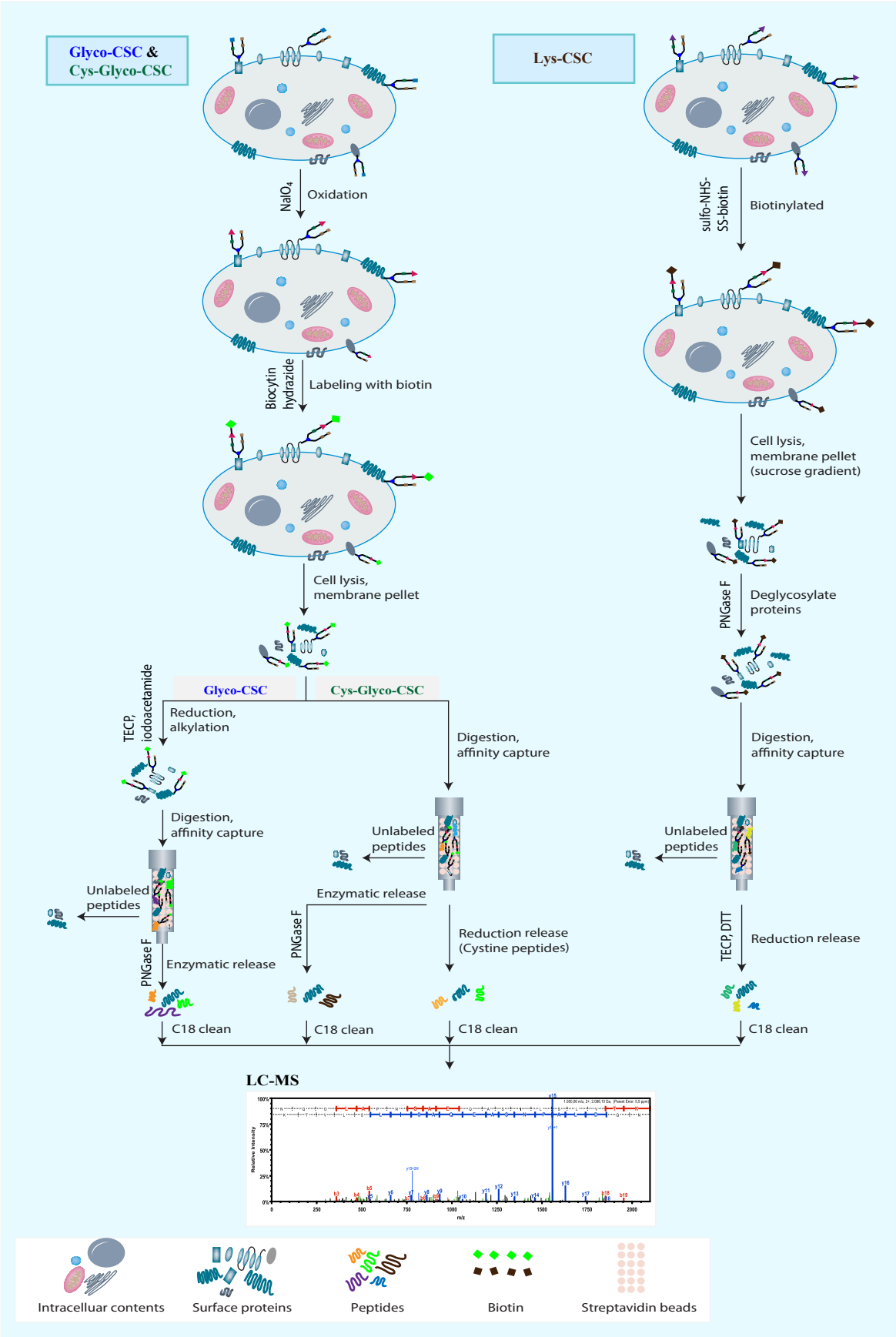


Figure 2-1. Workflow of Cell Surface Capturing (CSC) technology and it's 2 variants (Cys-Glyco-CSC and Lys-CSC). Modified from Wollscheid *et al.*, Nat Biotechnol., 2009 and Elschenbroich *et al.*, Exp Rev Pro., 2010.

2.2.4.2 Modifications to the protocol

Several strategies were applied to improve the original Glyco-CSC protocol in our lab. Experimental modifications are listed in the following Table 2-7. Cys-Glyco-CSC was performed using the same modifications (No.3 through No. 6) as Glyco-CSC. Since the Lys-CSC protocol uses a different biotin derivative for labeling, strategies only modifications No.4 through No.6 were applied to the Lys-CSC protocol.

Table 2-7. Strategies to improve the original CSC protocol.

Modification No.	Protocol Description
1	Technical Control (without biocytin hydrazide treated)
2	Original Glyco-CSC protocol
3	Optimization ①: lower concentration of biocytin hydrazide (5.4 mM)
4	Optimization ②: Optimization ① + bioruptor (instead of homogenizer)
5	Optimization ③: Optimization ② + homogenizer + 2 times digestion (firstly 2 hours, then overnight)
6	Optimization ④: Optimization ③ + digestion buffer (instead of pure NH_4HCO_3)

2.2.5 LC-MS:

Peptides in the sample were separated with an on-line 70 minutes reversed-phase nano HP-LC and analyzed on-line with a velos-pro orbitrap. MS1 spectra were acquired at 120000 resolution in data-dependent acquisition mode (top 10) and MS2 spectra at low resolution in the ion trap. The activation time was 10 ms; the AGC target value 10^4 ions; the maximum injection time 100 ms and the minimum ion count 500. Each sample was analyzed twice and the resulting technical replicates averaged.

Raw files were analyzed with MaxQuant using N-deamidation, protein n-terminal acetylation and methionine oxidation as a variable modification.

Carbamidomethylation on cysteines was used as a fixed modification. A maximum precursor mass error of 4.5 ppm and a fragment mass error of 0.5 Da were allowed. The final peptide and protein list were filtered at a 1% FDR. As database the whole reviewed human proteome from UniProt August-2015 was used.

The MaxQuant table containing the identified N-deamidations was processed further by incorporating annotations from UniProt and the Cell Surface Protein Atlas. Each deamidation was validated as and n-glycosylation based on the following criteria: i) the deamidation occurred in a NXS\T motif and had a MaxQuant localization probability greater than 0.75; ii) the deamidation occurred in a protein with at least one TM domain and/or signal peptide; and iii) the NXS\T motif did not overlap with a transmembrane (TM) domain. In this study, TM represented proteins with transmembrane domains or signal peptide, or that were present in the Cell Surface Protein Atlas, or that were annotated as Plasma Membrane proteins in Gene Ontology. Intensities between different samples were compared at the deamidation site level and deamidated-protein level. For the former n-glycosylations at the same site but in different tryptic peptides were summed. For the latter n-glycosylations at the same protein were averaged. Intensities were then log_2-transformed and median normalization between samples was applied. Clustering at the protein level was done using hiearchical clustering either using Euclidean distance or Cosine correlation distance. Edges in the network plot with a cosine correlation lower than 0.85 were filtered out. Finally, gene enrichment analysis was performed using DAVID.

Each of the samples were tested twice or three times. Intensity of the identified peptides were merged as average.

2.2.6 Flow cytometry

Peripheral blood (PB) or bone marrow (BM) aspirate samples of AML patients at initial diagnosis were collected for flow cytometric analysis. All samples were collected after written informed consent in accordance with the Declaration of Helsinki and approval by the Institutional Review Board of the Ludwig Maximilian University Munich. Patient characteristics are summarized in Table 2-9.

Antibody panel for the expression of candidate markers are shown in Table 2-8. Primary AML patient samples were analyzed by flow cytometry in the Laboratory of Leukemia Diagnostics of the Department of Internal Medicine III. Briefly, patient samples were thawed, cells were washed once with PBS and resuspended in FACS buffer. 10^6 cells in 100 µl of FACS buffer were used per tube. Appropriate amount of antibody or isotype control were added into the tube. Data was acquired using a Navios flow cytometer (Beckman Coulter, Brea, CA, USA).

Antigen expression intensity was determined by median fluorescence intensity (MFI) ratio. The MFI ratio is calculated by dividing the MFI value of the antigen-specific antibody by the MFI value of the respective isotype control, as previously described[52]. Positive expression was defined as MFI ratio ≥1.5. MFI values were determined using FlowJo software (Version 9.8.5) (Tree Star Inc., Ashland, Oregon).

2.2.7 Statistical analysis

Intensity based absolute quantification (iBAQ) of protein was calculated as the sum of all peptide peak intensities divided by the number of theoretically observable tryptic peptides.

Table 2-8. Surfaceome panel for FACS detection.

Tube	FITC	PE	ECD	PC7	Alexa Fluor 750	KO	Brilliant Violet 421
Isotype	IgG1	IgG1	IgG1	IgG1	IgG1	CD45	IgG1
1	CD38	**SIRBL (CD172b)**	CD34	CD33		CD45	**ITGA4 (CD49d)**
2	CD38	**PTPRJ (CD148)**	CD34	CD33		CD45	
3	**PLXA1**	**ITB7**	CD34	CD33	CD38	CD45	

Table 2-9. Patient characteristics for FACS tests.

Patient No.	Sex	Age (Y)	FAB	Blasts	Cytogenetics	NPM 1	FLT3-ITD	ELN	BM/PB
1	F	69	M4	68%	46,XX	Mut	FLT-ITD (low)	Fav	BM
2	M	68	NA	32%	NA	Mut	FLT-ITD (low)	Fav	BM
3	F	78	NA	20%	46,XX	Mut	WT	Fav	PB
4	M	86	M5b	47%	46,XY	Mut	WT	Fav	BM
5	M	52	M3	41%	46,XY,t(15;17)(q24;q21)[7]/46,XY,der(15)t(15;17)(q24;q21),ider(17)(q10)t(15;17)(q24;q21)[8]/46,XY[1]	WT	WT	Int-I	BM
6	M	68	M1	65%	46,XY	WT	WT	Int-I	BM
7	M	78	NA	21%	46,XY	WT	WT	Int-I	BM
8	F	59	M1	77%	46,XX	Mut	Mut	Fav	BM
9	M	78	NA	38%	46,XY	WT	WT	Int-I	BM
10	F	80	M2	23%	46,XX	WT	WT	Adv	BM
11	F	31	M5	32%	46,XX	Mut	WT	Fav	BM
12	F	60	M1	88%	46,XX	WT	WT	Int-I	BM
13	M	50	NA	26%	NA	Mut	WT	Fav	BM
14	F	61	M4	54%	46,XX,der(6)t(6;13)(p2?1;q?)[6]/46,XX[4]	WT	Mut	Int-I	PB
15	F	41	NA	NA	NA	WT	WT	Int-I	PB
16	M	32	M5a	96%	46,XY,t(9;11)(p21.3;q23.3)[3]/55,sl,+3,+8,+der(11)t(9;11)(p21.3;q23.3),+12,+13,+14,+18,+19,+20[13]	WT	WT	Int-I	BM
17	M	68	NA	36%	46,XY[20]	WT	WT	Adv	BM
18	F	87	M2	84%	46,XX[20]	Mut	FLT3-ITD(high)	Int -I	BM
19	F	54	NA	40%	46,XX,t(3;3)(q21.3;q26.2)[10]/46,XX[1].ish der(2)t(2;11)(q3?7;q23)(KMT2A+)[3/14]	WT	WT	Adv	BM
20	M	46	NA	59%	NA	NA	NA	NA	BM

Statistical data were analyzed using GraphPad Prism 7.1 (GraphPad software, La Jolla. CA). Data is displayed as mean ± standard error of the mean (SEM), using Two-tailed Student's *t* test to determine statistical significance. Graphs were generated with GraphPad Prism 7.1 (GraphPad software, La Jolla.CA), R Studio (R Studio, Boston, USA) and Adobe Illustrator CS6 (Adobe Systems, San José, USA).

3. Results

3.1 Surfaceome study on AML cell line and patient samples

3.1.1 Modifications to the Glyco-CSC protocol

Initially, we performed the original Glyco-CSC technology to capture N-glycosylated proteins using the OCI-AML3 cell line as described previously[158-160]. The protocol did work well with good peptides intensity detected and very little noise (Figure 3-1 A). 216 of peptides digested from 125 N-glycosylated proteins were identified. 54 were annotated as CD antigens. PANTHER association of the identified proteins revealed receptor function in about 1/3 of the proteins (Figure 3-1 B). While these initial results were encouraging, the number of identified proteins of these results lacked behind other publications on AML cell lines, where 237 and 230 proteins were identified in HL-60 and NB4 cell lines, respectively[160]. Moreover, only about 54% of identified proteins showed a mass shift (of 0.984 Da) at N-residues associated with N-glycosylated and had TM domains or signal peptide annotated in UniProt, indicating that the specificity was lower than expected. Therefore, we employed strategies to improve the standard protocol in order to increase the specificity of the technology and generate more surface proteins.

Cell viability during the oxidation step is very important to make sure the targeted proteins are cell surface glycoproteins. Ensuring high viability avoids the possibility of chemical reaction between sodium periodate and intracellular glycoproteins caused by cell lysis or permeabilized membranes. In our experiments, despite slightly decreased cell numbers, high cell viability was maintained before cell lysis was performed (Supplemental information, Figure S1 A and B). Comparable data were

also found in HL-60 cell line. As a benchmark CD33, a myeloid marker found on >99% of AML samples, was also detected in both two cell lines (Data from HL-60 was not shown).

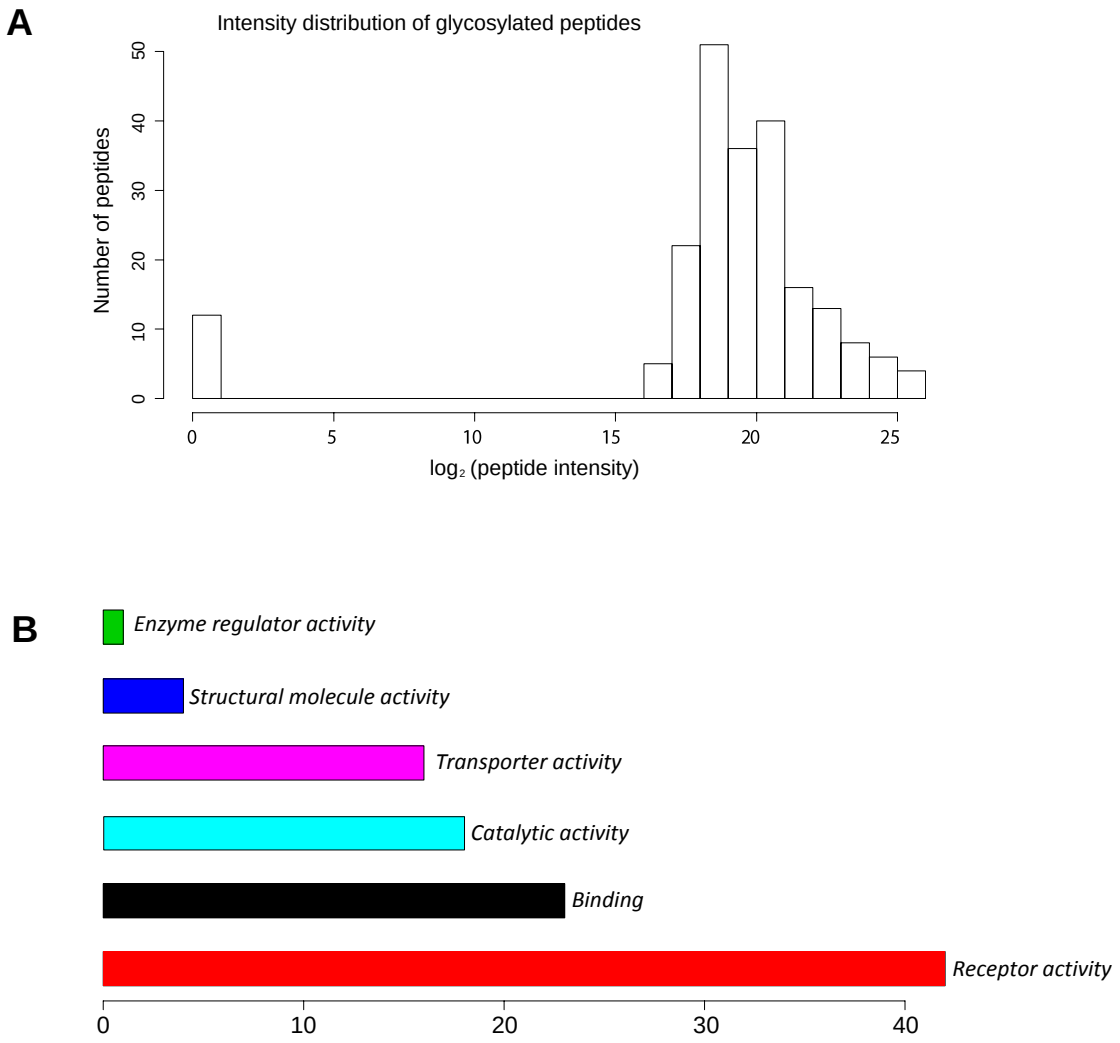


Figure 3-1. N-glycosylated proteins identified with original Glyco-CSC technology. (A) Distribution of identified glycosylated peptides on OCI-AML3 cell line. (B) PANTHER molecular function of identified surface proteins.

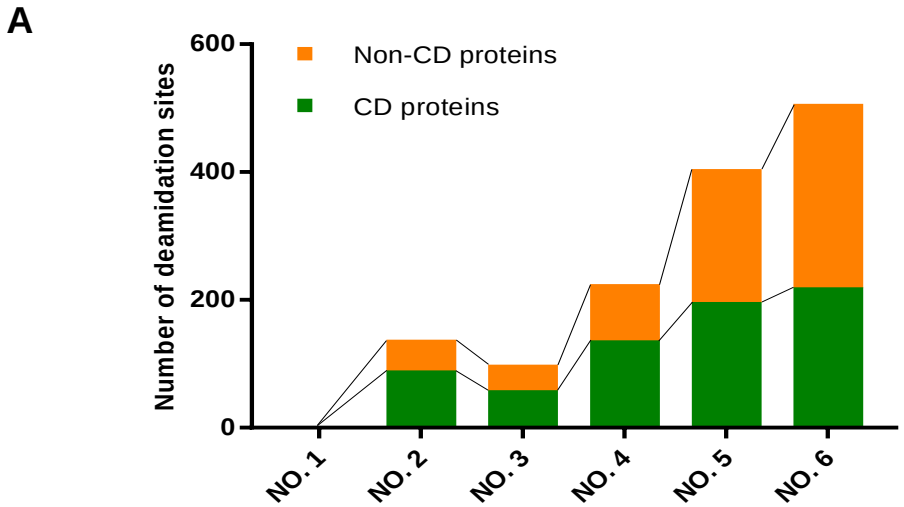
3.1.2 Strategies to improve the original Glyco-CSC protocol

In order to improve the experimental CSC protocol, we introduced several modifications (Table 2-7). After the vicinal diol groups on glycoproteins were oxidized to aldehyde functions by periodate, biocytin hydrazide can covalently link to the aldehydes and form the hydrazone. In out initial experiments, we detected cell aggregation during this step, which was eliminated by lowering the concentration of biocytin hydrazide (5.4 mM) since we found cell aggregation during our experiments, after we add it to the cell suspension or during the incubation. Sonication using a bioruptor and followed by cell homogenization assured the complete lysis of cells and increased our yield of membrane proteins after ultracentrifugation. Then, double digestion with trypsin and replacement of pure NH_4HCO_3 solution with digestion buffer (100 mM NH_4HCO_3, 1 mM iodoacetamide, 1 mM 2,2'-thiodiethanol) increased the peptide yield retrieved from LC-MS.

To evaluate the specificity of our modified method, we relied on the detection of the deamidation reaction detected by a mass shift (of 0.984 Da) at N-residues. Furthermore, the deamidation might be reduced by the three-dimensional structure of proteins and thus it can be enhanced by alkylation, tryptic digestion, and reduction[171]. Figure 3-2 shows the number of deamidation sites and the Loc. Prob. (probability of the PTM being localized at the claimed position, recommended threshold ≥0.75) of detected peptides. No glycosylated peptides were detected in our technical control without biocytin hydrazide (test NO. 1). Samples treated with lower concentration of biocytin hydrazide resulted in a slightly lower number of deamidation sites than the original concentration (test NO. 3 and test NO. 2, respectively). However, since cell aggregation affect the following cell lysis negatively and resulted in a decreased

amount of membrane protein and thus impacting specificity (data not shown), we preferred the modified protocol in the following experiments (test NO. 3). The number of deamidation sites increased in test group NO.4, where we performed cell lysis using the bioruptor rather than Dounce homogenizer, compared to the former 2 groups. Double digestion further improved the yield. Finally, the use of digestion buffer in test NO.6 worked better than pure NH_4HCO_3 in maintaining the peptides.

In summary, more than 500 deamidation sites, which represent 252 proteins, were identified using our final, modified protocol. In addition, the specificity of the technology was also increased. 80.4% of all identified proteins contained N-glycosylations and a transmembrane domain or a signal peptide annotated in UniProt compared to only 54% using the original protocol. Furhermore, 90.3% of the N-glycosylated proteins were annotated in UniProt as glycoproteins and 61.9% were annotated as plasma membrane proteins in Gene Ontology. These results showed the relative enrichment for N-glycosylated cell surface proteins in our study.



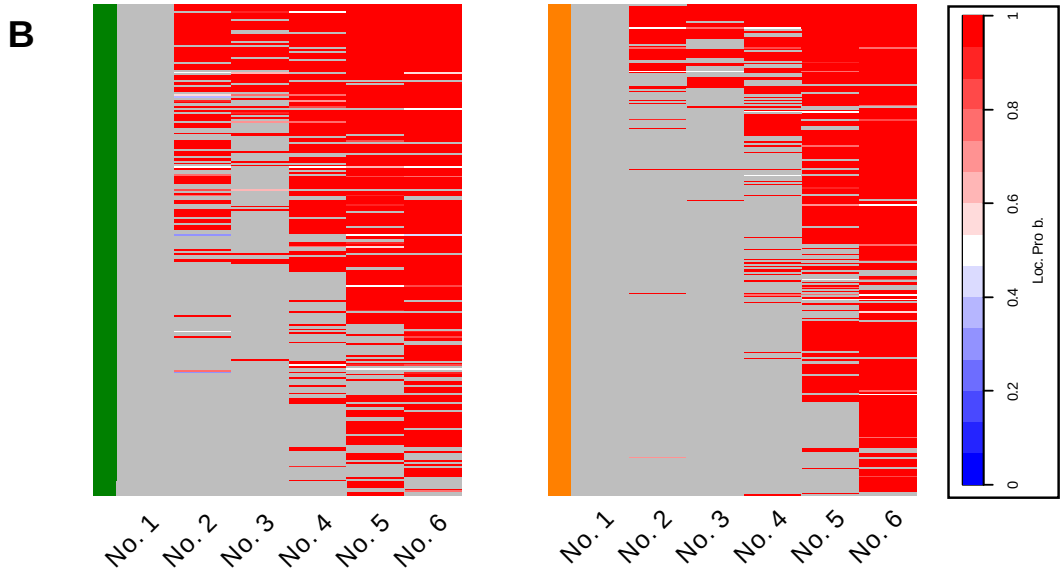
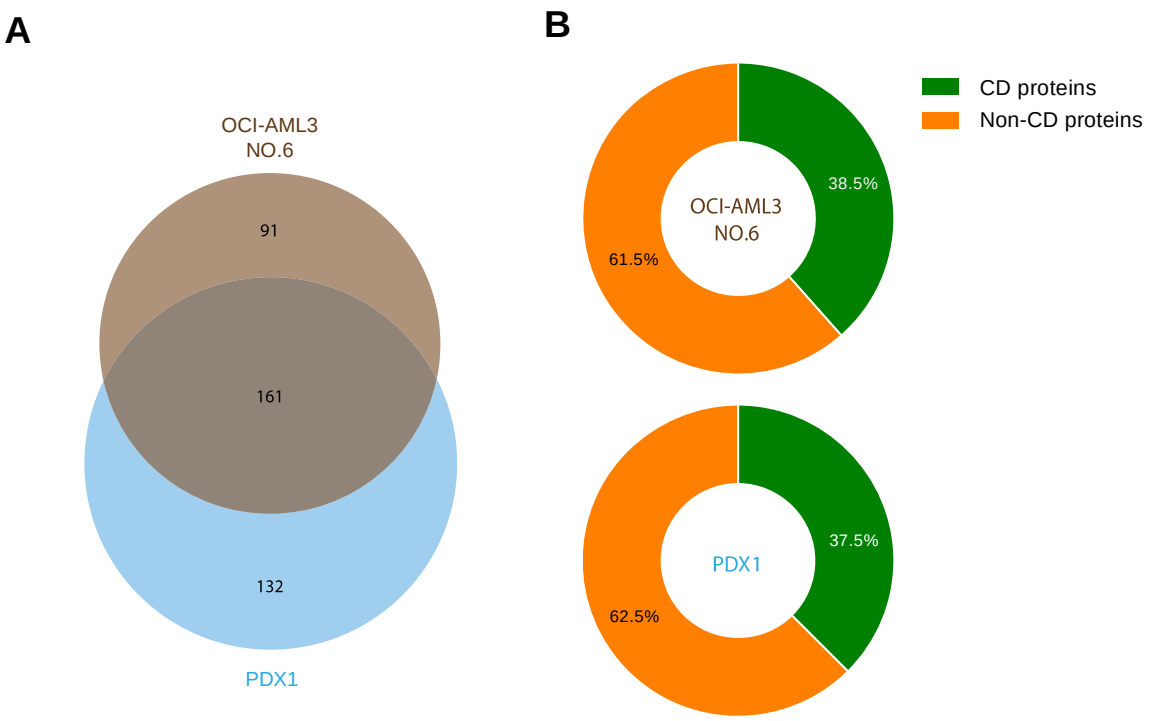


Figure 3-2. N-glycosylated peptides identified by improved strategies. (A) Deamidation sites detected on OCI-AML3 cell line with different improved strategies. (B) Probability of the post-translational modification (PTM) being localized at the claimed position (Loc. Prob., recommended threshold ≥ 0.75) of identified peptides in each group. Peptides representing CD proteins (labelling with green column) listed on the left while Non-CD proteins (labelling with brown column) listed on the right.

3.1.3 Surfaceome study on patient sample (PDX1) with improved Glyco-CSC protocol

In order to assure the effectiveness and robustness of the improved Glyco-CSC protocol, we began by employing the technology using a patient derived xenograft sample. 293 surface proteins were identified in this sample, slightly more than the from the OCI-AML3 cell line using our modified protocol (Figure 3-3 A). Similarly, almost the same percentage of CD and non-CD proteins were identified in the two samples (Figure 3-3 B). Comparable PANTHER protein classes[172], including receptors, signaling molecules, cell adhesion molecules and immunity proteins, were detected in these 2 samples (Figure 3-3 C). Abundance of identified protein was calculated as the sum of all peptide peak intensities divided by the number of the observed peptides in our experiments. Proteins were divided into 4 clusters by their

expression level (log_2 (Average of Abundance)). Cluster 1 showed proteins with low expression in both samples. Conversely, cluster 4 represents proteins with high expression in both samples (Figure 3-3 D). In fact, many well-known surface CD antigens, such as CD33 (P20138), CD45 (P08575), CD123 (P26951) and CD244 (Q9BZW8) were clustered in cluster 4 in our study. CD64 (P12314) and CLL-1 (Q5QGZ9) are two important surface markers for the differentiation of monocytes from myeloid progenitors[173]. CD64 showed high expression in both OCI-AML3 and PDX1 samples, while CLL-1 high expressed only in the PDX1 sample in our study.



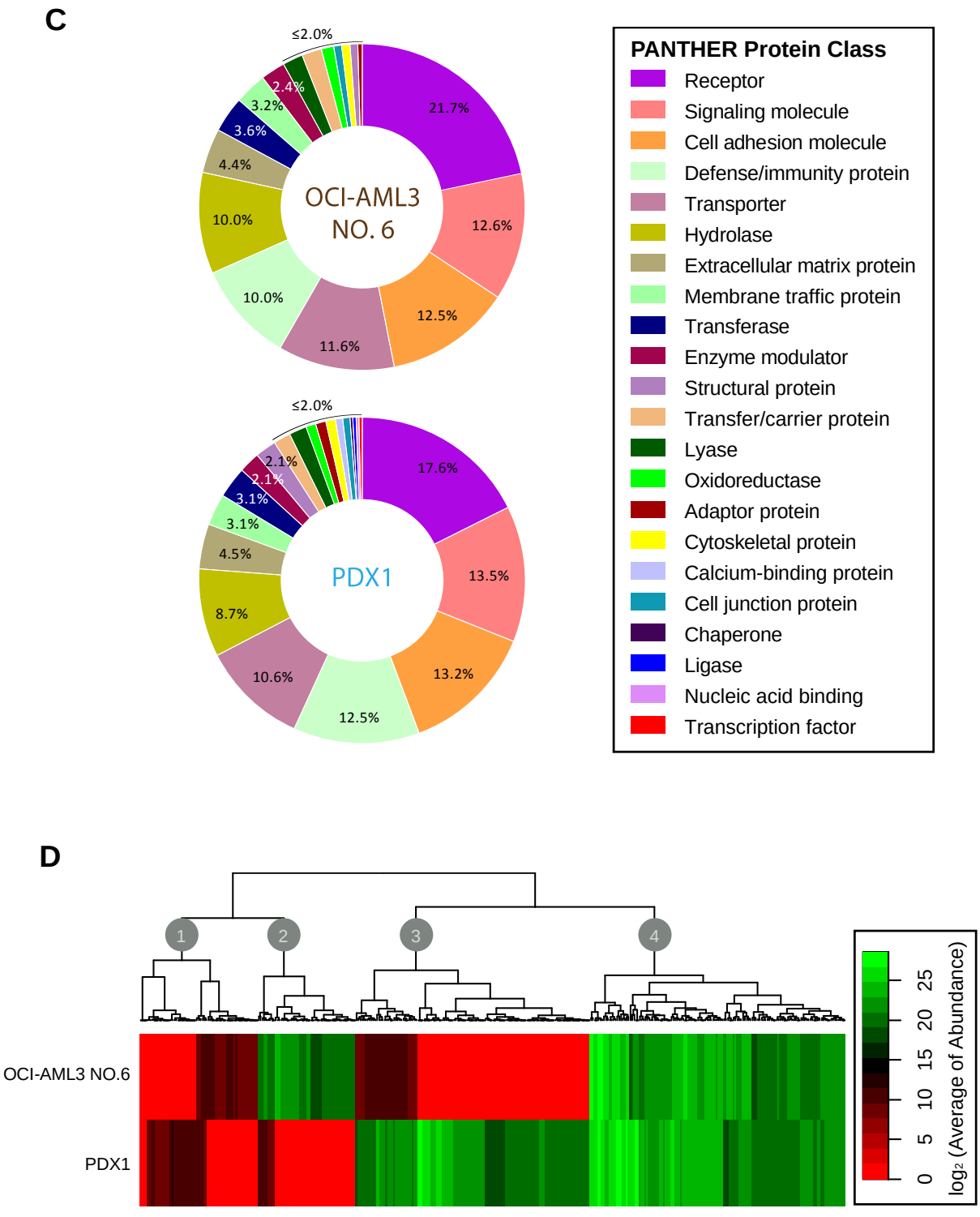


Figure 3-3 Cell surface proteins identified by improved Glyco-CSC technology. Totally 384 membrane proteins identified in 2 different cell types. (A) Area-proportional Venn diagram[174] of overlapping membrane proteins identified in OCI-AML3 and PDX cells. (B) More than one third of identified membrane proteins were annotated as CD antigens in both samples. (C) PANTHER proteins classifications of membrane proteins identified in AML samples (http://pantherdb.org/). (D) Expression level (log_2 (Average of abundance)) and hierarchical clusters of identified membrane proteins in OCI-AML3 and PDX samples. Green colour indicated high protein expression level, while red colour indicated low protein expression level.

3.1.4 Surfaceome study on OCI-AML3 cell line with improved Glyco-CSC, Cys-Glyco-CSC and Lys-CSC protocol

Buidling on the the results obtained above from the OCI-AML3 cell line and PDX1 sample using the Glyco-CSC technology, we next used the OCI-AML3 cell line to investigate the improved Cys-Glyco-CSC (n=3) and Lys-CSC (n=3) protocol, in parallel with additional 3 replicates of Glyco-CSC on this cell line. The step-to-step protocol is depicted in Figure 2-1 and the modifications are listed in Table 2-7. Each biological replicate was tested twice. Results from each technology were merged together.

496 cell surface proteins were identified using the Glyco-CSC protocol, 231 proteins were identified using Cys-Glyco-CSC, while only 126 proteins were identified with Lys-CSC (Figure 3-4 A). A network diagram illustrates the details of identified proteins using the 3 improved CSC protocols (Figure 3-4 B). Overall, 119 CD antigens (Green color nodes) were identified and 483 proteins were annotated as non-CD proteins. The width of the links indicated the peptide number of each identified proteins, ranging from 1 to 32. The size of the nodes increased with the number of TM domains, ranging from 0 to 36. Nevertheless, the size will not increase if there were more than 10 TM domains in that protein.

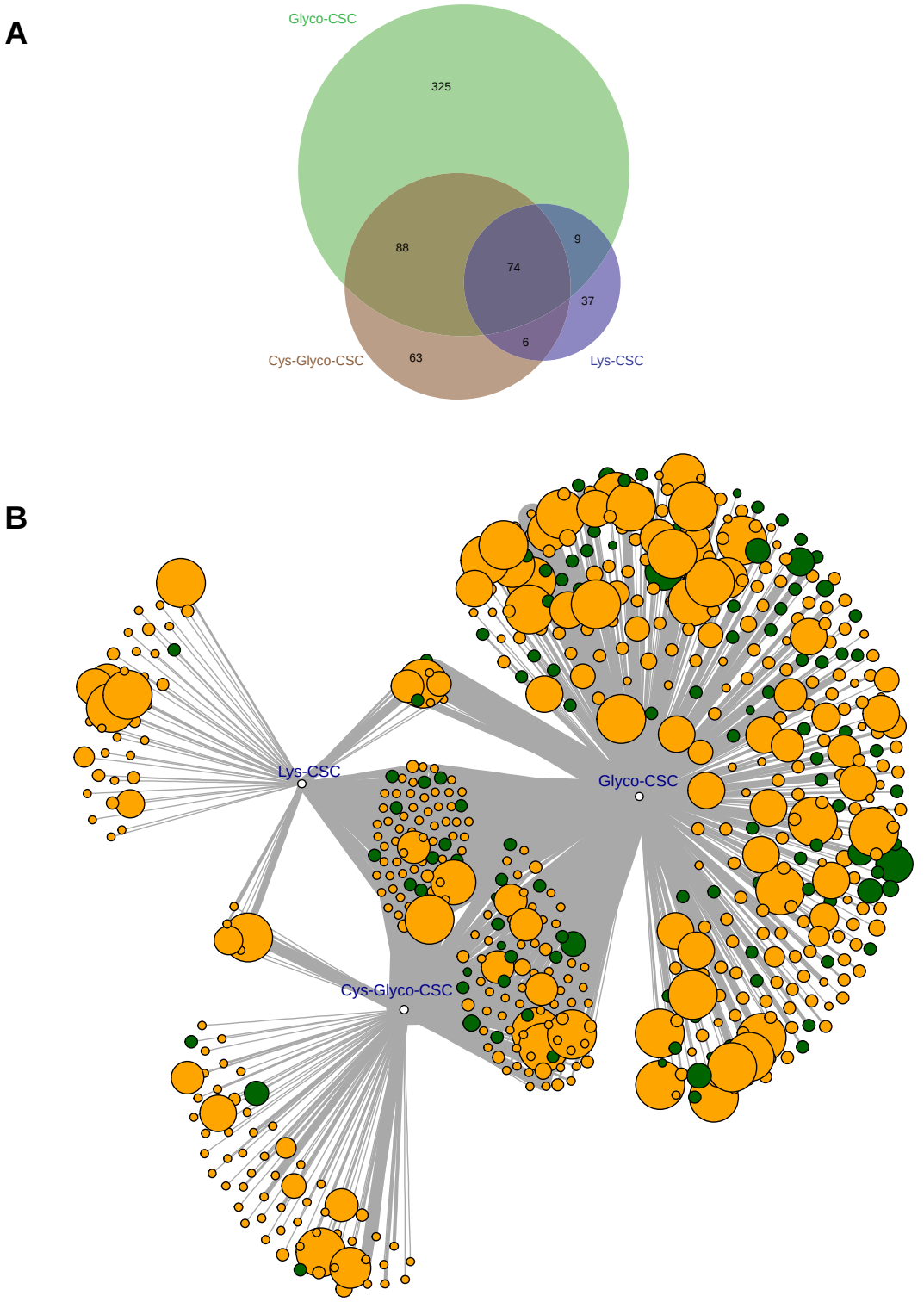


Figure 3-4 Comparison network of membrane proteins identified with improved Glyco-CSC, Cys-Glyco-CSC and Lys-CSC technology in OCI-AML3. (A) Area-proportional Venn diagram[174] of overlapping 602 membrane proteins identified with improved Glyco-CSC and its variant technology with OCI-AML3 cell line. (B) Nodes represented for CD proteins were in green colour and non-CD proteins were in brown colour. The thickness of the links connecting two nodes was proportional to the number of peptides identified for that protein. The size of the node indicated the number of transmembrane domains (TMs) in the protein. Proteins with more than 9 TMs were limited in size to 10.

3.1.5 Surfaceome study on clinical patient samples

Maintaining high viability of primary patient AML blasts while gathering sufficient cell numbers necessary for the CSC protocol is a significant challenge. Our pre-liminary work using clinical patient samples directly with our improved Glyco-CSC protocol also provided unsatisfied results. Specifically, cell viability decreased rapidly during oxidation and biotynilation steps before the cell lysis was performed (data not shown). Thus, in this study, we used two well studied methods to improve viability of clinical patient samples for to interrogate the AML surfaceome.

We used two PDX samples (PDX1 and PDX2) from Prof. Dr. med. Irmela Jeremias' group in Helmholtz Zentrum München[170]. For sample PDX1, we were able to perform the Glyco-CSC (n=1) and Cys-Glyco-CSC (n=1) protocol, while for PDX2, we were performed Glyco-CSC (n=1) only due to limited cell numbers isolated from mice spleen. Additionally, six clinical primary patient samples (PP01-PP06) were used to perform our improved Glyco-CSC protocol. Results from PP02 were retrospectively excluded in this study due to a change in the final clinical diagnosis from AML to ALL. To improve cell viability prior to CSC experiments, primary patient samples were maintained in a long-term culture system established in our lab[52]. Clinical characteristics of patient samples used for this study are listed in Table 2-6. Taken together, data from 22 samples (OCI-AML3 with Glyco-CSC (n=6, test NO.6 plus 5 replicates), OCI-AML3 with Cys-Glyco-CSC (n=5), OCI-AML3 with Lys-CSC (n=3), PDX1 with Glyco-CSC (n=1), PDX1 with Cys-Glyco-CSC (n=1), PDX2 with Glyco-CSC (n=1), PP01 with Glyco-CSC (n=1), PP03 with Glyco-CSC (n=1), PP04 with Glyco-CSC (n=1), PP05 with Glyco-CSC (n=1) and PP06 with Glyco-CSC (n=1))

were merged together as surfaceome results in this study. Intensity based absolute quantification (iBAQ) of protein was calculated as described in the Methods.

22.9% (165) of all identified proteins had CD annotations (Figure 3-5 A). In total, 23 types of PANTHER protein classes were associated with the 719 identified proteins (Figure 3-5 B). Furthermore, those 719 proteins were involved in 75 pathways found in the PANTHER database (Supplemental information, Figure S3). Most notably, these included the integrin signaling pathway (P00034) and inflammation related pathway (P00031), followed by the Wnt signaling pathway (P00057). Cancer associate and immune cells responses pathways, like p53 pathway (P00059), JAK/STAT signaling pathway (P00038), Wnt signaling pathway (P00057), Interferon-gamma signaling pathway (P00035) and Interleukin signaling pathway (P00036), B cell activation (P00010) and T cell activation (P00053), were also detected in our study. Expression levels ($\log_{10}$ (Average of iBAQ)) of the 165 CD proteins are shown in Figure 3-6.

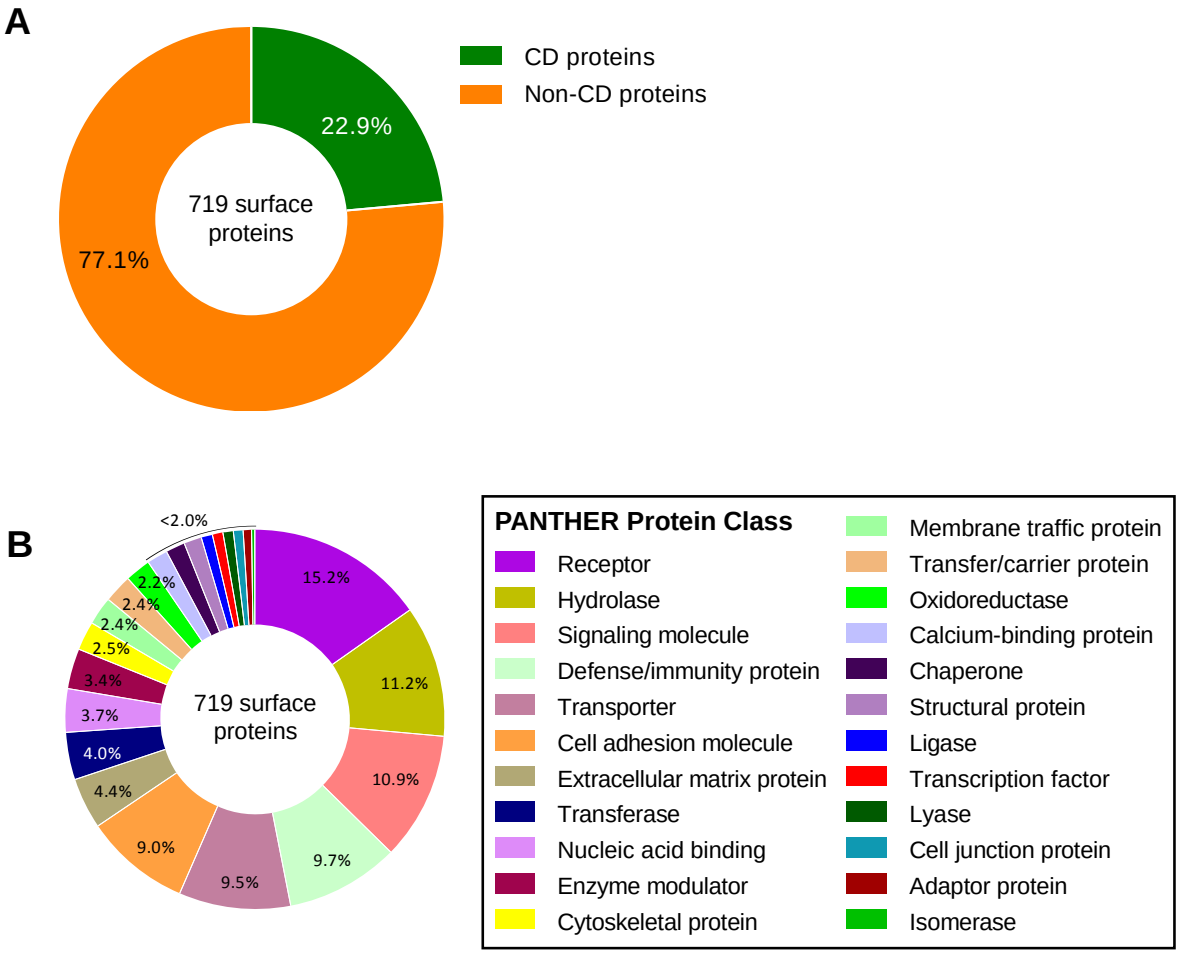


Figure 3-5. Totally 719 surface proteins identified with CSC and its variant technology in 1 AML cell line and 7 clinical samples. (A) 165 CD proteins and 554 non-CD proteins were identified in 1 AML cell line and 7 clinical patient samples with specific CSC technologies. (B) PANTHER proteins classifications of 719 membrane proteins identified in 8 AML samples (http://pantherdb.org/).

Figure 3-6. Expression of CD proteins identified in AML samples. Overview of all 165 CD antigens in 8 AML samples. Color of the heatmap indicated the value of $\log_{10}$ (Average of iBAQ).

3.2 Selection and verification of candidate surface markers

3.2.1 85 candidate proteins were filtered from 719 proteins as AML cell surface targeted markers

In order to curate a list of candidate markers we filtered the list of identified proteins to concentrate on those proteins which show low expression on healthy hematopoietic cells as well as other normal human tissues. To achieve this, we interrogated two databases to correlate gene expression of the 719 proteins on blood cells in different stages (BloodSpot) and on normal human tissues (Genotype-Tissue Expression Project (GTEx)).

We eliminated those proteins that showed a median value (log_2 (Expression)) of: (I), HSC_BM (Hematopoietic stem cells from bone marrow) <8; (II), early HPC_BM (Hematopoietic progenitor cells from bone marrow) <8 and (III), $CD14^+$ monocytes<10. Next, we eliminated proteins which showed median of RPKM in normal tissues excluding whole blood and spleen of ≥ 20 (rule IV) in the GTEx database.

For all of the 719 surface proteins, there was no expressiondata for 64 genes in BloodSpot and 40 genes in GTEx, respectively. Figures 3-8 (A)-(C) show the log_2 (Expression) of 655 genes in different stages of normal human hematopoiesis found in the BloodSpot (HemaExplorer) database. Genes were clustered by expression within the groups “HSC_BM” (Figure 3-8 A), “early HPC_BM” (Figure 3-8 B) and “$CD14^+$ monocytes” (Figure 3-8 C), respectively. Accordingly, Figures 3-8 D illustrate the expression values (RPKM) of 679 genes in normal human tissues, excluded spleen and whole blood. Expression of all 719 proteins identified in our samples as determined by LC-MS/MS and calculated as log_{10} (Average of iBAQ) is depicted in

Figure 3-8 E with values ranging from 0 to 8. 354 protein were identified (log_{10} (Average of iBAQ)>0) in at least 4 out of 8 samples, thus we only considered these proteins (rule V). Taken all five filter rules together, 85 cell surface proteins were selected as candidate biomarkers for further study on AML (Figure 3-8 F).

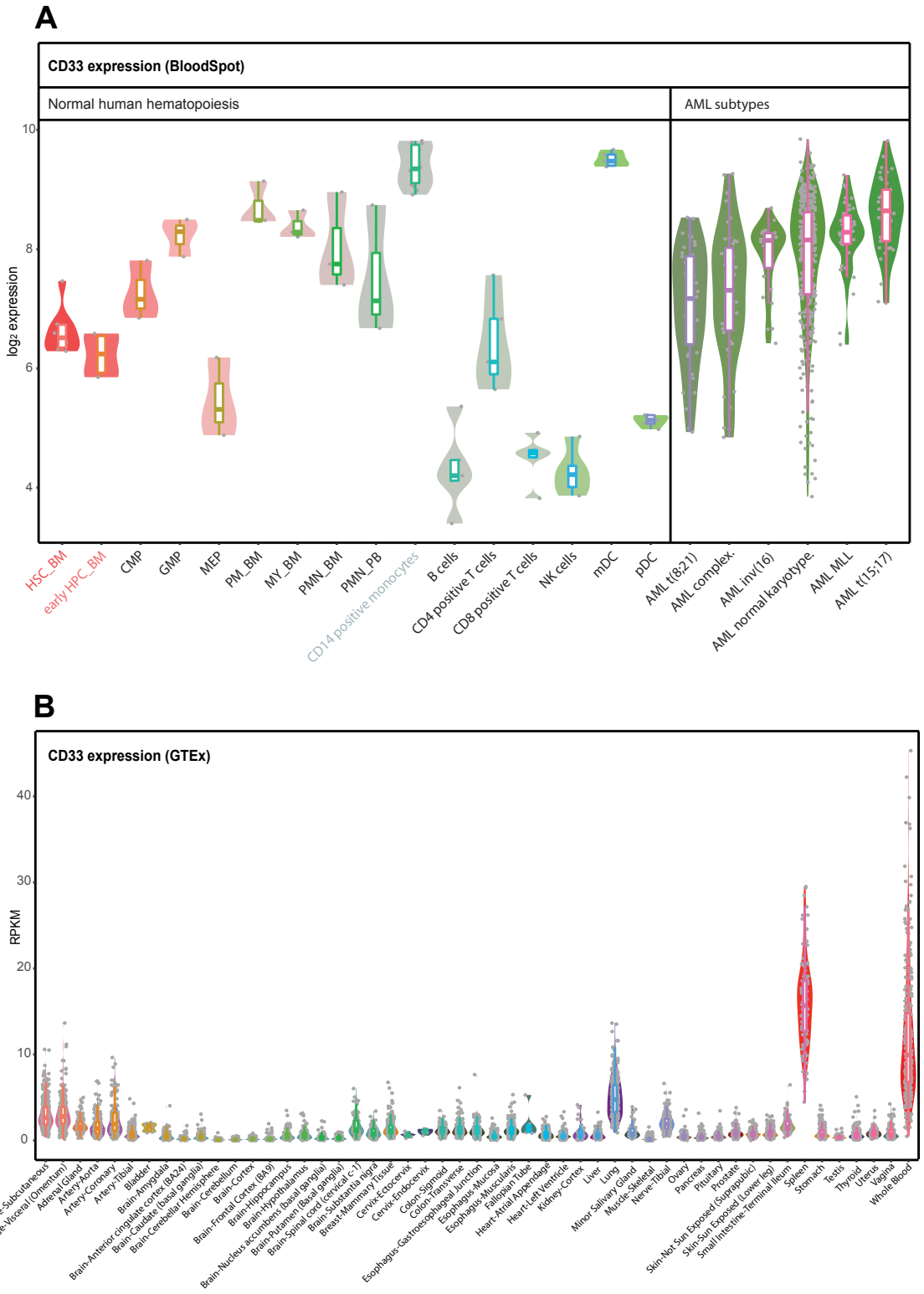
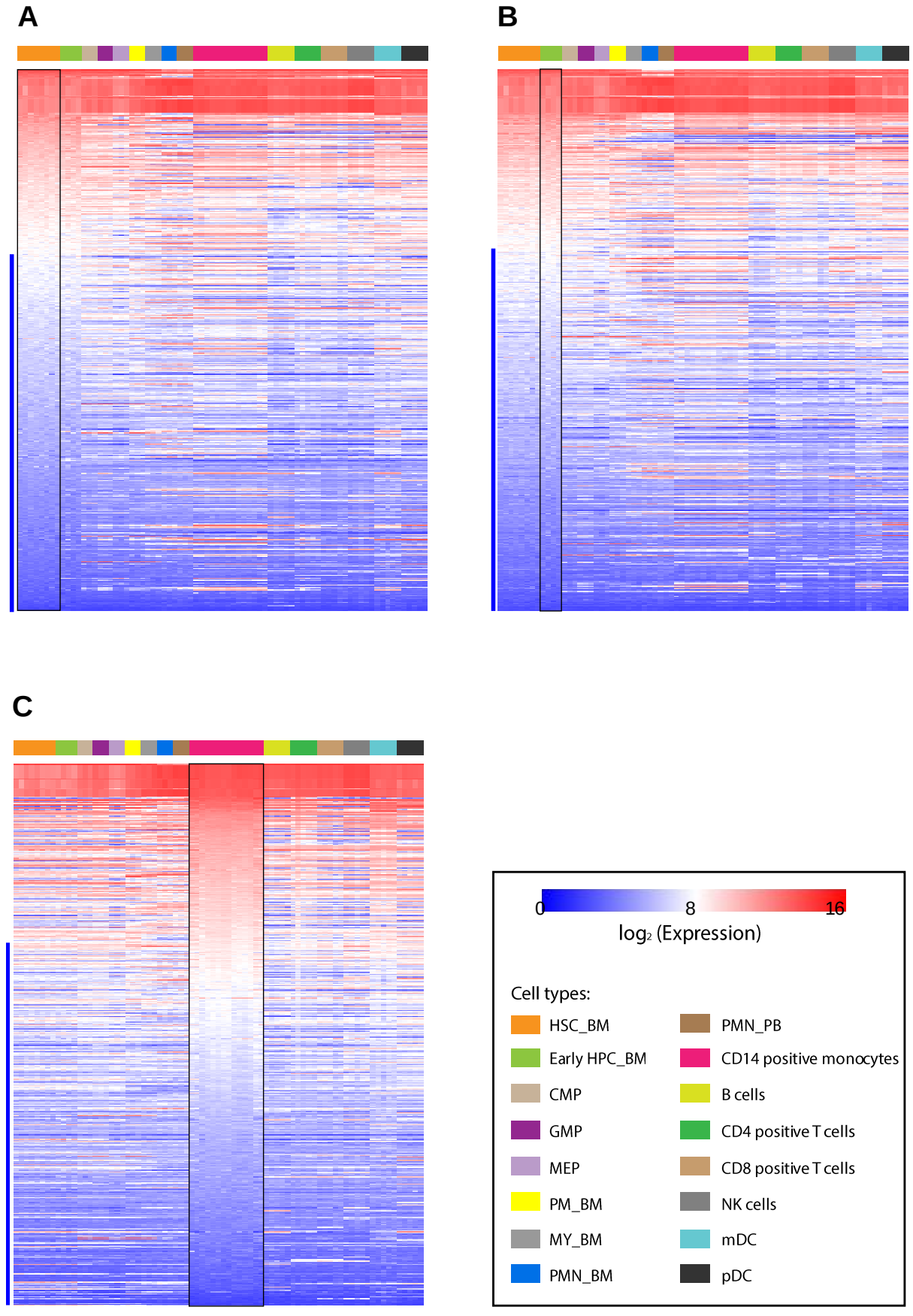
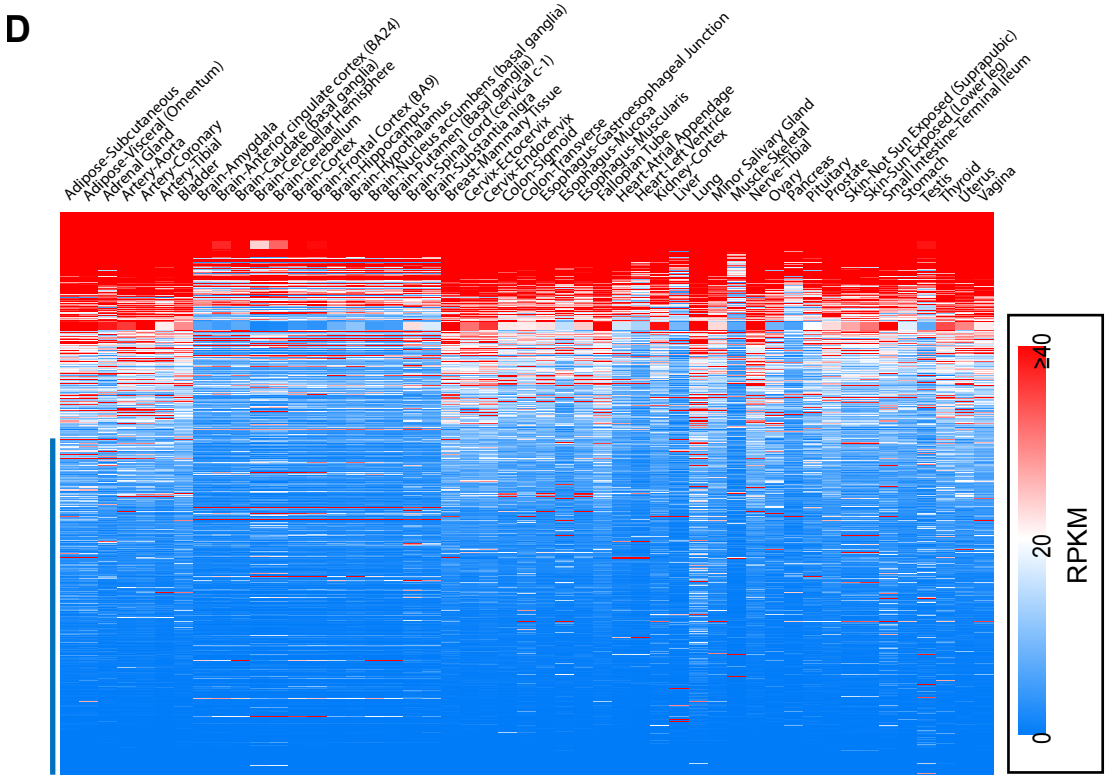
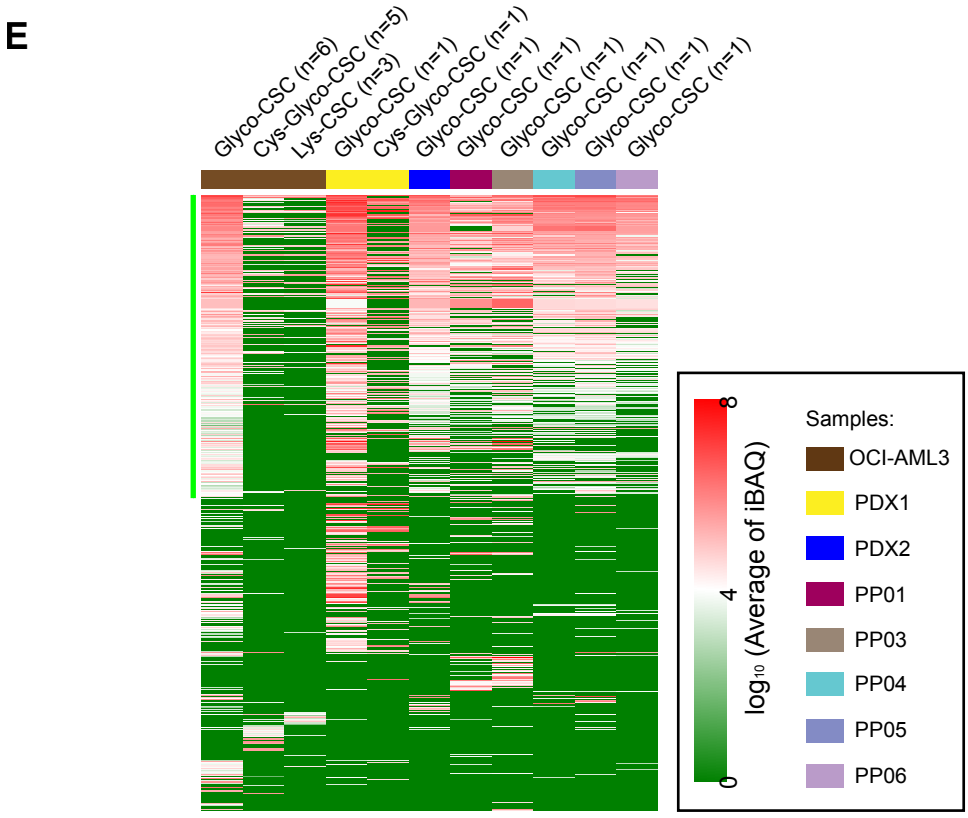


Figure 3-7. CD33 gene expression in hematopoietic cells, AML petients samples and normal human tissues. (A) Microarray data showed the expression level of CD33 in hematopoietic cells (left) and in AML patients (right). Data obtained from BloodSpot (http://servers.binf.ku.dk/bloodspot/) and violin diagram was generated with R. (B) RNA sequencing data of CD33 expression in normal human tissues. Data was downloaded from GTEx (https://gtexportal.org/home/) and violin diagram was generated with R Studio.
Abbreviations for Figure 3-7 A: AML complex., AML complex aberrant karyotype; AML inv(16), AML with inv(16)/t(16;16); AML MLL, AML with t(11q23)/MLL; AML normal karyotype., AML with normal karyotype + other abnormalities.; AML t(8;21), AML with t(8;21); AML t(15;17), AML with t(15;17).


A
B
C
0
8
16
log2 (Expression)
Cell types:
HSC_BM
Early HPC_BM
CMP
GMP
MEP
PM_BM
MY_BM
PMN_BM
PMN_PB
CD14 positive monocytes
B cells
CD4 positive T cells
CD8 positive T cells
NK cells
mDC
pDC


D
Adipose-Subcutaneous
Adipose-Visceral (Omentum)
Adrenal Gland
Artery-Aorta
Artery-Coronary
Artery-Tibial
Bladder
Brain-Amygdala
Brain-Anterior cingulate cortex (BA24)
Brain-Caudate (basal ganglia)
Brain-Cerebellar Hemisphere
Brain-Cerebellum
Brain-Cortex
Brain-Frontal Cortex (BA9)
Brain-Hippocampus
Brain-Hypothalamus
Brain-Nucleus accumbens (basal ganglia)
Brain-Putamen (basal ganglia)
Brain-Spinal cord (cervical c-1)
Brain-Substantia nigra
Breast-Mammary Tissue
Cervix-Ectocervix
Cervix-Endocervix
Colon-Sigmoid
Colon-Transverse
Esophagus-Gastroesophageal Junction
Esophagus-Mucosa
Esophagus-Muscularis
Fallopian Tube
Heart-Atrial Appendage
Heart-Left Ventricle
Kidney-Cortex
Liver
Lung
Minor Salivary Gland
Muscle-Skeletal
Nerve-Tibial
Ovary
Pancreas
Pituitary
Prostate
Skin-Not Sun Exposed (Suprapubic)
Skin-Sun Exposed (Lower leg)
Small Intestine-Terminal Ileum
Stomach
Testis
Thyroid
Uterus
Vagina
≥40
20
0
RPKM


E
Glyco-CSC (n=6)
Cys-Glyco-CSC (n=5)
Lys-CSC (n=3)
Glyco-CSC (n=1)
Cys-Glyco-CSC (n=1)
Glyco-CSC (n=1)
Glyco-CSC (n=1)
Glyco-CSC (n=1)
Glyco-CSC (n=1)
Glyco-CSC (n=1)
Glyco-CSC (n=1)
8
4
0
log10 (Average of iBAQ)
Samples:
OCI-AML3
PDX1
PDX2
PP01
PP03
PP04
PP05
PP06

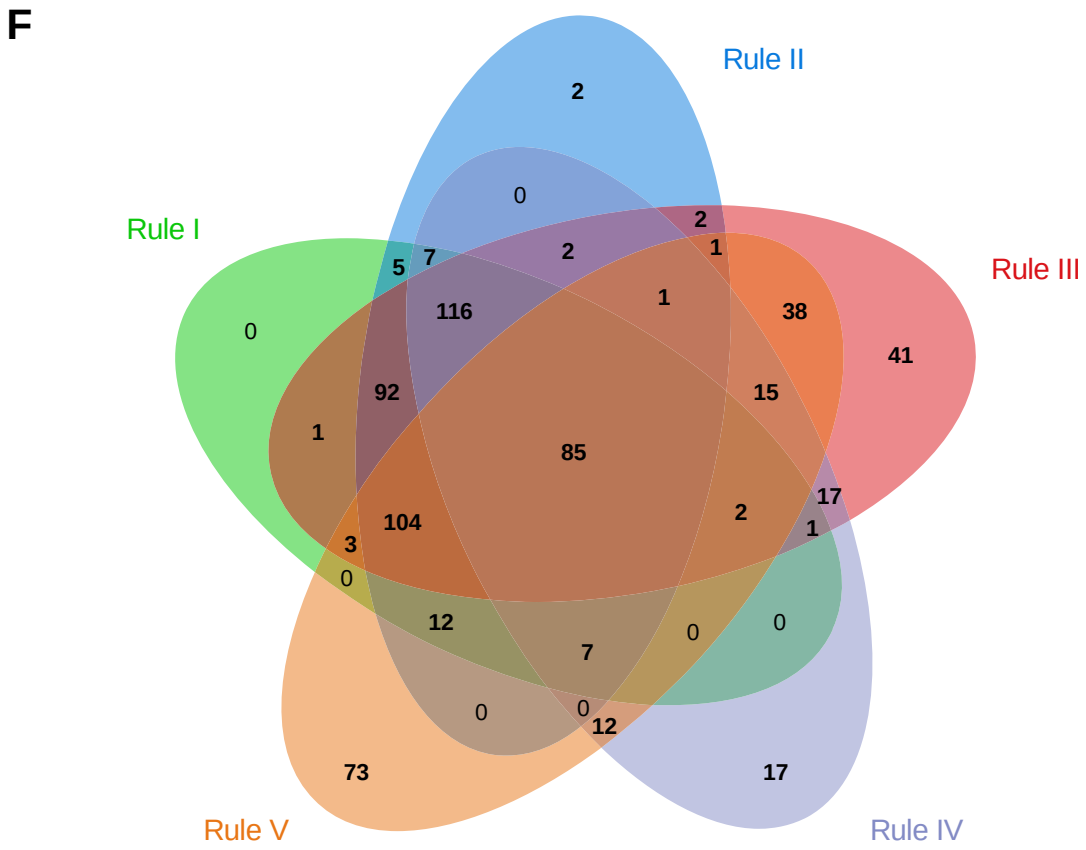


Figure 3-8. Integrated strategies to investigate new AML-associated markers from 719 identified surface proteins. Two databases were invited for further analyzation of potential new markers. Figure (A)-(C) showed the expression of 655 genes in hematopoietic cells at different maturation stages (Normal human hematopoiesis (HemaExplorer) based on curated microarray data (BloodSpot database: http://servers.binf.ku.dk/bloodspot/). Genes were hierarchical clustered by the value of $\log_2$ (expression). 436 and 437 targets were filtered out with strategy 1 and 2: median of $\log_2$ (expression) in HSC_BM<8 (A) and Early HPC_BM<8 (B), respectively. 522 targets were filtered out with strategy 3: median of $\log_2$ (expression) in $CD14^+$ monocytes<10 (C). Figure (D) demonstrated the expression of 679 genes in normal tissues based on RNA sequencing data (GTEx database: https://gtexportal.org/home/). Genes were hierarchical clustered by the value of RPKM. 283 candidates were filtered out with with strategy 4: RPKM of all other normal tissues (exclude two cell lines (EBV-transformed lymphocytes and Transformed fibroblasts), the whole blood and spleen) <20 in the GTEx database. Figure (E) described the expression level of all 719 proteins identified in our samples, with specific CSC technology. Proteins were hierarchical clustered by the value of $\log_{10}$ (Average of iBAQ). With strategy 5: commonly expressed in AML samples, 354 proteins were identified in no less than 4 out of 8 samples. (F) Symmetric Venn diagram[175] of five strategies above to select new candidate surface targets, which resulted in 85 candidates for further study on AML cell lines or patient samples.

Abbreviations for Figure 3-8 B: B cells, $CD19^+$ B cells; CMP, Common myeloid progenitor cell; early HPC_BM, Hematopoietic progenitor cells from bone marrow; GMP, Granulocyte monocyte progenitors; HSC_BM, Hematopoietic stem cells from bone marrow; mDC, $CD11c^+$ myeloid dendritic cells; MEP, Megakaryocyte-erythroid progenitor cell; MY_BM, Myelocyte from bone marrow; NK cells, $CD56^+$ natural killer cells; pDC, $CD123^+$ plasmacytoid dendritic cells; PM_BM, Promyelocyte from bone marrow; PMN_BM, Polymorphonuclear cells from bone marrow; PMN_PB, Polymorphonuclear cells from peripheral blood.

3.2.2 5 potential markers selected for further study on clinical samples

Finally, 3 CD proteins and 2 non-CD proteins were manually selected for further investigation following an extensive literature research based on novelty on potential for immunotherapeutic targeting. Expression levels of our 5 candidate markers and 6 well-known AML targeted-therapy markers also detected in our samples are depicted in Figure 3-9. 5 out of these 6 markers were identified in at least 6 AML of our samples, further validating our modified CSC protocol.

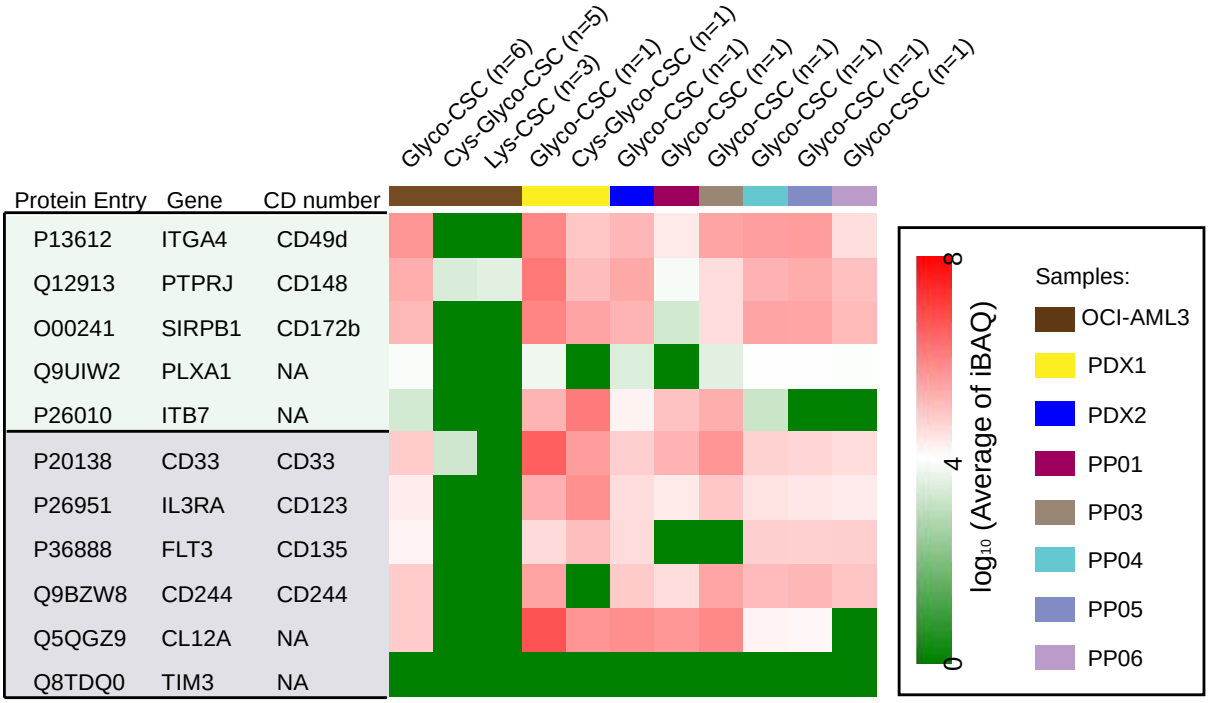
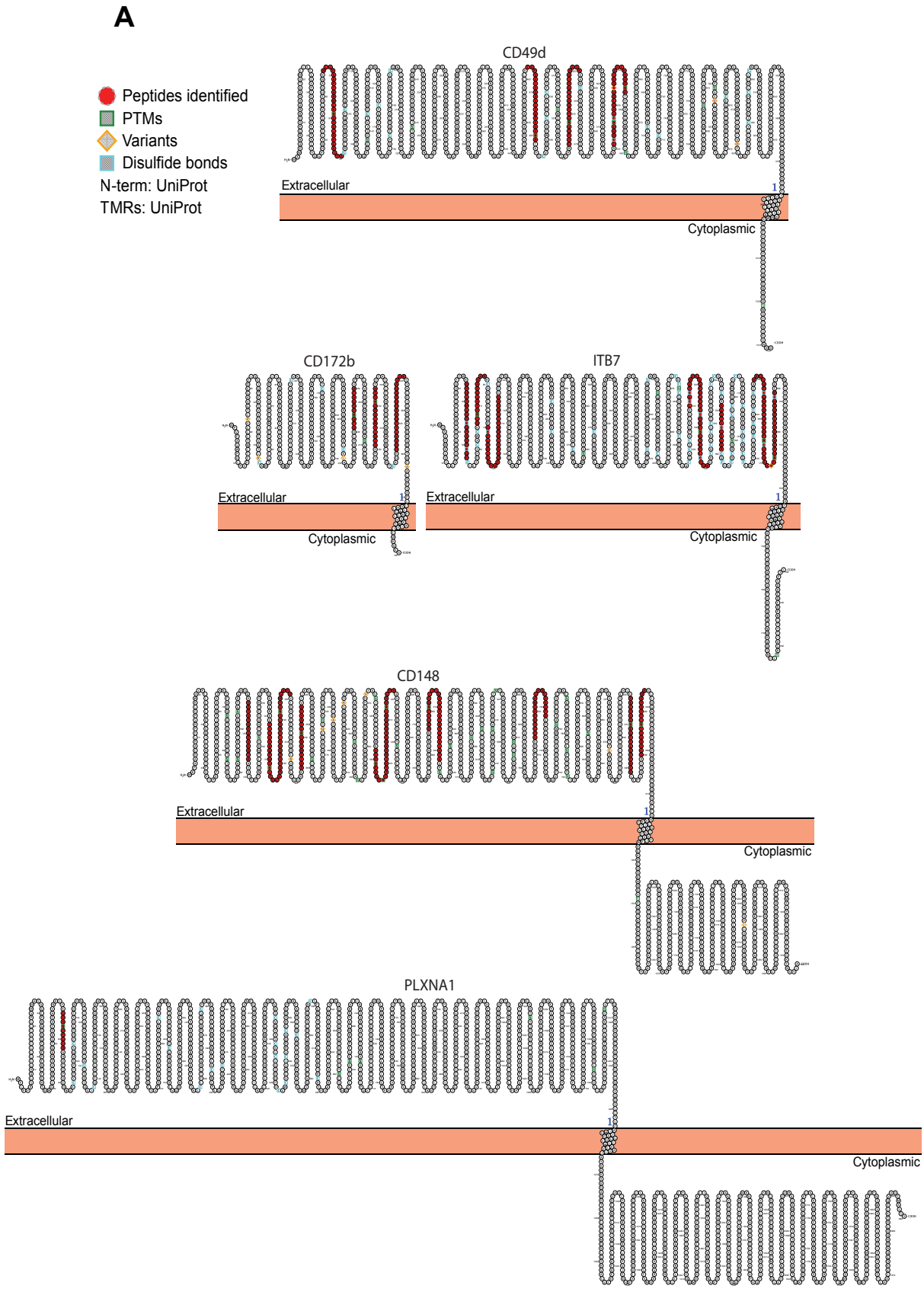


Figure 3-9. Protein expression of 5 new selected candidate markers and 6 novel markers in 8 groups of AML samples. O00241 and Q5TFQ8 belong to the same family, CD172b. 5 candidate markers, including CD49d, CD148, CD172b, PLXA1 and ITB7 were selected for further clinical detection on AML patient samples. 5 out of 6 novel AML targets (exclude TIM3) were identified in 75%-100% of our samples, which also indicate the modified CSC protocol worked efficiently in our lab.


A
Peptides identified
PTMs
Variants
Disulfide bonds
N-term: UniProt
TMRs: UniProt
CD49d
Extracellular
Cytoplasmic
CD172b
Extracellular
Cytoplasmic
ITB7
Extracellular
Cytoplasmic
CD148
Extracellular
Cytoplasmic
PLXNA1
Extracellular
Cytoplasmic

B

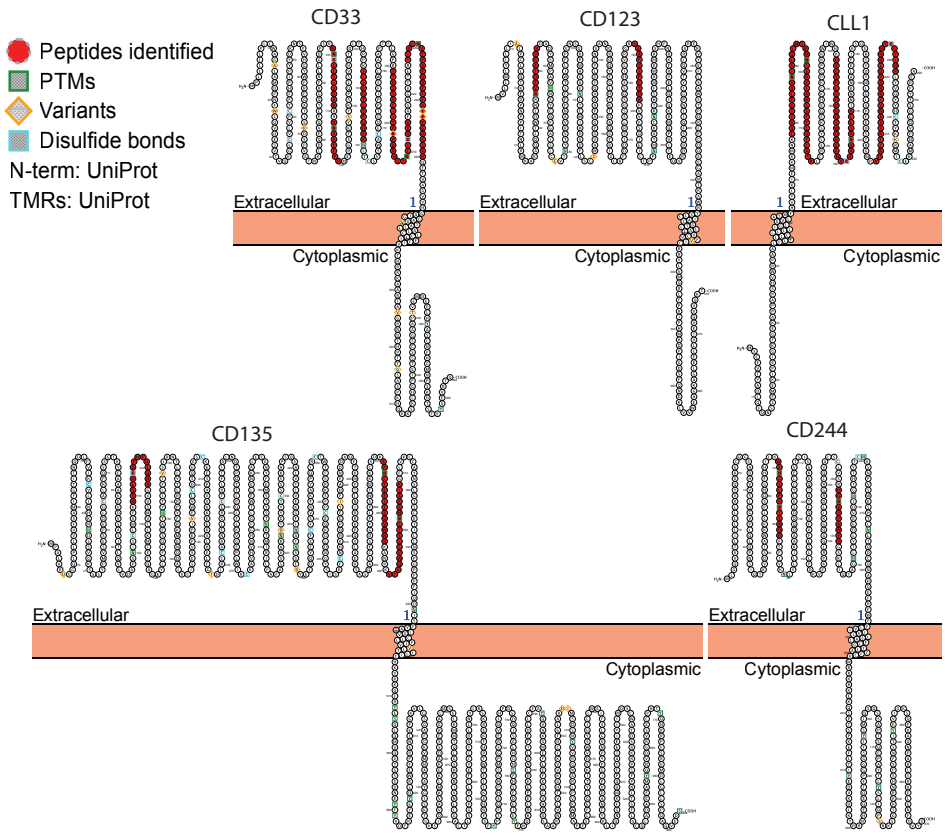


Figure 3-10. Schematic representation of identified surface proteins from AML samples. 5 new candidate markers (A) and 5 on-going studied proteins (B) were submitted to the protein database "Protter" (http://wlab.ethz.ch/protter/#). Peptides identified from each protein were highlighted in red. PTMs, variants and disulfide bonds were rounded in green, yellow and blue color, respectively. Brown band represented the membrane. All identified peptides were from extracellular particle of relative proteins.

Potential AML therapy markers, such as CD33, CD123, CD135, CD244 and CL12A, also have been identified in most (75%-100%) of our samples. However, we did not identify TIM3, which is an interesting antigen to target stem cells of AML[176], in any of our samples (Figure 3-9). The most possible reason might be the sequence of amino acids and the structure of the protein. There is a N-glycosylation for TIM3 at residue 172. The closest disulfide bonds to residue 172 might occur at residues 110 and 109, but there are many lysines/argines in between which can be cleaved by trypsin. So the chance of retrieving TIM-3 by piggy-peptides (Cys-Glyco CSC and Lys-CSC), or

putative N-glycosylation (Glyco-CSC) is either non-existent or of low abundance based on the mechanisms of CSC technologies[156,160] (Supplemental information, Figure S4).

Peptides identified with CSC and its variant technologies within the protein context from our 5 novel candidate markers as well as the 5 more established AML targets were reconfirmed using the protein database “Protter” (http://wlab.ethz.ch/protter/#) and labelled in red (Figure 3-10 A and B). All peptides identified in our samples were from the extracellular region of the proteins. These results also indicate that the CSC technologies worked efficiently in our lab and we chose appropriate strategies to filter candidate AML markers.

3.2.3 Flow cytometry analysis of 5 candidate markers with AML patient samples

To confirm the expression of our candidate markers, we performed multicolor flow cytometry analysis on independent AML samples in our clinical laboratory. Samples were stained with corresponded antibodies and isotype controls. Gating strategy of blast cells are shown in Figure 3-11 A. Median fluorescence intensity (MFI) ratio was used to measure the antigen expression intensity and it was calculated as described previously[52] (Figure 3-11 B).

MFI ratio value showed varying degrees of surface antigen density on AML patient samples (Figure 3-11 C). Most patients were positive for CD148 (90.0%, mean MFI ratio value=12.39, n=10), CD172b (65.0%, mean MFI ratio value=3.87, n=20), ITGA4 (72.7%, mean MFI ratio value=8.86, n=11) and ITGB7 (75.0%, mean MFI ratio

value=5.03, n=20); but remained negative for PLXNA1 (70.0%, mean MFI ratio value=1.48, n=20).

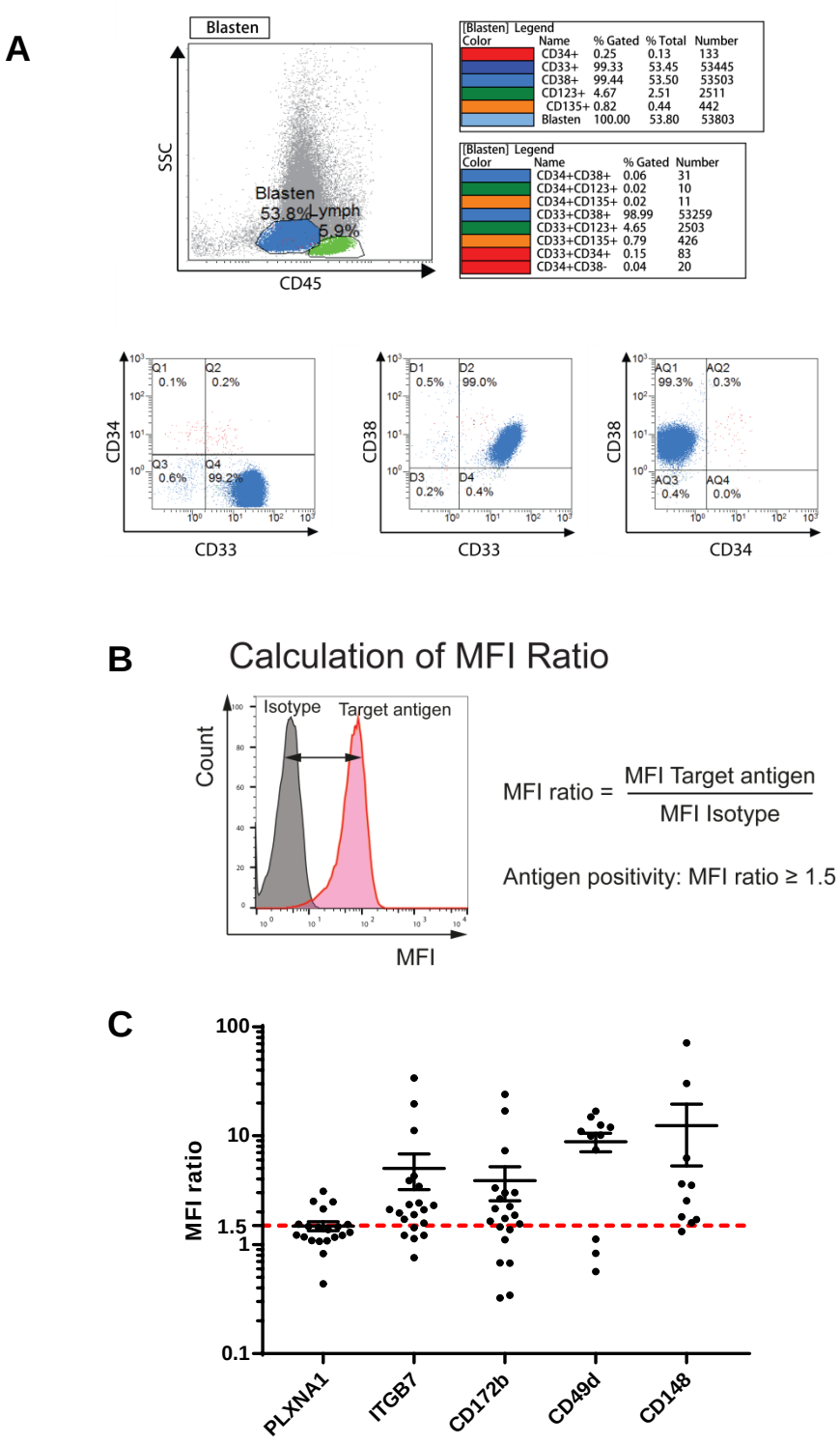


Figure 3-11. FACS analysis of 5 candidate antigens with AML patient samples. Flow cytometric gating strategy of blast cells (A) and calculation of MFI Ratio (B). Antigen expression of 5 candidate surface markers was illustrated as MFI ratio and 4 out of 5 (80%) candidates got mean MFI ratio>1.5 (C).

3.3 Supplemental information

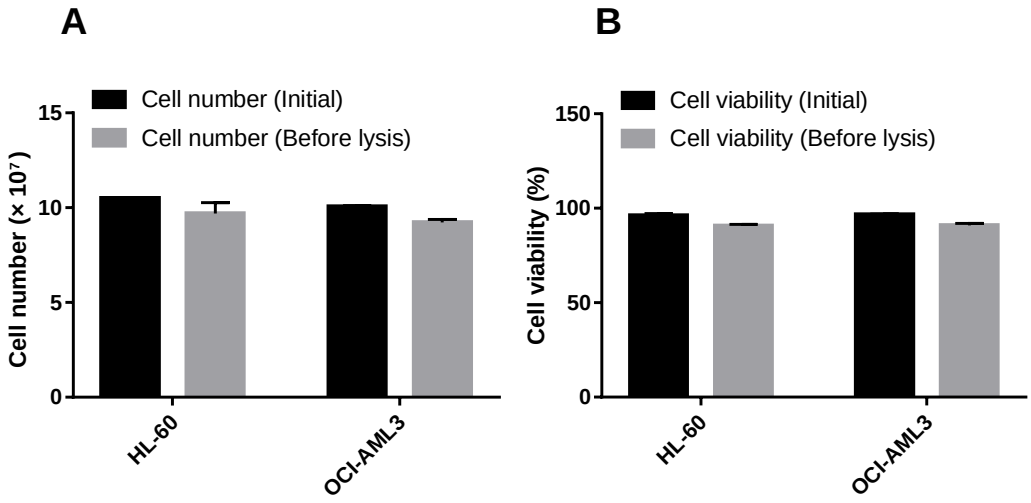


Figure S1. Stable cell morphology during the process of Glyco-CSC protocol. (A) Cells were slightly lost in both cell lines before cell lysis. (B) Cell viability was still very high after oxidation and biocytin hydrazide treatment.

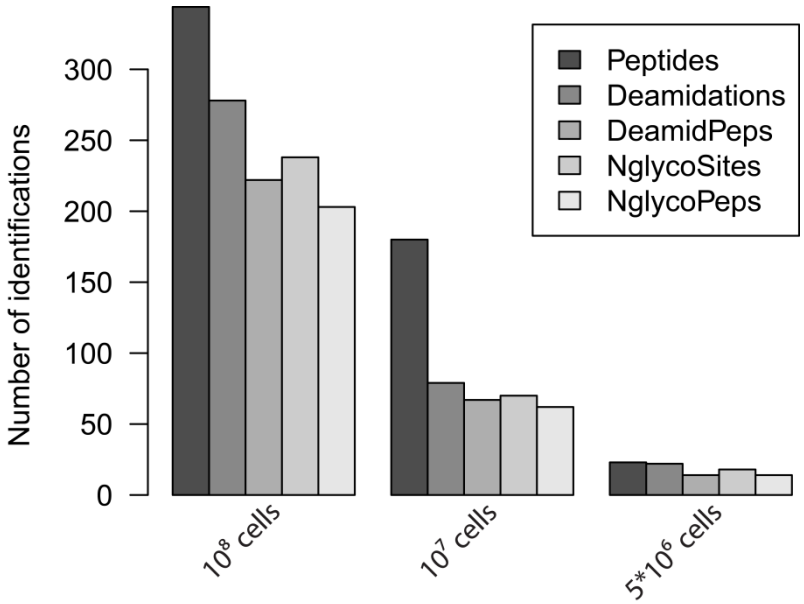
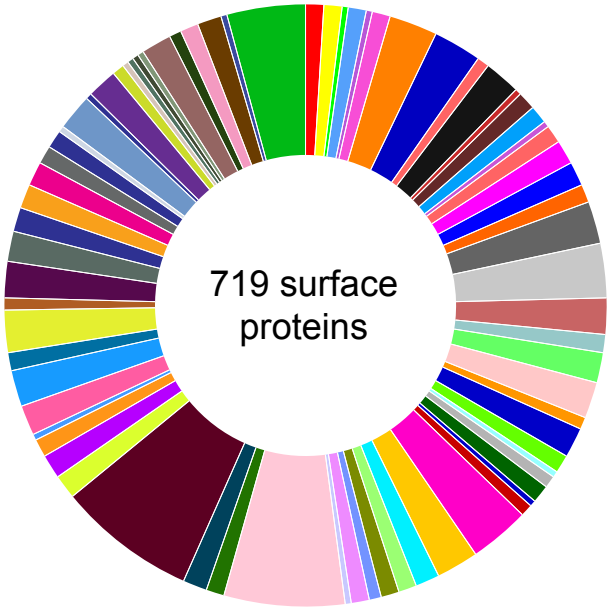


Figure S2. The number of identified peptides decreased with the reduction of cell number. Peptides represented all peptides identified, not necessary only from surface proteins. Deamidations standed for sites that may have had a glycosylation. DeamidPeps were deamidated peptides; however, a single deamidation site can have more than 1 single tryptic peptide. Nglycosites and Nglycopep (Nglycopeptides) had similar meanings, but only for N-glycosylations.



PANTHER pathway

- 5HT1 type receptor mediated signaling pathway (P04373)
- 5HT2 type receptor mediated signaling pathway (P04374)
- 5HT3 type receptor mediated signaling pathway (P04375)
- 5HT4 type receptor mediated signaling pathway (P04376)
- Adrenaline and noradrenaline biosynthesis (P00001)
- Alzheimer disease-amyloid secretase pathway (P00003)
- Alzheimer disease-presenilin pathway (P00004)
- Angiogenesis (P00005)
- Angiotensin II-stimulated signaling through G proteins and beta-arrestin (P05911)
- Apoptosis signaling pathway (P00006)
- ATP synthesis (P02721)
- Axon guidance mediated by netrin (P00009)
- Axon guidance mediated by semaphorins (P00007)
- Axon guidance mediated by Slit/Robo (P00008)
- B cell activation (P00010)
- Beta1 adrenergic receptor signaling pathway (P04377)
- Beta2 adrenergic receptor signaling pathway (P04378)
- Beta3 adrenergic receptor signaling pathway (P04379)
- Blood coagulation (P00011)
- Cadherin signaling pathway (P00012)
- CCKR signaling map (P06959)
- Cortocotropin releasing factor receptor signaling pathway (P04380)
- Cytoskeletal regulation by Rho GTPase (P00016)
- Dopamine receptor mediated signaling pathway (P05912)
- Endogenous cannabinoid signaling (P05730)
- Endothelin signaling pathway (P00019)
- Enkephalin release (P05913)
- FAS signaling pathway (P00020)
- FGF signaling pathway (P00021)
- GABA-B receptor II signaling (P05731)
- Glutamine glutamate conversion (P02745)
- Glycolysis (P00024)
- Gonadotropin-releasing hormone receptor pathway (P06664)
- Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway (P00026)
- Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway (P00027)
- Heterotrimeric G-protein signaling pathway-rod outer segment phototransduction (P00028)
- Histamine H1 receptor mediated signaling pathway (P04385)
- Histamine H2 receptor mediated signaling pathway (P04386)
- Huntington disease (P00029)
- Hypoxia response via HIF activation (P00030)
- Inflammation mediated by chemokine and cytokine signaling pathway (P00031)
- Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade (P00032)
- Insulin/IGF pathway-protein kinase B signaling cascade (P00033)
- Integrin signalling pathway (P00034)
- Interferon-gamma signaling pathway (P00035)
- Interleukin signaling pathway (P00036)
- Ionotropic glutamate receptor pathway (P00037)
- JAK/STAT signaling pathway (P00038)
- Metabotropic glutamate receptor group II pathway (P00040)
- Metabotropic glutamate receptor group III pathway (P00039)
- Muscarinic acetylcholine receptor 1 and 3 signaling pathway (P00042)
- Muscarinic acetylcholine receptor 2 and 4 signaling pathway (P00043)
- Nicotine pharmacodynamics pathway (P06587)
- Nicotinic acetylcholine receptor signaling pathway (P00044)
- Notch signaling pathway (P00045)
- Opioid prodynorphin pathway (P05916)
- Opioid proenkephalin pathway (P05915)
- Opioid proopiomelanocortin pathway (P05917)
- Oxytocin receptor mediated signaling pathway (P04391)
- p53 pathway (P00059)
- p53 pathway feedback loops 2 (P04398)
- Parkinson disease (P00049)
- PDGF signaling pathway (P00047)
- PI3 kinase pathway (P00048)
- Plasminogen activating cascade (P00050)
- Purine metabolism (P02769)
- Pyrimidine Metabolism (P02771)
- Pyruvate metabolism (P02772)
- Ras Pathway (P04393)
- T cell activation (P00053)
- TGF-beta signaling pathway (P00052)
- Thyrotropin-releasing hormone receptor signaling pathway (P04394)
- Toll receptor signaling pathway (P00054)
- VEGF signaling pathway (P00056)
- Wnt signaling pathway (P00057)

Figure S3. PANTHER pathway analyzation of all 719 surface proteins identified from AML samples. Totally 75 pathways generated from PANTHER database. 7.4% and 6.5% of identified proteins involved in the integrin signaling pathway (P00034) and inflammation related pathway (P00031), respectively. Followed by the Wnt signaling pathway (P00057), which also had about 4.2% of proteins in this group. Many other immune response and cancer related pathways, such as B cell activation (P00010), T cell activation (P00053), p53 pathway (P00059), JAK/STAT signaling pathway (P00038), Interferon-gamma signaling pathway (P00035) and Interleukin signaling pathway (P00036) and so on, were also described in the figure.

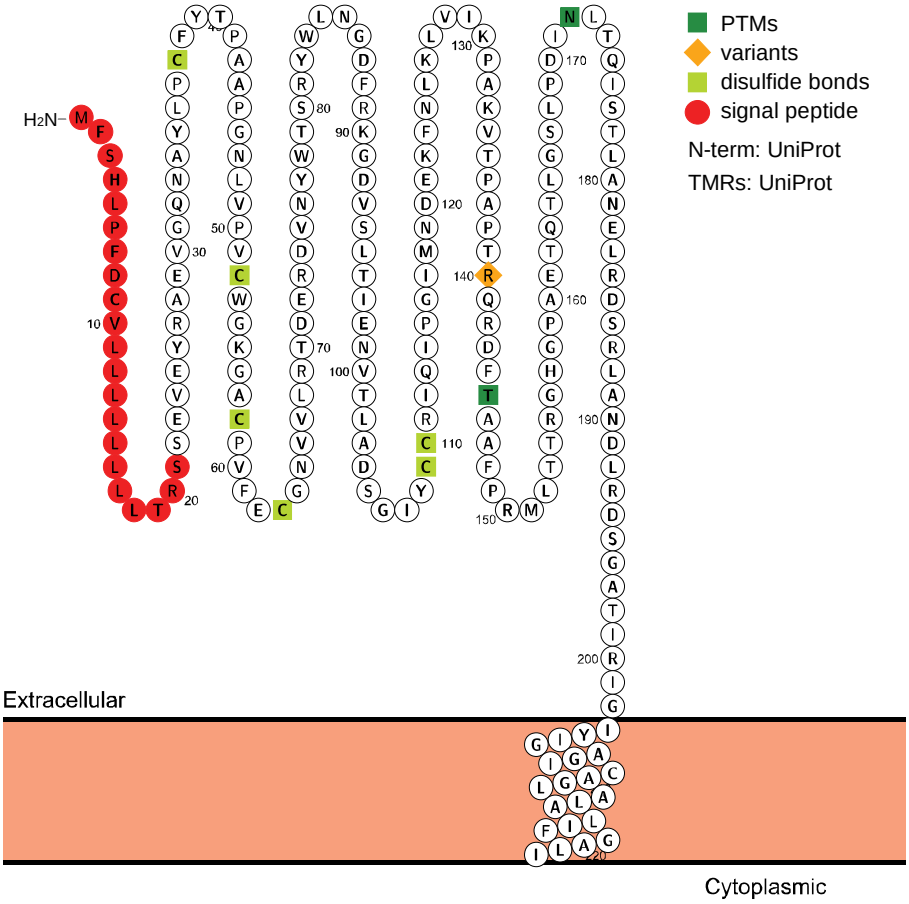


Figure S4. Schematic representation of the extracellular domain of Hepatitis A virus cellular receptor 2 (HAVR2, TIM3). Extracellular sequences of TIM3 has 202 amino acids, including 6 disulfide bonds, 2 PTMs and 1 variants. It has a TM domain from residue 203-223. Figure was modified from http://wlab.ethz.ch/protter/#.

4. Discussion

Acute myeloid leukemia (AML) is a disease with poor prognosis signified by short overall survival and a high risk of relapse after therapy. The disease is highly heterogeneous and despite some improvements current therapeutic strategies have not been successful. During the past few years, considerable advances have been made in the field of immunotherapy, however immunotherapy of AML has been challenging[52,114,115,177-180].

All of these novel therapy strategies, such as CAR T cells therapy, antibody based therapies and vaccines require target antigens that are as specific as possible for the malignant cell. Many surface-bound target antigens, such as CD33, CD123, CD47, CD244, CD7, CD96, CD157, TIM3, FLT3 and CLL1, are known to be over-expressed on AML cells and leukemic stem cells (LSC)[181-190]. However, for all of these antigens there is considerable overlap with expression detected on healthy cells.

CD33 and FLT3 are two well-characterised target antigens. CD33 is not a suitable target for conventional, non-conjugated antibody formats because of its rapid internalization into the cell upon receptor cross-linking. Moreover, CD33 is also high expressed in healthy cells, i.e. lung, spleen and whole blood. Recently, our group evaluated another candidate target, CD157, with a novel Fc-engineered antibody (MEN1112) in AML. MEN1112 could trigger NK cell-mediated cytotoxicity to against AML cell lines and primary AML cells[185]. In a similar study, Salih H.R. *et al*. also described another interesting target, CD133. In their study, monoclonal antibody 293C3-SIDE made degranulation and lysis of CD133-positive primary AML cells by activation of NK cells[191]. More recently in 2018, Salih H.R. *et al*. and colleagues published another NK cells-related AML therapy strategy with checkpoint modulator

OX40[192]. Taken those above and other researches, NK cells and its cell viability may play important role in AML immunotherapy[193,194]. Besides NK cells, regulatory T cells (T_{regs}) could be another candidate or auxiliary AML therapy strategy[195].

4.1 In combination of improved CSC workflow with *ex vivo* co-culture system provided impressive surfaceome data in AML

Because of the accessibility at the cell surface, there is great clinical interest to identify suitable surface antigens as therapeutic targets for cancer immunotherapy. A comprehensive analysis of the surface proteome promises to be helpful for the identification of additional targets, which may be considered for the development of antibody and CAR based immunotherapy in AML. However, until recently, a comprehensive mapping of the surface proteins from primary AML samples was technologically limited.

Here in this study, we improved the original CSC protocol and it's 2 variants to achieve this. The mass spectrometry-based CSC workflow capture bona fide cell surface proteins from living cells and provides the possibility to capture all surface proteins from any cell population. Wollscheid and colleagues described that as many as 95% of identified proteins were cell surface proteins[155]. In our study we adapted the protocol to increase the yield of identified proteins. Since we detected cell aggregation during the biotinylation step, we slightly reduced the concentration of hydrazide during this step for subsequent experiments. Several other modifications were made to increase the yield as well as the specificity of identified peptides, which include ultrasonic breakage, trypsinic digestion and a modified peptide digestion step (Table 2-7). Using our modified CSC protocol, we were able to double the yield of

identified proteins from AML cell lines, from initially 125 to 252. More importantly, the specificity of the protocol was significantly increased as well. 80.4% of all peptides identified with modified protocol fulfilled the criteria, a mass shift (of 0.984 Da) associated with successful N-glycosylation and had a transmembrane domain or signal peptide annotated in Uniprot, while only 54% of all peptides identified fulfilled these criteria in the original Glyco-CSC experiments.

Other studies also showed promising results for quantitative proteomics, such as CSC in combination with stable-isotope labeling with amino acids in cell culture (SILAC) technique[196], experimentally verified potential N-glycosylation sites which were not confirmed before[197], and a similar assay to capture cell surface proteins with both lectin-based affinity and glycocapture on hydrazide resins[198]. However, as the periodate oxidation/hydrazide method is highly specific for detecting glycoproteins, the CSC workflow will not work for minority of proteins which are non-glycosylated, such as the lymphoma target antigen, CD20[199]. Another disadvantage of chemical oxidation related techniques is the high cell number required. In this study, we found both the number of deamidations and peptides had a sharp decline with the reduction of the cell number (Supplemental information, Figure S2). To overcome this difficulty and maintain high viability in our samples, we performed *ex vivo* co-culture system prior to our CSC workflow from primary AML patient samples[52].

In combination with the modified CSC-workflow and *ex vivo* co-culture system, surfaceome data from 22 tests of primary and cell line samples were generated in our lab. Atotal of 719 surface proteins were identified and 22.9% of them had CD annotations. Of them all, 23 PANTHER proteins classes, which involved 75 PANTHER pathways were screened out (Figure 3-5 and Supplemental information,

Figure S3). Compare to our study, Bernd Wollscheid and his colleagues phenotyped more than 500 membrane proteins in two AML cell lines, HL-60 and NB4. However, they did not show any data from primary or relapsed AML patient samples[160]. In another surfaceome study, Dario Neri and colleagues identified about 320 membrane proteins, less than half of our identifications, with cell surface biotinylation related methods from acute leukemia cell lines and a normal human granulocytes (polymorphonuclear cells/PMN)[166]. Taken together, all data described above suggested that the modified CSC-workflow plus *ex vivo* co-culture system worked efficiently in our lab for the study of surfaceome in primary AML samples. This study is, to our knowledge, the first large scale of surfaceome identification from AML clinical patient samples.

4.2 5 suitable targets were selected for further clinical investigation

Next, we aimed to identify new un-studied surface targets for further evaluation in AML. Two databases were interrogated to filter all 719 proteins we identified above: a gene expression database (BloodSpot) of all blood cells during the development, differentiation and maturation and a gene expression database (Genotype-Tissue Expression Project , GTEx) of all normal human tissues. We identified and calculated the expression levels of all identified proteins, excluded those with significant expression in healthy HSCs, HSPCs and most healthy tissues. Finally, we only considered those proteins detected by CSC in at least half of the primary patient samples. 85 proteins were filtered and then subjected to manual screening. Of note, several antigens currently under investigation for AML immunotherapy (i.e. CD33,

CD123, CD135, CLL-1) were detected by the method as well. 5 interesting candidate novel markers were selected for the following tests in AML primary patient samples.

CD49d is a receptor for fibronectin and it may participate in cytolytic T-cell interactions with target cells. Most recently, two groups found it may also show positive characteristics in study on CLL/SLL[200,201]. CD148 is a transmembrane protein-tyrosine phosphatase with a single intracellular catalytic domain and an extracellular domain with structural similarities to cell adhesion molecules. Previous studies showed that CD148 was expressed but not functioning as a protein-tyrosine phosphatases for FLT3-ITD. The high ROS levels in FLT3 ITD–expressing cells led to partial inactivation of CD148 by reversible oxidation[202]. There were 3 proteins from signal-regulatory protein (SIRPB1, CD172b) family, identified in our samples (Figure 3-6). CD172a acted as inhibitory receptor by interacting with a broadly expressed transmembrane protein CD47 also called the "don´t eat me" signal[203]. Conversely, the molecular role of CD172g in immune response or cancer immunity is poorly understood to date. We have detected two isoforms of CD172b, signal-regulatory proteinβ -1 (O00241; Gene: SIRB1) and signal-regulatory proteinβ -1 isoform 3 (Q5TFQ8; Gene: SIRBL). Data from SIRPα/β- and SIRPβ -specific MoAbs revealed low / absent expression on HSC[204]. Since the natural ligand for SIRPβ is unknown, the *in vivo* functional role of SIRPβ remains unresolved[205]. We chose CD172b for further study on AML patient blasts in this work. PLXA1 is a coreceptor for SEMA3A, SEMA3C, SEMA3F and SEMA6D. In Lewis lung carcinoma, *Plxna1* knockdown caused an increased sensitivity to the EGFR-TKIs gefitinib and erlotinib[206]. 2 other members of the plexin family were detected to be differently regulated by ATRA. Increased protein abundances of CD232 (PLXC1) but decreased protein abundances of plexin D1 (PLXD1) were detected in both AML cell lines after ATRA stimulation[160].

PLXA1 might be a potential target in AML therapy after forming dimer with NRP-1[207,208]. ITB7 forms the heterodimer LPAM-1 with CD49d and plays role in homing to bone marrow and inflammation[209,210]. Vedolizumab (trade name Entyvio) is a monoclonal antibody binds to integrin α4β7 (LPAM-1), which can be used to treat inflammatory bowel disease after failure of TNFα-antagonists[211].

Results of our FACS analysis on independent patient samples shows that except for PLXNA1 (mean MFI ratio value=1.48), all the other 4 candidate targets, ITGB7, CD172b, ITGA4 and CD148, were found on primary AML patients (>1.5), indicating that these candidates might be potential targets for further development of antibody, CAR T cells, immune checkpoints or even combinatorial targeting approach in AML.

In conclusion, we successfully identified 719 surface proteins with the combination of modified CSC protocol with *ex vivo* culture system and subsequently selected 5 candidate markers for test on primary AML patient. 4/5 showed expression in independent primary patient samples. These candidates will be further evaluated as potential targets for immunotherapy and targeted therapy strategy in AML.

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