PROTEOMICS PROFILING OF THAI PATIENTS WITH MULTIPLE MYELOMA

BY

CAPTAIN DOLLAPAK APIPONGRAT

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
THE DOCTOR OF PHILOSOPHY (BIOMEDICAL SCIENCES)

GRADUATE PROGRAM IN BIOMEDICAL SCIENCES
FACULTY OF ALLIED HEALTH SCIENCES
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ENTITLED

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was approved as partial fulfillment of the requirements for the degree of the Doctor of Philosophy (Biomedical Sciences)

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Chairman _____
(Assistant Professor Major General Chantrapa Sriswasdi, M.D.)

Member and Advisor _____
(Professor Major General Oytip Nathalang, Ph.D.)

Member and Co-advisor _____
(Sittirak Roytrakul, Ph.D.)

Member and Co-advisor _____
(Assistant Professor Kamphon Intharanut, Ph.D.)

Member _____
(Sawang Petvises, Ph.D.)

Dean _____
(Associate Professor Plaiwan Suttanon, Ph.D.)

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Author	Captain Dollapak Apipongrat
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Dissertation Advisor	Professor Major General Oytip Nathalang (Ph.D.)
Dissertation Co-Advisor	Sittiruk Roytrakul (Ph.D.)
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ABSTRACT

Serum proteomic profiles could provide insight into disease pathogenesis and allow the discovery of reliable biomarkers for better diagnosis and prognosis for multiple myeloma (MM). This study aimed to characterize the serum proteomic profiles and identify potential serum prognostic biomarkers corresponding to MM disease activity and evaluate their impact on patient outcomes. Serum proteomic profiles of patients with MM and age-matched controls were performed using LC–MS/MS. In the verification and validation phases, the concentration of the candidate biomarkers was measured using an ELISA technique. In addition, the association of the proposed biomarkers with clinical outcomes was assessed. Among 465 serum samples obtained from 139 MM patients and 70 normal controls, 1,783 proteins were identified. Of these, 772, 581, 830, 1,425 and 1,301 proteins were identified in normal, patients with monoclonal gammopathy of unknown significance (MGUS), newly diagnosed MM (NDMM), MM with the response to treatment at least VGPR (RESP) and refractory/relapsed MM (RRMM) groups, respectively We identified 23 upregulated and 15 downregulated proteins differentially expressed in newly diagnosed and relapsed/refractory MM patients compared with MM patients who achieved at least a very good partial response to treatment (≥VGPR). The top two candidate proteins, metastasis-associated protein-2 (MTA2) and argonaute-2 (AGO2), were selected for further verification and validation studies. Both MTA2 and AGO2 showed significantly higher levels in the disease-active states than in the remission states ($p < 0.001$).

Regardless of the patient treatment profile, high MTA2 levels were associated with shorter progression-free survival (p = 0.044; HR = 2.48; 95% CI, 1.02 to 6.02). Conversely, high AGO2 levels were associated with IgG and kappa light-chains isotypes and an occurrence of bone involvement features (p <0.05) and were associated with prolonged time to response (p = 0.045; HR = 3.00; 95% CI, 1.03 to 8.76). Moreover, the analytic results using a publicly available NCBI GEO dataset revealed that AGO2 overexpression was associated with shorter overall survival among patients with MM (p = 0.032, HR = 1.60, 95% CI, 1.04 to 2.46). In conclusion, this study demonstrated the proteomic approach for characterizing and identifying serum biomarkers among patients with MM. Interestingly, MTA2 and AGO2 proteins were first identified as potential biomarkers that reflect disease activity, provide prognostic values and could serve as non-invasive indicators for disease monitoring and outcome predicting among patients with MM.

Keywords: Multiple myeloma, Proteomics profilling, Biomarkers

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Captain Dollapak Apipongrat

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (Cont.)

LIST OF FIGURES

LIST OF FIGURES (Cont.)

LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
β_2M	β_2-microglobulin
X^2	Chi-Square test
μΛ	Microliter (s)
μm	Micrometer (s)
2D-DIGE	Two-dimensional differential gel electrophoresis
2-DE	Two-dimensional gel electrophoresis
AGO2	Argonaute-2
AL	Amyloid light-chain
Alb	Albumin
AMBIC	Ammonium bicarbonate
ANG	Angiogenin
ANXA2	Annexin A2
ApoC1	Apolipoprotein C1
ApoD	Apolipoprotein D
ASCT	Autologous stem cell transplant
AUC	Area under the curve
BCR	B-cell receptor
b-FGF	Basic-fibroblast growth factor
BM	Bone marrow
BP	Biological process
BSA	Bovine serum albumin
CA	chromosome abnormalities
CBC	Completed blood counts
CC	Cellular component
CHSY1	Chondroitin synthase 1
CI	Confidence interval
CID	Collision-induced-dissociation

LIST OF ABBREVIATIONS (Cont.)

Symbols/Abbreviations	Terms
CLU	Clusterin
Cr	Creatinine
CR	Complete remission
CSR	Class-switch recombination
CT	Computed tomography
CTL	Cytotoxic T lymphocyte
CXCL-12	CXC chemokine ligand-12
CXCR4	CXC-chemokine receptor type 4
DKK1	Dickkopf homolog-1
DTT	Dithiothreitol
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
Ep-CAM	Epithelial transmembrane glycoprotein
ESI	Electrospray ionization
et al.	et alii
eV	Electron volt (s)
FDA	Food and Drug Administration
FDR	False discovery rate
FISH	Fluorescent in situ hybridization
GBP2	Interferon-induced guanylate-binding protein 2
GC	Germinal center
GO	Gene Ontology
Hb	Hemoglobin
HMM	Hyperdiploid multiple myeloma
HR	Hazard ratio
HSCs	Hematopoietic stem cells
HSP90	Heat shock protein 90

LIST OF ABBREVIATIONS (Cont.)

Symbols/Abbreviations	Terms
IAA	Iodoacetamide
ICAT	Isotope-coded affinity tag
IEC	Ion exchange chromatography
IFE	Immunofixation electrophoresis
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor-1
IL	Interleukin
iMIDs	Immunomodulatory imide drugs
IMWG	International Myeloma Working Group
IQR	Interquartile range
IRE1a	Inositol-requiring enzyme-1 alpha
ISS	International Staging System
iTRAQ	Isobaric tag for relative and absolute quantitation
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography tandem-mass spectrometry
LDH	Lactate dehydrogenase
LFQ	Label-free quantitative
LGAL1	Galactin-1
m/z	Mass-to-charge ratio.
MALDI	Matrix-assisted laser desorption ionization
MAPK	Mitogen-activated protein kinases
MDE	Myeloma defining events
MF	Molecular function

LIST OF ABBREVIATIONS (Cont.)

Symbols/Abbreviations	Terms
MGUS	Monoclonal gammopathy of unknown significance
MIP-1a	Macrophage inflammatory protein-1a
miRNAs	microRNAs
mL	Milliliter (s)
MM	Multiple myeloma
mm	Millimeter (s)
mM	Millimolar (s)
MM-EV	Multiple myeloma-derived extracellular vesicles
MPP	Metalloproteinase
MRD	Minimal residual disease
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MSCs	Mesenchymal stem cells
MTA2	Metastasis-associated protein-2
MVD	Microvessel density
NDMM	Newly diagnosed multiple myeloma
NF-κB	Nuclear factor-kappa B
NGF	Next-generation flow
NGS	Next-generation sequencing
NHMM	Non-hyperdiploid multiple myeloma
NK	Natural killer (cells)
NMR	Nuclear magnetic resonance
NuRD	Nucleosome remodeling and deacetylase
OPG	Osteoprotegerin
OS	Overall survival

LIST OF ABBREVIATIONS (Cont.)

Symbols/Abbreviations	Terms
PANTHER	Protein Analysis Through Evolutionary Relationships
PCL	Plasma cell leukemia
PD	Progressive disease
PD-1	Programmed cell death protein-1
PD-L1	Programmed cell death ligand-1
PET-CT	Positron emission tomography-computed tomography
PFS	Progression-free survival
PLLBs	Peptide ligand library beads
PPI	Protein-protein interaction
PR	Partial response
PRODH	Proline dehydrogenase
PSME	Proteasome activator complex
RANK	Receptor activator of nuclear factor-kappa B (NF-κB)
RB-1	Retinoblastoma-1
RESP	Multiple myeloma with the response to treatment at least very good partial response
RISC	RNA-induced silencing complex
R-ISS	Revised International Staging System
ROC	Receiver operating characteristic curve
RP-HPLC-ESI-MS/MS	Reverse-phase high-pressure liquid chromatography-electrospray ionization tandem mass spectrometry
RRMM	Relapsed/refractory multiple myeloma
SAA4	Serum amyloid A-4 protein

LIST OF ABBREVIATIONS (Cont.)

Symbols/Abbreviations	Terms
sCR	Stringent complete remission
SD	Stable disease
SD	Standard deviation
SDF-1	Stromal-derived factor-1
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SFLC	Serum free-light chains
SHM	Somatic hypermutation
SILAC	Stable isotope labeling with amino acids in cell culture
siRNAs	Short interfering RNAs
SMM	Smoldering multiple myeloma
SPEP	Serum protein electrophoresis
TD	T-cells dependent
TGF-b	Tumor growth factor-b
Th-1	T helper-1 (cells)
TI	T-cells independent
TMC1	Transmembrane channel-like protein 1
TOF	Time-of-flight
TP53	Tumor suppressor protien 53
TTR	Time to response
TXN	Thioredoxin
TXNL1	Thioredoxin-like protein 1
UPEP	Urine protein electrophoresis
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system

LIST OF ABBREVIATIONS (Cont.)

Symbols/Abbreviations	Terms
VCAM1	Vascular-cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VGPR	Very good partial response
VLA-4	Integrin alpha 4
WGS	Whole-genome sequencing
WHO	World Health Organization
XBP1	X box-binding protein 1
XPO1	Exportin 1

CHAPTER 1
INTRODUCTION

Multiple myeloma (MM) is a malignancy of the antibody-secreting plasma cells characterized by hyperproliferation of the malignant plasma cells (myeloma cells) in the bone marrow (BM). These myeloma cells can overproduce an abnormal monoclonal immunoglobulin (Ig), called paraprotein or M-protein, and secrete it into the plasma.[1] The elevation of M-protein is a cause of several pathophysiological abnormalities, including hyperviscosity, hypercalcemia, thrombocytopenia, anemia, and some neurological disorders. This M-protein is also a cause of specific end-organ damage, especially renal impairment and bone lytic lesions. Although, in recent years, the advanced therapeutic options to treat MM have markedly been improved, resulting in increased length of the patient's survival.[2] However, MM has still considered an incurable disease, and the etiology of MM is still unknown.

In the United States, MM is the second most common hematological malignancy, about 10% and about 1.8% of all cancer cases.[3] The incidence rates of MM are around 6.0 per 100,000 per year, and strongly related to age and gender.[4] The median age at diagnosis of MM is 69 years old, and a median survival of 5 to 7 years for newly diagnosed patients.[2] The disease is slightly more common in men than women (58% *vs.* 42%),[5] and the age-adjusted incidence rates are 6.9 and 4.5 per 100,000 per year in men and women, respectively.[4, 5] Geographically, the disease frequency is high in Australia/New Zealand, Europe, and North America, whereas its incidence and mortality seem to be low in Asia.[6, 7 8]

MM is one form of plasma cell dyscrasia. The disease progression of MM starts from the asymptomatic pre-malignant stage of monoclonal gammopathy, called monoclonal gammopathy of unknown significance (MGUS). According to the International Myeloma Working Group (IMWG) diagnostic criteria for MM and related plasma cell disorders,[9] MGUS can develop into the advanced asymptomatic stage, smoldering MM (SMM), that are both caused by a proliferation of monoclonal plasma cells leading to a detectable serum monoclonal protein and/or excess of plasma cells in the BM without clinical symptoms.[9, 10] The disease can progress to symptomatic MM

with the rate of progression around 0.5-1.0% per year for MGUS and 10% per year for SMM.[9, 11, 12] Despite advanced treatment modality, MM can progress to the most aggressive form, plasma cell leukemia (PCL), with a poor prognosis and high mortality rate.[13, 14] Most MM patients eventually relapse and/or refractory to drug treatment.[15]

The most common clinical symptoms in MM are fatigue and bone pain, which are found in approximately 32% to 58% of the patients.[16] Other common clinical findings, including pathological bone fracture, weight loss, and paresthesia, can be found in 34%, 24%, and 5% of MM patients, respectively.[4, 16] The important clinical features used to diagnose the disease are myeloma-related organ or tissue impairments, representing the end-organ damage.[4, 9, 16, 17]

The standard laboratory testing for MM screening includes total serum protein, serum and urine protein electrophoresis (SPEP and UPEP), immunofixation electrophoresis (IFE) and detection of serum free-light chains (SFLC).[18] SPEP is mainly used to detect abnormal M-protein, a tumor marker specific for monoclonal gammopathies reflecting the clonal proliferation of Ig. Approximately 97% of symptomatic MM patients have increased M-protein levels on SPEP.[4] The presence of serum M-protein is confirmed and characterized by an IFE, where the M-protein can be classified into IgG, IgA, IgM, IgD and IgE with their light chains: kappa (κ) or lambda (λ) isotypes. The elevation of SFLC and abnormal SFLC ratio are commonly observed and used as prognostic implications.[11, 19] Moreover, the BM aspiration and biopsy are needed to confirm the presence of myeloma cells and for further cytogenetic and chromosome study.[18] A diagnosis usually relies on the presence of at least 10% of nucleated cells being plasma cells in the BM.[9, 18] Computed tomography (CT) and/or magnetic resonance imaging (MRI) are recommended to assess bone and extramedullary disease.[20] The additional parameters, such as complete blood counts (CBC), serum creatinine (Cr), serum calcium, lactate dehydrogenase (LDH), serum albumin (Alb) and β_2-microglobulin (β_2M) level, are used to evaluate the disease stage and progression.[4, 9, 18]

Cytogenetic abnormalities were detected by fluorescent *in situ* hybridization (FISH) in newly diagnosed MM patients to assess the risk stratification.[21] The chromosome translocations involving the Ig loci and hyperdiploidy, such as t(11;14), t(4;14), t(14;16), t(6;14), t(14;20) and trisomy on chromosome 1, 13, and 21, are

commonly genetic aberrations found in MM and associated with clinical characteristics, prognosis and therapy responses.[22-24] The previous sequencing study has defined the mutation landscape of MM.[25, 26] Approximately 20% of the cases are *KRAS* and *NRAS* gene mutations, followed by *TP53*, *DIS3*, *FAM46C*, and *BRAF*, respectively. The association of these mutations with patient outcomes was demonstrated.[27-29] However, no single pathological, laboratory, or molecular testing can be used to distinguish between the premalignant and malignant stages, as well as to predict the progression of the disease. As a result, the diagnosis of MM has highly depended on overt clinical manifestations of serious end-organ damage.[30] Therefore, biomarkers from different sources for better diagnosis and prognosis are needed. Nevertheless, identifying these biomarkers remains a major challenge

Recently, the analysis of the global expression of proteins or proteomics has been widely employed for MM research studies. The proteomic analysis was used to identify known and novel expressed proteins among different tumor samples. The advantages of the proteomic analysis included the information of advanced pathogenesis, diagnosis, prognosis, response to therapy and provided novel biomarker discoveries.[31-37] Potential biomarkers associated with the patients' outcomes, for example, Annexin A2 (ANXA2) and galactin-1 (LGAL1) were identified from the BM extracellular matrix of newly diagnosed MM and associated with patients' overall survival (OS).[36] Moreover, a related study demonstrated that an increased level of integrin alpha-11 subunit and isoform-1 of the multimerin-1 in patient's serum samples are potential biomarkers for diagnosis.[32] Regarding the disease progression, the proteomic profiling may be changed in each stage. Since data of proteomic profiles associated with MM Thai patients remain unknown, further studies are suggested.

Therefore, in this study, we performed serum proteomic analysis to characterize the proteomic profiles in various disease states and to identify potential prognostic biomarkers corresponding to MM disease activity. In addition, the association between the proposed biomarkers and clinical outcomes among patients with MM were investigated.

CHAPTER 2
REVIEW OF LITERATURE

2.1 History of multiple myeloma

MM has been recognized since ancient times;[38-40] the first well-known case was reported in 1844 by Samuel Solly.[41] The 39-years-old woman, Sarah Newbury, presented with fatigue and bone pain from multiple fractures (**Figure 2.1A**). She died four years after the onset of symptoms, and the autopsy results revealed that almost of her BM were replaced with a red substance (**Figure 2.1B**). The disease was an inflammatory process and began with a "morbid action" of the blood vessels in which the "earthy matter of bone is absorbed and thrown out by the kidneys in the urine".[41]

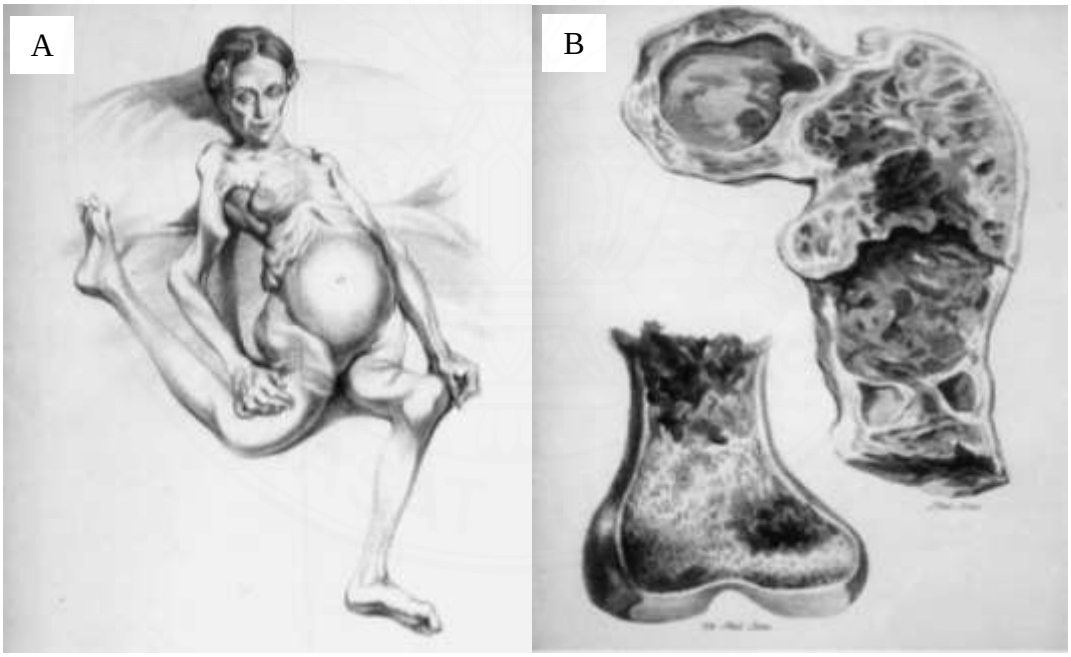

Figure 2.1 The first well-known multiple myeloma case, Sarah Newbury.[41] (A) The patient with fractured femurs and right humerus. (B) Destruction of femurs by myeloma tumor.

In 1845, Henry Bence Jones performed biochemical testing using the patients' urine samples, and a substance precipitated by the addition of nitric acid was observed. The precipitated substance was soluble in boiling water but re-precipitated after the

urine was cooled. Subsequently, the proteins were recognized as "Bence Jones proteins".[42] The term multiple myeloma was first introduced by von Rustizky in 1873.[43] Soft and reddish color consistency found in several sites of the BM obtained from eight patients at the autopsy and then called "multiple myeloma".[43] The term "Kahler's disease" was also used to describe myeloma resulting from a case report of a physician named Otto Kahler in 1889.[44, 45] The patient had progressive bone pain, proteinuria with Bence Jones protein, and the presence of large, round cells at autopsy similar to the first reported in 1844.[44, 45] In addition, the presence of pathologic fractures, Bence Jones proteinuria, anemia, and chronic renal complications were observed by the literature reviewing among 412 MM cases from 1848 to 1928. [46] In 1974, four cases of possible MM in American Indian skeletons from AD 200±1300 were reported. Discrete lytic lesions with sharply demarcated borders were described with no evidence of sclerosis or formation of new bone. This finding suggested that MM had probably been presented for more than centuries.[47]

In 1939, the electrophoresis technique was first applied to determine the protein patterns in serum and urine.[48] The tall narrow-based "church spire" peak or "M-spike" was observed only in MM patients but not in normal individuals.[48] Subsequently, the light chains of the Ig molecule in M-spike found in serum were identical to the Bence Jones protein which were detectected in the urine.[49] A narrow band of hyper-gammaglobulinemia on electrophoresis was also observed in patients with no evidence of malignancy. This finding is called "essential hyper-gammaglobulinemia" or a "benign monoclonal protein", presently the preferred term is MGUS. These patients have the potential to develop MM, amyloidosis, macroglobulinemia and other related diseases.[50] Additionally, the IFE technique was developed to determine the Ig subclasses and small monoclonal light chains not recognized by electrophoresis.[51]

2.2 Epidemiology of MM

Among hematological malignancy cases, 10 to 15% were MM cases.[3] Approximately 86,000 cases occur annually worldwide. Of these, 47,000 (54.7%) and 39,000 (45.3%) cases were males and females, with a male-to-female (M: F) ratio of 1.2: 1. Importantly, about 63,000 cases died from the disease each year.[3, 6, 8] The

incidence and mortality are higher in Australia/New Zealand, Europe, and North America than in Asia (**Figure 2.2**).[6-8]

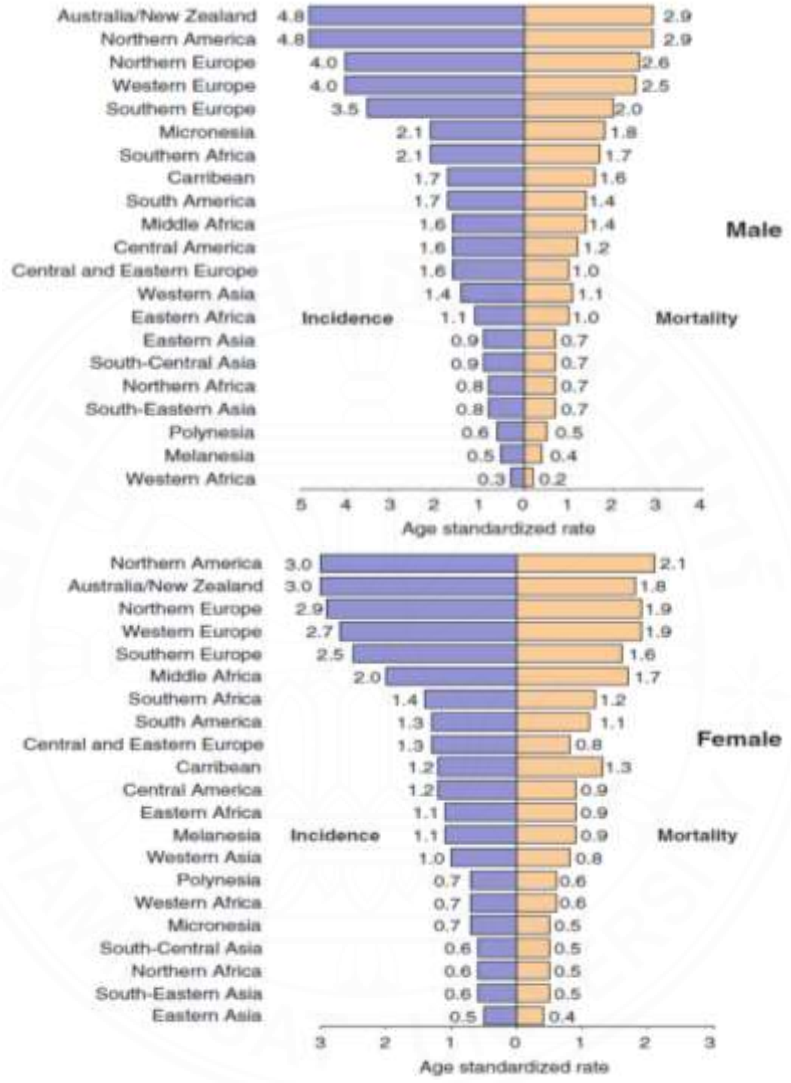

Figure 2.2 Age-standardized incidence and mortality rates per 100,000 for MM[6]

The incidence of new cases and mortality rates are increased by the patient's age. Based on data from 2012 to 2016 in the United States, the disease incidence is high in the age range of 65 to 74 years (30.2%), with a median age of diagnosis of 69 years (**Figure 2.3**). The highest percentage of deaths is in patients aged 75 to 84 (32.0%), with a median age of 75 years (**Figure 2.3**). Even though the patient survival rate trend to be low in the past ten years because of inappropriate therapies, currently, the median

survival rate is up 5 to 7 years, and the 5-year survival rate is 52.5% for newly diagnosed patients.[52]

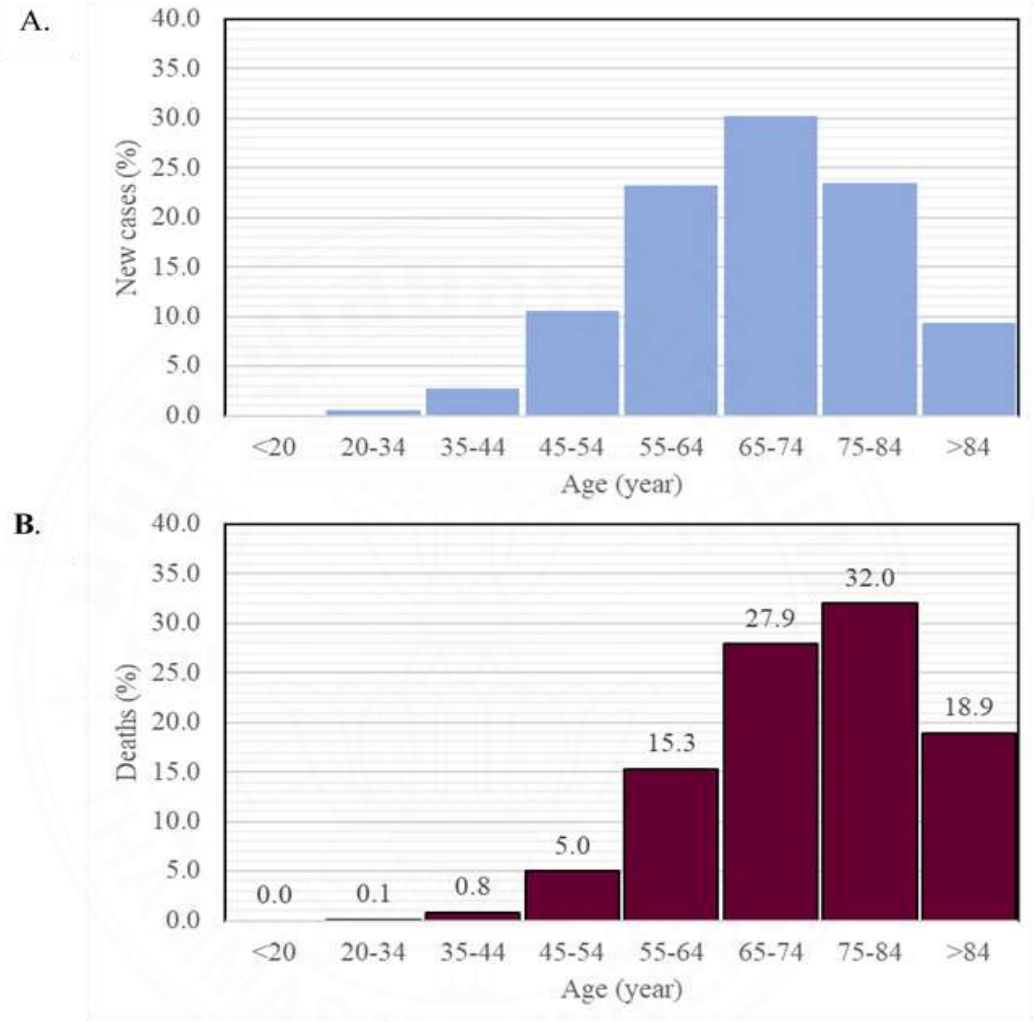

Figure 2.3 The percentage of new cases (A) and deaths (B) of MM patients classified by age groups from 2012 to 2016 (modified from the data of SEER cancer statistics review: National Cancer Institute, USA)[52]

In Thailand, the incidence rate of MM cases is around 0.4 to 0.9 per 100,000,[8] and the 5-year prevalence was 3.94%.[53] Moreover, the prevalence of MGUS was 2.35% in 2012.[54] In 2018, 1,251 newly diagnosed MM cases in Thailand were reported by The International Agency for Research on Cancer, World Health Organization (WHO).[53]

2.3 The pathogenesis of MM

MM is a B-cell malignancy characterized by over-proliferation and differentiation of monoclonal malignant plasma cells within the BM. The malignant plasma cells or myeloma cells are a subset of post-germinal center (post-GC) B-cells known as long-lived plasma cells, terminally differentiated and non-proliferating cells.[55-57] In general, plasma cells play a major role in the adaptive immune system, specifically in antibody production against specific pathogens. These cells can survive for months to years in the BM, representative for immunologic memory. Although the unknown etiology of MM, the myeloma cells produce abnormal Ig (IgG, IgM, IgA and rarely IgE or IgD), abnormal free-light chain proteins (κ or λ) and/or cytokines that stimulate osteoclasts, suppress osteoblasts and promote new blood vessel formation.[4, 57] The increase of those proteins is crucial in disease pathogenesis. An increase M and light chain proteins induce hyperviscosity and end-organ damage, especially in the kidney. Cytokines released from myeloma cells induce the invasion of osteoclasts in BM resulting in bone pain, osteoporosis, and hypercalcemia. Moreover, this invasion also leads to anemia by the hematopoietic interruption and immunologic alterations contributing to recurrent infections.[57]

2.3.1 B-cell development and myelomagenesis

B-cell is derived from hematopoietic stem cells (HSCs) that primarily develop in the BM. The HSCs can differentiate into multipotent and common lymphoid progenitors. The common lymphoid progenitors then differentiate through the stages pre-B, pro-B, immature B, and become mature B-cells.[56, 58] This process required the transcription factors, including E2A, PU.1, and PAX5, as well as cytokine signaling to stimulate the B cell maturation.[58-62] In addition, the two important enzymes, RAG1 and RAG2, are required for B-cell receptor (BCR) development.[60] These two enzymes are used to recombine the variable (V), diversity (D) and joining (J) segments of the heavy and light chains of immunoglobulin (*IGH* and *IGL*) genes leading to the genetic rearrangement of the BCR.[60]

The mature B cell circulate in the blood and are home to the follicular zone of lymph nodes. Activation of B-cell occurs after the exposure of pathogens and is divided into two pathways depending on antigen types; T-cell independent (TI) and

T-cell dependent (TD) antigens.[56-58, 60] In the TI activation pathway, naïve B-cells can recognize and activate directly without cytokine signals from T-cells. The B-cells are activated and undergone proliferation and differentiation to IgM-secreting short-lived plasma cells. On the contrary, in the TD activation pathway, B-cells require many cytokines and other complexe molecular signalings from T-cells. The activation by TD antigens results in a clonal selection of B-cells, somatic hypermutation (SHM) of the *IGH* and *IGL* genes and class-switch recombination (CSR). This allows B-cells to transform to be long-lived plasma cells that can produce the antigen-specific Ig with high-affinity and long-lasting immunity.[60] This process occurs in the germinal center (GC) of the lymphoid organs and also called GC reactions.[60] However, this GC reaction is prone to genomic errors contributing to oncogenesis. Several reports suggested that almost all myeloma cells are originated by mutations associated with GC reaction stimulated by TD antigens.[60, 63] Indeed, most genetic abnormalities in MM involve genetic mechanisms, such as V-(D)-J arrangement of the *IGH* and *IGL* genes during the SHM and CSR.[60]

2.3.2 Bone marrow microenvironment

The factors involved in the initiation of myeloma include genetic events and microenvironmental factors. The interaction between myeloma cells and the BM microenvironment is crucial for myeloma development, progression and treatment.[64-66] The microenvironment or niche comprises HSCs, osteoclasts and non-hematopoietic cells, including mesenchymal stem cells (MSCs), osteoblasts, fibroblasts, stromal cells and extracellular matrix (ECM).[64-67] These cellular compartments provide several factors, including cytokines, chemokines, growth factors and adhesion molecules, that promote angiogenesis, migration and proliferation of myeloma cells contributing to bone damage.[57, 65, 66] In addition, the ECM is related to myelomagenesis by providing structural support and facilitate cell adhesion and signaling to myeloma cells.[67]

The interactions between CXC-chemokine receptor type 4 (CXCR4) on the myeloma cells and stromal-derived factor-1 (SDF-1), or CXC chemokine ligand 12 (CXCL-12), expressed on stromal cells in the BM niches result in myeloma cells' migration from the secondary lymphoid organs to the BM by activation of intracellular downstream signaling molecules, Rho and Rac.[68, 69] Subsequently, adherence of

myeloma cell to BM is mediated by cell-adhesion molecules; vascular-cell adhesion molecule 1 (VCAM1) and integrin alpha 4 (VLA-4), CD40 and CD40 ligand (CD40L), and MUC-1 and ICAM-1, respectively (**Figure 2.4**).[71] Those interactions increase the production of growth factors, interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) from stromal cells and osteoclasts, which stimulate angiogenesis.[70, 71] In addition, the adhesion of myeloma cells to the ECM proteins, such as collagen, fibronectin, laminin and vitronectin, can also activate the up-regulation of cell-cycle regulatory proteins and anti-apoptotic proteins.[72]

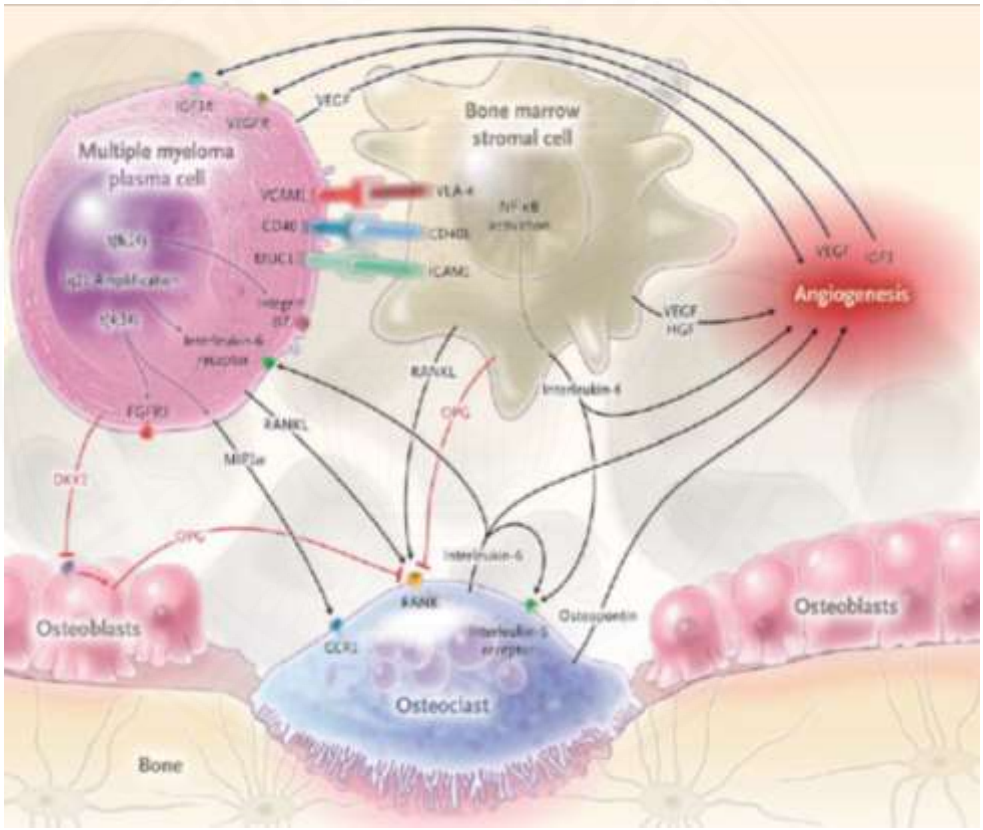

Figure 2.4 The interactions between myeloma cells, osteoclasts and stromal cells within the BM microenvironment[71]

The colonization of myeloma cells contributes to change in a microenvironment, and to create a unique niche in the BM, called BM remodeling, resulting in increased osteoclast activity and suppressed osteoblast-driven bone formation.[71, 73] The increased osteoclast activity is due to an imbalance ratio of the receptor activator of NF-κB (RANK) and osteoprotegerin (OPG), resulting from

increased production of RANK ligand (RANK-L) and decreased production of OPG (**Figure 2.4**). OPG is a potent inhibitor of osteoclast differentiation and is downregulated in myeloma-derived stromal cells. Moreover, macrophage inflammatory protein-1α (MIP-1α) is an inducer of osteoclasts in a RANKL-independent manner.[66] Osteoblast activity is also suppressed by the production of dickkopf homolog 1 (DKK1), a Wnt signaling pathway antagonist from myeloma cells. In addition, myeloma cells can inhibit a transcription factor, runt-related transcription factor 2, in osteoblasts leading to suppression of osteoblast differentiation.[71-74] The increased osteoclast activity and suppressed osteoblast activity are causes of pathologic fractures, bone pain and severe osteoporosis observed in MM patients.[64, 71-74]

Apart from cell-to-cell interaction, the interaction between myeloma cells and ECM is a dynamic process during the disease course. Changes in ECM are mediated by degrading proteins, such as metalloproteinase-1 (MMP-1), MMP-2 and MMP-9, correlating with disease progression.[64, 73, 76] MMP-9 is overproduced in myeloma cells resulting in induction and activation of MMP-1 and MMP-2. The increased MMP-1 level promotes collagen degradation, whereas the increased MMP-2 directly modulates myeloma cell invasion and spreading.[74, 75] Moreover, changes in ECM components of the BM samples from MGUS, MM and healthy donors were demonstrated in related studies. Losing of fibronectins, laminin and several kinds of collagens confirmed in both transcriptomic and proteomic levels were also observed in MGUS and MM patients.[36, 73, 76]

2.3.3 Angiogenesis and tumor micrometastasis

Angiogenesis is a prominent feature associated with disease progression and severity in several malignancies, including MM. In the plasma cell dyscrasias, increasing angiogenesis indicated by microvessel density (MVD) measuring within myeloma microenvironment are correlated with disease progression, severity and prognosis.[73] Increasing angiogenesis is found in the patients during disease progression from MGUS to symptomatic MM, suggesting its role in MM progression.[73] This process is triggered by angiogenesis-associated factors produced by stromal and myeloma cells, such as VEGF, basic-fibroblast growth factor (b-FGF), tumor growth factor-β (TGF-β) and IL-8. Those factors can stimulate endothelial cells to form new vessels.[64, 73, 75]

In most MM patients, not only the myeloma cells remain localized in the BM but they can disseminate and engraft multiple areas of the axial skeleton.[68] The disease may originates from a single local myeloma clone, called a solitary plasmacytoma.[77] Due to the continuous trafficking and increasing angiogenesis processes, the disease can progress from a solitary plasmacytoma to asymptomatic MGUS and subsequently to overt MM.[77] In cases of extramedullary MM, myeloma cells do not only home to the BM niches but also other organs, including subcutaneous sites, liver, gut, lungs and rarely the central nervous system.[68] Moreover, in the advanced stage, the myeloma cells are overproduced without the acquisition of dependence on the microenvironment resulting in the increased number of circulating myeloma cells and development of end-stage plasma cell leukemia.[68]

2.3.4 Immune dysregulation in MM

The progressive immune dysregulation is characterized by decreased antigen-presenting and effector cell function, that allows the myeloma cells to escape from immune surveillance by expressing inhibitory molecules, including programmed cell death ligand-1 (PD- L1) or CD274.[64, 78, 79] In general, the PD-L1 is expressed on antigen-presenting cells, such as macrophages and dendritic cells, as well as on non-hematopoietic cells, such as pancreatic islet cells and endothelial cells. [80] This molecule plays a role in protecting the tissue from immune-mediated injury.[80] The PD-1 or CD279, a receptor of the PD-L1, is expressed on T cells, B cells, monocytes and natural killer (NK) cells. The binding of PD-L1 to the PD-1 receptor results in decreased secretion of cytokines from T helper-1 (Th1), which induces T-cell apoptosis and inhibits T-cell proliferation and tumor cell killing by cytotoxic T lymphocyte (CTL).[78-81] In MM patients, PD-L1 is highly expressed on myeloma cells but not found in plasma cells isolated from MGUS patients and healthy individuals.[82-84] In addition, a significantly increased PD-L1 expression was described in relapsed/refractory MM (RRMM) patients and correlated with tumor burden and poor prognosis.[85]

2.4 Genetic events in MM

2.4.1 Primary genetic events in MM

Greater than 90% of MM patients harbor chromosome aberrations, including deletion, aneuploidy (mostly hyperdiploidy) and translocation.[55, 58] Based on chromosome abnormality, MM is divided into two groups, hyperdiploid MM (HMM) and non-hyperdiploid MM (NHMM). The HMM karyotypes are characterized by trisomy on odd-numbered chromosomes, including 3, 5, 7, 9, 11, 15, 19 and 21, which found in 50% to 60% of all cases.[55, 58] While the NHMM is involves translocation between *IGH* loci on chromosome 14 (14q32) and one of several partner oncogenes, including cyclins D (*CCND*), multiple myeloma SET domain-containing protein (*MMSET*) and v-avian musculoaponeurotic fibrosarcoma oncogene homologs (*MAF* or *MAFB*) genes.[86, 87] These chromosomal abnormalities are considered primary cytogenetic events, commonly found in plasma cell dyscrasia, especially in MGUS. Several studies demonstrated that cytogenetic abnormalities are associated with the prognosis and outcome of patients with MM.[86-91] Primary cytogenetic events are found independently with non-overlapping patterns, however, 15% of MM patients have both trisomies and *IGH* translocations (**Table 2.1**).[91] During disease progression and development, additional events caused by SHM, CSR, genetic mutations and chromosomal copy-number changes can occur s simultaneously with primary cytogenetic events.[91]

2.4.1.1 Hyperdiploidy

Approximately 50% of the MM cases carry hyperdiploid (48-75 chromosomes). Of these, multiple trisomies involving eight odd-numbered chromosomes, including 3, 5, 7, 9, 11, 15, 19 and 21, are the most common. Although the mechanisms remain unclear, the hyperploidy is hypothesized to occur during rapid B-cell proliferation in the GC resulting in chromosome segregation errors. Based on retrospective analyses, hyperdiploidy is associated with a better prognosis.[88-90]

2.4.1.2 Non-hyperdiploidy

(1) *IGH-CCND* translocation

IGH-CCND is the most common type of *IGH* translocation accounting for 50-60% of MM cases. This translocation involves t(11;14), t(12;14) and

t(14:16) translocation that juxtaposes the *IGH* enhancer(s) with *CCND1*, *CCND2* and *CCND3*, resulting in cyclin D dysregulation.[58, 86, 92] In general, the function of cyclin D involves the activation of downstream signaling molecules, CDK4 and CDK6, and the inactivation of the retinoblastoma (RB1) protein, which allows for cell cycle progression. Overexpression of *CCND* genes caused by *IGH* translocation induces susceptibility of myeloma cells to proliferative stimuli, such as IL-6 and insulin-like growth factor-1 (IGF-1), resulting in selective clonal expansion.[58, 92] These translocations occur at the switch region or chromosome breakpoints via the errors in V(D)J recombination during the B cell development.[93]

(2) *IGH-MMSET* translocation

IGH-MMSET or t(4;14) translocation is the second most common translocation occurring in up to 15% of MM cases.[88, 93] MMSET is a histone methyltransferase, which plays an important role in epigenetic regulation. The *MMSET* overexpression is associated with chromatin and gene dysregulations,[94] and strongly associated with increased expression and activating mutations of the fibroblast growth factor receptor 3 (*FGFR3*) gene contributing to MM pathogenesis.[95, 96]

(3) *IGH-MAF* and *IGH-MAFB* translocations

IGH-MAF and *IGH-MAFB* resulting from t(14;16) and t(14;20) translocations are found in 1-5% of MM cases. These translocations dysregulate the expression of a MAF transcription factor leading to increased expression of several genes, including *CCND2* and encoding genes of adhesion molecules, contributing to the ability of tumor cells to interact with the BM microenvironment.[58, 87]

Table 2.1 Primary cytogenetic events in MM patients[23, 30, 58, 87, 89-91, 95, 96]

Chromosome abnormalities	**Gene/chromosome defected**	**Prevalence (%)**
Hyperdiploidies	Recurrent trisomies involving odd-numbered chromosome	40-60
IGH translocation		
t(11;14)	*CCND1/IGH*	20
t(4;14)	*MMSET, FGFR3/IGH*	15
t(14;16)	*MAF/IGH*	3
t(14;20)	*MAFB/IGH*	1
IGH translocations with uncommon chromosome	*CCND3/IGH*, t(6p21;14) or *IRF4*/*IGH*, t(6p25;14)	4-5
Trisomies plus any *IGH* translocation	Presence of trisomies and any one of the recurrent *IGH* translocations in the same patient	15

CCND, cyclin D encoded gene; *FGFR3*, fibroblast growth factor receptor 3 encoded gene; *IGH*, immunoglobulin heavy chains encoded gene; *MAF*, v-avian musculoaponeurotic fibrosarcoma oncogene; *MAFB*, v-avian musculoaponeurotic fibrosarcoma oncogene homologue B; *MMSET*, multiple myeloma SET domain-containing protein encoded gene

2.4.2 Secondary genetic events in MM

Secondary cytogenetic abnormalities can occur during disease progression after normal plasma cell transitions to a clonal premalignant cell in the earlier stage of primary cytogenetic abnormalities (**Figure 2.5**).[91] Monosomy of chromosome 13 (monosomy 13) or deletion of chromosome 13q (del13q) is the most frequent secondary cytogenetic abnormality found in MM patients, followed by deletions of chromosome 17p (del17p), chromosome 1p (del1p) and amplification of chromosome 1q (amp1q), respectively.[87, 90, 91, 97]

2.4.2.1 Deletion of chromosome 13q (del13q)

Deletion of chromosome 13q (del13q) or monosomy 13 is detected in approximately 50% of MM patients and commonly found in MGUS, suggesting that del13q is a genetic event occurring in the early stage of clonal expansion.[97, 98] This abnormality may contribute to MM pathogenesis because several loci located on chromosome 13q are involved in the cell cycle and proliferation. Related studies revealed that del13q is a marker of poor prognosis in MM patients, especially in MM patients harboring del13q along with t(4;14).[97-99]

2.4.2.2 Deletion of chromosome 17p (del17p)

Deletion of chromosome 17p (del17p) is detected in about 10% of newly diagnosed MM, and high frequency is observed in the later stage of the disease.[100] Deletion of chromosome 17 is the cause of loss of several genes, especially the tumor suppressor *TP53* gene. The TP53 protein plays an important role in transcriptional regulators influencing cell-cycle arrest, DNA repair, apoptosis and response to DNA damage. Hence, the loss of this gene is associated with disease progression to an aggressive form and poor prognosis in MM patients.[100-102]

2.4.2.3 Deletion of chromosome 1p (del1p)

Approximately 30% of MM patients harbor deletion of chromosome 1p (del1p). This del1p is a marker associated with poor prognosis in MM patients.[103] The two common regions, 1p12 and 1p32.3, deletions involve MM pathogenesis. The 1p12 region contains a candidate tumor suppressor gene, *FAM46C*, a novel eukaryotic non-canonical poly (A) polymerase involving the regulation of gene expression, cell differentiation and development of several malignancies.[103-105] The *FAM46C* mutation is the cause of loss of gene function and is associated with disease progression in MM patients.[104, 105] The other 1p32.3 region contains two genes, *CDKN2C* and *FAF1*. The deletion of the 1p32.3 region can be found in either hemizygous or homozygous. *CDKN2C* encodes cyclin-dependent kinase-6 inhibitor involved in negative regulation of the cell cycle, whereas *FAF1* encodes a protein, inducing cell apoptosis through the Fas cell signaling pathway.[87, 103, 105]

2.4.2.4 Amplification of chromosome 1q (amp1q)

Amplification of the long arm of chromosome 1 (amp1q), with 4 or more copies, is detected in up to 45% of MM patients and is associated with a poor prognosis.[106] Of note, the number of 1q copies is positively correlated with clinical outcome. The candidate oncogenes, including *CKS1B*, *ANP32E*, *BCL9* and *PDZK1*, are identified in this region.[103, 106] Overexpression of those genes due to amp1q results in increased histone methylation and epigenetic upregulation, which are key to MM pathogenesis.[103, 106] Moreover, a BCL2- anti-apoptotic protein family encoded genes, such as the *MCL1* gene, promoting cell differentiation and survival of plasma cells and myeloma cells, is also identified in this region. High expression of the *MCL-1* gene causes tumorigenesis and resistance to several anticancer therapies.[107]

2.4.3 Genetic events involved in MM progression

2.4.3.1 Translocations involving *MYC* gene

Although the translocations affecting to MYC gene are a late secondary genetic event found in only 3-4% of patients with MGUS or SMM; however, their frequencies increased up to 15-20% in newly diagnosed symptomatic MM patients. In general, the proto-oncogene *MYC* is a key transcription factor involving in cell growth, proliferation, apoptosis, differentiation and transformation. Chromosomal abnormality involving the *MYC* locus is involved in disease progression to an aggressive form and poor prognosis.[108]

2.4.3.2 Recurrent gene mutation

Several genes are recurrently mutated and considered to be driver events in MM disease progression (**Figure 2.5**). Of these, KRAS, NRAS and BRAF mutations affecting the mitogen-activated protein kinases (MAPK) pathway are observed in up to 20-50% of MM patients and associated with disease progression.[87, 91, 109, 110] Mutations of *KRAS* and *NRAS* are less found in MGUS (>10%), but the frequencies are higher in symptomatic MM (~50%) and plasma cell leukemia (64-70%).[87, 110] The mutations of the gene involved in DNA-repair processes, such as *TP53*, *ATM* and *ATR*, are found in up to 15%, whereas other genes, including *DIS3*, *FAM46C*, *PRDM1*, *IRF4*, *ERG1* and *SP140*, are found in less than 10% of MM cases.[87, 91] In addition, gene mutations encoding components of the NF-κB pathway, such as *TRAF3*, *CYLD* and *LTB*, are observed in 3.0-5.5% of MM cases with disease progression.[111-113]

These gene mutations can promote proliferation, survival, immune evasion and drug-resistance of myeloma cells, which subsequently activate secretion of several cytokines and growth factors, enhancing cell growth, angiogenesis and inflammation.[111, 112]

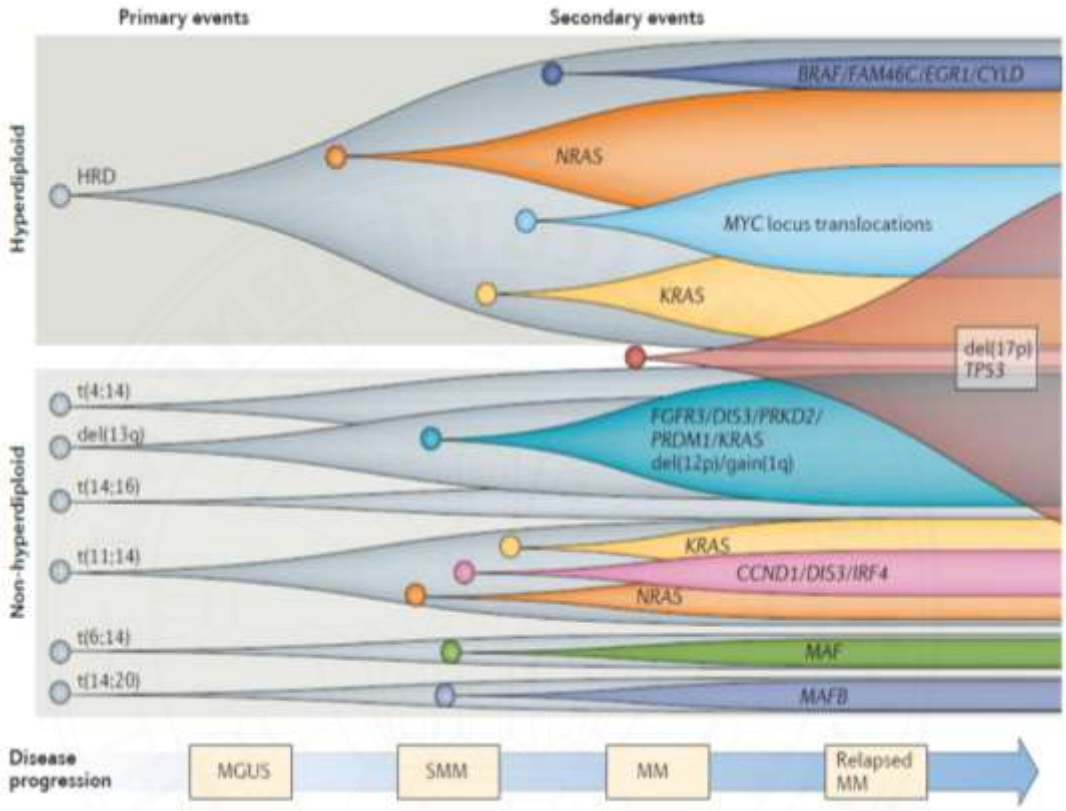

Figure 2.5 Genetic events occurred in multiple myeloma[87]

2.5 Diagnosis of MM

2.5.1 Disease development and progression

Approximately 80% of MM cases originate from an asymptomatic premalignant stage called MGUS, which is found in 3-4% among populations aged over 50 years.[5-6] MGUS can progress to symptomatic MM or related malignancy at a rate of 1% per year.[5, 9-12, 17] Some MGUS patients can evolve into an advanced asymptomatic stage called SMM. The risk of progression from SMM to symptomatic MM is about 10% per year in the first five years after diagnosis.[9-12] This rate depends on underlying cytogenetic types, especially in patients with t(4;14) and del17p, who are at higher risk

of disease progression from MGUS or SMM to MM.[88, 93, 100-102] Almost MM patients can develop into RRMM after treatment and only few patients can evolve to an aggressive stage, PCL.[9-12]

2.5.2 Clinical presentation and diagnosis

The most common clinical presenting symptoms in MM patients are fatigue and unexplained bone pain, which found in approximately 32% to 58% of the cases.[16] Other common clinical findings, including pathologic bone fracture, weight loss and paresthesia, can be found in 34%, 24% and 5%, respectively.[4, 16] The important clinical features used to diagnose the disease are myeloma-related organ or tissue impairments, representing the end-organ damage. These specific end-organ damage features are recognized as "CRAB" features. The CRAB features include hypercalcemia (serum calcium greater than 11.0 mg/dL), renal insufficient (serum creatinine greater than 2.0 mg/dL), anemia (hemoglobin level less than 10.0 g/dL) and bone lytic or osteolytic lesions.[4, 9, 16, 17] Other impairments, such as symptomatic hyperviscosity, amyloidosis, neurological complications and recurrent bacterial infections (more than two consecutive times within 12 months), can be often observed in MM patients.[4, 16, 17] Because of impairment of the immune system, MM patients are susceptible to bacterial infections, especially pneumonia and pyelonephritis, which are a common cause of death in MM patients.[4, 16, 17]

According to the 2014 Revised International Myeloma Working Group Criteria for the diagnosis of multiple myeloma and related disorders (**Table 2.2**),[17] the diagnosis of MM required the presence of 10% to 60% clonal plasma cells on BM examination or a biopsy-proven plasmacytoma together with the specific myeloma defining events (MDE).

Table 2.2 International myeloma working group diagnostic criteria for multiple myeloma and related plasma cell disorders[17]

Disorder	Disease definition
Non-IgM monoclonal gammopathy of undetermined significance (Non-IgM MGUS)	All 3 criteria must be met: - Serum M protein (non-IgM type) <3.0 g/dL - Clonal BM plasma cells <10.0% - Absence of end-organ damage such as hypercalcemia, renal insufficiency, anemia and bone lesions (CRAB) that can be attributed to the plasma cell proliferative disorder
Smoldering multiple myeloma (SMM)	Both criteria must be met: - Serum M protein (IgG or IgA) ≥3.0 g/dL, or urinary M protein >500 mg per 24 hours and/or clonal BM plasma cells 10%-60% - Absence of myeloma defining events or amyloidosis
Multiple myeloma (MM)	Both criteria must be met: - Clonal BM plasma cells ≥10% or biopsy-proven bony or extramedullary plasmacytoma - Any one or more of the following myeloma defining events: • Evidence of end organ damage that can be attributed to the underlying plasma cell proliferative disorder, specifically: - Hypercalcemia: serum calcium >0.25 mmol/L (>1 mg/dL) higher than the upper limit of normal or >2.75 mmol/L (>11 mg/dL)

Table 2.2 International myeloma working group diagnostic criteria for multiple myeloma and related plasma cell disorders (Cont.)[17]

Disorder	Disease definition
Multiple myeloma (MM) continued	- Renal insufficiency: Cr clearance <40 mL per minute or serum Cr >177 µmol/L (>2.0 mg/dL) - Anemia: hemoglobin (Hb) value of >2 g/dL below the lower limit of normal, or a Hb value <10 g/dL - Bone lesions: one or more osteolytic lesions on skeletal radiography, CT, or positron emission tomography-CT (PET-CT) • Clonal BM plasma cell percentage ≥60% • Involved: uninvolved SFLC ratio >10 (involved SFLC level must be >100 mg/L) • More than one focal lesion on MRI studies (at least 5 mm in size)
IgM monoclonal gammopathy of undetermined significance (IgM MGUS)	All 3 criteria must be met: - Serum IgM-M protein <3.0 g/dL - BM lymphoplasmacytic infiltration <10% - No evidence of anemia, constitutional symptoms, hyperviscosity, lymphadenopathy, or hepatosplenomegaly that can be attributed to the underlying lymphoproliferative disorder.

Table 2.2 International myeloma working group diagnostic criteria for multiple myeloma and related plasma cell disorders (Cont.)[17]

Disorder	Disease definition
Light chain MGUS	All criteria must be met: - Abnormal SFLC ratio (<0.26 or >1.65) - Increased level of the appropriate involved light chain (increased kappa FLC in patients with ratio >1.65 and increased lambda FLC in patients with ratio <0.26) - No immunoglobulin heavy chain expression on IFE - Absence of end-organ damage that can be attributed to the plasma cell proliferative disorder - Clonal BM plasma cells <10% - Urinary M-protein <500 mg/24 hours
Solitary plasmacytoma	All 4 criteria must be met - Biopsy proven solitary lesion of bone or soft tissue with evidence of clonal plasma cells - Normal BM with no evidence of clonal plasma cells - Normal skeletal survey and MRI (or CT) of spine and pelvis (except for the primary solitary lesion) - Absence of end-organ damage (CRAB) that can be attributed to a lympho-plasma cell proliferative disorder

Table 2.2 International myeloma working group diagnostic criteria for multiple myeloma and related plasma cell disorders (Cont.)[17]

Disorder	Disease definition
Solitary plasmacytoma with minimal marrow involvement*	All 4 criteria must be met - Biopsy proven solitary lesion of bone or soft tissue with evidence of clonal plasma cells - Clonal BM plasma cells <10% - Normal skeletal survey and MRI (or CT) of spine and pelvis (except for the primary solitary lesion) - Absence of end-organ damage (CRAB) that can be attributed to a lympho-plasma cell proliferative disorder

* Solitary plasmacytoma with 10% or more clonal plasma cells is considered as multiple myeloma.

2.5.3 Staging and prognosis

According to the International Staging System (ISS),[114] MM is divided into 3 stages based on the levels of serum β_2M and Alb. The elevation of β_2M level reflects high tumor mass and reduced renal function. While the depression of serum Alb level is mainly caused by inflammatory cytokines, such as IL-6, secreted by the myeloma microenvironment.[114] The median OS of the patient is 62, 44 and 29 months for stage I, II and III, respectively (**Table 2.3**).[114]

Table 2.3 The International Staging System (ISS) criteria for multiple myeloma[114]

Stage	Criteria	Median OS (months)
I	Serum β_2M <3.5 mg/L Serum Alb ≥3.5 g/dL	62
II	Not stage I or III*	44
III	Serum β_2M ≥5.5 mg/L	29

* There are two categories for stage II: serum β_2M <3.5 mg/L but serum Alb < 3.5 g/dL; or serum β_2M 3.5 to < 5.5 mg/L irrespective of the serum aAlb level.

The chromosomal abnormalities detected by the FISH technique are the key element defining the clinical outcomes in MM patients. Patients with del17p, t(14;16) and t(14;20) are considered a high-risk, while patients with t(4;14) translocation and amp1q are considered an intermediate-risk MM. The other chromosomal abnormalities are considered standard-risk.[115] In addition, serum LDH is another relevant biomarker in MM because an increased serum LDH is associated with disease aggressiveness, suggesting high proliferation of tumor mass and involvement in the extramedullary disease.[116, 117] Therefore, the combination of ISS, chromosome abnormalities (CA) and LDH was recommended in the Revised ISS (R-ISS) criteria to improve the predictive value and was used to stratification in newly diagnosed MM patients (**Table 2.4**).[118]

Table 2.4 The Revised International Staging System criteria[118]

R-ISS stage	Criteria	Median OS (months)
I	ISS stage I and standard-risk CA by FISH and normal serum LDH	82
II	Not R-ISS stage I or III	62
III	ISS stage III and either high-risk CA by FISH or high serum LDH	40

CA, chromosome abnormalities; FISH, fluorescence *in situ* hybridization; LDH, lactase dehydrogenase; OS, overall survival; R-ISS, Revised International Staging System

2.5.4 Refractory and/or relapsed multiple myeloma (RRMM)

Based on a recent American Society of Hematology–Food and Drug Administration (FDA) panel on endpoints in myeloma,[119] there are two categories of refractory myeloma, relapsed and/or refractory myeloma (RRMM) and primary refractory myeloma. RRMM is defined as a disease that becomes non-responsive or progressive on therapy or within 60 days of the last treatment in patients who had achieved a minimal response (MR) or better on prior therapy, whereas primary refractory myeloma is defined as a disease that is non-responsive in patients who never achieved an MR or better with any therapy.[119]

2.5.5 Laboratory testing in MM

Laboratory assessment for diagnostic work-up in patients with monoclonal gammopathy is recommended by several expert groups. After completing a medical history and physical examination, when MM is suspected clinically, patients should be evaluated for presence of M-protein using a combination of tests, including SPEP, IFE and SFLC.[17, 18] The 24-hr-urine collection and M-protein analysis by UPEP are recommended. Serum electrolytes, serum calcium, CBC and renal function tests are indicators providing CRAB symptoms. In addition, imaging techniques, including CT, MRI and PET, are required to detect osteolytic bone lesions and determine the disease stage.

2.5.5.1 Serum proteins electrophoresis (SPEP)

In SPEP, proteins are separated according to their respective electrical charges on agarose gel using both electrophoretic and electroendosmotic forces presented in the system. Then, the proteins are stained with a visible stain, mostly Amido black, Coomasie blue and acid blue stains. Based on their electrical charge at a particular pH and molecular weight, the serum proteins are fractionated into five classical fractions: Alb, alpha1 (α1), alpha2 (α2), beta (β1 and β2) and gamma (γ) proteins.[48] The pattern of the protein fractions is scanned with a densitometer to obtain a semi-quantitative estimating of specific fractions and then visualized by a densitometric graph (**Figure 2.6**). Although the relative proportions of those fractions identified by SPEP are helpful in diagnosis and prognosis of plasma cell dyscrasias; however, abnormal patterns can be observed in pregnancy and other disorders, including inflammatory response, rheumatic diseases, liver diseases, protein-loss-disorders and genetics deficiencies.[120]

2.5.5.2 Immunofixation electrophoresis (IFE)

When a restricted band is identified in SPEP, the IFE should be performed to definitively identify the M-protein isotype. IFE is a procedure, in which the serum proteins are separated by electrophoresis, and treated with specific antiserum against human IgG, IgA, IgM, kappa, lambda and rarely for IgE and IgD. The gel is washed with saline to remove all unprecipitated antibodies, followed by staining, destaining and drying processes. The positive result is presented with a precipitin band. As compared with SPEP, IFE could improve test sensitivity by 10-folds to detect specific isotypes of the M-protein.[121] Therefore, IFE is suggested in the case of a patient with suspected monoclonal gammopathy with negative SPEP result. Normal and abnormal patterns of IFE test results are shown in **Figure 2.7**.

2.5.5.3 Serum free light chains (SFLC)

SFLC is an important tumor marker presented in the serum and urine of patients with monoclonal gammopathies. In healthy individuals, light chains found in the serum are bounded form with the heavy chain. However, low levels of free-light chains are found in the serum of normal individuals due to the overproduction and secretion by plasma cells.[122] The quantitative measurement of SFLC was performed using the immunoturbidimetric or immunonephelometric assay, in which the polyclonal

or monoclonal antibodies specifically recognized the epitope in the hidden constant region of the light chains, are used to detect the κ and λ FLC in serum separately. The SFLC concentrations are calculated by comparing the optical density with the reference standard curve.[122-124] The elevation of the SFLC is associated with plasma cell dyscrasia, amyloid light-chain (AL) amyloidosis and other light-chain deposition diseases.[122-124]

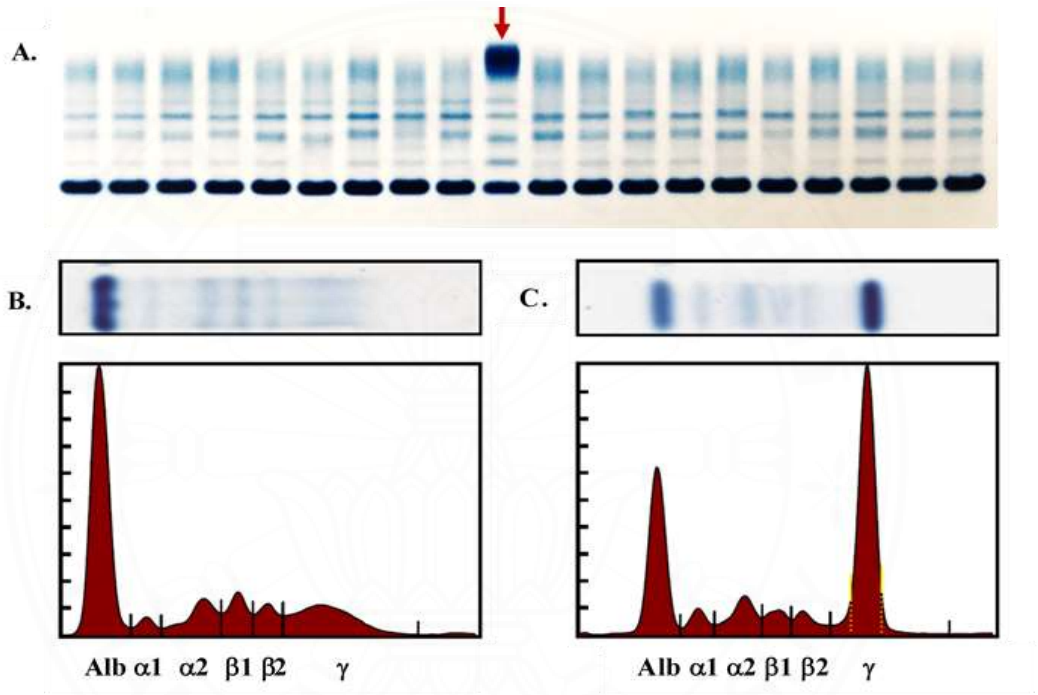

Figure 2.6 The results of SPEP on agarose gel. (A) The SPEP results from a MM patient demonstrating a restricted band on the gamma globulin zone (red arrow) compared with healthy individuals. (B) A densitometric graph showed the normal pattern of the serum proteins. (C) A densitometric graph showed the "M-spike" on the gamma globulin zone in MM patient.

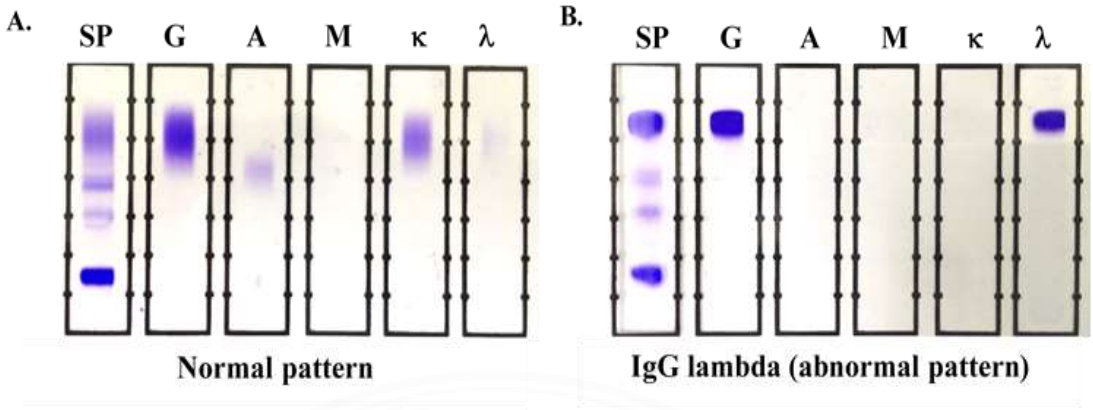

Figure 2.7 The results of IFE on agarose gel. (A) Normal pattern showed the fainted bands on each lane with specific antibodies to immunoglobulin isotypes. (B) An abnormal pattern (IgG lambda pattern) from MM patient showed the restricted precipitin bands on anti-IgG and anti-lambda light chain lanes.

2.6 Treatment of MM

The treatment of MM depends on the patient's experiencing symptoms, staging and the patient's overall health. Although MM is considered an incurable disease; however, the treatment goals are to eliminate myeloma cells, control tumor growth, reduce pain, and allow patients to have a better life. The treatment options typically include chemotherapy and targeted therapy, BM stem cell transplantation and other types of treatments, such as radiation therapy and surgery.[125, 126]

2.6.1 Treatment of newly diagnosed MM

Treatment should be initiated in all patients with MM according to the updated definition proposed by the IMWG. The recommended common treatment regimens in MM are shown in **Table 2.5.**[125, 126] The approach to treat a symptomatic newly diagnosed MM is outlined in **Figure 2.8**.[16] There are 3 important phases of therapy; initial therapy (with or without autologous stem cell transplant, ASCT), consolidation/maintenance therapy and treatment of relapse.[16, 17]

Table 2.5 Common front-line treatment regimens in multiple myeloma[125, 126]

Regimens	Suggested doses	Overall response rate (%)	Estimated CR+VGPR rate (%)
Thalidomide–dexamethasone (TD)	Thalidomide 200 mg oral days 1–28; dexamethasone 40 mg oral days 1, 8, 15, 22; repeated every 4 weeks	65	30
Lenalidomide–dexamethasone (Rd)	Lenalidomide 25 mg oral days 1–21 every 28 days; dexamethasone 40 mg oral days 1, 8, 15, 22 every 28 days; repeated every 4 weeks	70	40
Bortezomib–dexamethasone (VD)	Bortezomib 1.3 mg/m^2 intravenous days 1, 8, 15, 22; dexamethasone 20 mg on day of and day after bortezomib (or 40 mg days 1, 8, 15, 22); repeated every 4 weeks	80	40
Melphalan–prednisone–thalidomide (MPT)	Melphalan 0.25 mg/kg oral days 1–4 (use 0.20 mg/kg per day oral days 1–4 in patients over the age of 75 years); prednisone 2 mg/kg oral days 1–4; thalidomide 100–200 mg oral days 1–28 (use 100 mg dose in patients >75 years); repeated every 6 weeks	70	30
Bortezomib–melphalan–prednisone (VMP)	Bortezomib 1.3 mg/m^2 intravenous days 1, 8, 15, 22; melphalan 9 mg/m2 oral days 1–4; prednisone 60 mg/m2 oral days 1–4; repeated every 35 days	70	40
Melphalan–prednisone–lenalidomide (MPR)	Melphalan 0.18 mg/kg oral days 1–4; prednisone 2 mg/kg oral days 1–4; lenalidomide 10 mg oral days 1–21; repeated every 4 weeks	67	33

Table 2.5 Common front-line treatment regimens in multiple myeloma (Cont.)[125, 126]

Regimens	Suggested doses	Overall response rate (%)	Estimated CR+VGPR rate (%)
Bortezomib–thalidomide–dexamethasone (VTD)	Bortezomib 1.3 mg/m^2 intravenous days 1, 8, 15, 22; thalidomide 100–200 mg oral days 1–21; dexamethasone 20 mg on day of or after bortezomib (or 40 mg days 1, 8, 15, 22); repeated every 4 weeks × 4 cycles as pre-transplant induction therapy	95	60
Bortezomib–cyclophosphamide–dexamethasone (VCD)	Cyclophosphamide 300 mg/m^2 orally on days 1, 8, 15 and 22; bortezomib 1.3 mg/m2 intravenously on days 1, 8, 15, 22; dexamethasone 40 mg orally on days on days 1, 8, 15, 22; repeated every 4 weeks	90	70
Bortezomib–lenalidomide–dexamethasone (VRD)	Bortezomib 1.3 mg/m^2 intravenous days 1, 8, 15; lenalidomide 25 mg oral days 1–14; dexamethasone 20 mg on day of and day after bortezomib (or 40 mg days 1, 8, 15, 22); repeated every 3 weeks	100	70

CR, complete response; VGPR, very good partial response

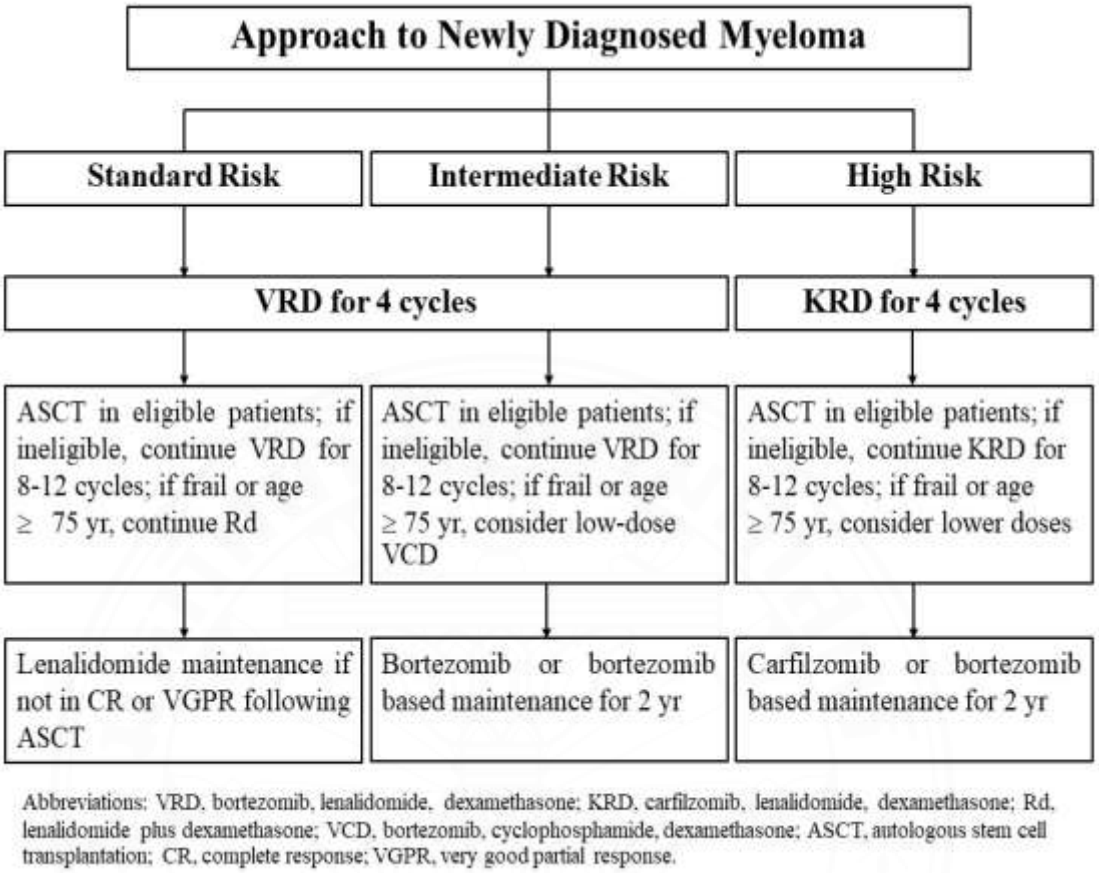

Figure 2.8 An approach for treatment in newly diagnosed multiple myeloma[16]

For the initial or induction therapy, the drugs option varies across the country depending on drug availability. The standard initial treatment for symptomatic MM patients depends on their eligibility for high dose therapy (HDT) and ASCT, which is based on the patient's age, comorbidities and functional status. In the USA, the upper age limit for ASCT is approximately 75 years. In most Asian countries, including Thailand, patients 65 years and older are not considered candidates for ASCT.

In clinical practice, the triplet regimens using a combination of bortezomib (V), dexamethasone (D), cyclophosphamide (C) and thalidomide (T), including bortezomib-thalidomide-dexamethasone (VTD) regimen, bortezomib-cyclophosphamide-dexamethasone (VCD) regimen and bortezomib-lenalidomide-dexamethasone (VRD) regimen, are widely used in the treatment of newly diagnosed MM.[16] In addition, the triplet regimen of carfilzomib, lenalidomide and dexamethasone (KRD) is recommended only in patients with high-risk MM. In ASCT eligible group,

patients are treated with approximately 3–4 cycles of induction therapy before stem cell harvest.[16, 17] Long-term melphalan-based treatment is inappropriate for patients considering ASCT candidate because it can interfere with adequate stem cell mobilization.[126]

The consolidation phase is generally defined as treatment given for a short duration, about 2 to 4 cycles, after induction therapy or after ASCT. In this phase, the patients were usually treated with the same regimen used for induction therapy, followed by high-dose therapy. The goal of the consolidation therapy is to further better or deepen response.[127] Several studies demonstrated that consolidation treatment could improve the depth of response; however, there are insufficient data to establish that consolidation treatment prolongs OS in MM patients.[126, 127]

Maintenance therapy is usually given after an ASCT or induction therapy in non-transplant candidate MM patients. In the maintenance phase, a low dose chemotherapy is used over a long period to maintain or prolong remission and to prevent or delay the relapsed disease.[126] In post-ASCT patients, a meta-analysis of randomized trials revealed that maintenance therapy using immunomodulatory imide drugs (iMIDs), such as thalidomide and lenalidomide, improved progression-free survival (PFS) and OS.[128] However, the benefit of maintenance therapy in non-transplant candidate patients remains unclear. [17]

2.6.2 Autologous stem cell transplantation (ASCT)

ASCT has been considered the standard approach for frontline therapy in eligible MM patients. ASCT could improves CR rates and prolongs median OS in MM by approximately 12 months, with 2% of mortality rate.[129, 130]

2.6.3 Response criteria of treatment in MM patients

The uniform response criteria for MM was established by IMWG to facilitate precise comparisons of the efficacy of treatment strategies in trials and to provide the definition and classification for the responses in MM patients (**Table 2.6**).[119, 131]

Table 2.6 The IMWG uniform response criteria by response subcategories for multiple myeloma[119, 131]

Response category	Criteria
Stringent complete remission (sCR)	CR as defined above plus normal SFLC ratio and absence of clonal cells in BM by immunohistochemistry or immunofluorescence
Complete remission (CR)	• Negative IFE on the serum and urine and • disappearance of any soft tissue plasmacytomas and • <5% plasma cells in BM
Very good partial response (VGPR)	Serum and urine M-protein detectable by IFE but not on electrophoresis or > 90% reduction in serum M-protein plus urine M-protein level <100 mg/24 h
Partial response (PR)	• 50% reduction of serum M-protein and reduction in 24 h urinary M-protein by >90% or to <200 mg/24 h • If the serum and urine M-protein are unmeasurable, >50% decrease in the difference between involved and uninvolved SFLC levels is required in place of the M-protein criteria • If serum and urine M-protein are not measurable and SFLC assay is also not measureable, >50% reduction in plasma cells is required in place of M-protein, provided baseline BM plasma cell percentage was >30% • In addition to the above listed criteria, if present at baseline, a >50% reduction in the size of soft tissue plasmacytomas is also required

Table 2.6 The IMWG uniform response criteria by response subcategories for multiple myeloma (Cont.)[119, 131]

Response category	Criteria
Stable disease (SD) or no change	Not meeting criteria for CR, VGPR, PR, or PD
Progressive disease (PD)	Increase of >25% from lowest response value in any one or more of the following: • Serum M-component and/or (the absolute increase must be >0.5 g/dL) • Urine M-component and/or (the absolute increase must be >200 mg/24 h) • Only in patients without measurable serum and urine M-protein levels; the difference between involved and uninvolved SFLC levels. The absolute increase must be >10 mg/dL • BM plasma cell percentage; the absolute percentage must be >10% • Definite development of new bone lesions or soft tissue plasmacytomas or definite increase in the size of existing bone lesions or soft tissue plasmacytomas • Development of hypercalcaemia (corrected serum calcium >11.5 mg/dL or 2.65 mmol/L) that can be attributed solely to the plasma cell proliferative disorder

Table 2.6 The IMWG uniform response criteria by response subcategories for multiple myeloma (Cont.)[119, 131]

Response category	Criteria
Relapse	Clinical relapse requires one or more of: Direct indicators of increasing disease and/or end organ dysfunction (CRAB features). It is not used in calculation of time to progression or PFS but is listed here as something that can be reported optionally or for use in clinical practice • Development of new soft tissue plasmacytomas or bone lesions • Definite increase in the size of existing plasmacytomas or bone lesions. A definite increase is defined as a 50% (and at least 1.0 cm) increase as measured serially by the sum of the products of the cross-diameters of the measurable lesion • Hypercalcemia (>11.5 mg/dL) [2.65 mmol/L] • Decrease in hemoglobin of >2.0 g/dL [1.25 mmol/L] • Rise in serum creatinine by 2.0 mg/dL or more [177 mmol/L or more]
Relapse from CR (To be used only if the end point studied is disease-free survival)	Any one or more of the following: • Reappearance of serum or urine M-protein by IFE or electrophoresis • Development of >5.0% plasma cells in the BM • Appearance of any other sign of progression (i.e., new plasmacytoma, lytic bone lesion, or hypercalcemia)

More recently, the updated IMWG response criteria have now included minimal residual disease (MRD) status defined by the absence of BM plasma cells by next-generation flow (NGF) or next-generation sequencing (NGS), as shown in **Table 2.7**.[132]

Table 2.7 The updated IMWG response criteria 2016[132]

Response subcategory	Criteria
Sustained MRD-negative	MRD-negative in the marrow (NGF and/or NGS) and by imaging as defined below, confirmed one year apart. Subsequent evaluations can be used to further specify the duration of negativity (e.g. MRD-negative at 5 years)
Flow MRD-negative	Absence of phenotypically aberrant clonal plasma cells by NGF on BM aspirates using the EuroFlow standard operation procedure for MRD detection in MM (or validated equivalent method) with a minimum sensitivity of 1 in 10^5 nucleated cells or higher
Sequencing MRD-negative	Absence of clonal plasma cells by NGS on BM aspirates in which presence of a clone is defined as less than two identical sequencing reads obtained after DNA sequencing of BM aspirates using the Lymphosight VR platform (or validated equivalent method) with a minimum sensitivity of 1 in 10^5 nucleated cells or higher
Imaging + MRD-negative	MRD-negative as defined by next-generation flow cytometry or NGS plus Disappearance of every area of increased tracer uptake found at baseline or a preceding PET-CT or decrease to mediastinal blood pool SUV or decrease to less than that of surrounding normal tissue

IMWG, International Myeloma Working Group; MRD, minimal residual disease; NGF, next-generation flow cytometry; NGS, next-generation sequencing; PET-CT, positron emission tomography-computed tomography; SUV, standardized uptake value.

2.7 Proteomics studies

Although the current genomics studies provided information about gene expression during several physiological and pathological processes, the relationship between the *in vivo* concentration of an mRNA and its encoded protein is inconsistent.[133] In humans, the genome contains about 25,000-30,000 genes. Most of them were transcribed and generated different variants of mRNA, which can be translated into diverse functional proteins. The posttranslational modifications, including phosphorylation, glycosylation and acetylation, are vital keys contributing to diversity in protein structure, function and activity.[134] In clinical, studying proteins provide disease information and is also used to determine mechanisms, elucidate pathways and indicate disease status. Thus, the studies of proteins are crucial for disease diagnosis, prognosis and monitoring of disease development.[134]

Proteomics is the studies involved in the applications of technologies to identify and quantify overall proteins in a particular cell, tissue, or organism. The term proteome was first used in 1994 by Mark Wilkins to denote the "PROTein complement of a genOME".[135] The "proteome" can be defined as the overall protein content of a cell characterized by its localization, interactions, post-translational modifications, interactions and turnover at a particular time.[134]

2.7.1 Methods in proteomics

Proteome analysis provides the complete description of structural and functional information of cells using single or multiple proteomics techniques. Several technologies are used to separate, purify, characterize, identify and quantify of the proteins. Moreover, bioinformatics tools are employed to analyze the protein interaction. An overview of the proteomics techniques is shown in **Figure 2.9**.[134]

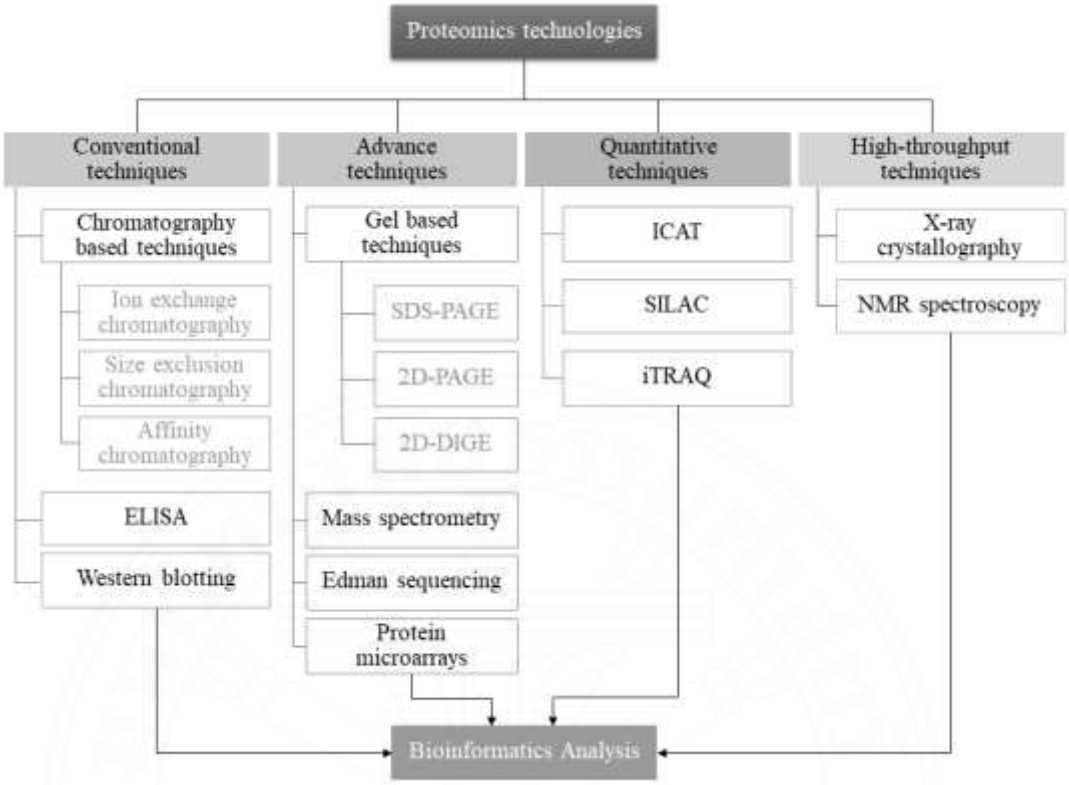

Figure 2.9 An overview of the proteomics approaches[134]

In the conventional proteomic techniques, chromatography-based techniques, including ion exchange chromatography (IEC), size exclusion chromatography (SEC) and affinity chromatography, are used to purify proteins in the first step. For analysis of selective protein, enzyme-linked immunosorbent assay (ELISA) and Western blotting can be used to restrict and define the expression level of the interested protein. [134]

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional gel electrophoresis (2-DE) and two-dimensional differential gel electrophoresis (2D-DIGE) techniques are advanced proteomics techniques used for separation of complex protein samples. Edman sequencing has been developed to determine the amino acid sequence of a particular protein. In addition, X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy techniques are high-throughput techniques providing a three-dimensional (3D) structure of the protein that might be helpful to understanding its biological function. The protein microarray

techniques, including analytical, functional and reverse-phase microarrays, have been established for high throughput and rapid expression analysis.[134, 136, 137]

Mass spectrometry (MS) based techniques are the most effective tools for proteomics study. A mass spectrometer determines the mass of a molecule by measuring its mass-to-charge (*m/z*) ratio.[31] Each mass spectrometry analyzer consists of three components; the source, mass analyzer and detector. The digested peptides obtained from an intersted sample are ionized either by matrix-assisted laser desorption ionization (MALDI) or electrospray ionization (ESI) in the source. The charged peptides are sorted according to their m/z ratio and measured by the detector[31] Applications of MS techniques include not only mass determination and protein identification but also provide a detailed characterization of protein isoform, post-translational modification and quantification of protein levels.[134, 137] Quantitative protein analysis by mass spectrometry is an effective way to discover the biomarkers.[138] Quantitative protein analysis by mass spectrometry is an effective way to discover the biomarkers. The label-free quantitative (LFQ) protein analysis and stable isotope labeling techniques, including isotope-coded affinity tag (ICAT) labeling, stable isotope labeling with amino acids in cell culture (SILAC) and isobaric tag for relative and absolute quantitation (iTRAQ) techniques, are used to quantify proteins within a sample and widely used for biomarker discovery.[138]

2.7.2 Proteomic analysis in MM research

Proteomics has been employed in MM research for many specific purposes, including the identification of the mechanisms of MM pathogenesis, the discovery of novel diagnostic and prognostic biomarkers and the discovery of potential targeting molecules for MM treatment. Several published studies demonstrated the advantages of proteomic approaches in MM research. Examples of proteomic studies in MM research are shown in **Table 2.8**.

Table 2.8 Examples of proteomic studies in multiple myeloma research

Approach	Study	Sample type	Number of MM subject	Method	References
Study MM pathogenesis	Interaction between myeloma cells and osteoclasts	Separated myeloma cells and osteoclasts	50	SELDI–MS	Yin, 2005 [139]
	The proteome alterations during disease transformation from MM to secondary plasma cell leukemia	BM samples from same patient at MM and sPCL stages	1	SILAC LC–MS/MS	Zatula et al., 2017 [140]
	Proteomic characterization of BM extracellular matrix in MM patients	BM samples	16	LC–MS/MS	Glavey et al., 2018 [36]
	The role of the unfolded protein response pathway in osteoclastogenesis in MM	MM cell lines	-	ESI–MS/MS	Raimondi et al., 2020 [141]
Biomarker discovery	A reference map for proteomics comparison in BM of MM patients	Separated plasma cells from BM	8	2DE MALDI–TOF/TOF MS	Lu et al., 2010 [142]
	Identifying pathogenic factors and potential biomarkers in sera of MM patients.	Serum samples	8	LC MS/MS	Zhang et al., 2015 [32]
	Development of a specific model for predicting the presence of MGUS	Serum samples	103	MALDI-TOF MS/MS	Barceló et al., 2018 [143]
Drug monitoring and predicting the response	Comparative proteomic profiles in BM of RRMM patients responding to bortezomib-based chemotherapy	BM samples	77	iTRAQ LFQ	Dytfeld et al., 2016 [144]
	Novel biomarkers to predict the response to bortezomib-based chemotherapy in MM patients	Serum samples	67	LFQ using LC–MS/MS	Łuczak et al., 2017 [33]
	Discovery of biomarkers to predict response to treatment containing bortezomib	Serum samples	17	LFQ using LC–MS/MS	Ting et al., 2017 [37]
	The role of exportin 1 (XPO1) in bortezomib resistance	MM cell lines	-	iTRAQ LFQ	Chanukuppa et al., 2019 [145]

2DE, two-dimensional gel electrophoresis; ESI, electrospray ionization; iTRAQ, isobaric tag for relative and absolute quantitation; LC, liquid chromatography; LFQ, label-free quantitation; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; SELDI, surface-enhanced laser desorption/ionization; SILAC, stable isotope labeling with amino acids in cell culture; TOF, time-of-flight

2.7.2.1 Proteomic studies of MM pathogenesis and disease progression

To study the MM pathogenesis, focused on the pathophysiology of bone involvement in myeloma, Yin[139] used SELDI–MS analysis to demonstrate the *in vivo* interaction between myeloma cells and osteoclasts. The secreted proteome obtained from myeloma cells separated from 50 MM patients, osteoclasts and co-cultured myeloma cells with osteoclasts, were analyzed and compared. The results showed a significantly increased level of the secreted protein, chondroitin synthase 1 (CHSY1), observed in co-cultured myeloma cells with osteoclasts compared with the other groups.[139] This protein plays an important role in the activation of the Notch2 signaling pathway and inhibition of the Notch1 signaling pathway, that known to be involved in the interaction between myeloma cells and the BM microenvironment and promoted bone destruction in MM.[139]

In 2017, Zatula et al.[140] used proteomics approaches to elucidate the proteome alterations during disease transformation from MM to secondary PCL. In this study, SILAC quantitative technique was used to analyze the proteomic profile of six human myelomas and two human B-cell lymphoma cell lines. In addition, myeloma cells separated from the same patient at both MM and PCL stages were subjects for LC–MS/MS analysis. The results revealed that 795 differentially expressed proteins were identified among MM and PCL samples. Gene ontology analysis indicated that proteins involved in aerobic glycolytic metabolism were upregulated, while the proteins involved in glycan synthesis were downregulated in PCL. Most upregulated proteins, including serum amyloid A-4 protein (SAA4), apolipoprotein D (ApoD) and interferon-induced guanylate-binding protein 2 (GBP2), were observed in PCL with the fold changes of 21.2, 9.75 and 5.92-fold, respectively.

The related study was conducted by Glavey et al.[36] in 2018. The proteomic analysis was performed to characterize the BM-ECM in patients with MGUS and MM compared with healthy donor-derived BM-ECM. The results revealed that the tumor ECM was remodeled at mRNA and protein levels in MGUS and MM to allow the development of a permissive microenvironment. Notably, two ECM-affiliated proteins, ANXA2 and LGALS1, are more abundant in MM, and higher levels of those proteins were associated with inferior overall survival. This finding suggested that BM-

ECM remodeling plays an important role in MM and provides prognostic implications for MM patients.

In a recent study by Raimondi et al.[141], reverse-phase high-pressure liquid chromatography-electrospray ionization tandem mass spectrometry (RP-HPLC-ESI-MS/MS) was employed to demonstrate the role of the unfolded protein response (UPR) pathway in osteoclastogenesis induced by multiple myeloma-derived extracellular vesicles (MM-EV). In human MM cell lines, the myeloma cells can produce extracellular vesicles or exosomes that can activate osteoclast differentiation and promote osteoclast function in bone resorption activity.[146] The results revealed that MM-EV induced osteoclastogenesis through the activation of the inositol-requiring enzyme-1 alpha (IRE1α)/x box-binding protein 1 (XBP1) axis of the UPR pathway.

2.7.2.2 The use of proteomic analysis to discover novel diagnostic biomarkers

In 2010, Lu et al.[142] used proteomic techniques to identify the abundant proteins in plasma cells separated from the BM of eight newly diagnosed MM patients. The proteomics analysis was accessed using 2DE and MALDI-TOF/TOF MS techniques. The results demonstrated that a total of 517 spots were identified by 2DE, corresponding to 268 proteins characterized by mass spectrometry. These identified proteins correspond to different functional categories and represent a preliminary functional profile of the MM proteome. This database can serve as a reference map for proteomics comparison and could be a useful source for potential biomarker identification.

In 2015, Zhang et al.[32] applied proteomic analysis to identify pathogenic factors and potential biomarkers in the sera of MM patients. Pooled serum samples obtained from 8 MM patients and pooled serum samples from eight healthy donors were subjects for this analysis. The high abundance proteins, such as albumin and immunoglobulin, were depleted using peptide ligand library beads (PLLBs)-based affinity method. The low abundance serum proteins were identified using 1D gel-LC MS/MS. Altogether 789 and 849 proteins were identified in MM patients and healthy controls, respectively. Of these, 22 proteins, including serum amyloid A protein, vitamin D-binding protein isoform-1 precursor, kallikrein, apolipoprotein A-I, integrin alpha-1 and isoform-1 of multimerin-1, were differentially expressed. In addition, the

serum levels of integrin alpha-1 and isoform-1 of multimerin-1 were validated by Western blotting to demonstrate the diagnostic value of these biomarkers.

To identify the early diagnostic biomarkers, the study by Barceló et al.[143] in 2018 demonstrated the use of the MALDI-TOF technique to examine sera obtained from 103 MGUS patients and 108 healthy volunteers. Their ultimate objective is to develop a specific model for predicting the presence of MGUS. In this study, their developed model can classify unknown serum samples as belonging to either MGUS patients or healthy individuals with 88% average model accuracy, 89% average sensitivity and 86% average specificity.

2.7.2.3 Proteomics analysis to identify the potential biomarkers for predicting the response and resistance to chemotherapy

Dytfeld et al.[144] reported alteration in the BM proteomic profile in MM patients who responded to bortezomib-based chemotherapy. Comparative proteomic analysis was conducted using three independent quantitative approaches, MALDI-iTRAQ, ESI-iTRAQ and LFQ techniques. The proteomic data were compared among healthy control, patients who responded less than CR or VGPR and patients who achieved CR or VGPR. The proteins associated with proteasome function and protein folding, such as proteasome activator complex (PSME) subunit 1 (PSME1), PSME2 and heat shock protein 90 (HSP90), exhibited upregulation in RRMM patients. Similarly, the proteins involved in oxidative stress and cell hemostasis, such as thioredoxin (TXN), thioredoxin-like protein 1 (TXNL1) and lactoylglutathione lyase, were upregulated, whereas proteins involved in the regulation of apoptotic processes and programmed cell death, such as ANXA1, ANXA6 and LGALS1, were downregulated in RRMM patients.

Another report was conducted by Łuczak et al.[33] in 2017. This group reported the alteration in several proteins, including apolipoprotein C1 (ApoC1), complement components and sulfhydryl oxidase 1, involved in crucial biological processes, such as regulation of hydrolase activity and cellular response to the stimulus. These novel molecules can be used as biomarkers to predict the response to bortezomib-based chemotherapy in MM patients In the same year, Ting et al.[37] performed the proteomic analysis in serum samples obtained from 17 MM patients (9 responders and 8 non-responders) using the LFQ technique to discover biomarkers to predict response

to treatment containing bortezomib. An additional 20 MM patient serum samples were used for the ELISA-based validation phase. The results showed that the levels of clusterin (CLU) and angiogenin (ANG) concentration were significantly higher in the responders group, while complement C1q had a higher level in the non-responders group. Interestingly, this study demonstrated the use of these novel biomarkers in combination with standard biomarkers, including Alb, β_2M, M-protein and κ/λ ratio, as a potential panel of biomarkers to predict response to treatment containing bortezomib.

A recent study by Chanukuppa et al.[145] demonstrated the role of exportin 1 (XPO1) in bortezomib resistance. Here, 112 differentially expressed proteins were identified using iTRAQ and LFQ proteomic approaches in bortezomib-resistant MM cell lines (RPMI 8226-R), and the interesting candidate protein, XPO1, was selected for further study. XPO1 is a well-known nuclear exporter, and previous reports suggested that overexpression of XPO1 was a common feature observed in both solid and hematological malignancies.[147] Using functional studies like cell count assay, flow cytometry assay and soft agar assay, the results proved that XPO1 knockdown in RPMI 8226-R cell line results in re-sensitization to bortezomib drug. This result indicates that XPO1 plays a crucial role in emerging bortezomib resistance and could be a potential therapeutic target for MM treatment.

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Study population

3.1.1 Patients

Patients diagnosed with MGUS and MM presented at the Division of Hematology, Department of Medicine, Phramongkutklao Hospital (Bangkok, Thailand) between May 2020 and December 2021 were enrolled. Patient demographic data, including age, sex and date of diagnosis, were collected by retrospective medical record reviews. Only newly diagnosed patients, laboratory parameters, such as M-protein levels, IFE, SFLC ratio, hematocrit, total protein, albumin, creatinine, serum calcium, β_2M, LDH levels and cytogenetic detection by FISH technique, were collected at first diagnosis. All patients were treated with appropriated drug regimen according to the status of their MM. Patients' responses were assessed according to IMWG response criteria.[119, 131] Clinical events, including disease progression, relapsed or refractory to treatment and death, were recorded by prospective following. Additional 70 serum samples were collected from age-matched healthy donors and were used as normal control serum. Informed consent was obtained from all subjects under the Declaration of Helsinki. The study protocol was approved by Institutional Review Board Royal Thai Ammy Medical Department, Bangkok, Thailand (approved No. IRBRTA 433/2563, Subject No. S076h/62) and The Human Research Ethics Committee of Thammasat University, Pathumthani, Thailand (HREC-TUSc, COE No. 015/2564, **Appendix A**).

3.1.1.1 Inclusion criteria

Subjects eligible for inclusion in this study must meet all of the following criteria:

(1) Patients must be diagnosed with MGUS or MM based on the 2014 revised IMWG criteria for the diagnosis of MM and related disorders.[17]

(2) Patients must be evaluated for MM staging based on the ISS criteria[114] or R-ISS crteria[118] for MM.

(3) All patients must be evaluated the based-line SPEP, IFE and SFLC at first diagnosis before the treatment.

3.1.1.2 Exclusion criteria

Subjects meeting any of the following criteria are not eligible for inclusion in this study

(1) Subjects with prior malignancy.

(2) Subjects with a history of organ transplant or allogeneic hematopoietic stem cell transplant before MGUS or MM diagnosis.

(3) Subjects who received any blood component, especially plasma or platelet transfusion, within 90 days before blood sample collection.

3.1.2 Serum samples

In this study, 70 age-matched healthy donors, 20 MGUS and 139 MM patients were enrolled. A total of 465 serum samples, including 70, 20, 57, 228 and 90 serum samples were obtained from healthy donors, patients with MGUS, newly diagnosed MM (NDMM), MM with the response to treatment at least VGPR (RESP) and refractory/relapsed MM (RRMM), respectively. The time points of sampling are shown in **Figure 3.1.**

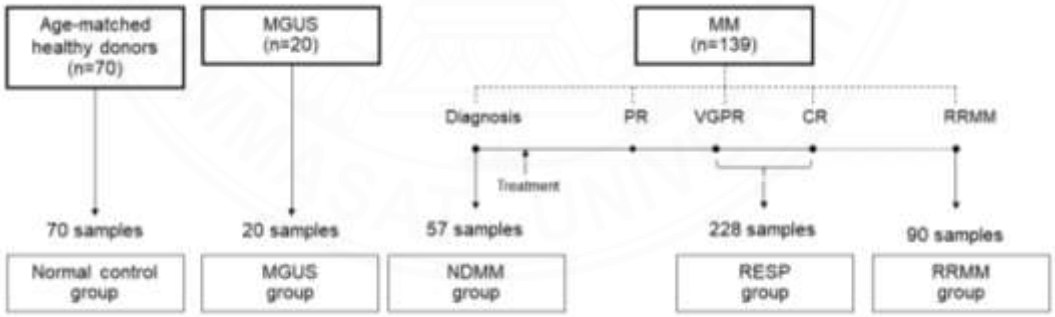

Figure 3.1 A schematic diagram demonstrating the time points of sampling

All serum samples were obtained from Special Hematology Laboratory, Phramongkutklao Hospital, as leftover specimens. Four milliliters of blood samples were collected in a serum clot activator tube (VACUTTE®, Greiner Bio-One, Thailand), incubated for 30 minutes at room temperature to allow completely clotting, and then centrifuged at 1,500 × *g* for 10 minutes to separate the serum. These serum

samples were divided into five groups based on the time point of sampling, including MGUS, NDMM, RESP, RRMM and normal control samples (**Figure 3.1**). Each serum sample was aliquoted into 3 microtubes (200 μL per microtube), and all serum samples were stored at −80ºC until used for proteomic analysis.

3.1.3 Pooled serum samples

To reduce variation which may occur among individual samples, 10-50 serum samples were pooled in each study group. Briefly, each individual sample was diluted by a 1:50 ratio using distilled water (4 μL of serum + 196 μL of distilled water). The protein concentration of each diluted sample was determined, then 5 μg of protein from each sample was pooled. The detail of each pooled sample is listed in **Table 3.1**.

Table 3.1 Pooled serum sample preparation

Sample No.	Number of samples	Pooled sample	Description
N001-N025	25	Normal-1	Age-matched healthy donors (Normal control)
N026-N050	25	Normal-2	
N051-N070	20	Normal-3	
MGUS001-MGUS020	20	MGUS-1	MGUS patients
NDMM001-NDMM020	20	NDMM-1	Newly diagnosed MM patients
NDMM021-NDMM040	20	NDMM-2	
NDMM041-NDMM057	17	NDMM-3	
RESP001-RESP050	50	RESP-1	MM patients with response >VGPR
RESP051-RESP100	50	RESP-2	
RESP101-RESP150	50	RESP-3	
RESP151-RESP200	50	RESP-4	
RESP201-RESP228	28	RESP-5	

Table 3.1 Pooled serum sample preparation (Cont.)

Sample No.	Number of samples	Pooled sample	Description
RRMM001-RRMM020	20	RRMM-1	Refractory/relapsed MM patients
RRMM021-RRMM040	20	RRMM-2	
RRMM041-RRMM060	20	RRMM-3	
RRMM061-RRMM080	20	RRMM-4	
RRMM081-RRMM090	10	RRMM-5	

3.2 Materials

3.2.1 Proteomic analysis using nano-LC–MS/MS

Table 3.2 Plastics used for LC–MS/MS

Plastics	Supplier
Microtubes (0.2/0.5/1.5 mL) Axygen®	Corning Life Science, China
Falcon tubes (15 mL)	Fred Baker Scientific, UK
Pasteur pipette (3 mL)	Greiner bio one, UK
Pipette tips (10/20/100/200/1000 μL)	Greiner bio one, UK

Table 3.3 Chemicals and reagents used for LC–MS/MS

Reagents	**Supplier**
Bovine serum albumin (BSA)	Sigma-Aldrich, USA
10 mM dithiothreitol (DTT)	Thermo Scientific, UK
10 mM ammonium bicarbonate (AMBIC)	Thermo Scientific, UK
30 mM iodoacetamide (IAA)	Thermo Scientific, UK
Sequencing grade trypsin	Promega, Germany
0.1 % formic acid	Sigma-Aldrich, USA
80% acetonitrile	Sigma-Aldrich, USA

Table 3.4 Equipment used for LC–MS/MS

Equipment	**Supplier**
Eppendorf BioSpectrometer® basic	Eppendorf, Germany
Dry bath incubator (HB-96D)	Daihan Scientific, Korea
Ultimate-3000 Nano/Capillary LC System	Thermo Scientific, UK
Hybrid Quadrupole Q-Tof Impact II™	Bruker Daltonics, Germany
µ-Precolumn holder, with 300 µm i.d. × 5 mm	Thermo Scientific, UK
µ-Precolumn holder, with 75 µm i.d. × 15 mm	Thermo Scientific, UK
Acclaim PepMap100 C18, 5 µm, 100 ºA	Thermo Scientific, UK
Acclaim PepMap RSLC C18, 2 µm, 100 ºA, nanoViper	Thermo Scientific, UK

3.2.2 ELISA

3.2.2.1 Human MTA2 ELISA commercial kit

Table 3.5 Reagents and materials provided in the MTA2 ELISA commercial kit

Reagents	Quantity (per kit)
Pre-coated, ready to use 96-well strip plate	1
Standard material	2
Detection reagent A	1 × 120 μL
Detection reagent B	1 × 120 μL
TMB substrate	1 × 9 mL
Wash buffer (30 × concentrate)	1 × 20 mL
Plate sealer for 96 wells	4
Standard diluent	1 × 20 mL
Assay diluent A	1 × 12 mL
Assay diluent B	1 × 12 mL
Stop solution	1 × 6 mL

3.2.2.2 Human AGO2 ELISA commercial kit

Table 3.6 Reagents and materials provided in the AGO2 ELISA commercial kit

Reagents	Quantity (per kit)
Pre-coated, assay plate (12 × 8 Microwells)	1
Standard material	2
Biotin-antibody (100 × concentrate)	1 × 120 μL
HRP-avidin (100 × concentrate)	1 × 120 μL
Biotin-antibody diluent	1 × 15 mL
HRP-avidin diluent	1 × 15 mL
Sample diluent	1 × 50 mL
TMB substrate	1 × 10 mL
Wash buffer (25 × concentrate)	1 × 20 mL
Plate sealer for 96 wells	4
Stop solution	1 × 10 mL

3.2.2.3 Other material required for ELISA

Table 3.7 Other materials required for ELISA

Materials	Supplier
Microplate reader, Synergy™ HT	Bio-Tek instrument, USA
Dry bath incubator (HB-96D)	Daihan Scientific, Korea
Multi-channel pipette, Axypet®	Corning Life Science, China
Pipette tips (10/20/100/200/1000 μL)	Greiner bio one, UK
Microtubes (0.5/1.5 mL) Axygen®	Corning Life Science, China
UltraPure™ distilled water	Invitrogen, USA

3.3 Experimental design

The overview workflows of this study are shown in **Figure 3.2**. In the discovery phase, five groups of pooled plasma samples obtained from age-matched healthy donors, MGUS, NDMM, RESP and RRMM patients were included. The plasma proteins were in-solution digested by trypsin and injected into a mass spectrometer (LC–MS/MS). The LC–MS/MS analysis of each pooled sample is done in triplicate. The proteomic profiles for each study group were analyzed using bioinformatics and statistical tools. The candidate serum biomarkers were identified and verified using publicly available gene expression databases and a literature review. In the verification phase, additional serum samples were obtained separately from age-matched healthy donors (n = 20), MGUS (n = 15), NDMM (n = 35), RESP (n = 50) and RRMM (n = 40). The serum concentrations of the interesting biomarkers were measured by ELISA technique and were compared among the study groups. In the validation phase, paired-serum samples collected from the same patient but with the difference in time point, were obtained from MM patients, including paired-serum samples collected at first diagnosis and at the time of response (NDMM-RESP, n = 30) and paired-serum samples collected at the time of response and at the time of relapse (RESP-RRMM, n = 30). The serum concentrations of the interesting biomarkers were measured and compared individually.

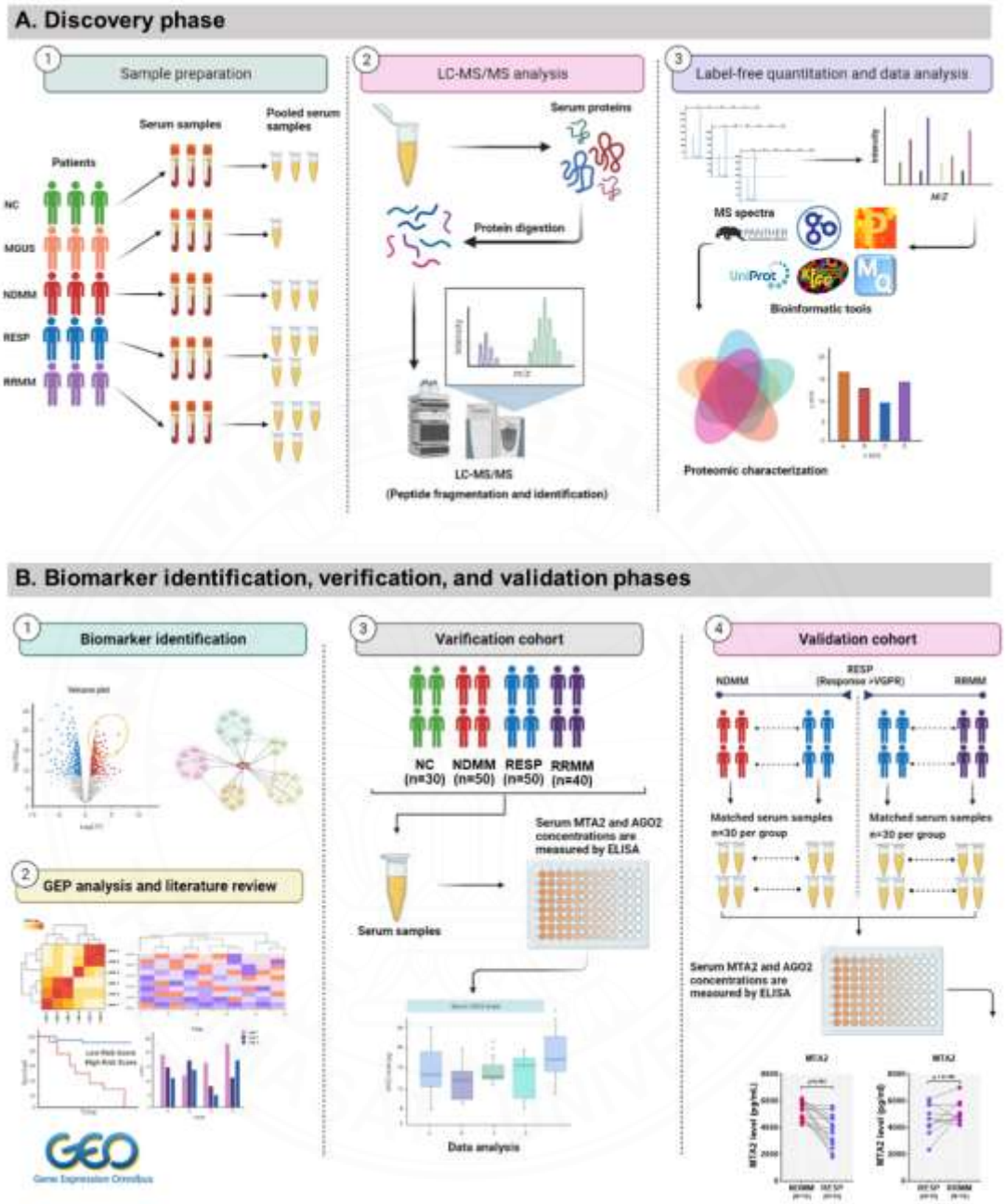

Figure 3.2 The overview workflows of this study. (A) Discovery phase. (B) Biomarker identification, verification and validation phases.

3.4 Proteomic analysis using nano-LC–MS/MS

3.4.1 Sample preparation

The protein concentration of pooled serum samples was determined by Lowry assay using bovine serum albumin (BSA) as a standard protein.[148] Briefly, 10 mM dithiothreitol (DTT) in 10 mM ammonium bicarbonate (AMBIC) is added into the protein solution to reduce disulfide bonds. The reformation of disulfide bonds in the protein is blocked by alkylation by adding 30 mM iodoacetamide (IAA) in 10 mM AMBIC.

3.4.2 Protein digestion

Five micrograms of each protein sample were subjected to in-solution digestion. Samples were completely dissolved in 10 mM AMBIC. To reduce disulfide bonds, 5 mM DTT in 10 mM AMBIC was added, then incubated at 60ºC for 1 hour, followed by alkylation of sulfhydryl groups by adding 15 mM IAA in 10 mM AMBIC and left at room temperature for 45 minutes in the dark. For digestion, samples were mixed with 50 ng/µl of sequencing grade Trypsin (Promega, Germany) by 1:20 ratio and incubated at 37ºC overnight. Before LC–MS/MS analysis, the digested samples must be dried and protonated with 0.1 % formic acid.

3.4.3 LC/MS-MS

The tryptic peptide samples were prepared for injection into an Ultimate 3000 Nano/Capillary LC System (Thermo Scientific, UK) coupled to a Hybrid Quadrupole Q-Tof Impact II™ (Bruker Daltonics, Germany) equipped with a Nano-captive spray ion source. Briefly, 1 µL of peptide digests were enriched on a µ-Precolumn 300 µm i.d. × 5 mm C18 Pepmap 100, 5 µm, 100 ºA (Thermo Scientific, UK), separated on a 75 µm I.D. × 15 cm, and packed with Acclaim PepMap RSLC C18, 2 µm, 100 ºA, nanoViper (Thermo Scientific, UK). The C18 column was enclosed in a thermostatted column oven set to 60°C. Solvents A and B containing 0.1% formic acid in water and 0.1 % formic acid in 80% acetonitrile, respectively, were supplied in the analytical column. A gradient of 5–55% solvent B was used to elute the peptides at a constant flow rate of 0.30 µL/min for 30 minutes. Electrospray ionization was carried out at 1.6 kV using the CaptiveSpray. Nitrogen was used as a drying gas (flow rate of 50 L/hour). Collision-induced-dissociation (CID) product ion mass spectra were

obtained using nitrogen gas as the collision gas. Mass spectra and MS/MS spectra are obtained in the positive-ion mode at 2 Hz over the range (*m/z*) 150–2200. The collision energy was adjusted to 10 eV as a function of the *m/z* value. The LC-MS analysis of each sample was done in triplicate.

3.4.4 Bioinformatics and data analysis

MaxQuant 1.6.6.0 were used to quantify the proteins in individual samples using the Andromeda search engine to correlate MS/MS spectra to the Uniprot *Homo sapiens* database.[149] Label-free quantitation with MaxQuant's standard settings was performed: maximum of two miss cleavages, a mass tolerance of 0.6 daltons for the main search, trypsin as digesting enzyme, carbamidomethylation of cystein as fixed modification, and the oxidation of methionine and acetylation of the protein N-terminus as variable modifications. Only peptides with a minimum of 7 amino acids, as well as at least one unique peptide, were required for protein identification. Only proteins with at least two peptides and at least one unique peptide, were considered as being identified and used for further data analysis. Protein false discovery rate (FDR) was set at 1% and estimated by using the reversed search sequences. The maximal number of modifications per peptide was set to 5. As a search FASTA file, the proteins presented in the *Homo sapiens* proteome were downloaded from Uniprot. Potential contaminants presented in the contaminants.fasta file that comes with MaxQuant were automatically added to the search space by the software.

The MaxQuant ProteinGroups.txt file was loaded into Perseus Version 1.6.6.0,[150] potential contaminants that did not correspond to any UPS1 protein was removed from the data set. Max intensities were $\log_2$ transformed, and pairwise comparisons between conditions were done via *t*-tests. Missing values were also imputed in Perseus using constant value (zero). The visualization and statistical analyses were conducted using the MultiExperiment Viewer (MeV) in the TM4 suite software.[151]

To explore the potential functions of differentially expressed proteins and the pathways that these proteins might be involved, the Protein Analysis Through Evolutionary Relationships (PANTHER Version 11.1, available from: http://www.pantherdb.org/) analysis was performed by keeping the *Homo sapiens* as a selected organism.[152] Bioinformatics analyses, including Gene Ontology (GO)

annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and protein domain functional enrichment, were performed to classify the functions of the identified proteins. When FDR was <1.0%, GO terms and KEGG pathways were significantly enriched. In addition, a protein-protein interaction (PPI) network was constructed using STITCH Version 5.0 (available from: http://stitch.embl.de/) to analyze the common and the forecasted functional interaction networks between identified proteins and small molecules.[153]

3.5 MTA2 and AGO2 levels measurement using ELISA

The concentrations of MTA2 and AGO2 in serum samples were measured using commercial ELISA kits (Human MTA2 [MBS2705865] and Human Proteins Agonaute-2/EIF2C2 [MBS910054] ELISA kits, MyBioSource, CA, USA). The assay procedure was performed according to the manufacturer's instruction.

3.6 Statistical analysis

Continuous variables were presented as mean, median, standard deviation (SD) and/or interquartile range (IQR) and compared using Student t-test, paired t-test, Mann–Whitney U test, Kruskal–Wallis test and ANOVA test. Categorical variables were described as frequency and percentage. The receiver operating characteristic (ROC) with the area under the curve (AUC) was used to determine the optimal cut-off providing high sensitivity and specificity for each protein. Clinical data and laboratory parameters were compared among diverse groups using Chi-Square (χ^2) or Fisher's exact tests, as appropriate. A Kaplan–Meier plot for time to response (TTR) and PFS was analyzed to determine the association between biomarkers and patients' outcomes. The hazard ratio (HR) with a 95% confidence interval (CI) was calculated using univariate and/or multivariate Cox regression analysis. Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism, Version 9 (GraphPad Software, CA, USA). A p-value of less than 0.05 was considered statistically significant.

CHAPTER 4
RESULTS

4.1 Serum proteomics characterization

4.1.1 Baseline characteristics of the study population

In this study, 70 age-matched healthy donors, 20 patients with MGUS and 139 patients with MM were enrolled. The baseline characteristics of the study population are shown in **Table 4.1**. No significant difference was observed in age and gender among the study groups. In MGUS patients, the most common heavy and light chain isotypes were IgG and lambda light chain, which were found in 55.0% and 60.0% of the cases, respectively. Similarly, in MM patients, the most common heavy chain isotype was IgG, which was found in 61.9%, followed by IgA, found in 24.5% of the cases. MM with free-light chains was observed in 12.8% of MM cases. Kappa light chain was the most common isotype found in 56.1% of MM cases. The ISS stages were evaluated for MM patients. Most of them were ISS-stage III (66.2%), followed by ISS-stage II (20.1%) and ISS-stage I (13.7%), respectively.

Table 4.1 Baseline characteristics of the study population

Characteristics	Study groups		
	Healthy donors (n = 70)	**MGUS (n = 20)**	**MM (n = 139)**
Age, years			
Mean ± SD	60.4 ± 10.2	65.1 ± 7.4	62.2 ± 12.0
Gender, n (%)			
Male	37 (52.9)	11 (55.0)	80 (57.5)
Female	33 (47.1)	9 (45.0)	59 (42.5)
Heavy chain type, n (%)			
IgG	N/A	11 (55.0)	86 (61.9)
IgA	N/A	9 (45.0)	34 (24.5)
Other (IgM, IgD and IgE)	N/A	0 (0.0)	1 (0.8)
None	N/A	0 (0.0)	18 (12.8)
Light chain type, n (%)			
Kappa (κ)	N/A	8 (40.0)	78 (56.1)
Lambda (λ)	N/A	12 (60.0)	61 (43.9)
ISS stage, n (%)			
I	N/A	N/A	19 (13.7)
II	N/A	N/A	28 (20.1)
III	N/A	N/A	92 (66.2)

Ig, immunoglobulin; ISS, The International Staging System; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; N/A, not applicable.

4.1.2 The overviews of serum proteomic profiles

A total of 1,783 proteins were identified using LC–MS/MS with FDR <1.0%. Of these, 772, 581, 830, 1,425 and 1,301 were serum proteins identified in normal, MGUS, NDMM, RESP and RRMM groups, respectively. The overlapping of the protein expression among the study groups was visualized by Venn's diagram (**Figure 4.1A**). The results revealed that 126 proteins were common proteins identified in all study groups, and 80 proteins were common proteins identified only in disease groups. Interestingly, 8, 10, 17, 67 and 70 were signature proteins identified in normal, MGUS, NDMM, RESP and RRMM groups, respectively.

To explore the potential functions of differentially expressed proteins and the pathways these proteins might be involved, the PANTHER Version 11.1 (available from: http://www.pantherdb.org/) analysis was performed by keeping the *Homo sapiens* as a selected organism. Bioinformatics analyses, including GO annotation and KEGG functional enrichment, were performed to classify the functions of the identified proteins. Significant GO terms and KEGG pathways enrichment were considered when FDR was <1.0%. In addition, a PPI network was constructed using STITCH Version 5.0 (available from: http://stitch.embl.de/).

Using the PANTHER database, only 1,281 of 1,738 expressed proteins were described in GO analysis. The proteins were classified based on their respective biological process (BP), molecular function (MF) and cellular component (CC), as shown in **Figure 4.1B**. According to the BP classification, most of them (383 of 1,281, or 29.9%) were associated with cellular processes involving cellular component organization, cellular response to stimuli and signal transduction processes. Approximately 19.0% (244 of 1,281) and 18.4% (236 of 1,281) of the expressed proteins were associated with metabolic and biological regulation processes, respectively. Based on the MF, most identified proteins were involved in molecular binding, catalytic activity and molecular regulators. Furthermore, most identified proteins were classified as structural, membrane and intracellular or cytoplasmic proteins. Only a few of them were protein-containing complexes, such as ribonucleoprotein complex, catalytic complex and membrane protein complex.

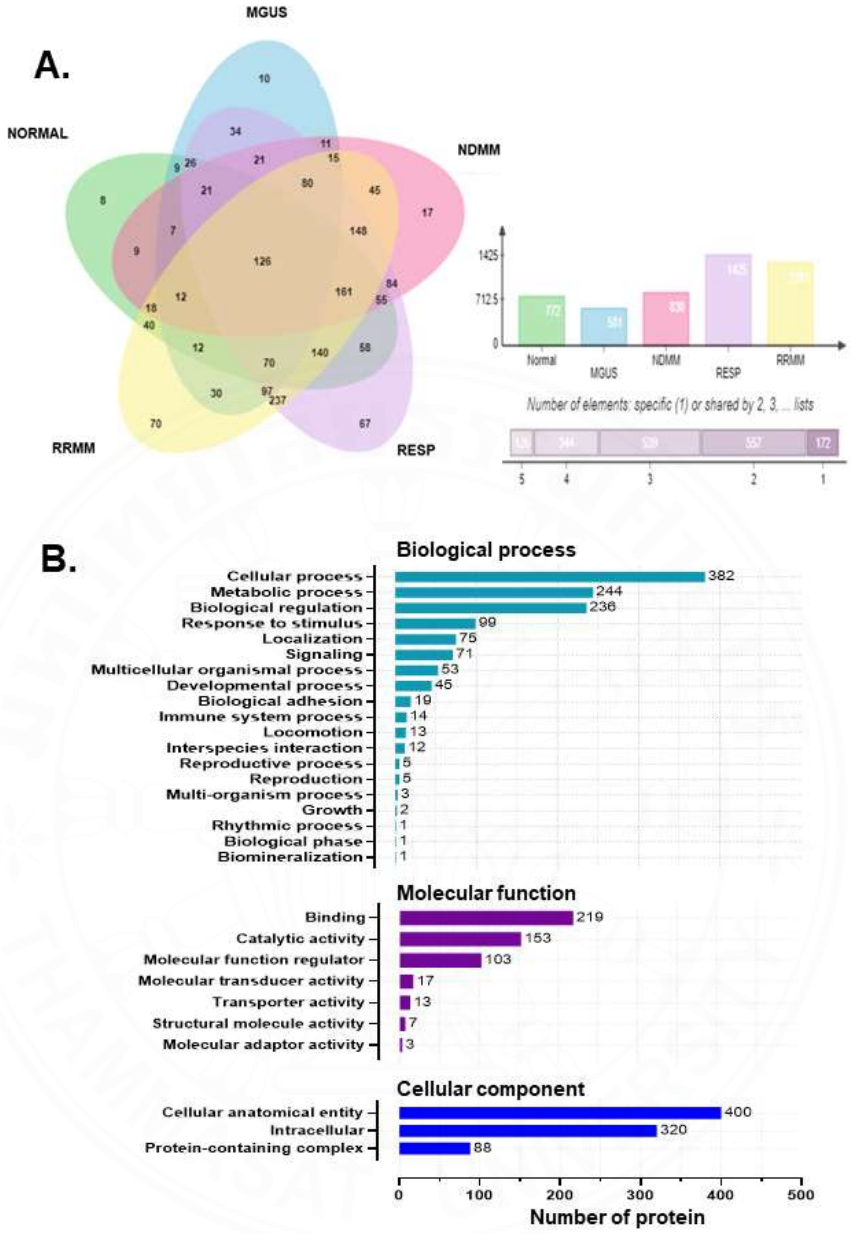

Figure 4.1 Differentially expressed proteins identified by LC–MS/MS and GO classification. (A) Venn diagram illustrating the overlap between the serum proteins detected in normal, MGUS, NDMM, RESP and RRMM. (B) PANTHER GO protein classification of the differentially expressed proteins.

4.1.3 The common serum proteins

Altogether 126 common proteins were identified in all study groups. Consistent with the basic serum protein components, the most abundant serum protein detected in this analysis was Alb, which showed the highest log_2 intensity in all study groups, followed by several kinds of immunoglobulins and apolipoproteins (**Figure 4.2**). The GO classification of the common proteins is shown in **Figures 4.3A** and **4.3B**. Moreover, to compare the relative expression levels of the identified proteins, the row Z-score was calculated, and a heatmap was plotted to illustrate the comparative expression levels among the study group (**Figure 4.3C**).

Based on their BP, the proteins involved in the metabolic process and cellular component organization or biogenesis were majority proteins accounting for 26.9% and 19.2%, respectively. In terms of MF, these proteins functioned as catalytic (43.7%), binding (37.5%) and functional regulatory molecules (18.8%). In addition, the majority of the common proteins were classified as cellular structural proteins (45.6%) and intracellular proteins (**Figure 4.3B**).

Accession No.	Protein name	Score	Normal	MGUS	NDMM	RESP	RRMM
A8K9P0	Albumin	323.3	22.4	24.3	23.2	24.8	23.8
S6B291	IgG H chain	152.9	20.3	22.3	22.4	20.3	20.9
Q6PJF2	IGK@ protein	323.3	20.2	22.5	22.3	21	22.9
Q6N092	DKFZp686K18196 (Fragment)	323.3	20	22.5	20.5	20.4	21.9
Q6N093	DKFZp686I04196 (Fragment)	7.5	19.9	19.1	22.9	20.8	20.3
U5KJ79	dC->dU-editing enzyme APOBEC-3G	15.0	19.4	18.6	10.8	17.9	19
O95347	SMC protein 2(SMC-2)	10.3	19.2	16.3	14.3	12.8	16
Q92824	Proprotein convertase subtilisin/kexin type 5	13.4	19.1	13.9	9.89	16.4	16.2
A0A2R8YEM9	TPR and ankyrin repeat-containing protein 1	21.6	18.8	19.7	15.1	19.1	19.3
Q6PJR7	IGL@ protein	166.4	18.6	20.1	21.2	19.5	22.2
P51512	Matrix metalloproteinase-16 (MMP-16)	10.4	18.6	12.9	13.4	20	16.5
A0A024R3E3	Apolipoprotein A-I, isoform	114.3	18.4	19.8	18	22.3	19.4
Q6AI59	DKFZp781H0795 (Fragment)	22.0	18.4	13.2	13.9	17.2	19
A0A2X0U2G7	ARHGEF28 (Fragment)	14.4	18.4	14.5	12.4	19.3	16.5
A0A1W2PQ71	Potassium voltage-gated channel KQT3	9.1	18.3	13.4	19.7	19.8	18.6
E9KL23	Serpin peptidase inhibitor clade A member 1	187.0	18	21.2	19.2	20.5	20.4
B7ZVX0	DOCK9 protein	13.1	17.6	14.7	16.4	19.4	17.4
A0A0U1RRH1	Ryanodine receptor 3	12.5	17.6	12.8	17.3	19.7	19.7
P01023	Alpha-2-macroglobulin (Alpha-2-M)	8.5	17.5	20.5	17.4	19.9	20.4
A0A0U1RRM1	Transcriptional repressor p66-beta	11.8	17.4	13.6	11.6	19.2	21.1
Q15172	PP2A B subunit isoform B'-alpha	6.1	17.2	15.5	12.2	14.2	18.1
A0A2S0BDD1	Antithrombin-III (Serpin C1)	6.6	17.1	14.5	17.6	18.2	17.4
A0A024RDC2	WD repeat and FYVE domain containing 3	8.1	17.1	16.7	14.8	19.4	14.2
B0I1T2	Unconventional myosin-Ig	8.1	17	13.3	16.7	18.5	16.7
P0CG39	POTE ankyrin domain family member J	8.8	16.9	13.9	19.9	12.4	18.7
Q53H26	Beta-1 metal-binding globulin	30.0	16.9	20.3	18.6	20.8	19.7
A8K8U1	CAND1	12.7	16.8	19.4	17.5	16.1	18.1
Q2M389	WASH complex subunit 4	9.4	16.8	11.3	17.3	15.5	15.9
J3KRA9	Non-specific serine/threonine protein kinase	10.5	16.6	18.6	17.1	15.5	15.4
Q5EBM2	Uncharacterized protein	7.8	16.5	20	12.1	17.8	18
A0A5C2GFZ5	IG c514 (Fragment)	178.2	16.5	18	18.2	16.3	14.9
A0A7P0TBE7	WD repeat-containing protein 62	9.0	16.4	16.9	12.2	14.8	15.9
F5GXT3	Anoctamin	6.3	16.2	14.6	13.2	14.6	17.9
P00738	Haptoglobin	5.6	16.1	20.6	17.6	17.5	18.9
Q13402	Unconventional myosin-VIIa	7.9	16.1	11.3	15.3	20	13.9
Q5FWF6	Zinc finger protein 789	8.8	16	16	15.4	17.9	17.3
C6EMX8	DNA replication licensing factor MCM7	6.7	16	15.9	16.4	16.4	17.1
Q5T7N2	ES cell-associated protein 11	12.0	16	10.2	16.5	19.5	20.4
Q6UXN9	WD repeat-containing protein 82	5.7	15.9	16.8	17.8	18.2	18.3
Q96M86	Dynein heavy chain domain 1-like protein	20.9	15.9	12.2	19.4	18.5	16.3
Q5SWA1	Protein phosphatase 1 regulatory subunit 15B	9.9	15.9	11.2	18	13.1	15.1
A2RTY6	Inter-alpha (Globulin) inhibitor H2	16.5	15.8	15.8	15.5	18.7	17.4
J3KNG8	Folliculin-interacting protein 1	13.9	15.6	13	12.3	16.4	15.6
A0A384P5S9	Epididymis secretory sperm binding protein	6.7	15.6	14.4	14.1	15.1	15
Q7Z7G8	Vacuolar protein sorting-associated p13B	22.9	15.5	13.1	12.2	13.8	17.4
P02790	Hemopexin (Beta-1B-glycoprotein)	9.5	15.4	18.1	17.6	17.3	17.1
A0A024R3R7	HEAT repeat-containing protein 1	12.0	15.3	11.3	20.3	16.4	17.1
A0A3B3IT03	Zinc finger protein 600	11.7	15.1	12.5	11.1	20.1	17
A0A1S5UZ31	TRIO and F-actin-binding protein	23.1	15.1	13.8	13.1	11.2	13.4
Q14008	Cytoskeleton-associated protein 5 (	6.6	15	14	14.6	17.4	17.7
Q6ZRZ4	Uncharacterized protein C9orf47	6.0	15	14.6	15.3	16	15.6
B4DXR5	Golgi autoantigen	10.7	14.9	11.5	14.5	14.8	16.2
B3KMH8	Autophagy protein 5	7.6	14.9	13.1	13.4	16.9	15.6
Q9H2K8	Serine/threonine-protein kinase TAO3	8.3	14.9	12.9	14.5	16.7	14
Q86SQ0	Protein LL5-beta	7.7	14.9	15.5	10.6	16.3	16.6
Q9C0I3	Coiled-coil serine-rich protein 1	6.3	14.8	15.2	15.1	17.3	13.5
Q9NPG3	Ubinuclein-1 (HIRA-binding protein)	14.0	14.8	12.1	14.2	14.8	16.9
E7EVJ3	Glucosamine N-sulfotransferase	11.4	14.7	13.1	10.3	16.4	14.8
Q7Z2Z1	Treslin	10.3	14.7	13.6	20.1	14.6	13
P51532	Transcription activator BRG1	13.6	14.7	20.9	18.6	15.8	15.5
B4E3R6	Squamous cell carcinoma antigen	7.9	14.7	10.4	16.1	14.1	12.5
O14617	AP-3 complex subunit delta-1	12.4	14.5	13.8	10	13	20.3
Q9HD74	Zinc finger protein SBZF3	15.8	14.5	11.3	12.2	14.7	15.7

Accession No.	Protein name	Score	Normal	MGUS	NDMM	RESP	RRMM
A0A2S1ZR87	Structural maintenance of chromosomes 6	6.7	14.5	12.5	15.1	16.3	12.7
A0A7P0Z491	Abnormal SLM-associated protein	6.1	14.5	13.2	11.6	17.6	21
G8JLP4	Limkain-b1	15.3	14.4	17.7	14.4	15.9	16.1
P11498	Pyruvate carboxylase	9.7	14.4	14.6	14.2	15.3	16.5
B4DDI2	Neuronal guanine nucleotide exchange factor	7.7	14.3	12	16.8	15.2	12.6
H0YJZ6	Endoribonuclease Dicer	8.1	14.2	16.2	15.6	13.6	13.4
Q5T011	KICSTOR complex protein SZT2	6.1	14	15.3	12.7	14.6	18.4
Q12913	Protein-tyrosine phosphatase eta	6.4	14	13.3	19.2	17.7	15.6
Q59GK3	RAS p21 protein activator 1 isoform 1	11.8	13.9	17	17.5	14.2	15.7
H3BQQ2	E3 ubiquitin-protein ligase ZNF598	6.0	13.8	12.9	19.1	15.9	13.9
Q7KZY0	Matrix metalloproteinase 15	6.9	13.7	16.1	13.3	11.3	10.8
J3KNV1	Zinc finger protein 292	10.0	13.7	16.3	18.3	14.4	17.9
Q50KC8	Taste receptor type 2 (Fragment)	8.5	13.7	11.1	15	15.2	15.8
B4E015	G patch domain-containing protein 3	10.7	13.4	12.6	11.6	18.5	13.4
C9JEV4	Tectonin beta-propeller repeat-containing 1	6.6	13.4	11.4	21	14.3	12.8
O75901	Ras association domain-containing protein 9	8.9	13.4	12.5	16.7	16.8	14.4
Q8TF21	Ankyrin repeat domain-containing protein 24	6.8	13.3	15.7	14.5	18.1	14.3
Q9Y3R5	Protein dopey-2	12.2	13.2	10.8	13.7	18.4	15.2
Q4R1Q4	HAPRIN-a2 (Fragment)	17.3	13	12.7	14.1	19.4	16.1
Q02383	Semenogelin-2 (Semenogelin II) (SGII)	7.6	12.9	18.3	14.7	12	13.8
P42166	Lamina-associated polypeptide 2,	7.6	12.9	11.6	18.2	13.3	15.6
Q7LBC6	Lysine-specific demethylase 3B	12.7	12.8	13.3	14	12.6	14.7
Q5T5P2	Sickle tail protein homolog	9.6	12.8	9.68	12.3	13.8	12.9
Q96KG3	AKAP350C	8.1	12.7	13.5	19.7	17.8	13.3
O75182	Histone deacetylase complex subunit Sin3b	6.7	12.5	14	13	11.3	13.9
Q53HH4	Ras-GTPase-activating protein SH3	6.3	12.5	11.3	12.5	14	11.1
A0A2R8YDB0	CMP-N-acetylneuraminate-beta-1,4-galactoside	9.8	12.5	12.2	10	15.9	15
M0QY65	Sialic acid-binding Ig-like lectin 7 (Fragment)	8.2	12.4	13	14	15.8	14.8
B4DYA4	Kelch domain-containing protein 3	9.6	12.4	14.2	13.4	12.6	12.7
Q9NP71	ChREBP	6.4	12.4	17.2	12.6	12.3	13.6
Q9NS98	Semaphorin-3G (Semaphorin sem2)	7.4	12.4	11	13.5	14.2	11.4
A0A6Q8PGY3	Sodium channel protein type 11 subunit alpha	14.1	12.3	17.1	15.2	17.2	16.3
Q5JV89	Uncharacterized protein DKFZp434F1622	7.2	12.2	12.6	14.9	16	15.1
Q8N7W2	BEN domain-containing protein 7	6.0	12.1	15.1	12.9	11.9	12
H0Y6I0	Golgin subfamily A member 4 (Fragment)	18.9	12.1	15.5	11.7	12.8	19.2
A0A669KAW2	Uncharacterized protein	7.1	12	12.3	13.3	14.9	14.3
B4DW26	Interstitial collagenase	6.2	11.7	14.9	14.1	15.1	11.3
O15060	Zinc finger protein 39	7.4	11.6	16.8	12.8	16.3	15.9
Q2VF42	Glucose-6-phosphate 1-dehydrogenase	6.9	11.6	13.2	15.1	18.6	19.2
A0A024R663	Kinectin 1 (Kinesin receptor)	11.3	11.6	15	11.7	13	13.5
Q6ZTY9	LINC02902	7.8	11.5	13	12.2	11.4	11.8
Q6ZN50	Oxysterol-binding protein	8.5	11.3	13.9	13	12.3	15.5
P24557	Thromboxane-A synthase	9.4	11.2	17.6	11.8	11.3	12.8
Q9BV73	Centrosome-associated protein CEP250	11.6	11.1	14.4	13.6	12.6	14.5
O75691	Novel nucleolar protein 73 (NNP73)	19.9	11.1	18.2	12.7	16.7	14.2
O94782	Ubiquitin carboxyl-terminal hydrolase 1	10.1	11	11	16.5	15.4	13.3
Q96IR2	Zinc finger protein 845	7.8	11	13.7	12.8	15	14.5
J3QT09	Sentrin-specific protease 7	5.8	11	14	12.4	15.3	13.4
G3V3F7	X-linked retinitis pigmentosa GTPase p1	6.2	10.9	18.2	15.3	15	12.1
D3DSS6	Dedicator of cytokinesis 5, isoform CRA_a	13.2	10.9	15.4	15	15.7	16.5
Q9H2M9	Rab3 GTPase-activating protein	7.5	10.8	13.1	13.3	15.2	13.3
O14544	Suppressor of cytokine signaling 6 (SOCS-6)	9.1	10.7	11.8	14.7	17.3	13.3
H0YDJ3	PRP4 pre-mRNA-processing factor 4	7.4	10.7	13.5	16.8	14.3	12.2
C9JT67	Coiled-coil domain-containing protein 144A	7.8	10.6	10.2	16.2	13.4	14.8
Q7Z6P3	Ras-related protein Rab-44	6.5	10.5	12.3	17.1	13.2	13
A0JNW5	UHRF1-binding protein 1-like	11.0	10.3	13	17.7	15.1	14.3
Q5FBX5	Interleukin-7	8.2	10.2	11.4	12	12	13.2
Q8TEQ8	GPI ethanolamine phosphate transferase 3	7.8	10.2	13.2	11.9	14.1	12.1
Q2TAZ0	Autophagy-related protein 2 homolog A	9.6	9.98	12.5	12.2	11	12.9
P13533	Myosin-6 (Myosin heavy chain 6)	10.8	9.97	14.7	18.5	20.1	12.2
Q8IY85	EF-hand Ca-binding domain-containing p13	6.4	9.91	12.3	12	19.3	12.3
Q96SE4	DNA-directed DNA polymerase	8.8	9.87	14.1	13.1	13.3	12.9
C9JLR9	Zinc finger translocation-associated protein	9.8	9.78	15.6	17.1	13.7	19.9

Figure 4.2 The relative expression levels of 126 common proteins

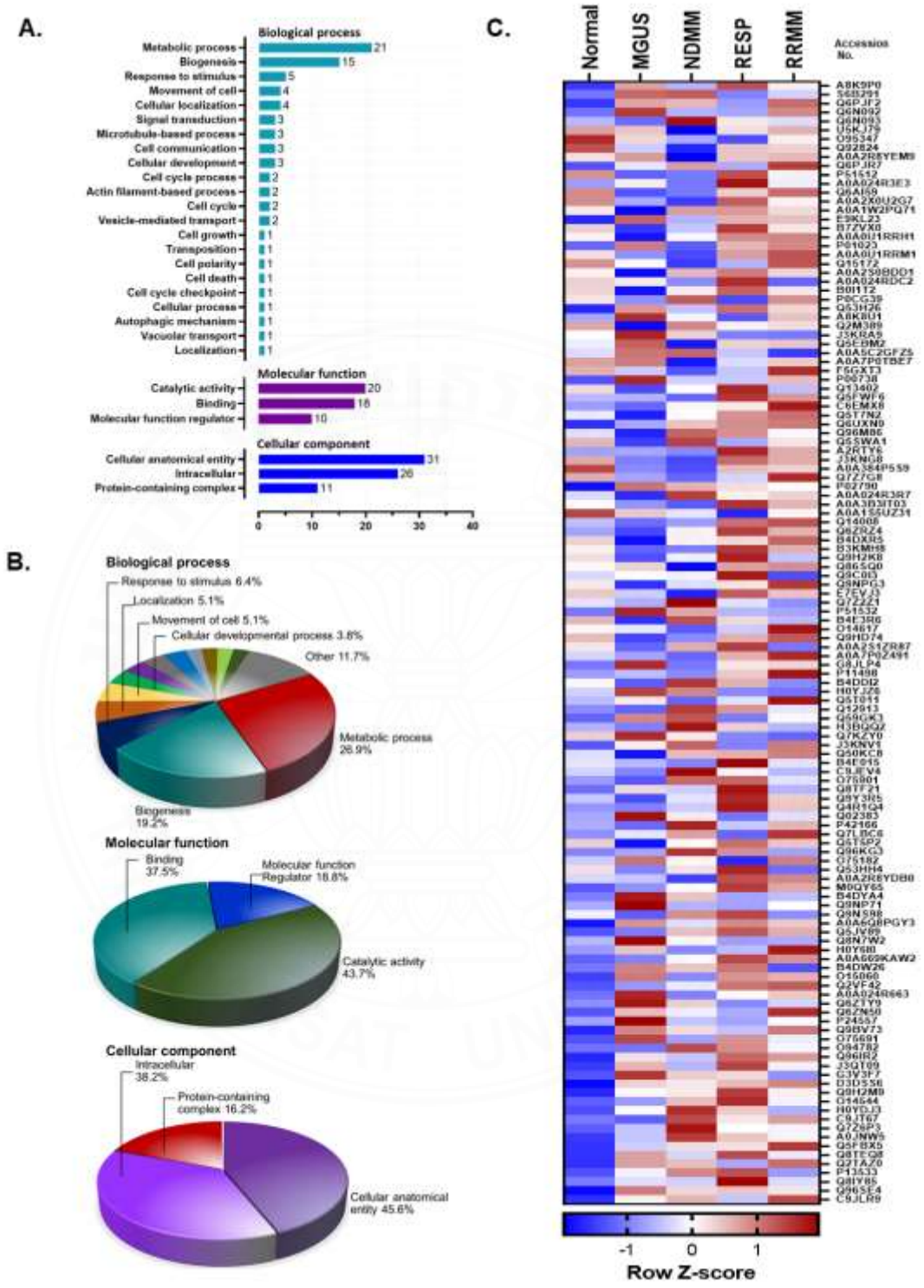

Figure 4.3 The common serum proteins differentially expressed among the study groups. (A-B) PANTHER GO protein classification of 126 common proteins. (C) Heatmap of 126 differentially expressed proteins showing the relative levels of expression among normal, MGUS, NDMM, RESP and RRMM groups.

We further analyzed the common proteins by individually comparing a fold-change of those between normal and NDMM groups. The fold-change was calculated using the difference between the log_2-intensity of NDMM and normal (log_2 [NDMM]-log_2 [normal]). We found that 81 proteins showed up-regulation and 45 proteins showed down-regulation in NDMM patients compared to the normal controls.

4.1.3.1 Upregulated common proteins

Consistent with the salient clinical picture of MM patients, several isotypes of immunoglobulin showed higher expression in MM compared to normal group. According to GO functional enrichment analyses (**Figure 4.4**), the common upregulated proteins were significantly related to cellular component organization or biogenesis, organelle organization and cell cycle process, as shown in **Figure 4.4A**.

The proteins involved in the positive regulation of the cell cycle process, such as treslin or TopBP1-interacting checkpoint and replication regulator and transcription activator BRG1, as well as the proteins involved in DNA replication, such as DNA polymerase, endonuclease dicer1, DNA replication licensing factor MCM7, were significantly upregulated in NDMM group. Almost upregulated proteins functioned in the DNA replication process, including nucleoside-triphosphate activity, helicase activity, chromatin binding, DNA helicase activity and DNA replication origin binding (**Figure 4.4B**). Based on CC classification, the majority of the upregulated proteins are the proteins located in the nucleus, nucleoplasm, nuclear lumen and chromosome (**Figure 4.4C**). Notably, we found that the proteins involved in the Ras family, including Ras-related protein Rab-44, Ras p21 protein activator 1 and Ras association domain-containing protein 9, were upregulated in MM serum. All Ras protein family belongs to a member of small GTPase proteins and are involved in the cell signaling process. The upregulation of these proteins is involved in the positive regulation of cell growth, differentiation and survival. Furthermore, the proteins involved in the ubiquitin-proteasome system (UPS), such as ubiquitin carboxyl-terminal hydrolase 1 and E3 ubiquitin-protein ligase, were also upregulated in MM patients.

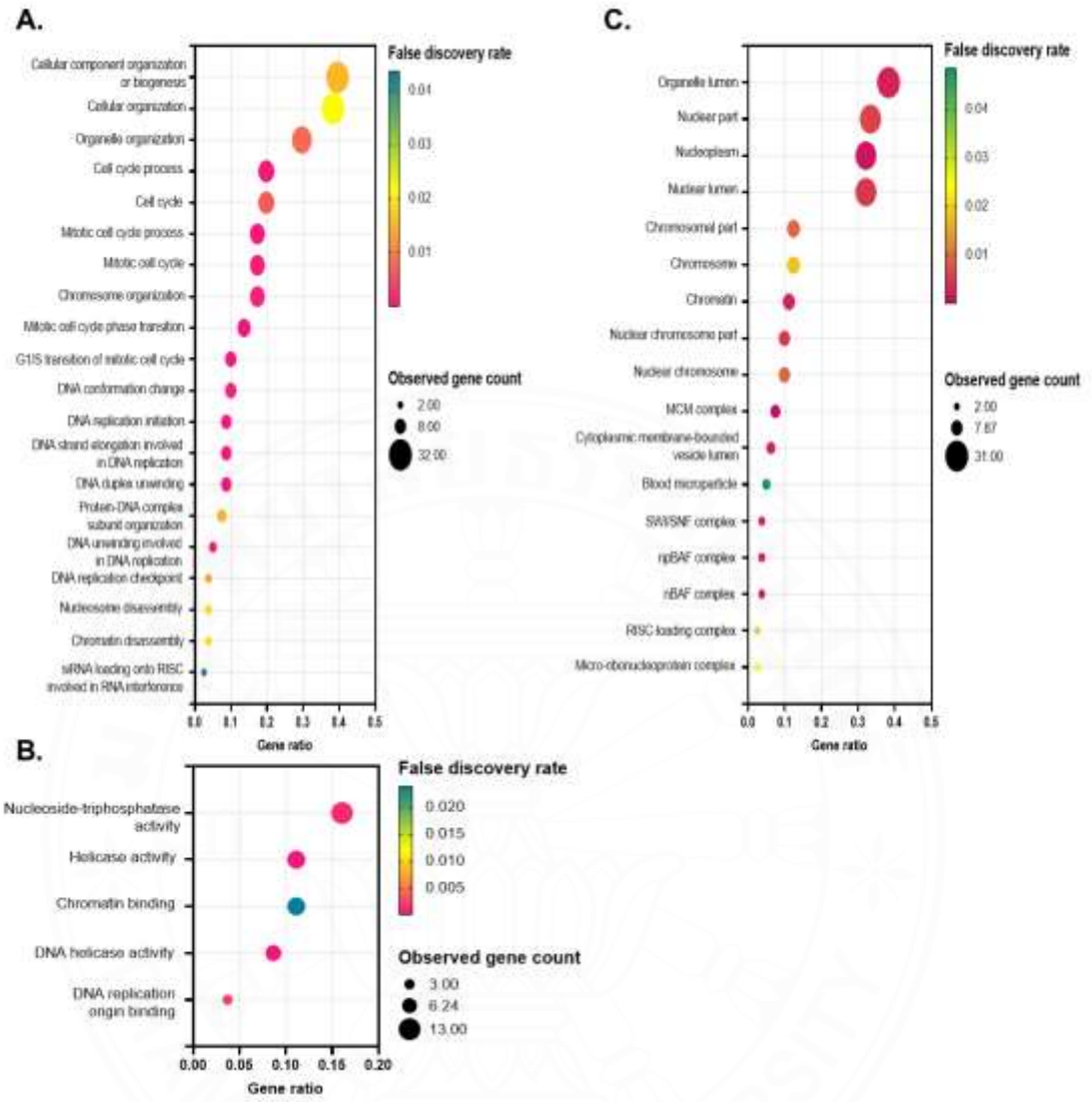

Figure 4.4 GO functional enrichment analysis of the 81 upregulated common proteins in NDMM. The proteins were classified according to the following GO categories: (A) biological process, (B) molecular function and (C) cellular component. The y-axis indicates the functional classification or pathway, and the x-axis indicates the protein-encoded gene ratio (observed gene count/total upregulated genes) of the respective function type. The color key that accompanies the bubble plot indicates the false discovery rate, and the bubble size indicates the observed gene count of the proteins in the respective functional class or pathway.

4.1.3.2 Downregulated common proteins

Among 45 downregulated proteins, the most abundant proteins involved in the negative regulation of catalytic activity and cellular protein metabolic process (**Figure 4.5A**). Most of these proteins are located on chromosome part, chromosome and condensin complex (**Figure 4.5B**). However, no significant respective MF was observed among these downregulated proteins.

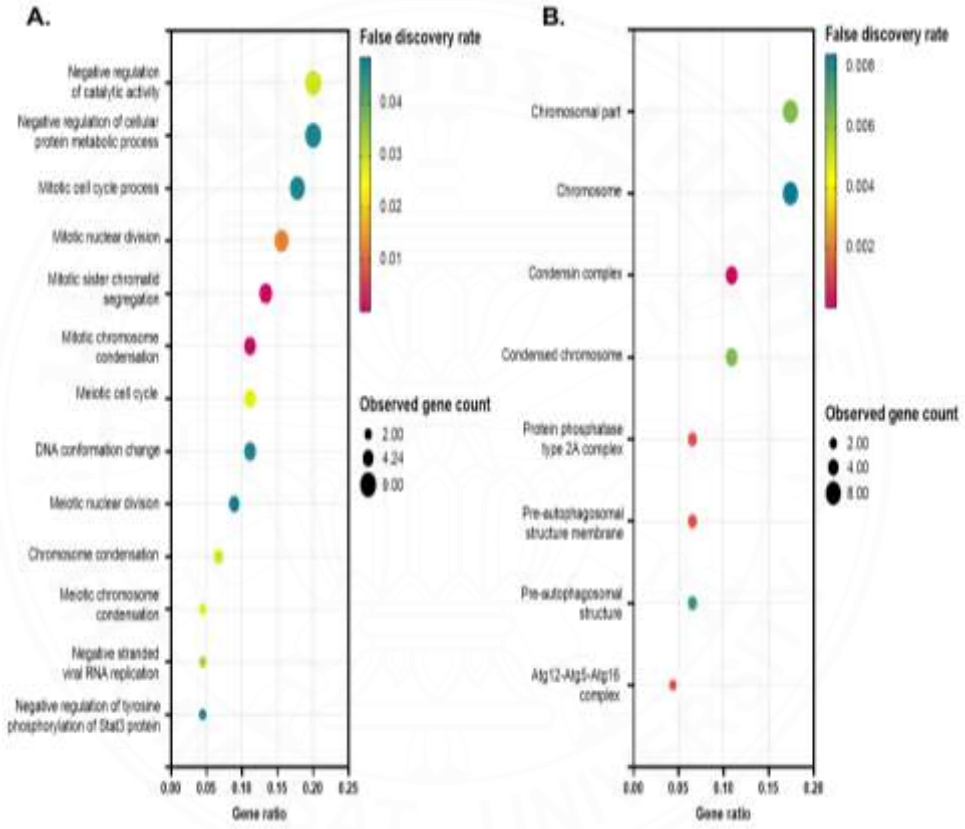

Figure 4.5 GO functional enrichment analysis of the 45 downregulated common proteins in NDMM. The proteins were classified according to the following GO categories: (A) biological process and (B) cellular component. The y-axis indicates the functional classification or pathway, and the x-axis indicates the protein encoded gene ratio (observed gene count/total downregulated genes) of the respective function type. The color key that accompanies the bubble plot indicates the false discovery rate, and the bubble size indicates the observed gene count of the proteins in the respective functional class or pathway.

4.1.4 The common serum proteins expressed in the disease groups

We found 80 serum proteins expressed in all disease groups but not normal serum. The lists and relative expression levels of these disease-associated proteins are shown in **Figure 4.6**.

Accession No.	Protein name	Score	Log2 intensity			
			MGUS	NDMM	RESP	RRMM
H3BRD5	Unconventional myosin-IXa	13.0	19.2	19.4	20.1	17.4
Q96RL1	BRCA1-A complex subunit RAP80	7.7	20.2	16.2	20.9	14.7
P98170	E3 ubiquitin-protein ligase XIAP	7.3	17.0	18.1	18.8	17.2
A0A1X7SBR9	Thrombospondin motifs 19	10.4	14.2	20.5	16.4	16.2
P05023	Sodium/potassium-transporting ATPase alpha-1	6.3	17.7	17.1	16.7	14.6
O75923	Dysferlin	11.0	15.7	16.0	16.2	17.3
A0A494C181	CCA tRNA nucleotidyltransferase 1	7.1	22.0	13.4	14.2	14.3
A0A0A0MRJ7	Coagulation factor V	9.1	12.3	16.9	16.4	18.3
Q9UKV8	Protein argonaute-2 (Argonaute2)	12.7	13.6	13.6	17.7	18.6
Q9HBG6	Intraflagellar transport protein 122 homolog	9.2	15.0	18.0	12.9	17.4
Q9Y4C4	Malignant fibrous histiocytoma-amplified seq 1	9.8	16.0	20.3	13.1	12.8
A0A6Q8PF19	Sodium leak channel non-selective protein	7.7	17.3	13.6	15.1	16.0
A0A1B0GXK6	Disks large-associated protein 2	11.8	13.7	15.8	17.4	14.9
F5H2D0	Complement subcomponent C1r	11.3	20.1	12.4	12.4	16.0
B7Z855	Ubiquitin carboxyl-terminal hydrolase 7	5.7	11.2	21.6	15.4	12.5
Q6P2Q9	Pre-mRNA-processing-splicing factor 8	6.7	15.3	14.5	15.8	15.1
Q9NT68	Teneurin-2 (Ten-2)	5.9	14.3	11.6	16.4	18.1
Q9NQV6	PR domain zinc finger protein 10	7.4	9.9	12.5	19.3	18.6
B7ZKX2	Uncharacterized protein	13.2	12.5	18.2	12.8	16.4
P02549	Spectrin alpha chain, erythrocytic 1	11.4	15.1	18.7	14.2	11.9
Q8IVM0	Coiled-coil domain-containing protein 50	8.1	14.1	12.7	18.9	14.1
Q02388	Collagen alpha-1(VII) chain	6.7	13.3	15.4	15.6	15.5
O75339	Cartilage intermediate layer protein 1	7.1	17.3	12.2	10.4	19.5
B4DZQ2	Alpha-actinin-3	10.3	18.3	11.7	14.8	14.6
A0A7P0T8N2	Cytoplasmic dynein 1 heavy chain 1	6.8	11.3	15.3	16.0	16.6
Q4G0X9	Coiled-coil domain-containing protein 40	9.5	11.2	16.1	14.0	18.0
Q96JB5	CDK5 regulatory subunit-associated protein 3	5.6	17.4	12.0	12.5	17.2
B4DVW8	Nuclear body protein SP140	7.8	10.5	14.4	17.6	16.3
A0A1W2PPX1	Cellular communication network factor 6	6.6	16.5	16.0	13.4	12.8
E5RJN3	Condensin-2 complex subunit H2	7.3	11.4	18.4	13.6	15.1
Q6UWX4	HHIP-like protein 2	9.3	11.4	16.2	17.0	13.2
C9JG08	Uncharacterized protein C2orf16	7.0	10.5	18.5	11.5	17.1
D3DP75	Rab3 GTPase-activating protein	6.8	14.2	17.7	12.6	13.1
Q96BY2	Modulator of apoptosis 1 (MAP-1)	6.9	17.5	12.5	14.7	12.7
P01871	Immunoglobulin heavy constant mu	28.8	18.8	12.9	12.6	12.9
Q8WWQ0	PH-interacting protein (PHIP)	9.9	12.9	13.5	15.1	15.7
Q69YQ0	Cytospin-A	5.9	13.1	14.0	15.2	14.8
Q5JY77	GASP-1	11.0	12.4	11.0	17.6	16.1
B2R969	MGAT3	6.5	11.9	15.9	11.3	17.8
Q8N4P8	Nucleolar GTP-binding protein 1 (Fragment)	8.3	15.1	15.5	13.1	13.2

Accession No.	Protein name	Score	Log2 intensity			
			MGUS	NDMM	RESP	RRMM
A8K6U0	IPLA2(GAMMA)	9.2	12.4	16.8	13.1	14.6
Q58F21	Bromodomain testis-specific protein	10.7	11.7	14.9	15.2	14.9
C9JSP0	Protein downstream neighbor of Son	11.4	13.8	15.3	12.7	14.9
Q9BRZ2	E3 ubiquitin-protein ligase TRIM56	5.9	11.7	11.2	12.6	20.8
E9PFB9	Coiled-coil domain-containing protein 18	6.2	13.0	10.1	14.7	18.2
B2RBS8	Albumin-like protein	7.2	18.7	10.0	14.0	13.4
Q9H2P0	Activity-dependent neuroprotective protein	7.0	11.9	13.8	15.7	14.4
Q8NDX6	Zinc finger protein 740	9.3	12.5	10.8	16.1	16.2
B4DZE1	Eukaryotic translation initiation factor 4	7.1	14.8	11.7	11.0	18.0
B4DMS0	Cysteine-rich protein 2-binding protein	8.3	13.5	16.5	11.9	13.5
P35579	Myosin-9	6.3	14.1	13.1	14.7	13.3
P51587	Breast cancer type 2 susceptibility protein	13.0	12.7	11.8	14.4	16.2
H0YNN7	Notch2-binding receptor 1	6.0	13.8	15.5	12.5	13.0
A0A087WTF0	Protein tyrosine phosphatase protein 1	9.9	15.0	14.8	13.1	11.7
B7Z6K4	Zinc finger protein 406	7.3	14.5	10.3	13.7	15.7
Q9H5L6	DNA transposase	9.3	13.2	13.8	13.9	13.0
I6L994	Serine/threonine-protein kinase RIO3	6.7	13.4	11.8	15.6	12.9
O00300	Osteoprotegerin	7.4	13.0	16.2	12.2	12.1
A0A0C4DGN6	ARF GTPase-activating protein GIT1	7.9	13.6	12.7	14.0	13.2
E7EUH7	Pseudouridylate synthase 7 homolog	7.8	13.5	15.0	12.5	12.3
Q5SQS8	Uncharacterized protein C10orf120	8.4	10.3	11.5	14.1	17.4
Q9Y2F5	Little elongation complex subunit 1	6.0	13.9	12.8	12.7	13.7
Q96DX9	Amyloid beta (A4) protein-binding	7.7	13.8	16.5	10.3	12.4
B7ZAX9	SWI/SNF-related matrix-associated actin	8.5	12.6	14.1	14.3	11.0
B0AZQ4	Structural maintenance of chromosome protein	20.9	13.2	10.0	14.3	13.3
E9PBC6	Transforming acidic coiled-coil-containing p2	10.8	12.5	12.3	14.0	12.1
A0A0G2JK05	LY6G6F-LY6G6D readthrough	7.3	14.3	11.6	12.8	12.0
Q5VY43	Platelet endothelial aggregation receptor 1	9.2	12.3	11.7	14.2	12.3
Q5JRA6	Transport and Golgi organization protein 1	12.2	16.4	12.6	10.0	11.5
B2R6H3	Kinesin-like protein	8.4	13.0	11.0	11.7	14.5
Q59F82	C21orf2 protein variant (Fragment)	6.5	13.2	9.9	14.6	12.3
A0A024RAC9	Zinc finger, UBR1 type 1, isoform CRA_c	11.0	10.6	11.4	14.5	13.5
Q9BWF3	RNA-binding protein 4	5.7	14.6	11.7	12.1	11.4
Q6VY07	Phosphofurin acidic cluster sorting protein 1	6.0	12.7	14.2	11.7	11.1
Q63HN4	Uncharacterized protein DKFZp686J1732	6.4	11.4	12.7	12.6	12.9
E9PGA6	C1QTNF3-AMACR readthrough	6.9	13.2	12.8	11.8	11.9
A0A087X0B9	Protein-tyrosine-phosphatase	7.8	10.4	9.5	14.8	14.6
I3L4W6	ZSCAN32 (Fragment)	6.4	11.4	10.3	12.8	13.1
Q07973	Cytochrome P450 24A1	8.1	9.3	11.4	13.0	12.8
Q13769	THO complex subunit 5 homolog	8.1	10.4	12.8	12.4	10.7

Figure 4.6 The relative expression levels of 80 common proteins identified in disease groups

Based on GO classification, the respective BP, MF and CC of the disease-associated proteins are provided in **Figures 4.7A** and **4.7B**. The BP classification, including cellular process, biological process, metabolic process, response to stimuli and localization, were observed in 28.6%, 20.2%, 18.2%,9.1% and 9.1%, respectively. Based on their MF, these disease-associated proteins were classified into binding (37.5%), catalytic activity (23.5%), molecular regulator (11.8%) and transport activity (5.9%). This results suggested that almost these proteins acted as catalysts, regulators and transporter proteins involved in cell activities via many biological pathways. The CC classification of the 80 disease proteins is also demonstrated in **Figure 4.7B**. They were classified as cellular anatomical entities (48.8%), intracellular (36.6%) and protein-containing complex (14.6%). In addition, the protein expression pattern among the disease groups was represented by the heatmap (**Figure 4.7C**).

The results from GO functional enrichment analysis indicated that proteins involved in the regulation of microRNAs (miRNAs)-dependent gene expression and RNA processing, including pre-mRNA-processing-splicing factor 8, eukaryotic translation initiation factor 4, RNA-binding protein 4, argonaute-2 (AGO2), bromodomain testis-specific protein and THO complex (THOC) subunit 5, were the major upregulated proteins in this disease groups (**Figure 4.8A**). Depending on MF, most of them are involved in nucleotide triphosphate activity and mRNA binding (**Figure 4.8B**). Moreover, the predominant proteins in disease groups were located in the nucleus, macromolecular complex and nucleoplasm (**Figure 4.8C**). Furthermore, the proteins involving UPS, including ubiquitin carboxyl-terminal hydrolase-7 and E3 ubiquitin-protein ligase XIAP, were also observed with higher abundance in the disease groups.

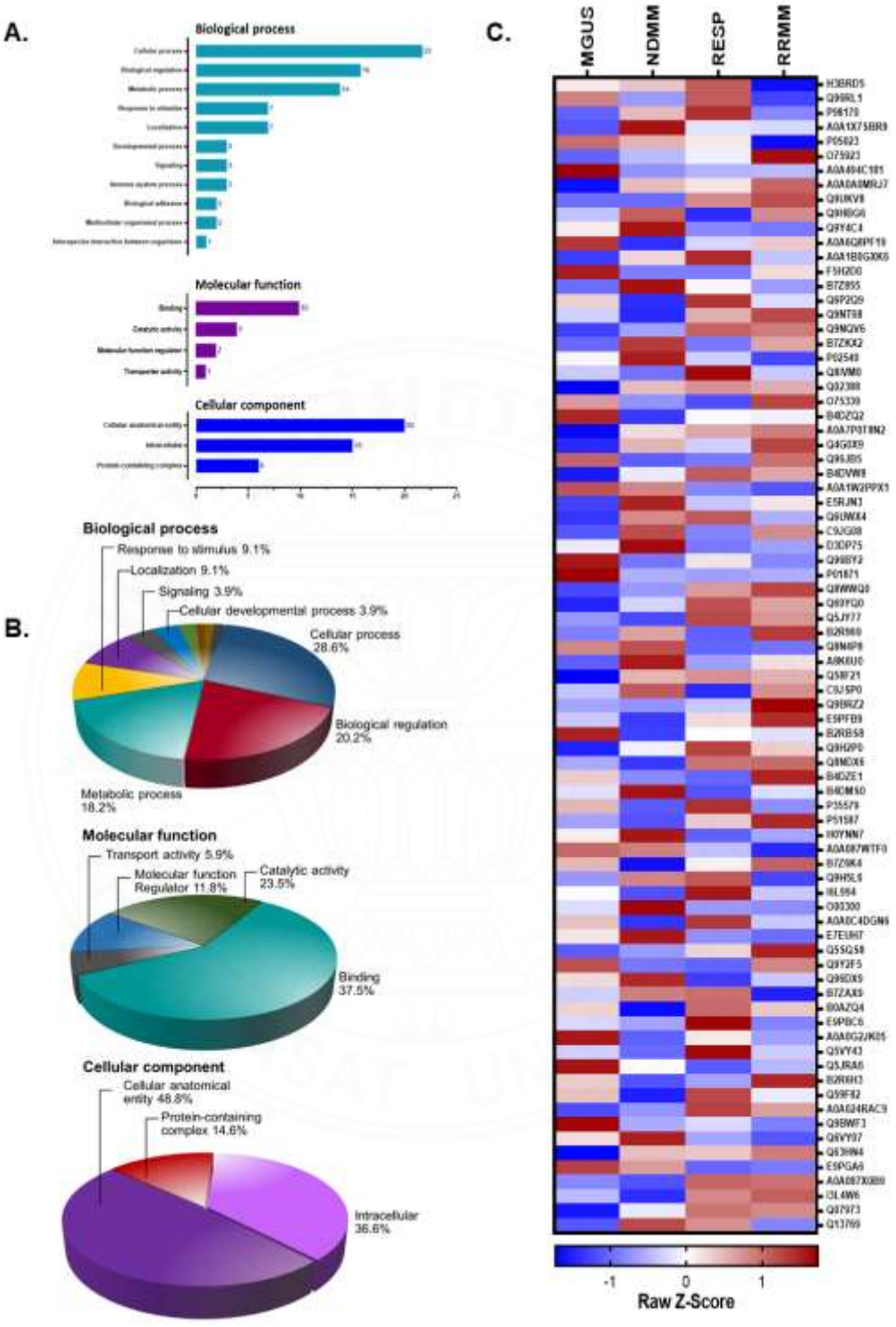

Figure 4.7 The common serum proteins expressed in disease groups. (A.-B.) PANTHER GO protein classification of 80 common proteins in disease groups. (C.) Heatmap of 80 differentially expressed proteins showing the levels of expression among MGUS, NDMM, RESP and RRMM groups.

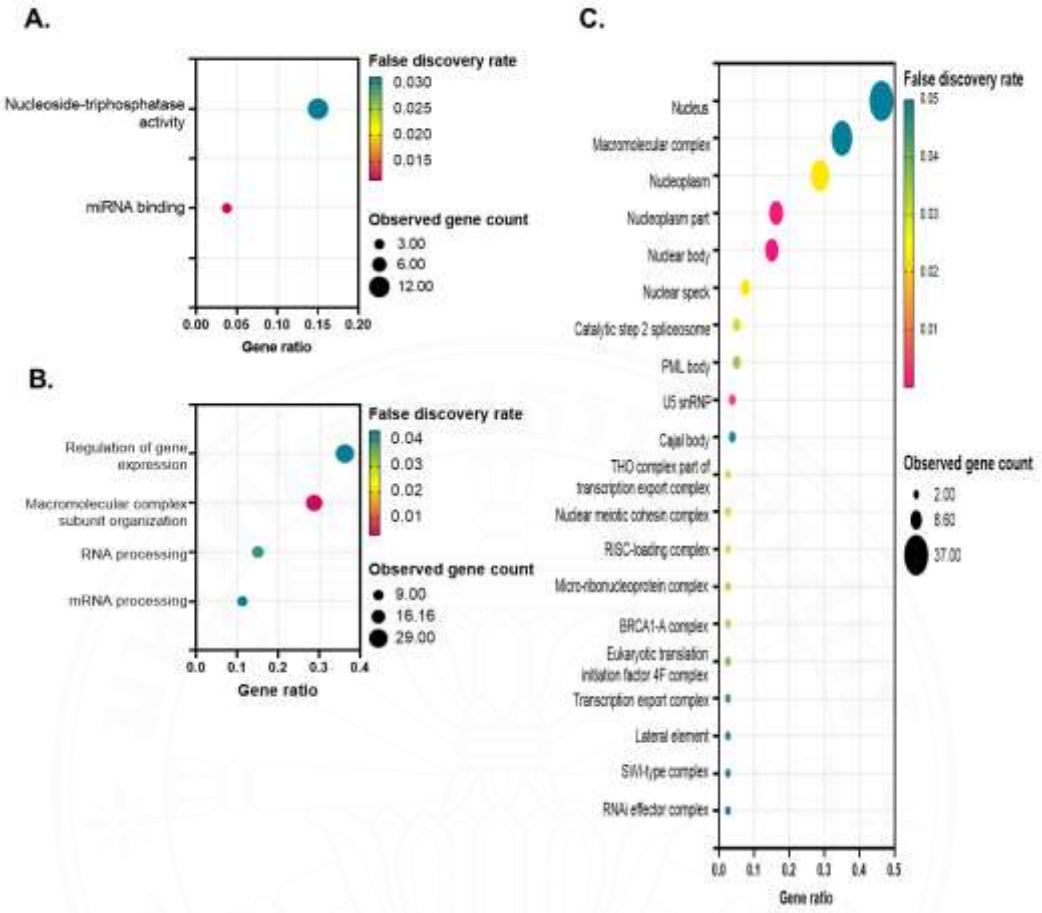

Figure 4.8 GO functional enrichment analysis of the 80 common proteins in the disease groups. The proteins were classified according to the following GO categories: (A) biological process, (B) molecular function and (C) cellular component. The y-axis indicates the functional classification or pathway, and the x-axis indicates the protein-encoded gene ratio (observed gene count/total common protein encoded genes) of the respective function type. The color key that accompanies the bubble plot indicates the false discovery rate, and the bubble size indicates the observed gene count of the proteins in the respective functional class or pathway.

4.1.5 The signature proteins

We found 8, 10, 17, 67 and 70 signature proteins identified in normal, MGUS, NDMM, RESP and RRMM groups, respectively. The lists of these proteins are shown in **Tables 4.2-4.6**.

Table 4.2 List of 8 signature proteins identified in normal serum

No.	Accession No.	Gene	Protein name	Log_2 intensity
1	H7BXS4	*CAMK2B*	Calcium/calmodulin-dependent protein kinase type II subunit beta	18.8784
2	B4DNZ4	*TIMP3*	Metalloproteinase inhibitor 3	17.6022
3	Q8WVV4	*POF1B*	Premature ovarian failure protein 1B	16.2047
4	A0A286YEQ8	*SCN1A*	Sodium channel protein	15.3285
5	Q15697	*ZNF174* *ZSCAN8*	Zinc finger protein 174 (Zinc finger and SCAN domain-containing protein 8)	15.0755
6	I0CE67	*FHL2*	Four-and-a-half LIM domains 2	14.9009
7	B7Z951	*DAGK*	Diacylglycerol kinase (DAG kinase)	14.2253
8	B3KTS4	*ARHGEF16*	Rho guanine nucleotide exchange factor 16	11.2516

Table 4.3 List of 10 signature proteins identified in MGUS serum

No.	Accession No.	Gene	Protein name	Log_2 intensity
1	A0A087WXZ6	*FCGR1B*	High affinity immunoglobulin gamma Fc receptor IB (Fragment)	17.020
2	Q53T94	*TAF1B*	TATA box-binding protein-associated factor RNA polymerase I subunit B	15.223
3	A4D248	-	Hypothetical gene supported by AK124321	15.196
	Q9NVM9	*INTS13*	Integrator complex subunit 13	14.913
5	B4DMM4	*RABEP1*	Rab GTPase-binding effector protein 1	13.632
6	D3DSM4	*COL18A1*	Collagen, type XVIII, alpha-1 isoform	13.371
7	Q8N2X6	*C5orf55*	Chromosome 5 open reading frame 55	13.181
8	A6NK75	*ZNF98*	Zinc finger protein 98 (ZNF98)	12.970
9	B4DE95	*ZNF187*	Zinc finger protein 187 (ZNF187)	12.361
10	A0A024R7I8	*IL27RA*	Interleukin 27 receptor-alpha-isoform	11.924

Table 4.4 List of 17 signature proteins identified in NDMM serum

No.	Accession No.	Gene	Protein name	Log_2 intensity
1	P24347	*MMP11* *STMY3*	Matrix metalloproteinase-11	19.699
2	Q9ULL1	*PLEKHG1* *KIAA1209*	Pleckstrin homology domain-containing family G member 1	19.226
3	Q9UF56	*FBXL17* *FBL17* *FBX13* *FBXO13*	F-box/LRR-repeat protein 17	18.923
	Q5VVK9	*TRMT13*	X modification enzyme TRM13	18.394
5	Q9HD33	*MRPL47* *NCM1* *CGI-204*	Mitochondrial 39S ribosomal protein L47	16.878
6	A0A0D9SFD2	*THEMIS*	Protein THEMIS	16.154
7	B4DR62	*LMLN*	Leishmanolysin-like peptidase	15.865
8	A0A3B3IRY8	*EPHB1*	Receptor protein-tyrosine kinase	15.504
9	Q08EN2	*TPH1*	TPH1 protein	14.148
10	A0A140VKH5	-	Testis tissue sperm-binding protein Li 92mP	13.954
11	Q9BT22	*ALG1* *HMAT1* *HMT1* *PSEC0061*	Asparagine-linked glycosylation protein 1 homolog	13.665
12	B4DEA8	*ACADVL*	Very-long-chain specific acyl-CoA dehydrogenase	13.423
13	Q12986	*NFX1* *NFX2*	Transcriptional repressor NF-X1	12.579
14	Q8NA66	*CNBD1*	Cyclic nucleotide-binding domain-containing protein 1	12.109
15	B3KXD1	*PLCXD3*	Phosphatidylinositol-specific phospholipase C	11.882
16	A0A024R7X9	*UBE2W*	Ubiquitin-conjugating enzyme E2W	11.460
17	Q6IPW4	*NDUFV2*	NADH dehydrogenase [ubiquinone] flavoprotein 2	11.316

Table 4.5 List of 67 signature proteins identified in RESP serum

No.	Accession No.	Gene	Protein name	Log_2 intensity
1	B3KMW2	*USP36*	Ubiquitin carboxyl-terminal hydrolase 36	21.286
2	Q2M2A3	*GANC*	Glucosidase, alpha neutral C	21.093
3	B4DWS9	*SERPINB5*	Serpin B5	20.469
4	A1L447	*USP29*	Ubiquitin carboxyl-terminal hydrolase	20.230
5	A0A024QZZ4	*ZNF322A*	Zinc finger protein 322A	19.712
6	A0A0G2JPT5	*RAD17*	Cell cycle checkpoint protein RAD17	19.604
7	M0R181	*RPL21*	60S ribosomal protein L21	19.549
8	Q8IYF3	*TEX11*	Testis-expressed protein 11 (ZIP4H)	19.281
9	P12757	*SKIL*	Ski-like protein (Ski-related oncogene)	19.118
10	Q4LE36	*ACLY*	ATP-citrate (pro-S-)-lyase	18.867
11	Q6ZSS3	*ZNF621*	Zinc finger protein 621	18.621
12	V9HWG4	*HEL37* *PIP5K3*	1-phosphatidylinositol-3-phosphate 5-kinase	18.590
13	B4DP06	*ATIC*	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase	18.305
14	A0A087WUX1	*ENKUR*	Enkurin	17.841
15	B7Z592	*ZC3H15*	Zinc finger CCCH domain-containing protein 15	17.815
16	Q6P142	*LIN9*	LIN9 protein	16.606
17	Q5JZY3	*EPHA10*	Ephrin type-A receptor 10	16.605
18	A0A1B0GV63	*ARID1B*	AT-rich interactive domain-containing protein 1B	16.516
19	A0A024R1I0	*NF2*	Neurofibromin 2	16.434
20	Q9UDR5	*AASS*	Alpha-aminoadipic semialdehyde synthase	16.272

Table 4.5 List of 67 signature proteins identified in RESP serum (Cont.)

No.	Accession No.	Gene	Protein name	Log_2 intensity
21	Q9Y3A0	*COQ4* *CGI-92*	Ubiquinone biosynthesis protein COQ4 homolog	16.161
22	A0A024QZN9	*VDAC2*	Outer mitochondrial membrane protein porin 2	16.110
23	G3V5E1	*CCNK*	Cyclin-K	16.084
24	A0A2X0SFI5	*ECT2L*	ECT2L	15.913
25	C9JKS0	*NR2C2*	Nuclear receptor subfamily 2 group C member 2	15.722
26	B4DYX8	*PDP1*	Pyruvate dehydrogenase (lipoamide)-phosphatase 1	15.330
27	A0A0U1RQB8	*SERPINA2*	Putative alpha-1-antitrypsin-related protein	15.257
28	Q9H176	*ZNF143*	ZNF143 protein (Fragment)	15.219
29	Q6IBA9	*TNFSF10*	Tumor necrosis factor ligand superfamily member	15.181
30	C9J069	*AJM1* *C9orf172*	Apical junction component 1 homolog	15.096
31	O75243		R30783_1	15.007
32	Q59FM8	*HECTD1*	RBR-type E3 ubiquitin transferase	14.998
33	A0A024RBG0	*C12orf48*	PARP-1 binding protein (PARP1-binding protein)	14.966
34	B2R6S9	*LRPAP1*	Low density lipoprotein receptor-related protein associated protein 1 (LRPAP1)	14.935
35	B7Z8S8	*CAPN1*	Calpain inhibitor (Calpastatin)	14.904
36	Q6ZNA1	*ZNF836*	Zinc finger protein 836	14.842
37	Q9NX65	*ZSCAN32* *ZNF434* *HCCS5*	Zinc finger and SCAN domain-containing protein 32	14.640
38	O14958	*CASQ2*	Calsequestrin-2 (Calsequestrin, cardiac muscle isoform)	14.379
39	E9PCD7	*MAN2B2*	Alpha-mannosidase	14.379
40	Q8N6Q8	*METTL25* *C12orf26*	Methyltransferase-like protein 25	14.339

Table 4.5 List of 67 signature proteins identified in RESP serum (Cont.)

No.	Accession No.	Gene	Protein name	Log_2 intensity
41	A8MW78	*GRAP*	GRB2-related adapter protein	14.245
42	A0A7S5EWW8	-	IGH c918_heavy_IGHV3-15_IGHD6-19_IGHJ4	14.186
43	B4DIH2	*GDAP1*	Ganglioside-induced differentiation-associated protein 1	14.121
44	Q6PG37	*ZNF790*	Zinc finger protein 790	14.033
45	Q6PKG0	*LARP1* *KIAA0731* *LARP*	La-related protein 1 (La ribonucleoprotein domain family member 1)	13.846
46	B0QYC0	*IL2RB*	Interleukin-2 receptor subunit beta	13.838
47	Q9NVU7	*SDAD1* *NUC130*	Protein SDA1 homolog (Nucleolar protein 130)	13.813
48	Q08495	*DMTN* *DMT* *EPB49*	Dematin (Dematin actin-binding protein)	13.628
49	A0A024RBM7	*PRDM4*	PR domain zinc finger protein 4 (PR domain-containing protein 4)	13.573
50	Q5JQQ2	*DPCD*	Protein DPCD	13.457
51	J3QL54	*NUP85*	Nuclear pore complex protein Nup85	13.357
52	B4DYC6	*SGT1*	SGT1	13.180
53	Q8N8P6	-	Putative uncharacterized protein FLJ39060	13.068
54	F5H617	*RTN3*	Reticulon	12.901
55	Q8IWZ5	*TRIM42*	Tripartite motif-containing protein 42	12.834
56	A0A494C0K8	*IGSF1*	Immunoglobulin superfamily member 1	12.814
57	Q7Z524	-	HUMEEP	12.595
58	B0BCZ3	*ENPEP*	Aminopeptidase	12.574
59	Q5U058	*GAP43*	Axonal membrane protein GAP-43 (Growth-associated protein 43)	12.458
60	O60583	*CCNT2*	Cyclin-T2 (CycT2)	12.422

Table 4.5 List of 67 signature proteins identified in RESP serum (Cont.)

No.	Accession No.	Gene	Protein name	Log_2 intensity
61	B5MCF9	*PES1*	Pescadillo homolog	12.301
62	B3KPP7		Autophagy-related protein 9	12.273
63	A0A286YF60	*SCYGR3 KRTAP28-3*	Small cysteine and glycine repeat-containing protein 3 (Keratin-associated protein 28-3)	11.948
64	A0A494C1I7	*ZNF587B*	Zinc finger protein 587B	11.790
65	D6RG18	*CCNH*	Cyclin-H	11.225
66	A0A024RCX2	*PRRT1*	Proline-rich transmembrane protein 1	11.179
67	M0QZC2	*PRKD2*	Serine/threonine-protein kinase D2	10.892

Table 4.6 List of 70 signature proteins identified in RRMM serum

No.	Accession No.	Gene	Protein name	Log_2 intensity
1	A0A2R8YEQ5	*NEXMIF*	Neurite extension and migration factor	20.749
2	X6R3B1	*F11*	Coagulation factor XI	20.342
3	B5M0C0	*DUOXA1*	Dual oxidase maturation factor 1	19.760
4	Q5SQS7	*SH2D4B*	SH2 domain-containing protein 4B	19.582
5	B7Z1N6	*ALDOA*	Fructose-bisphosphate aldolase	19.369
6	Q8N7U5		cDNA FLJ40332 fis, clone TESTI2031760	19.152
7	A0A0U1RQC7	*CFAP99*	Cilia- and flagella-associated protein 99	18.817
8	A0A0C4DGE7	*EVC2*	Ellis van Creveld syndrome 2 (Limbin)	18.664
9	J3KP02	*LEKR1*	Leucine-, glutamate- and lysine-rich protein 1	18.538
10	B4DF67	*MAPK5*	Mitogen-activated protein kinase 5	18.109
11	Q96NH3	*TBC1D32 BROMI C6orf170 C6orf171*	PTBC1 domain family member 32	17.638
12	Q6PFW1	*PPIP5K1 HISPPD2A IP6K IPS1 KIAA0377 VIP1*	Inositol hexakisphosphate and diphosphoinositol-pentakisphosphate kinase 1	17.569
13	A0A024QZ95	*OLFM4*	Olfactomedin 4	17.516
14	A0A024R2I4	*C3orf20*	Chromosome 3 open reading frame 20	17.293
15	Q9ULA1	*ZNF667*	Zinc finger protein 667	17.217
16	Q2NL82	*TSR1 KIAA1401*	Pre-rRNA-processing protein TSR1 homolog	17.085
17	A0A0A0MQS0	*PHF20L1*	PHD finger protein 20-like protein 1	17.028
18	F8W9B8	*EXOC5*	Exocyst complex component 5	17.008
19	A0A087WYG4	*MROH5*	Maestro heat-like repeat family member 5	16.734
20	Q6ZMU5	*TRIM72 MG53*	Tripartite motif-containing protein 72	16.658

Table 4.6 List of 70 signature proteins identified in RRMM serum (Cont.)

No.	Accession No.	Gene	Protein name	Log_2 intensity
21	Q9BY89	*KIAA1671*	Uncharacterized protein KIAA1671	16.655
22	A0A2R8Y6J6	*SCN10A*	Sodium channel protein	16.531
23	B2RAM6	*KIF11*	kinesin family member 11	16.449
24	U3KQG0	*TARS2*	Threonyl-tRNA synthetase	16.384
25	Q12899	*TRIM26 RNF95 ZNF173*	Tripartite motif-containing protein 26	16.381
26	Q7L5A3	*FAM214B KIAA1539*	Protein FAM214B	16.274
27	A0A024R1Y2	*ACLY hCG_19718*	ATP-citrate synthase	16.253
28	Q9Y2H9	*MAST1 KIAA0973 SAST*	Microtubule-associated serine/threonine-protein kinase 1	16.125
29	Q8NB16	*MLKL*	Mixed lineage kinase domain-like protein (hMLKL)	15.952
30	A0A024R2L8	*CTDSPL*	Carboxy-terminal domain, RNA polymerase II	15.847
31	Q9NXH8	*TOR4A C9orf167*	Torsin-4A (Torsin family 4 member A)	15.786
32	A0A024R9R5	*ZFP106*	Zinc finger protein 106 homolog	15.785
33	Q13467	*FZD5 C2orf31*	Frizzled-5	15.755
34	F5H2N6	*KIAA1586*	E3 SUMO-protein ligase	15.729
35	Q68DW7	*STAG1 D*	Cohesin subunit SA-1	15.629
36	Q96GD3	*SCMH1*	Polycomb protein SCMH1	15.604
37	E9PJN0	*ACOT8*	Acyl-coenzyme A thioesterase 8	15.469
38	A0A0K0K1J0	*HEL-S-292*	Epididymis secretory protein Li 292	15.194
39	Q14692	*BMS1 BMS1L KIAA0187*	Ribosome biogenesis protein BMS1 homolog	15.175
40	Q8IV76	*PASD1*	Circadian clock protein PASD1	15.050

Table 4.6 List of 70 signature proteins identified in RRMM serum (Cont.)

No.	Accession No.	Gene	Protein name	Log_2 intensity
41	A0A087X254	*ZNF615*	Zinc finger protein 615	15.008
42	B3KVF0		DNA helicase	14.863
43	Q8TDG4	*HELQ HEL308*	Helicase POLQ-like	14.600
44	A5PLL1	*ANKRD34B*	Ankyrin repeat domain-containing protein 34B	14.529
45	Q86Y64	*ZNF354A*	Zinc finger protein 354A	14.527
46	C9JSJ3	*MEIOSIN BHMG1*	Meiosis initiator protein	14.358
47	B3KP14	*GRSF1*	G-rich sequence factor 1	14.324
48	B3KU60	*FTO*	Alpha-ketoglutarate-dependent dioxygenase FTO	14.324
49	Q9BQ24	*ZFYVE21*	Zinc finger FYVE domain-containing protein 21	14.280
50	F8VZA0	*PUS7L*	Pseudouridylate synthase 7 homolog-like protein	13.993
51	Q5JWV1	*UCKL1*	Uridine-cytidine kinase-like 1	13.886
52	A0A0C5BIK5		NOK mRNA	13.785
53	C9J1J2	*NME6*	Nucleoside diphosphate kinase 6	13.717
54	Q9NR61	*DLL4*	Delta-like protein 4	13.702
55	Q6ICI8	*APOL4*	APOL4 protein (	13.651
56	Q495X7	*TRIM60 RNF129 RNF33*	Tripartite motif-containing protein 60	13.568
57	Q96NW4	*ANKRD27 PP12899*	Ankyrin repeat domain-containing protein 27	13.483
58	A8MUV8	*ZNF727 ZNF727P*	Putative zinc finger protein 727	12.815
59	A0A494C077	*GIPR*	Gastric inhibitory polypeptide receptor	12.640
60	Q569K6	*CCDC157 KIAA1656*	Coiled-coil domain-containing protein 157	12.639

Table 4.6 List of 70 signature proteins identified in RRMM serum (Cont.)

No.	Accession No.	Gene	Protein name	Log_2 intensity
61	B7Z970	*INTS9*	Integrator complex subunit 9	12.484
62	Q5T457	*ANKRD2*	Ankyrin repeat domain-containing protein 2	12.234
63	C9JX88	*PSMC2*	26S proteasome AAA-ATPase subunit RPT1	12.040
64	Q9BR84	*ZNF559*	Zinc finger protein 559	11.806
65	A0A3B3ITQ6	*NEK5*	Serine/threonine-protein kinase Nek5	11.737
66	A0A024RAE1	*C1orf33*	Ribosome assembly factor mrt4	11.388
67	A0A0S2Z486	*NDN*	Necdin-like protein isoform 2	11.204
68	B4DW48	*TPRC4AP*	Trpc4-associated protein	10.597
69	Q6ZT31		BRAWH3014609	10.536
70	B2RU13	*KCNA7*	Potassium voltage-gated channel, shaker-related subfamily, member 7	10.198

4.2 Biomarker identification

4.2.1 Identification of candidate protein biomarkers

In this chapter, we aimed to identify potential serum biomarkers indicating disease activity in MM patients. The differentially expressed proteins identified in normal, NDMM, RESP and RRMM were subjects for further exploration to discover the biomarkers. Based on MM disease activity, the ideal biomarkers should be present in the active states of the disease, such as newly diagnosed or relapsed/refractory disease, but they exhibited downregulation or were absent in disease remission states (RESP). Therefore, the differentially expressed proteins identified in normal group (772 proteins) were filtered out. To identify potential biomarkers indicating MM disease

activity, 966 differentially expressed proteins identified among patients were analyzed. Only 288 differentially expressed proteins identified in disease-active states (NDMM and RRMM) were compared with RESP. Proteins with fold change (disease-active states *vs.* remission states) greater than 1.5 or less than -1.5 and adjusted *p*-values less than 0.05 were considered potential candidate biomarkers. Among these, 38 dysregulated proteins including 23 up-regulated and 15 down-regulated proteins were compatible with the criteria (**Figure 4.9**). The upregulated and downregulated candidate proteins are listed in **Tables 4.7** and **4.8**.

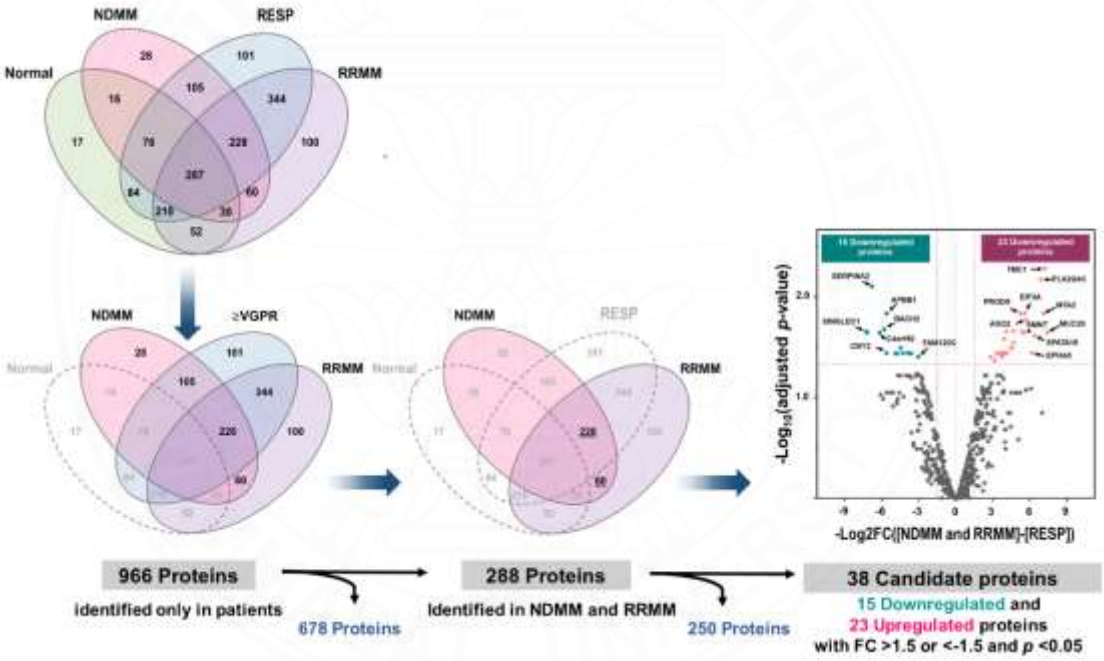

Figure 4.9 The overview of candidate biomarker screening processes. Volcano plot demonstrating the change in the relative abundance of 288 proteins between disease-active states (NDMM and RRMM) and disease remission states (RESP). The x-axis represents $\log_2$ fold changes of proteins, and the y-axis represents $-\log_{10}$ FDR-adjusted *p*-values. The rose pink and blue-green dots represent significantly upregulated and downregulated proteins with a $\log_2$-fold change >1.5 and <-1.5, respectively.

Table 4.7 The significant upregulated candidate proteins identified in NDMM and RRMM serum samples with a fold change >1.5

No.	Accession No.	Gene name	Peptide sequence	Log_2-FC	p-value
1	Q8TDI8	*TMC1*	ESLRPKRK	7.99	0.001
2	A0A024QZH0	*PLA2G4C*	QEWDLAKSLQKTIQAAR	7.61	0.024
3	A0A0G2JRT2	*MUC20*	ASPTIVPHPGDSSASSESR	6.96	<0.001
4	A0A024R534	*MTA2*	AECSIRLPKAAK	6.78	<0.001
5	E7EVV3	*SPATA18*	QLQNIEEEAALLSIAR	6.19	0.005
6	F8W9W0	*EPHA5*	IPIRWTAPEAIAFR	6.04	0.002
7	X5D7N2	*PRODH*	HALLAPWACRLLVLLR	5.50	0.003
8	Q6ZNE1	*EIF3A*	DRPDLSAPESLQLYLDTR	5.43	<0.001
9	B3KY41	*SMNT*	ENWLHSQQREAEQR	5.33	0.017
10	Q9UKV8	*AGO2*	AALAQHRDGNSQGSTHSGE-ASDHK	5.02	0.024
11	P51587	*BRCA2*	CLSPERVLLQLKYR	4.48	0.007
12	Q86XN6	*ZNF761*	CEECDKAFHFK	3.85	0.006
13	A0A2R8YG28	*OTOA*	ISPIEIGLFISYDNATK	3.75	0.007
14	A0A090N8Y2	*ERP70*	DIASDQTSGQVDMMHR	3.41	0.019
15	O14917	*PCDH17* *PCDH68* *PCH68*	GGGGLQPHSYEARICNYR	3.35	0.005
16	Q5SXM1	*ZNF678*	AGCEECPYKPEGSHK	3.17	0.014
17	F5H450	*FZD10*	ADVHRAGLYPHPR	3.07	0.015
18	Q14CC5	*TPH2*	ELSKLYPTHACR	2.92	0.043
19	Q7KZN9	*COX15*	APARAPALLPLYRQK	2.35	0.003
20	A0A0C4DG03	*SULT6B1*	GFLFPGVAGHTDQK	2.25	0.024
21	Q7Z6I6	*ARHGAP30*	AYFRELPDPLLTYR	1.87	0.010
22	J3KN75	*TBC1D8B*	EDDPEKFR	1.73	0.002
23	A0A024RAC0	*LUZP1*	AANGLEADNSCPNSK	1.57	0.023

Table 4.8 The significant downregulated candidate proteins identified in NDMM and RRMM serum samples with a fold change <-1.5

No.	Accession No.	Gene name	Peptide sequence	Log2-FC	p-value
1	Q9HCE0	*EPG5*	DWPKRLYTSHFAYLIFEPK	-1.94	0.001
2	Q9UKT9	*IKZF3*	AEMSNGAPQELEKK	-2.16	0.008
3	Q13470	*MKRN2*	AQEPDFPHLVQRLCIECVEG-HLYVCADGR	-2.28	0.001
4	O60500	*NPHS1*	KSLILNVK	-2.81	0.000
5	C6KE32	*P2RX7*	CCQPCVVNEYYYR	-2.94	0.001
6	Q9ULX3	*NOB1* *PSMD8BP1*	TFCGHRCLIYR	-3.07	0.001
7	Q9NX05	*FAM120C* *CXorf17*	DRLAEWGRR	-3.11	0.031
8	A0A0S2Z4T5	*RIN1*	LELEQVRQKLLQLLR	-3.26	0.033
9	Q9BV57	*ADI1* *MTCBP1* *HMFT1638*	DKLPNYEEKIK	-3.66	0.006
10	A0A1W2PRI9	*C4orf50*	AEWGSHKWQAFSWNER	-4.24	0.008
11	Q9BYV9	*BACH2*	AGDVEMDRK	-4.31	0.006
12	A0A140VJV5	*CBY2*	KDVVELSASKDHLLPSPR	-4.79	0.004
13	B4E100	*APBB1*	ESKETNEKMNAK	-4.89	0.014
14	P20848	*SERPINA2*	AEELHPAGTAETK	-6.25	0.002
15	Q9BZZ2	*SIGLEC1*	ADTGFYFCEVQNVHGSER	-6.53	0.016

4.2.2 Functional analyses of the candidate proteins

To evaluate the functional relevance of the 38 dysregulated proteins, GO functional and KEGG pathway enrichment analyses were performed. The proteins were classified based on their respective biological processes, molecular functions and cellular components (**Figure 4.10A**). The KEGG pathway enrichment analysis revealed that most dysregulated proteins were significantly enriched in RNA transport, pathways in cancer, miRNAs in cancer and the p53 signaling pathway (**Figure 4.10B**). In addition, the genetic constitution, functional and drug–protein interactions of the 38 candidate proteins was described on PPI network (**Figure 4.11**).

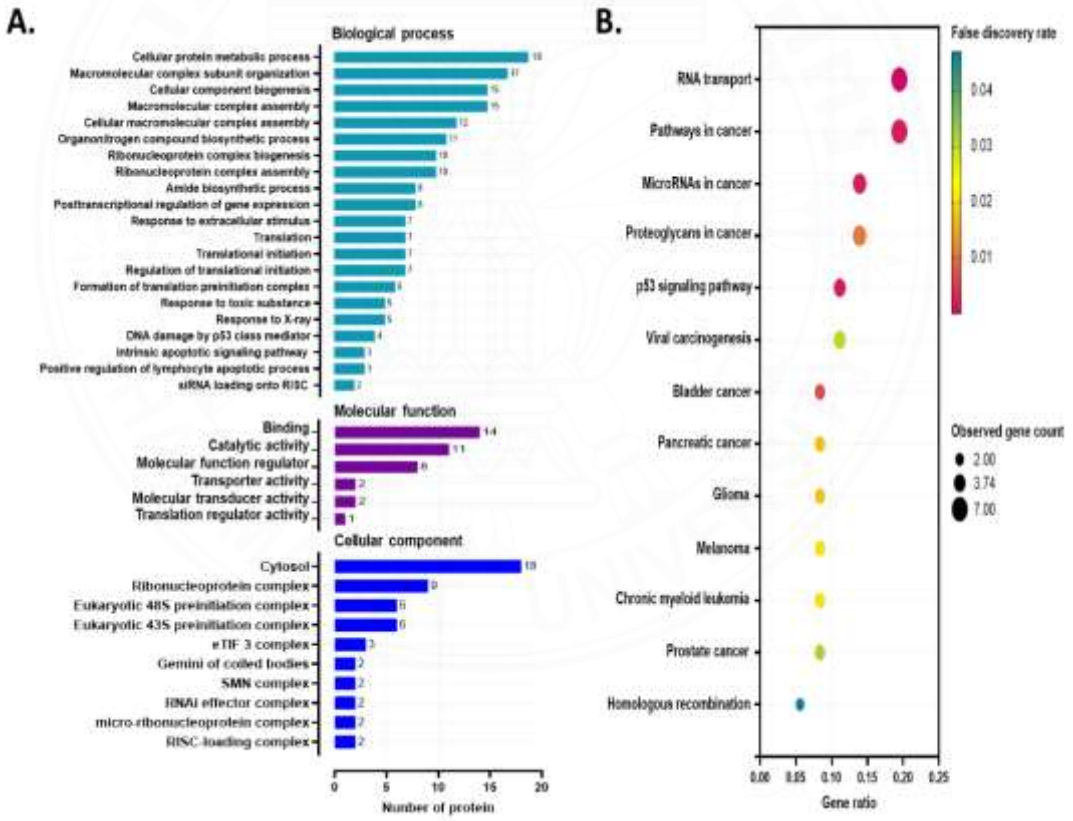

Figure 4.10 GO functional and KEGG pathway enrichment analysis of the 38 candidate proteins. (A) Bar charts demonstrating the distribution of the proteins according to GO classification. (B) KEGG pathway enrichment analysis.

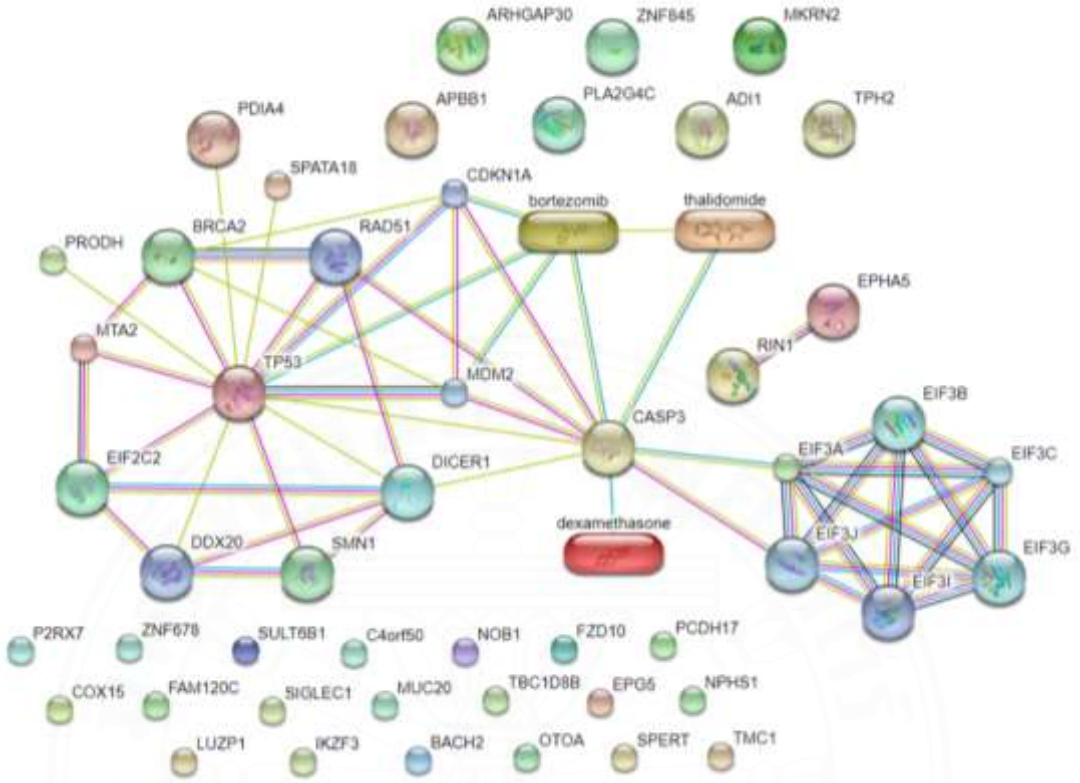

Figure 4.11 The PPI network of 38 candidate proteins (search on Sep 7, 2022, PPI enrichment *p*-value<0.001)

4.2.3 Analysis of gene expression profiles

To confirm whether the dysregulated proteins were expressed by myeloma cells, the NCBI GEO microarray data set (accession no.GSE47552)[154] of plasma cells isolated from 41 patients with MM and 5 normal controls were analyzed. A comparison of normalized gene expression values among 38 dysregulated proteins encoding genes is shown in **Figure 4.12A**. Gene levels were expressed by normalized expression values and two-tailed *t*-test were calculated to compare between MM and healthy control groups. The results were shown in **Table 4.9**. We found that 12 of 38 genes were significantly expressed among patients with MM. However, only five genes—metastasis-associated protein-2 (*MTA2*), argonaute-2 (*AGO2*), proline dehydrogenase (*PRODH*), transmembrane channel-like protein 1 (*TMC1*) and protein FAM120C (*FAM120C*)—showed significant expression patterns consistent with our

results from LC–MS/MS. Regarding our criteria for biomarker selection consisting of (a) fold change >1.5 or <-1.5 and p <0.05 by LC–MS/MS and (b) fold change >1.2 or <-1.2 and p <0.05 using data from the microarray dataset,[154] only MTA2 and AGO2 were selected for further analyses as potential biomarkers (**Figure 4.12B**).

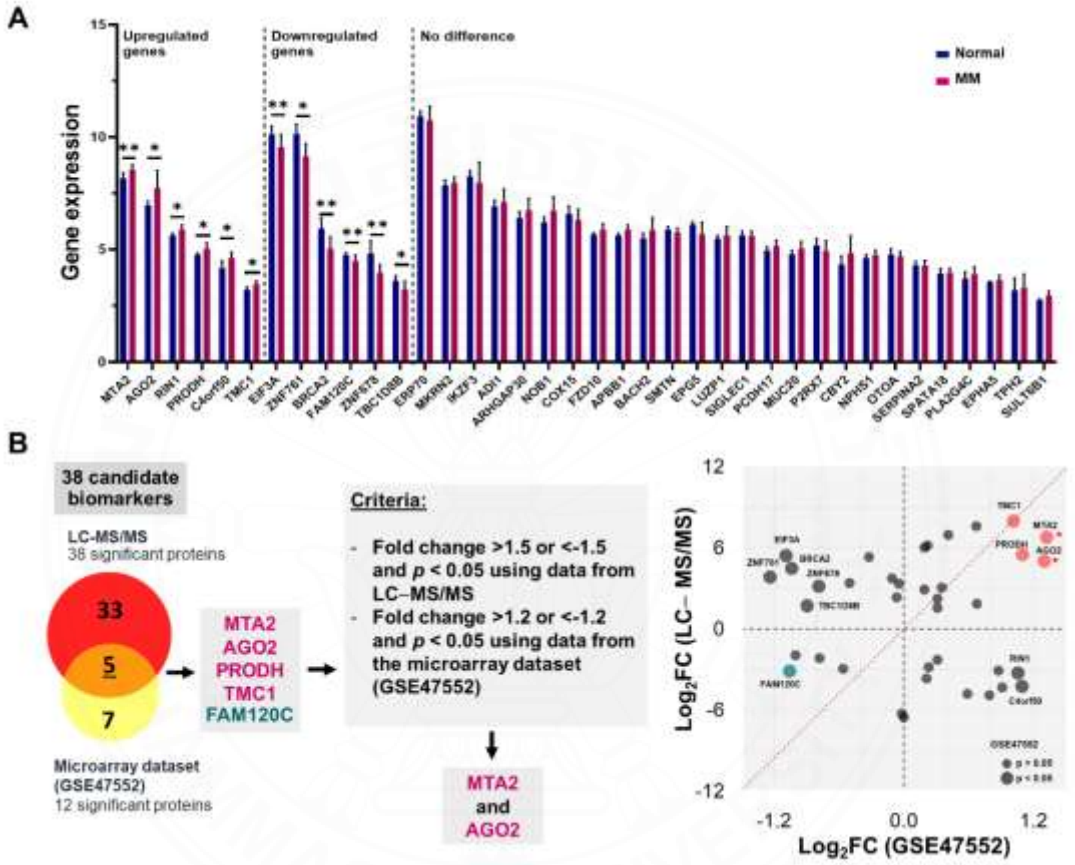

Figure 4.12 The expression profiles of the genes involving 38 dysregulated proteins among 41 patients with MM compared with 5 normal controls using the data from GSE47552.[154] (A) Bar chart showing the relative gene expression levels of genes involving 38 dysregulated proteins. The significant p-value less than 0.05 and 0.01 are represented with * and **, respectively. (B) Selecting of the candidate biomarkers.

Table 4.9 The gene expression profiles of the genes involving 38 candidate proteins compared between MM patients and normal controls using data from GSE47552

No.	Gene name	Type of regulation	MM *vs*. Control		*p*-value
			Fold change	t (df=44)	
1	*TMC1*	Up	1.015	2.153	0.037*
2	*PLA2G4C*	-	0.951	1.219	0.229
3	*MUC20*	-	1.052	1.755	0.086
4	*MTA2*	Up	1.185	3.567	<0.001*
5	*SPATA18*	-	0.991	0.329	0.744
6	*EPHA5*	-	1.026	1.001	0.322
7	*PRODH*	Up	1.059	2.220	0.032*
8	*EIF3A*	Down	0.945	2.103	0.041*
9	*SMTN*	-	0.945	1.420	0.163
10	*AGO2*	Up	1.110	2.177	0.035*
11	*BRCA2*	Down	0.850	3.567	<0.001*
12	*ZNF761*	Down	0.902	3.760	<0.001*
13	*OTOA*	-	0.983	0.731	0.469
14	*ERP70*	-	1.001	1.673	0.071
15	*PCDH17*	-	1.043	1.812	0.077
16	*ZNF678*	Down	0.821	4.801	<0.001*
17	*FZD10*	-	1.039	1.931	0.059
18	*TPH2*	-	1.032	0.358	0.722
19	*COX15*	-	0.958	1.291	0.229
20	*SULT6B1*	-	1.058	2.654	0.111
21	*ARHGAP30*	-	1.053	1.403	0.167
22	*TBC1D8B*	Down	0.895	2.233	0.031*
23	*LUZP1*	-	1.027	0.835	0.408
24	*EPG5*	-	0.930	1.749	0.087
25	*IKZF3*	-	0.967	0.648	0.520

* significant *p*-value

Table 4.9 The gene expression profiles of the genes involving 38 candidate proteins compared between MM patients and normal controls using data from GSE47552 (Cont.)

No.	Gene name	Type of regulation	MM *vs*. Control		*p*-value
			Fold change	t (df=44)	
26	*MKRN2*	-	1.014	0.257	0.798
27	*NPHS1*	-	1.029	1.291	0.204
28	*P2RX7*	-	0.952	1.136	0.262
29	*NOB1*	-	1.082	1.801	0.079
30	*FAM120C*	Down	0.819	7.838	<0.001*
31	*RIN1*	Up	0.964	2.684	0.010*
32	*ADI1*	-	1.027	0.674	0.504
33	*C4orf50*	Up	1.110	3.648	0.001*
34	*BACH2*	-	1.066	1.401	0.168
35	*APBB1*	-	1.016	0.714	0.479
36	*CBY2*	-	1.120	1.444	0.156
37	*SIGLEC1*	-	0.998	0.113	0.910
38	*SERPINA2*	-	0.998	0.076	0.939

* significant *p*-value

4.2.4 KEGG enrichment-based network analysis of MTA2 and AGO2

We further analyzed KEGG enrichment-based PPI network analysis, which revealed MTA2 and AGO2 as a central mediator of multiple signaling pathways, including apoptosis, miRNAs in cancer, ubiquitin mediated proteolysis, nucleosome remodeling and deacetylase (NuRD) complex, the *TP53*, the MAPKs and FOXO signaling pathways, as shown in **Figure 4.13**.

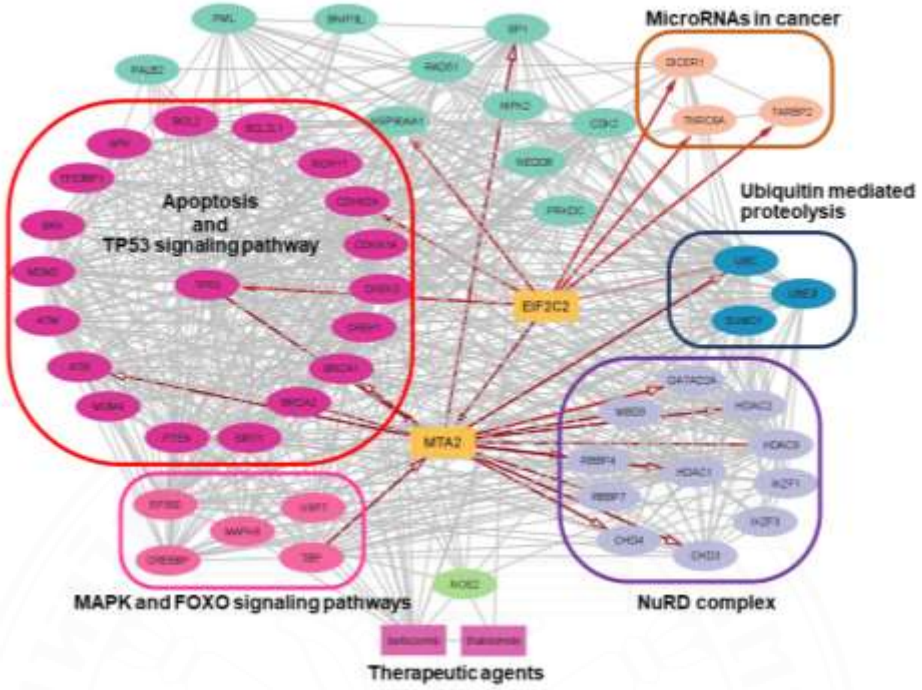

Figure 4.13 KEGG pathway enrichment-based network analysis. The top candidate proteins, MTA2 and AGO2 (gold yellow), were predicted to be a central mediator of multiple signaling pathways.

4.3 Biomarker verification and validation

4.3.1 Biomarkers verification

4.3.1.1 Serum MTA2 and AGO2 levels

In the verification phase, the sample size was calculated using a pooled SD of $\log_2$ intensity from LC–MS/MS (SD of 17.6 and 18.4 for MTA2 and AGO2); a level of significance of 5% and a level of the estimation error of 5%. We estimated that at least 50 samples were required for each group. The simple randomized sampling was performed using a random sample of cases function in IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA), in which each sample has an exactly equal chance of being selected. However, due to insufficient volume existing in some samples, only 160 samples from NDMM (n = 50), RESP (n =

50), RRMM (n = 40) and normal controls (n = 20) were used for MTA2 and AGO2 measurement by enzyme-linked immunosorbent assay (ELISA).

MTA2 and AGO2 concentrations were measured using an ELISA assay. Median (IQR) MTA2 and AGO2 levels in each group are shown in **Table 4.10**, and comparison of MTA2 and AGO2 levels among the study group are shown in **Figure 4.14**.

Table 4.10 Median (IQR) MTA2 and AGO2 levels measured by ELISA

	Median (IQR), pg/mL			
	Normal (n = 20)	**NDMM (n = 50)**	**RESP (n = 50)**	**RRMM (n = 40)**
MTA2	2,274.0 (1,750.0–3,332.0)	5,001.0 (4,356.0–6,069.0)	2,889.0 (2,091.0–4,256.0)	4,395.0 (3,720.0–5,149.0)
AGO2	46.2 (15.7–64.2)	123.4 (90.9–208.9)	55.6 (34.0–73.9)	80.1 (48.2–127.7)

IQR, inter-quartile ranges; NDMM, newly diagnose multiple myeloma; RESP, multiple myeloma patients with the response to treatment at least very good partial response; RRMM, relapsed/refractory multiple myeloma.

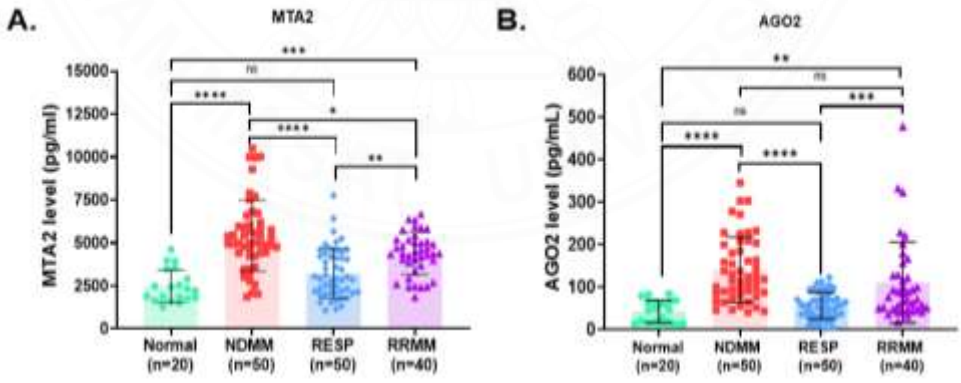

Figure 4.14 Serum MTA2 and AGO2 levels measured by ELISA. Comparison of (A) MTA2 levels and (B) AGO2 levels among normal control, MGUS, NDMM, RESP and RRMM patients. * represents *p*-value <0.05, ** represents *p*-value <0.01, *** represents *p*-value <0.001, **** represents *p*-value <0.0001 and ns represents not significant.

4.3.1.2 Specific cut off for serum MTA2 and AGO2

The diagnostic performance and specific cut-off for MM discrimination were analyzed based on MTA2 and AGO2 levels from controls and NDMM. Sensitivity, specificity and ROC with AUC were evaluated. The MTA2 level of 4,101.0 pg/mL could provide a sensitivity of 82.6% and a specificity of 95.0%; while the AGO2 level of 83.0 pg/mL could provide a sensitivity of 80.0% and a specificity of 95.0% (**Figure 4.15**). These levels were considered the optimal specific cut-offs.

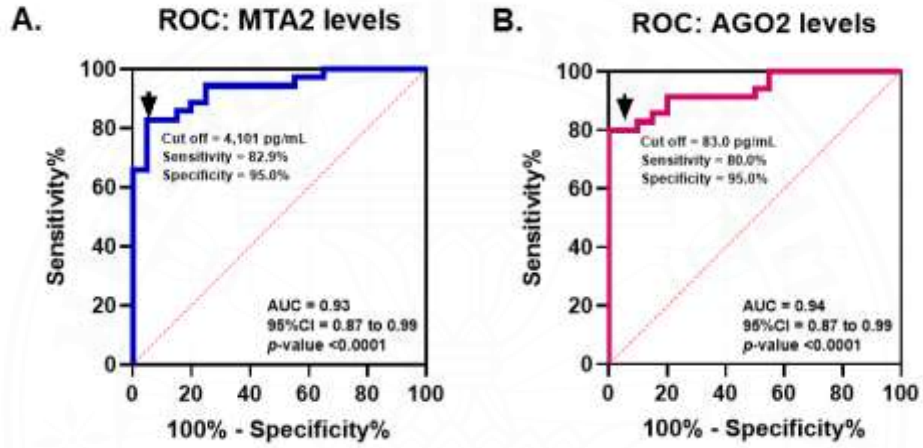

Figure 4.15 The ROC analysis demonstrating the specific cut off for serum (A) MTA2 and (B) AGO2

4.3.2 Biomarkers validation

To confirm our findings, 30 NDMM and 30 RRMM patients were enrolled to the validation cohorts. Paired serum samples were collected from each patient at the time of diagnosis and at the time of VGPR/CR for NDMM cohort, and at the time of VGPR/CR and the time of relapsed/refractory disease for RRMM cohort. Demographic data of the NDMM and RRMM patients are summarized in **Table 4.11**.

Table 4.11 Patient demographics and baseline disease characteristics of 60 patients in validation cohorts

Characteristics	**NDMM cohort (n = 30)**	**RRMM cohort (n = 30)**
Age, year		
Mean ± SD	58.93 ± 11.96	62.20 ± 10.01
Sex, n (%)		
Male	16 (53.3)	20 (66.7)
Female	14 (46.7)	10 (33.3)
Heavy chain type, n (%)		
IgG	13 (43.3)	24 (80.0)
IgA	11 (36.7)	6 (20.0)
Other (IgM, IgD and IgE)	0 (0.0)	0 (0.0)
None	6 (20.0)	0 (0.0)
Light chain type, n (%)		
Kappa (κ)	17 (56.7)	18 (60.0)
Lambda (λ)	13 (43.3)	12 (40.0)
ISS stage, n (%)		
I or II	14 (46.7)	7 (23.3)
III	16 (53.3)	23 (76.7)
ASCT, n (%)		
ASCT eligible (≤65 yrs.)	20 (66.7)	20 (66.7)
Non-ASCT eligible (>65 yrs.)	10 (33.3)	10 (33.3)
First-line treatment regimen, n (%)		
Bortezomib based	25 (83.3)	23 (76.7)
Non-Bortezomib based	5 (16.7)	7 (23.3)
Following time, days		
Median (range),	194 (97–793)	456 (119–2,192)

ASCT, autologous stem cell transplantation; Ig, immunoglobulin; IQR, inter quartile range; ISS, International Staging System; NDMM, newly diagnosed multiple myeloma; RRMM, relapsed/refractory multiple myeloma, SD, standard deviation.

The serum MTA2 and AGO2 levels were determined using the ELISA technique, and were shown in **Figure 4.16**. In the NDMM cohort, the MTA2 levels measured at the time of diagnosis were significantly higher than those measured at the time of response, with a median (IQR) of 4,825.0 (4,064.0-5,952.0) *vs.* 3,281.0 (2,154.0-4,654.0) pg/mL, Wilcoxon matched-pairs signed rank test *p*-value <0.0001 (**Figure 4.16A**). Similar to MTA2, the AGO2 levels measured at the time of diagnosis were significantly higher than those measured at the time of response, with a median of 114.9 (78.4-180.4) vs. 59.1 (32.8-96.9) pg/mL, Wilcoxon matched-pairs signed rank test *p*-value <0.0001 (**Figure 4.16B**).

In the RRMM cohort, both MTA2 and AGO2 levels measured at the time of response were significantly lower than those measured at the time of relapsed/refractory disease (both *p*-values <0.001, **Figure 4.16C-D**). The median of serum MTA2 levels measured at the time of response and at the time of relapsed/refractory disease were 3,548.0 (2,181.0-4,620.0) and 4,435.0 (3,226.0-5,012.0) pg/mL, respectively. The median of serum AGO2 levels measured at the time of response and at the time of relapsed/refractory disease was 48.3 (30.9-73.9) and 70.1 (47.1-96.9) pg/mL, respectively.

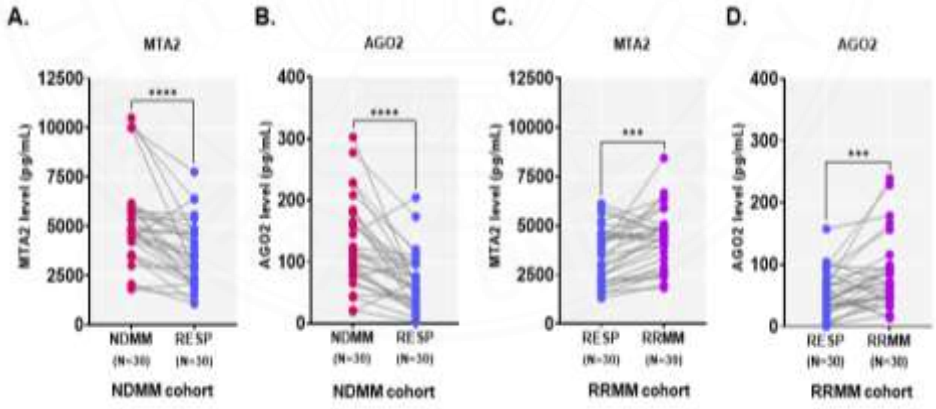

Figure 4.16 Serum MTA2 and AGO2 levels measured by ELISA technique in NDMM and RRMM cohorts. (A-B) Scatter plots demonstrating the serum MTA2 and AGO2 levels of paired serum samples collected at the time of diagnosis compared with those at the time of response. (C-D) Scatter plots demonstrating the serum MTA2 and AGO2 levels of paired serum samples collected at the time of response compared with those at the time of relapsed/refractory disease. *** represents *p*-value <0.001 and **** represents *p*-value <0.0001.

4.3.3 Association between serum MTA2 and AGO2 levels and disease characteristics

To evaluate the effects of serum MTA2 and AGO2 levels on disease characteristics, we analyzed the association between disease features and serum MTA2 and AGO2 levels using the specific cut-offs established for those markers; 4,101.0 pg/mL for MTA2 and 83.0 pg/mL for AGO2. The serum levels at the time of diagnosis and response were used for the analysis in the NDMM and RRMM cohorts, respectively. The patient with a serum biomarker level more than the cut-off is considered a high protein expression group, and those with a serum biomarker level less than or equal to the cut-off are considered a low protein expression group. Characteristics data were analyzed and compared between high and low protein expression groups. The results are shown in **Table 4.12**.

Table 4.12 Association between patient's demographics and disease characteristics and the proteins levels

Characteristics	MTA2 levels						AGO2 levels					
	NDMM cohort			RRMM cohort			NDMM cohort			RRMM cohort		
	Low (n = 7)	High (n = 23)	*p*-value	Low (n = 19)	High (p 11)	*p*-value	Low (n = 8)	High (n = 22)	*p*-value	Low (n = 19)	High (n = 11)	*p*-value
Age, Mean ± SD	56.0±15.1	59.8±11.1	0.465	60.30±10.3	65.6±8.9	0.167	61.0±13.5	58.2±11.6	0.577	60.6±9.9	68.5±8.5	0.850
Sex, n (%)												
Male	6 (85.7)	10 (43.5)	*0.050**	11(57.9)	9 (81.8)	0.180	7 (87.5)	9 (40.9)	*0.024**	15 (62.5)	5 (83.3)	0.333
Female	1 (14.3)	13 (56.5)		8 (42.1)	2 (18.2)		1 (12.5)	13 (59.1)		9 (37.5)	1 (16.7)	
ISS stage, n (%)												
I or II	3 (42.8)	11 (47.8)	0.818	4 (21.1)	3 (27.3)	0.698	5 (62.5)	9 (40.9)	0.295	6 (25.0)	1 (16.7)	0.666
III	4 (57.2)	12 (52.2)		15 (78.9)	8 (72.7)		3 (37.5)	13 (59.1)		18 (75.0)	5 (83.3)	
ASCT, n (%)												
ASCT eligible	5 (71.5)	15 (65.2)	0.760	5 (26.3)	5 (45.5)	0.284	4 (50.0)	16 (72.7)	0.243	17 (70.8)	3 (50.0)	0.333
Non-ASCT eligible	2 (28.5)	8 (34.8)		14 (73.7)	6 (54.5)		4 (50.0)	6 (27.2)		7 (29.2)	3 (50.0)	
Treatment, n (%)												
Bortezomib based	N/A	N/A	N/A	3 (15.8)	4 (36.4)	0.199	N/A	N/A	N/A	20 (83.3)	3 (50.0)	0.084
Non-Bortezomib based	N/A	N/A		16 (84.2)	7 (63.6)		N/A	N/A		4 (16.7)	3 (50.0)	
Bone involvement, n (%)												
Positive	3 (42.8)	16 (69.6)	0.199	5 (26.3)	6 (54.5)	0.389	2 (25.0)	17 (77.3)	*0.009**	6 (25.0)	5 (83.3)	*0.080**
Negative	4 (57.2)	7 (30.4)		14 (73.7)	5 (45.5)		6 (75.0)	5 (22.7)		18 (75.0)	1 (16.7)	

*, significant *p*-value; ASCT, autologous stem cell transplantation; Ig, immunoglobulin; IQR, inter quartile range; ISS, International Staging System; NDMM, newly diagnosed multiple myeloma; RRMM, relapsed/refractory multiple myeloma, SD, standard deviation.

For MTA2, no significant difference was found between high and low serum MTA2 level groups in terms of age, sex, ISS stage, ASCT eligibility, treatment group and bone involvement features in both NDMM and RRMM cohorts (*p*-value >0.05). For AGO2, high AGO2 level was frequently observed in females than males in NDMM patients (*p*-value = 0.024), but this effect was not observed in RRMM patients. Similar to MTA2, no significant difference was found between high and low serum AGO2 level groups in terms of age, ISS stage, ASCT eligibility and treatment group in both NDMM and RRMM cohorts (*p*-value >0.05). Notably, we found that a high level of AGO2 was associated with bone involvement features in both NDMM and RRMM patients, with a *p*-value of 0.009 and 0.080, respectively.

4.3.4 Impact of serum MTA2 and AGO2 levels on patient's outcomes

Regarding the treatment response and MM disease progression as events of interest, in the NDMM cohort, the TTR was counted from the start of treatment to the first observation of the response of at least VGPR. The impact of the biomarker levels on patients' TTR was evaluated using the biomarker levels when newly diagnosed. For the RRMM cohort, the PFS was counted after the patient achieved a response of at least VGPR until diagnosis for disease progression, and the levels of the biomarkers at response were used to evaluate the impact on patients' PFS. The OS was not assessed due to the low number of events (deaths) at the time of analysis.

Proportion of patients with high and low MTA2 and AGO2 levels among NDMM and RRMM cohorts are shown in **Figures 4.17A** and **4.17C**. In the NDMM cohort, no significant difference in TTR was observed among patients with NDMM with high and low MTA2 levels. Interestingly, high AGO2 levels were associated with prolonged TTR (median TTR 230 *vs.* 153 days, $p = 0.045$, HR = 3.00, 95% CI, 1.02 to 8.76) compared with those with low levels (**Figure 4.17B** and **Table 4.13**). In contrast, in the RRMM cohort, high MTA2 levels at response at least VGPR were associated with shorter PFS (median PFS 306 *vs.* 628 days, $p = 0.044$, HR = 2.48, 95% CI, 1.02 to 6.02) compared with those with low levels. This effect was not observed at high and low AGO2 levels (**Figure 4.17D** and **Table 4.13**). Moreover, univariate Cox regression analysis was used to analyze the independent clinical variables associated with TTR and PFS of patients with MM, including age, ISS stage III vs. I/II, high LDH level, bone involvement features and treatment regimens. No association was observed

between these variables and patients' TTR and PFS in this study population. This implied that high AGO2 and MTA2 levels were independent factors associated with prolonged TTR and shorter PFS among patients with MM (**Figures 4.17E** and **4.17F**).

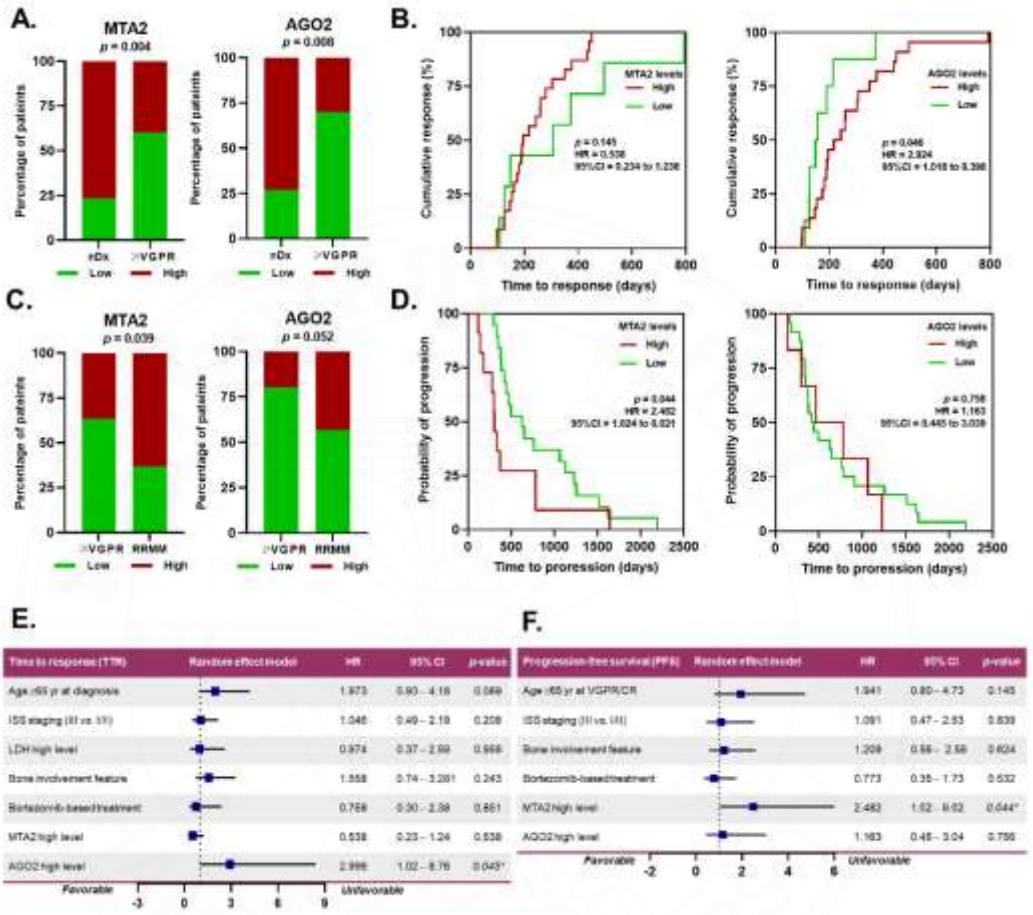

Figure 4.17 Impact of serum MTA2 and AGO2 levels on patients' outcomes. (A and C) Proportion of patients with high and low MTA2 and AGO2 levels among NDMM and RRMM cohorts. (B and D) Kaplan-Meier plots demonstrating TTR and PFS of patients according to levels of serum MTA2 and AGO2.The *p*-value is that corresponding to the Log-rank test. (E-F) Forest plots of univariate Cox models with probabilities for each factor associated with TTR and PFS of MM based on clinical variables.

Table 4.13 Univariate Cox-type cause specific hazard models of the TTR and PFS

Biomarker	Group	n	Time to response (TTR)			n	Progression-free survival (PFS)		
			HR (95%CI)	Median TTR (range), days	*p*-value		HR (95%CI)	Median PFS (range), days	*p*-value
MTA2	High	23	0.54 (0.23–1.23)	196 (97–450)	0.145	11	2.16 (1.01–5.11)	306 (119–1,645)	***0.044***
	Low	7		308 (108–793)		19		628 (298–2,192)	
AGO2	High	19	2.92 (1.02–8.40)	230 (97-793)	***0.045***	6	1.16 (0.45–3.04)	625 (141–1,231)	0.758
	Low	11		153 (108-372)		24		432 (162–219)	

A significant *p*-value is showed in bold and italic letter.
HR, hazard ratio; CI, confidence interval

4.3.5 Association of MTA2 and AGO2 gene expressions and patients with MM survival using the NCBI GEO data set

Furthermore, the effects of *MTA2* and *AGO2* gene expressions on patients with MM survival were evaluated by analyzing GSE2658[155]. No significant difference in OS was observed between patients with high and low *MTA2* expression levels (Log-rank test $p = 0.455$, HR = 1.18, 95% CI, 0.76 to 1.82). Notably, a significantly reduced OS was observed among patients with high *AGO2* expression levels compared with those with low expression levels (Log-rank test $p = 0.032$, HR = 1.60, 95% CI, 1.04 to 2.46, **Figure 4.18**).

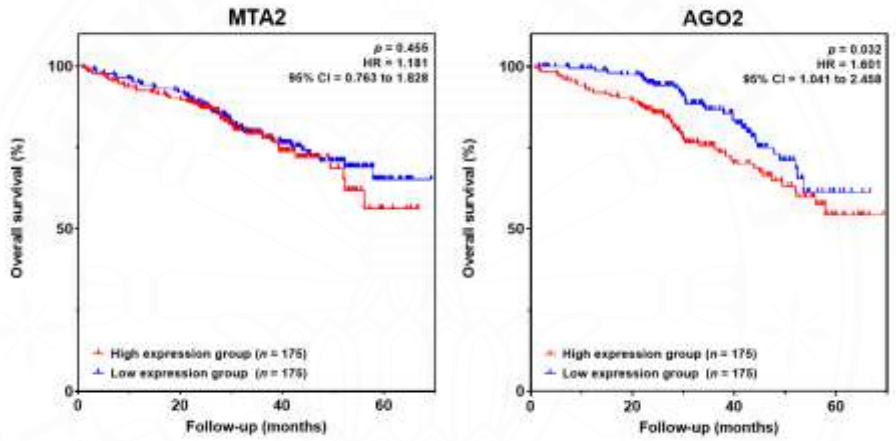

Figure 4.18 Kaplan-Meier analysis of patient outcome data from the GSE2658 [28] data set (n = 350) demonstrated the OS of patients with MM expressing higher levels of *MTA2* and *AGO2* compared with those with lower expression levels based on the median expression of the cohort.

CHAPTER 5
DISCUSSIONS AND CONCLUSIONS

5.1 Discussion

In the present study, we used LC–MS/MS technique to characterize serum proteomic profiles in all disease states of MM patients and those of age-matched normal controls. In addition, bioinformatics tools were used to analyze the enriched biological functions, interactions and pathways of the identified proteins.

As well-known, blood serum or plasma proteome is a gold mine of disease biomarkers because it contains several low-abundant proteins reflecting the overall state of the disease.[156, 157] During the disease course, which comes along with inflammation, necrosis, apoptosis and hemolysis, contents of cells may be released into the serum. The presence of those components in the serum reinforces the benefits of using a proteomic approach to discover biomarkers for disease states. Another advantage of using serum as a source for proteomic analysis is the minimally invasive and low-risk sample collection procedure, which also facilitates sequential testing in a simple routine laboratory. However, analysis of this biofluid is highly challenging due to the complexity and wide dynamic range of its proteomes.[156, 157]

In general, 99% of serum proteins constitute high-abundant proteins, including Alb, Ig, antitrypsin, transferrin, haptoglobin, alpha2-macroglobulin, alpha1-acid glycoprotein and apolipoprotein.[158] Only the remaining 1% contains many low-abundant circulatory secreted proteins that are the potential to be disease-associated biomarkers. In practice, quantitative detection of the low-abundant proteins is quite difficult because the more abundant proteins can often mask the detection of the low-abundant proteins.[158-160] Several high-abundant serum proteins depletion techniques, such as electrophoresis, chromatography and immunoaffinity-based assays, have been employed to reduce sample complexity and to enrich the low-abundant proteins before proteomics analysis.[159, 160] Although the removals of high-abundant proteins are necessary as they can increase a chance of detection of the low-abundant proteins; however, the discovery of serum biomarkers can be obstructed during this process due to protein-protein interactions between some low- and high-abundant proteins.[161, 162]

Therefore, in this study, we used whole serum protein as a source for proteomics analysis.

A total of 1,738 differentially expressed proteins were identified among the study groups. Of these, 126 proteins were common proteins expressed in all study groups. In concordance with the basic serum protein contents, high-abundant proteins, including Alb, Igs and apolipoproteins, were observed among all groups, with high relative intensity. As compared to normal controls, 81 proteins showed up-regulation, and 45 proteins showed down-regulation in the NDMM group. We found that the proteins involved in the positive regulation of the cell cycle process and the proteins involved in the DNA replication were significantly up-regulated, whereas the proteins involved in the negative regulation of catalytic activity and cellular proteins metabolic process were down-regulated in the NDMM group. This result suggested that the cell proliferation and cell turnover rates increased during the disease course in MM patients.

In the patients' serum, we found 80 proteins expressed in all states of the disease. The GO functional enrichment analysis results indicated that proteins involved in the microRNAs (miRNAs)-dependent gene expression and RNA processing were significantly up-regulated in the patient's serum. In general, miRNAs are noncoding RNAs that play a critical role in the regulation of global gene expression, and their dysregulation can contribute to the initiation and progression of a variety of cancers.[163, 164] In MM, several studies have demonstrated the overexpression of miRNAs in transcriptomic level and their association in disease pathophysiology.[165-167] Our finding confirms the alteration of miRNAs at proteomic level in MM patients.

We also found that the proteins involved in the UPS were upregulated in many states of MM patients' serum. The ubiquitin-proteasome pathway represents the major pathway for intracellular protein degradation.[168] Due to the uncontrolled production of immunoglobulins and the high rate of protein synthesis, myeloma cells subsequently required an increased demand for protein turnover and degradation. Thus, the burden of the proteins involved in the UPS is increased in myeloma cells to accelerate protein degradation and prevent the accumulation of misfolded proteins in the cells.[168-170] The proteasome also activates the NF-kB signaling, which promotes the expression of anti-apoptotic factors, cell adhesion molecules, cytokines and growth factors, resulting in increased survival of myeloma cells.[168-170] Therefore, the increasing levels of those

proteins observed in our study are consistent with the disease pathophysiological mechanisms and also confirms the important role of UPS in MM pathogenesis.

Next, we defined the signature proteins of serum obtained from normal, MGUS, NDMM, RESP and RRMM. Almost the signature proteins identified in the disease groups are mainly associated with proteasome activity, regulation of the cell cycle process, signal transduction and programmed cell death. The presence of these proteins was consistent throughout the disease spectrum from MM to RRMM. Although the signature proteins are predominately expressed in each disease stage; however, further investigation of diagnostic and prognostic values of these molecules as the potential biomarker for MM is needed.

Because MM is a disease of the elderly; therefore, we used serum samples obtained from aged-matched healthy donors as the normal control group in this study. However, the impact of aging on expression of these proteins cannot be excluded. Further study with comparison of proteins expression between younger and older is needed to elucidate the age effects on the protein expression. This study clearly demonstrated the alteration of serum proteomics profiles in each stages of MM. These results could serve as useful information for understanding the MM pathogenesis and could be used as the resource for biomarker discovery in the future.

To identify potential serum biomarkers indicating the disease activity, we analyzed the 228 differentially expressed proteins in MM patients. The GO functional, KEGG pathways enrichment, and PPI analyses were employed to discover the potential biomarkers. Our results revealed that 38 proteins, including 23 up-regulated and 15 down-regulated proteins, were considered potential candidate biomarkers. However, only the top 2 candidate proteins, MTA2 and AGO2, were chosen for further validation study.

In general, MTA2 belongs to a member of the family of transcriptional regulators, metastasis tumor-associated (MTA), which plays crucial roles in the regulation of cytoskeleton organization at the transcriptional level. MTA2 is also a central component of the NuRD complex, which plays a role in transcriptional regulation via histone deacetylation and chromatin remodeling.[171] MTA2 overexpression has been observed in several carcinomas, including gastrointestinal,

lung, renal, breast and hepatocellular carcinomas, and was associated with tumor invasion capacity, metastasis and unfavorable prognosis.[171-176] In gastric cancer, MTA2 expression was regulated by specificity protein 1 (Sp1) at the transcriptional level, and MTA2-knockdown demonstrated impairment invasion and metastasis of gastric cancer cells.[173] In non-small-cell lung cancer, MTA2 promotes the metastasis of cancer through the inhibition of the cell adhesion molecule epithelial transmembrane glycoprotein (Ep-CAM) and E-cadherin, and its increased expression is associated with poor prognosis.[174] In renal cell carcinoma, increased MTA2 expression was significantly associated with high-grade tumor and was an independent prognostic factor for unfavorable OS.[175] Moreover, in estrogen receptor-alpha–negative breast cancer, elevated MTA2 expression is associated with poor prognosis and enhanced both *in vitro* and *in vivo* tumor metastasis through Rho pathway activation.[176]

In hematological malignancies, studies relating to MTA2 are limited. A large sample size study using the whole-genome sequencing (WGS) technique revealed an association of mutation on the MTA2 gene with abnormal clonal hematopoiesis but has no known involvement in myeloid neoplasias.[177] Moreover, the related study using a mouse model has demonstrated the loss of MTA2 leading to defective BM and splenic B cell development.[178] However, studying of role and function of MTA2 involving in MM is inadequate.

The another candidate protein, AGO2 or EIF2C2, is a member of the Argonaute (AGO) protein family, which plays important roles in epigenetic gene regulation via short interfering RNAs (siRNAs) or miRNAs guided gene silencing processes, including RNA interference, translation repression and hetero-chromatinization. Furthermore, AGO2 is found to be a core component of the RNA-induced silencing complex (RISC),[179] and acts as a regulator of miRNAs function and maturation.[179, 180] AGO2 overexpression has been reported in several carcinomas, including breast, head and neck squamous cells, nasopharyngeal, urothelial, ovarian and colorectal carcinomas.[181-185] As a key regulator of miRNAs function and maturation, AGO2 is associated with myeloma cell growth and survival, angiogenesis and drug resistance mechanisms.[186-188] The related study by Zhou Y et al. reported the association between the overexpression of AGO2 and high-risk MM patients. In addition, the silencing of

AGO2 dramatically decreased viability in MM cell lines.[167] More recent study conducted by Misiewicz-Krzeminska I, et al. demonstrated a high level of AGO2 expression was significantly associated with a shorter time to progression in MM patients.[189]

In the verification phase, the serum concentrations of MTA2 and AGO2 measured by ELISA were consistent with the results from LC–MS/MS. Those protein levels were significantly higher in the disease-active states, NDMM and RRMM, compared with RESP and control groups. When the diagnostic performance of those biomarkers was evaluated, we found an excellent diagnostic value of both MTA2 and AGO2, with high sensitivity and specificity, to discriminate patients with MM from normal individuals.

In the validation phase, our results confirmed a significant alteration in the serum levels of MTA2 and AGO2 in both NDMM and RRMM cohorts. We further investigated the association between serum MTA2 and AGO2 levels and patient disease characteristics and outcomes. Regardless of the patient treatment regimens, high MTA2 levels measured at response were associated with shorter PFS. High AGO2 levels were frequently observed among females and were associated with IgG and kappa light chain isotypes. Notably, high AGO2 levels were also associated with the occurrence of bone involvement features and were an independent factor associated with prolonged TTR among patients with NDMM. A related study demonstrated that AGO2 overexpression was significantly associated with a shorter PFS among patients with MM.[189] However, this finding was not observed in our study.

The limitations of this study are first, it is a single-center study with a relatively small sample size. Some clinical features, including OS, were not evaluated due to the limited number of event and narrow-following time. Second, cytogenetic abnormalities, which are known factors affecting protein expression, were not investigated in our study. Therefore, further studies with larger sample sizes, completed cytogenetic profiles and analysis of these two biomarkers compared with traditional biomarkers for disease monitoring are suggested.

5.2 Conclusion

In conclusion, this study demonstrated the proteomic approach to characterizing and identifying serum biomarkers among patients with MM. Interestingly, MTA2 and AGO2 proteins were first identified as potential serum biomarkers providing prognostic value and potential in clinical applications. Elevated levels of these biomarkers correlated with disease activity and were associated with adverse outcomes among patients with MM. Nevertheless, validation and standardization of the proposed biomarkers before implementing in clinical practice are recommended.

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APPENDICES

APPENDIX A

ETHICAL APPROVAL STATEMENTS

1. Certificate of Approval provided by Institutional Review Board Royal Thai Ammy Medical Department, Bangkok, Thailand.

RL 01_2560

คณะอนุกรรมการพิจารณาโครงการวิจัย กรมแพทย์ทหารบก

317/5 ถนนราชวิถี เขตราชเทวี กรุงเทพฯ 10400

ที่ IRBRTA 433 /2563 รหัสโครงการ: S076h/62

ชื่อโครงการวิจัย: การวิเคราะห์โปรตีโอมิกส์ของผู้ป่วยคนไทยที่เป็นโรคมะเร็งไขกระดูกชนิดมัลติเปิลมายอีโลมา
[Proteomics profiling of Thai patients with multiple myeloma]

เลขที่โครงการวิจัย: -

ชื่อผู้วิจัยหลัก: ร้อยเอก คฑาภค อภิพงศ์วัฒน์

สังกัดหน่วยงาน: กองอายุรกรรม โรงพยาบาลพระมงกุฎเกล้า

สถานที่ทำการวิจัย: 1. ห้องปฏิบัติการโลหิตวิทยาพิเศษ กองอายุรกรรม โรงพยาบาลพระมงกุฎเกล้า
2. ห้องปฏิบัติการเทคนิคการแพทย์ คณะสหเวชศาสตร์ มหาวิทยาลัยธรรมศาสตร์
3. ห้องปฏิบัติการโปรตีโอมิกส์ สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ (สวทช.)

เอกสารรับรอง:

(1) แบบรายงานการส่งโครงร่างการวิจัยเพื่อพิจารณา ฉบับที่ 2 ลงวันที่ 25 กุมภาพันธ์ 2563
(2) โครงร่างการวิจัย ฉบับที่ 2 ลงวันที่ 25 กุมภาพันธ์ 2563
(3) เอกสารชี้แจงข้อมูลแก่ผู้เข้าร่วมโครงการวิจัย และหนังสือแสดงเจตนายินยอมเข้าร่วมการวิจัย ฉบับที่ 2 ลงวันที่ 25 กุมภาพันธ์ 2563
(4) แบบบันทึกข้อมูล ฉบับที่ 2 ลงวันที่ 25 กุมภาพันธ์ 2563
(5) ประวัติผู้วิจัย ร.อ.คฑาภค อภิพงศ์วัฒน์ ฉบับที่ 2 ลงวันที่ 25 กุมภาพันธ์ 2563
(6) ประวัติผู้ร่วมวิจัย พ.ต.ภาสกิษฎ์ ประยงค์วัฒน์ ฉบับที่ 2 ลงวันที่ 25 กุมภาพันธ์ 2563
(7) ประวัติที่ปรึกษา พล.ต.หญิง ศ.อ้อยทิพย์ ณ ถลาง ฉบับที่ 2 ลงวันที่ 25 กุมภาพันธ์ 2563

ขอรับรองว่าโครงการดังกล่าวข้างต้นได้ผ่านการพิจารณารับรองจากคณะอนุกรรมการพิจารณาโครงการวิจัยกรมแพทย์ทหารบกว่าสอดคล้องกับแนวทางจริยธรรมสากล ได้แก่ ปฏิญญาเฮลซิงกิ รายงานสภาองค์แนวทางจริยธรรมสากลสำหรับการวิจัยในมนุษย์ขององค์การสากลด้านวิทยาศาสตร์การแพทย์ (CIOMS) และแนวทางการปฏิบัติการวิจัยที่ดี (ICH GCP)

วันที่รับรองด้านจริยธรรมของโครงร่างการวิจัย: 28 มีนาคม 2563
วันสิ้นสุดการรับรอง: 27 มีนาคม 2564
ความถี่ของการส่งรายงานความก้าวหน้าของการวิจัย: 1 ปี

พันเอก
(สุชี พานิชกุล)
ประธานคณะอนุกรรมการพิจารณาโครงการวิจัย
กรมแพทย์ทหารบก

2. Certificate of Exemption provided by The Human Research Ethics Committee of Thammasat University (Science), HREC-TUSc.

ScF 03_02 (Eng)

The Human Research Ethics Committee of Thammasat University (Science), (HREC-TUSc)
Room No. 110, Piyachart Building, 1st Floor, Thammasat University Rangsit Campus,
Prathumthani 12121, Thailand, Tel: 0-2986-9213 ext.7358 E-mail: ecsctu3@Staff.tu.ac.th

COE No. 015/2564

Certificate of Exemption

Project No. : 085/2564
Protocol Title : Proteomics profiling of Thai patients with multiple myeloma
Principle Investigator : DOLLAPAK APIPONGRAT
Place of Proposed Study/Institution: Faculty of Allied Health Sciences, Thammasat University

The Human Research Ethics Committee of Thammasat University (Science), Thailand, has approved the above study project, in accordance with the compliance to the Declaration of Helsinki, the Belmont report, CIOMS guidelines and the International practice (ICH-GCP).

The Human Research Ethics Committee of Thammasat University (Science), decided to exempt the above study. These decision has been reported in 8/2564 meeting.

Signature:.....
(Assoc. Prof. Jinda Wangboonskul, Ph.D.)
Chairman of The Human Research Ethics Committee of Thammasat University (Science)

Signature: .....
(Assoc. Prof.Laksana Laokiat, Ph.D.)
Secretary of The Human Research Ethics Committee of Thammasat University (Science)

Date of issue : 5 August 2021

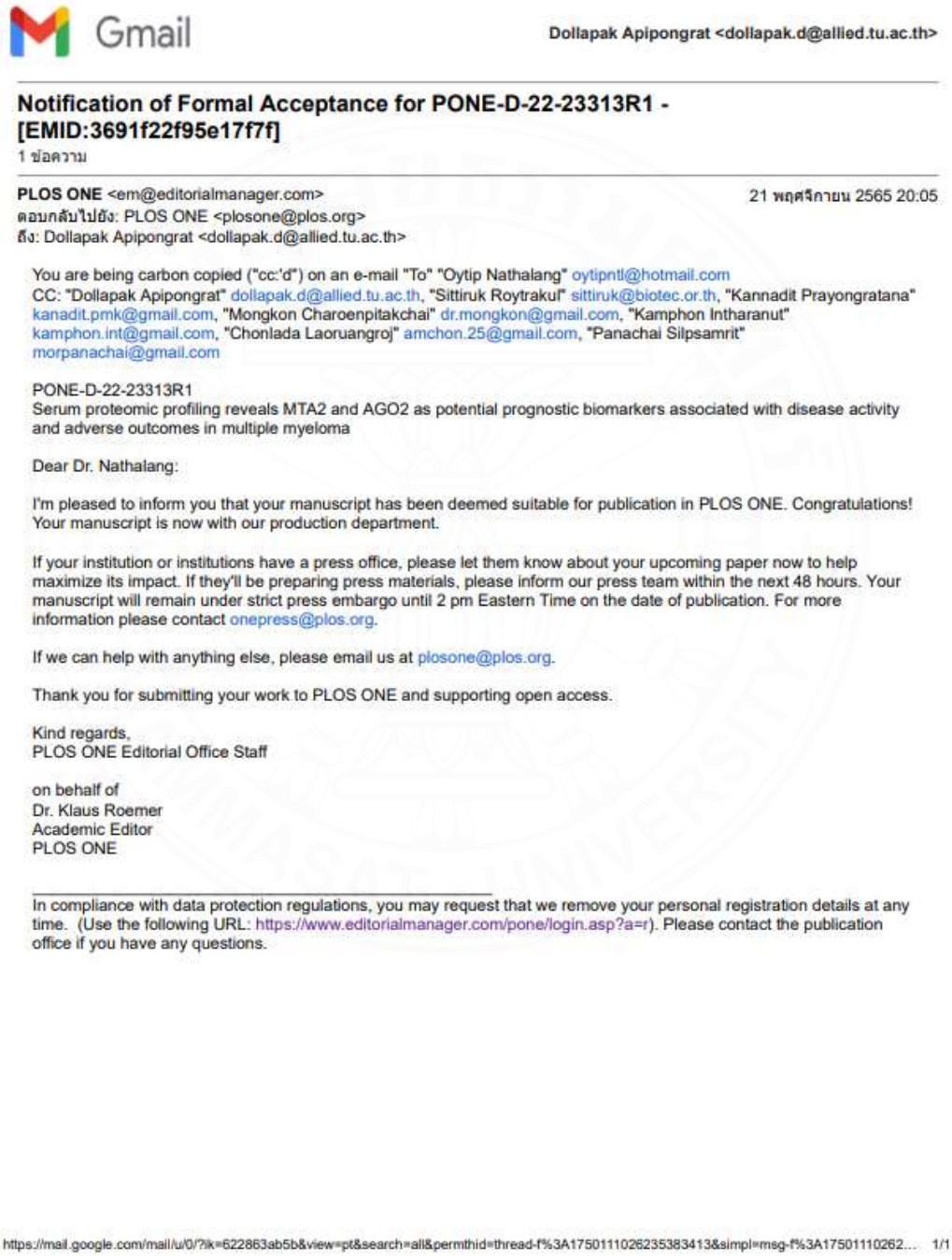
The approval documents including

1) Research proposal
2) Principal investigator's Curriculum Vitae

APPENDIX B

ACCEPTANCE LETTER FOR PUBLICATION

This study has been accepted for publication in the *PLOS ONE* journal.



Gmail

Dollapak Apipongrat <dollapak.d@allied.tu.ac.th>

Notification of Formal Acceptance for PONE-D-22-23313R1 - [EMID:3691f22f95e17f7f]

1 ข้อความ

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ถึง: Dollapak Apipongrat <dollapak.d@allied.tu.ac.th>

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PONE-D-22-23313R1
Serum proteomic profiling reveals MTA2 and AGO2 as potential prognostic biomarkers associated with disease activity and adverse outcomes in multiple myeloma

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BIOGRAPHY

Name	Captain Dollapak Apipongrat
Educational Attainment	2009: Bachelor of Science (Medical Technology) Second Class Honours 2019: Master Degree of Medical Technology (M.MT)
Scholarship	2019: The Excellent Research Graduate Scholarship – EreG Scholarship Program Under the Memorandum of Understanding between Thammasat University and the National Science and Technology Development Agency (NSTDA)

Publications

- Apipongrat D, Police P. Laboratory detection of lupus anticoagulant. RTA Med J.2019;72(4):271-7.

- Apipongrat D, Numbenjapon T, Prayoonwiwat W, Arnutti P, Nathalang O. Association between *SLC44A2* rs2288904 polymorphism and risk of recurrent venous thromboembolism among Thai patients. Thrombo Res. 2019;174:163-5.

- Apipongrat D. The *JAK2V617F, CALR* and *MPL* gene mutations in myeloproliferative neoplasms. Asian Arch Pathol. 2021:3(5);5-12.

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- Apipongrat D, Police P, Lamool R, Butthep P, Chantkran W. Validation of high concentrated thrombin time assay for unfractionated heparin monitoring. J Clin Lab Anal. 2022;36:e24695.