INVESTIGATING STATUS OF TUMORIGENIC BARRIERS IN MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE (MGUS) AND MULTIPLE MYELOMA (MM)

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Summary

Multiple myeloma (MM), an incurable late stage B-cell malignancy characterized by the presence of monoclonal plasma cells in the bone marrow, is the second most common haematological malignancy after non-Hodgkin's lymphoma. It is mostly preceded by the pre-malignant tumor stage, monoclonal gammopathy of undetermined significance (MGUS). MGUS progresses sporadically to MM with a probability of about 0.6-3% per year. Until now, all the disease-initiating genetic abnormalities are found in MGUS at a similar frequency as in MM. Thus, the genetic abnormalities causing the transformation of MGUS to MM are still unknown. Recent studies have shown that two important tumorigenic barriers, DNA damage response (DDR) and oncogene-induced senescence (OIS), are activated in various premalignant tumors, and malignant transformation is accompanied by defects in these barriers. The aim of my thesis project is to study whether defects in one or both of these barriers might also mediate transformation from MGUS to MM.

Double staining IHC method was optimized and applied to compare the differential status of DDR checkpoint and OIS between MM and MGUS. As activation of DDR and OIS give rise to senescence or apoptosis in cells, different markers of cell cycle checkpoint, proliferation, apoptosis, and senescence such as Cyclin D1, Ki67, p53, Bax, Bcl-2, CC3, and p16 were combined with CD138 as plasma cell markers and optimized in the Ventana automated stainer (Roche). The double staining IHC was important in determining the pattern of expression of these specific markers in the bone marrow plasma cells, as CD138 stained the membrane of the plasma cells whilst the other markers had either cytoplasmic or nuclear staining.

Our results showed that CC3 expression as a phenotypic marker of apoptosis was significantly (p-value=0.03) increased in MM compared to MGUS samples. However,

we did not see overexpression of the apoptosis and senescence markers in MGUS compared to MM. For example, p16 expression as a senescence marker did not change in both groups (p-value=0.09). Due to failure to find primary evidence of DDR or OIS activation in MGUS, we are not able to suggest that defects in OIS or DDR causes transformation of MGUS to MM, based on our work thus far.

Furthermore, Bax and Bcl-2 overexpression was observed in MM samples compared to MGUS (Bax p-value=0.001, Bcl-2 p-value<0.001). Whilst Bcl-2 overexpression was also associated with short overall survival (log-rank p-value=0.04) in our MM patients, Bax overexpression was not similarly associated (log-rank p-value=0.46). Expression of Cyclin D1, Ki67, p53 did not vary between MGUS and MM samples (p-values were 1, 0.18, and 0.10, respectively). Neither was overall survival of MM patients associated with expression of Cyclin D1, Ki67, p16, and CC3. In contrast, p53 expression showed significant association with poor prognosis (lon-rank p-value=0.003).

In conclusion, increased levels of pro- and anti-apoptotic markers were found in MM compared to MGUS patients; however, the senescence and proliferation markers were not varied between these two groups of patients.

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List of Abbreviations

MM	**Multiple Myeloma**
MGUS	**Monoclonal Gammopathy of Undetermined Significance**
PC	**Plasma Cell**
PCL	**Plasma cell leukemia**
SMM	**Smoldering Multiple Myeloma**
FISH	**Fluorescence In Situ Hybridization**
Ig	**Immunoglobulin**
TLC	**Translocation**
DAB	**3.3'-diaminobenzidine**
IHC	**Immunohistochemistry**
PAP	**Peroxidise-anti-peroxidise**
ABC	**Avidin-Biotin-Complex**
LSAB	**Labelled Streptavidin-biotin**
HRP	**Horseradishes peroxidise**
AEC	**3-amino-9-ethylcarbazole**
AP	**Alkaline Phosphatase**
CGH	**Comparative Genomic Hybridization**
OIS	**Oncogene-induced Senescence**
DDR	**DNA Damage Response**
PCR	**Polymerase Chain Reaction**
BMB	**Bone Marrow Biopsy**
ASCT	**Autologous Stem Cell Transplantation**

Statement of problems encountered during the project

(1) Prior to coming to Singapore to begin my M.Sc. program, I started to collect bone marrow biopsy BMB of myeloma patients from Iran to add to the Singaporean sample pool for my proposed thesis project, after consulting with my supervisor. Collecting the clinical data and BMB of 80 myeloma patients prospectively in different hospitals and pathology laboratories took 4 months, since there were no appropriate paraffin-embedded archival tissues in Iran to meet with my requirements. The 1200 sections were cut and transferred to Singapore, because we did not have permission to export the BMBs to Singapore. However, after preliminary trials, I found that these myeloma samples were not usable for this project as the quality of paraffin embedded tissues was not good for double staining immunohistochemistry (IHC) work. Indeed, the calcification of bone marrow samples and fixation process carried out at the Iranian histology laboratories were not done well.

(2) In 2008 when I commenced the IHC bench work the selection and optimization of various markers such as p53, p-p53, p-CHK2, ki67, Bcl-2, CD20, and MDM2 were done in six months and all markers were applied for single IHC staining in myeloma and MGUS samples. Unfortunately, we experienced a lot of problems to score any of the above-mentioned markers, since bone marrow has different cells and identification of plasma cells by morphology is not accurate method. In particular, the scoring of the MGUS samples was exceedingly difficult, because there are very few plasma cells in the bone marrow of MGUS patients. Thus, we decided to optimize the double staining protocol for some markers, but we were not able to do double staining without an autostainer. Finally, the Department of Pathology had a BondMax autostainer installed and we were then able to begin optimization of p53/CD138, Ki67/CD138, Bcl-2/CD138, CD20/CD138, and MDM2/CD138 in a few bone marrow

samples. With some initial success with the double-staining protocols, we subsequently bought reagents for the double staining IHC for 600 tests. Unfortunately, the specific new red kit which we acquired did not work properly and some of the bone marrow cells were not stained for red color. As the vendor had stopped production of the old kit, and were not able to resolve the problem of inadequate staining by the new replacement kit, we ended up losing 5 more months, which was the time taken to perform the optimization of various markers on the Bond-Max stainer.

(3) Following the failure with the Bond-Max staining protocols, we started to optimize markers on the Ventana autostainer, which we found to be a more suitable stainer for double staining IHC of our selected markers. We proceeded to optimize double staining for various markers including p-p53, p53, p-CHK2, DCR2, p21, p16, Ki67, Bax, Bcl-2, CD20, MDM2, Cyclin D1, and CC3. But, due to constraints of time (I only had a two-year scholarship contract provided by A-STAR, which ends in January 2010), we were just able to optimize some of the above, namely, p53/CD138, Ki67/CD138, Cyclin D1/CD138, p16/CD138, Bax/CD138, and CC3/CD138 markers.

In conclusion, our results included in my thesis write-up are based on double staining IHC performed on the Ventana stainer only. I did not include the results of the single staining for p-p53 and p-CHK2 in this thesis; however, the expressions of these two markers in single staining were negative in all samples (data not shown).

1. Introduction

1.1 Multiple Myeloma

1.1.1 Definition

Multiple myeloma (MM) is a plasma cell malignancy that is derived from a single clone and distributed at several sites of bone marrow. In many cases, it is preceded by a pre-malignant tumor stage, monoclonal gammopathy of undetermined significance (MGUS), which is the most frequent lymphoid tumor in humans.

The elevated production of monoclonal antibodies and bone disruptions are two prominent features of MM. The host response to plasma cell infiltration into different organs also leads to multiple organ dysfunctions and symptoms of renal failure, hypercalcemia, anemia, hyperviscosity, susceptibility to infections, bone pain or fractures, and neurological symptoms [1].

1.1.2 Epidemiology

Multiple myeloma is an incurable cancer with a prevalence of 20,000 new cases per year in the United States. This is the second most common hematological malignancy after non-Hodgkin's lymphoma (NHL) and accounts for approximately 1% of neoplastic diseases and 13% of hematological cancers. The incidence differs globally from 1 case per 100,000 people in China, to about 4 cases per 100,000 people in the developed countries. In addition, the incidence adjusted for gender and race is 7-10 per 100,000 in men and 4-6 per 100,000 in women, and it is two times higher in black Americans than white Americans. In Singapore, the prevalence of MM was reported as 0.6% in men and 0.5% in women from 1998 to 2002. Also, the risks for Malay females and Indian males were slightly higher than for Chinese [2].

There is no known reason for this unequal sex and race distribution. This malignancy is a disease of the elderly, with the age range of 20-92 years and the median age of 62 and 61 years in men and women respectively; with only 2% of patients being younger than 40 years old [3,4].

MM accounts for almost 20% of deaths from hematological malignancies and approximately 2% of deaths from cancers. Depending on different treatments, the survival rate varies from 3 to 7 years, for example, after conventional treatment the median survival is about 3 to 4 years. However, high dose chemotherapy treatment plus autologous bone marrow transplantation (BMT) can extend the median survival of MM patients by 5 to7 years [5,6].

1.1.3 Clinical Manifestation

Bone pain is the most common symptom in MM and affects the quality of life adversely in approximately 70% of the patients. Bone pain is precipitated by movement and typically involves the ribs and the backbone. The lytic bone lesions are caused by proliferation of plasma cells in bone, activation of osteoclasts and suppression of osteoblasts. In myeloma patients, the localized and persistent pain often indicate frequency of pathologic fracture [7].

The next common clinical manifestation is bacterial infection, which is often the major cause of death in these patients. The lung and urinary tract are the most susceptible organs to infections. *Streptococcus pneumonia*e, *Klebsiella pneumoniae*, and *Staphylococcus aureus* are common pathogens in the lung and *Escherichia coli* and other gram-negative bacteria are the most frequent pathogens in the kidney [8].

Around 20-40% of newly diagnosed patients have renal failure. This complication is generally caused by the cytotoxic effects of monoclonal light chain deposition in the renal anatomical structure, primarily in the tubules and to a lower extent the glomeruli. Other contributory factors include hypercalcemia, dehydration, and use of nephrotoxic drugs, contrast agents, and occasional infiltration of the kidney by plasma cells [9].

Approximately 20-60 % of patients have mild to moderate anemia at the time of diagnosis and almost all patients with uncontrolled disease become anemic. The anemia in MM patients is a normocytic, normochromic anemia due to replacement of BM hematopoietic cells by plasma cells and inhibition of haematopoiesis by tumor factors. Megaloblastic anemia is also present in some patients due to vitamin B12 or folate deficiency [10].

Another clinical feature of myeloma is the accompanying clotting abnormalities. This is caused by failure of antibody-coated platelets to function appropriately or interaction of the M-protein with clotting factors. MM patients are at risk of developing deep venous thrombosis (DVT), especially when receiving lenalidomide or thalidomide chemotherapy, in combination with dexamethasone.

The neurologic symptoms that occasionally occur in MM patients have many causes. Hyperviscosity may cause fatigue, headache, visual disturbances, and retinopathy. Hypercalcemia may produce lethargy, depression, weakness, and confusion. Bone damage may lead to cord compression, and loss of bladder and bowel control. Amyloid infiltration in peripheral nerves can cause sensory motor neuropathies such as carpal tunnel syndrome. In addition, sensory neuropathy can be a side effect of Bortezomib and thalidomide therapy [11].

1.1.4 Diagnosis

MM is diagnosed based on three criteria:

1- Monoclonal plasma infiltration (CD138+) cell into the bone marrow, which is assessed in bone marrow aspiration (BMA) or bone marrow biopsy (BMB).

2- Presence of M-protein in serum or urine, detected by immunofixation and electrophoresis.

3- Bone lytic lesions, screened by MRI and skeletal survey.

The most common differential diagnosis in myeloma patients is monoclonal gammopathy of undetermined significance (MGUS). MGUS can be distinguished from myeloma by an M-protein level of 0.5 to 3 g/dL, with less than 10% plasma cell in bone marrow [4].

The second differential diagnosis in myeloma patients is smoldering multiple myeloma (SMM), which contains 10-30% plasma cell infiltration in bone marrow without osteolytic lesions or any other secondary manifestations of symptomatic myeloma, and an M-protein level ≥3 g/dL. However, symptomatic MM patients also present with more than 10% monoclonal plasma cells in their bone marrow, an M-protein level of ≥3 g/dL in serum or urine, and presence of end organ damages such as osteolytic bone lesion, renal failure, and other secondary manifestations of myeloma(Table 1) [1].

Exteramedullary MM involves extramedullary sites of the bone marrow such as skin, pleural fluid, and blood. It is an aggressive malignancy, and when involving the blood is also known as plasma cell leukemia (PCL) [12].

Table 1: Diagnostic criteria for multiple myeloma and monoclonal gammopathy of undetermined significance

Monoclonal gammopathy of undetermined significance (MGUS)
M protein in serum <30 g/L Bone marrow clonal plasma cells <10% No evidence of other B cell proliferative disorders No myeloma-related organ or tissue impairment (no end organ damage, including bone lesions)
Asymptomatic myeloma (smouldering myeloma)
M protein in serum >30 g/L and/or Bone marrow clonal plasma cells >10% No myeloma-related organ or tissue impairment (no end organ damage, including bone lesions) or symptoms
Symptomatic multiple myeloma
M protein in serum and/or urine
Bone marrow (clonal) plasma cells or plasmacytoma
Myeloma-related organ or tissue impairment (end organ damage, including bone lesions)

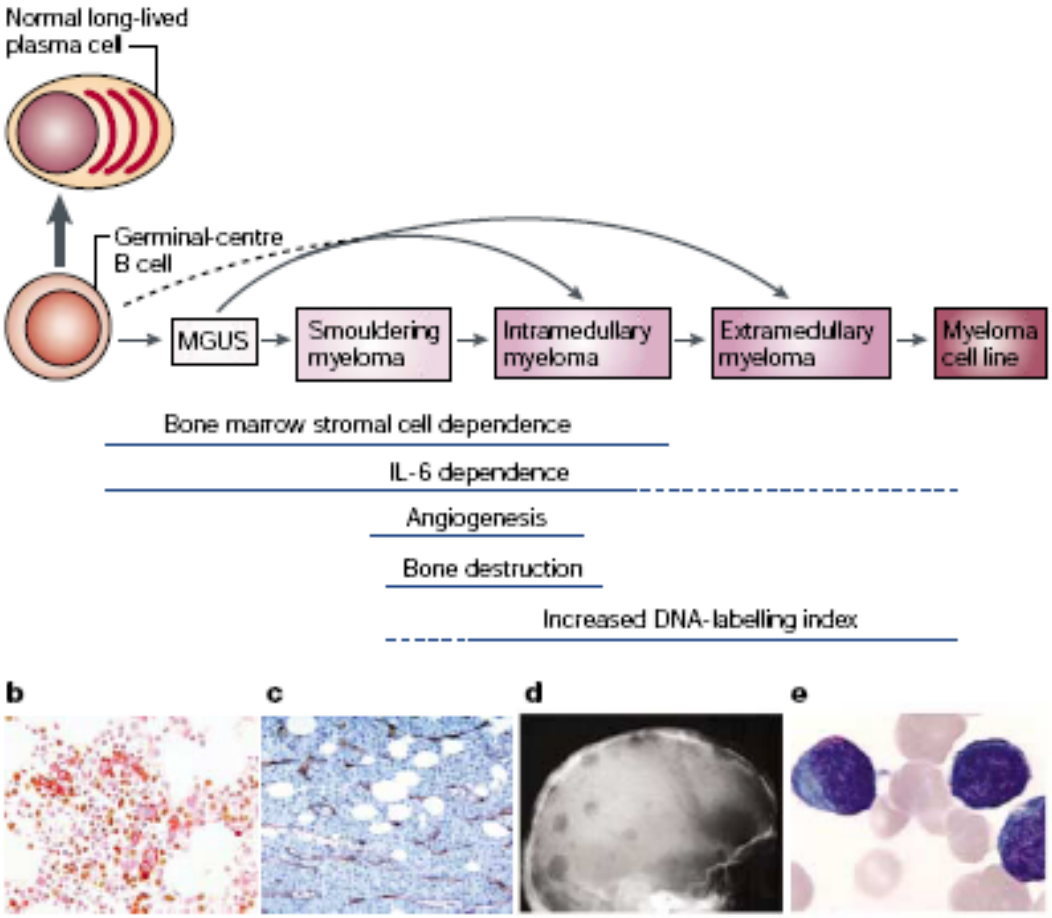


Figure 1: Staging of multiple myeloma a) MM arises from a normal germinal center of B-cell. Around 30% of MM seems to arise from MGUS without passing through SMM. The intramedullary myeloma is just localized in bone marrow, but with time the tumor can acquire the ability to invade extramedullay locations (skin, blood, and pleural fluid) and develops the extramedullary myeloma. Also, most MM cell lines are derived from extramedullary myeloma. **b)** The low proliferative index in MGUS. IHC staining for ki67 and CD138. **c)** Bone marrow staining of CD34 to show an increase vascularity in MM. **d)** Punched-out bone lesions in MM**. e)** Peripheral blood smear with plasma cells circulating in Plasma cell leukemia. *Adapted from Kuehl WM, Bergsagel PL: Multiple myeloma: evolving genetic events and host interactions. Nat Rev Cancer 2002, 2(3):175-187, with modification.*

1.1.5 Staging

There are multiple staging systems for predicting the survival of myeloma patients based on clinical data and laboratory tests. The first popular system introduced by Durie and Salmon in 1975 was based on various factors such as level and type of

monoclonal protein (M-protein), calcium level, hemoglobin, and number of bone lesions. The Durie and Salmon (DS) staging has three stages (I, II, III) and each stage is subdivided in two substages based on renal function (**A**: serum creatinine less than 2 mg/dL and **B**: serum creatinine more than 2 mg/dL). Median survival for patients in stage IA is more than 5 years and for stage IIIB is about 15 months (Table 2).

However, emerging novel targeted therapies and high dose chemotherapies in myeloma treatment made the DS staging system less applicable. An additional detracting factor is the number of lytic lesions, an important factor in DS staging, is observer dependent. Therefore, the international staging system (ISS) was proposed to overcome the limitation of DS staging.

ISS, the most powerful and reproducible classification in myeloma, is based on serum β2microglobulin (β2M) and albumin levels, which make it as a simple system with widespread use. The ISS consists of three stages: Stage I (β2M less than 3.5 mg/L and serum albumin ≥3.5 g/dL with median survival of 62 months), Stage II (neither stage I nor III with median survival of 44 months), and stage III (β2M≥ 5.5 mg/L with median survival of 29 months) (Table3) [13].

Table 2: Durie-Salmon staging system

Stage	**Criteria**	**Estimated tumor burden, × 10^{12} cells/m^2**
I	All of the following:	
	1. Hemoglobin >100 g/L (>10 g/dL) 2. Serum calcium <3 mmol/L (<12 mg/dL) 3. Normal bone x-ray or solitary lesion 4. Low M-component production a. IgG level <50 g/L (<5 g/dL) b. IgA level <30 g/L (<3 g/dL) c. Urine light chain <4 g/24h	<0.6 (low)
II	Fitting neither I nor III	0.6-1.20 (intermediate)
III	One or more of the following: 1. Hemoglobin <85 g/L (<8.5 g/dL) 2. Serum calcium >3 mmol/L (>12 mg/dL) 3. Advanced lytic bone lesions 4. High M-component production a. IgG level >70 g/L (>7 g/dL) b. IgA level >50 g/L (>5 g/dL) c. Urine light chains >12 g/24h	
Level	Stage	Median survival, months
Subclassification based on serum creatinine levels		
A<177 µmol/L (<2 mg/dL)	IA	61
B>177 µmol/L (<2 mg/dL)	IIA, B	55
	IIIA	30
	IIIB	15

Table 3: International staging system

Level	Stage	Median survival, months
β_2M<3.5, alb ≥ 3.5	I (28%)	62
β_2M<3.5, alb<3.5 or β_2M=3.5-5.5	II (39%)	44
β_2M>5.5	III(33%)	29

*Note: β_2M, serum β_2-microglobulin in mg/L; alb, serum albumin in g/dL; (#), % patients presenting at each stage.

1.1.6 Treatment

Smoldering multiple myeloma (SMM) and MGUS do not require any treatment. However, symptomatic or progressive myeloma needs therapeutic intervention, which can significantly improve the quality of life and also prolong survival of patients. Myeloma treatments are based on systemic therapy and symptomatic supportive care to control the progression of MM and prevent severe morbidity from the MM complications, respectively.

The initial standard therapy for newly diagnosed patients is based on whether or not the patient is a candidate for autologous stem cell transplantation (ASCT) plus high dose chemotherapy. In transplant candidate, melphalan should be avoided as it will damage stem cells. High-dose pulsed glucocorticoids alone or in combination VAD chemotherapy (vincristine, doxorubicin, dexamethasone) have been used for cytoreduction in newly diagnosed patients. In addition, a combination of novel agents such as thalidomide, lenalidomide, and Bortezomib with dexamethasone has rapid responses in transplant candidates [14].

In patients older than 65 years and those who are not transplant candidates, the melphalan and prednisone (MP) alone or in combination with thalidomide (MPT) are the standard therapies.

By conventional therapy, the complete response (CR) rate was very low in myeloma patients, but introduction of high-dose therapy (HDT) with ASCT increased the partial response as well as the CR rate, which led to a prolonged progression-free survival in patients. Moreover, two consecutive HTDs (with tandem ASCT) are more effective than a single HTD (transplant) in myeloma patients who do not achieve a good partial response or complete response [15].

Novel agents in myeloma treatment such as lenalidomide, bortozemib, and liposomal doxorubicin, which are effective on tumor cells and bone marrow stromal cells, can be used to treat the relapsed/refractory myeloma patients. In addition, thalidomide can achieve good responses in refractory cases if it is not used as initial therapy [16].

Supportive care of complications in myeloma are as important as primary anti-tumor treatments. For instance, the hypercalcemia responds well to Bisphosphonates, hydration, and glucocorticoids therapy. The gold standard of hyperviscosity syndrome is plasmapheresis. Since myeloma patients are immune deficient and at risk of infections, it is really important to control the lung and urinary tract infections by vaccination, prophylactic IV immunoglobulin and aggressive treatments. Supportive treatments of bone fractures, pain, fatigue, anemia, and emotional distress as well as treatment of renal failure are critical parts of the therapeutic management of MM patients, which can significantly improve their quality of life [10].

1.1.7 Pathogenesis

MM is a malignancy of incompletely differentiated plasma cells. But, the relationship between normal plasma cell and malignant plasma cell has not been identified yet, as the normal physiological plasma cell differentiation is a complex and multi-step process.

B-cells follow three optional fates after activation by antigens: elimination by apoptosis due to self-reactivity or low affinity, differentiation to memory B-cells following affinity maturation or terminal differentiation to immunoglobulin secreting plasma cells. Memory B-cells are long-lived cells, their activation by antigens leads to rapid antibody response. Plasma and memory B-cells are arrested in G1 phases of the cell cycle, prompts an important question of how the cell fate and divergent differentiation control the cell cycle [16].

Plasma cells are important for the antibody response as they produce large numbers of immunoglobulins (Igs), which are specific for antigens. In addition to Igs, the CD138 (Syndecan-1) is the only protein that increases on the surface of plasma cells during the early stages of differentiation and is an excellent marker for plasma cell identification. CD138 also presents in malignant plasma cells and its function has not been identified in normal and malignant plasma cells yet. Through homeostasis of plasma cells, primary plasma cells are not able to divide and they are arrested in the G1 phase of the cell cycle. These plasma cells are eliminated by apoptosis following Ig secretion. But, certain long-lived plasma cell populations in lamina propria of intestines and bone marrow are exempted from apoptotic elimination and they provide long-term immunity in the body. Consequently, these long-lived plasma cells are more relevant to the development of malignant myeloma cells, which also accumulate in the bone marrow [17].

Plasma cell differentiation occurs in the extra-follicular foci, because the activated B-cell leaves the germinal center (GC). Post-germinal center or germinal center B-cells have modified their immunoglobulin (Ig) genes by IgH switch recombination, sequential rounds of somatic hypermutation, and also antigen selection. Two specific DNA modifications can cause double strand DNA breaks or mutations near non-Ig genes and oncogenes in (GC) B-cells (Figure 2) [12]. Furthermore, the post-germinal center B-cells can produce plasma blasts (PBs) that have successfully finished somatic hypermutation, antigen selection, and also IgH switching before moving to the bone marrow , where stromal cells facilitate the plasma blast terminal differentiation into long-lived Plasma Cells (PCs).

The origin of long-lived and short-lived plasma cells and how they migrate to bone marrow are still mysterious. Moreover, better understanding of myeloma pathogenesis depends on complete explanation of plasma cell differentiation process. Therefore, more studies are needed to answer the underlying mechanisms of plasma cell differentiation and its relationship to tumors pathogenesis in MM.

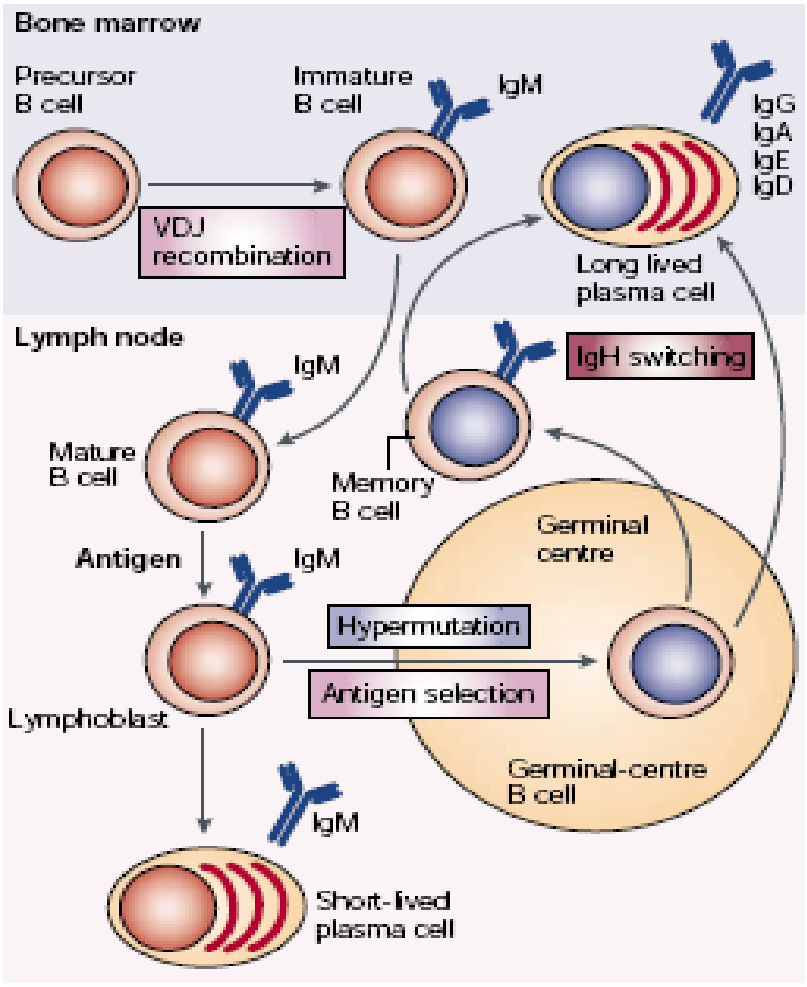


Figure 2: B-cell development. The VDJ recombination, somatic hypermutation and Ig heavy chain (IgH) switch recombination are three B-cell-specific DNA remodelling mechanisms. Sequential and regulated VDJ recombination assembles combinations of the many Ig V, D and J segments in precursor bone-marrow B-cells to create Ig heavy chain (H)/Ig light chain (L) antigen receptors. After VDJ recombination step, immature B-cells that express functional surface IgM exit the bone marrow and home to secondary lymphoid tissues as mature B-cells.
Productive interaction of mature B-cells with antigen results in proliferation and differentiation. Indeed, the primary immune response generates pre-germinal-center plasma cells that typically are short lived, and usually secrete IgM but can secrete other Igs as a result of IgH switch recombination.
Germinal centres are generated during the primary immune response. Antigen-activated lymphoblasts that enter a germinal centre are subjected to multiple rounds of somatic hypermutation of IgH and IgL V(D)J sequences and antigen selection. Cells that express high-affinity antigen receptors are selected for survival, with subsequent differentiation to memory B cells or post-germinal-centre plasma cells. Then, post-germinal-centre plasmablasts/plasma cells that undergo IgH switch recombination typically home to the bone marrow. *Adapted from Kuehl WM, Bergsagel PL: Multiple myeloma: evolving genetic events and host interactions. Nat Rev Cancer 2002, 2(3):175-187, with modification.*

1.1.8 Genetics

The majority of recent myeloma studies focus on the genetic events driving the pathogenesis of myeloma disease. Fluorescence in-situ hybridization (FISH) and cytogenetics have confirmed numerous abnormalities that involve the genes of the myeloma cells leading to both hyperdiploid and non-hyperdiploid patterns. Hyperdiploid abnormalities, caused by several trisomies (3, 5, 7, 9, 11, 15, 19, and 21), are associated with better outcome compared to non-hyperdiploid abnormalities that are associated with reduced life span because of partial loss of chromosome 17 (p13) and high risk translocations of chromosome 13. There are several recurrent IgH translocations that are presented in the primary oncogenic events and early pathogenesis of MM. These primary translocations most frequently happen at IGHR on chromosome 14 (q23.33), which is typically juxtaposed to MAF (t[14;16][q32.33;23]), CCND1 (t[11;14][q13;q32.33]), CCND3 (t[6;14][p21;q32.33]), FGFR3 and WHSC1 (t[4;14][p16.3;q32.33]), and MAFB t[14;20] [q32.33;q11.1]) [4]. The secondary translocation abnormalities that have caused disease progression are complex abnormalities of MYC, secondary Ig translocations, FGFR3 mutations, inactivation of RB1, TP53, and PTEN by mutations or deletions, activation of NRAS, KRAS, and inactivation of cyclin-dependent kinase inhibitors CDKN2C and CDKN2A (Figure3) [4].

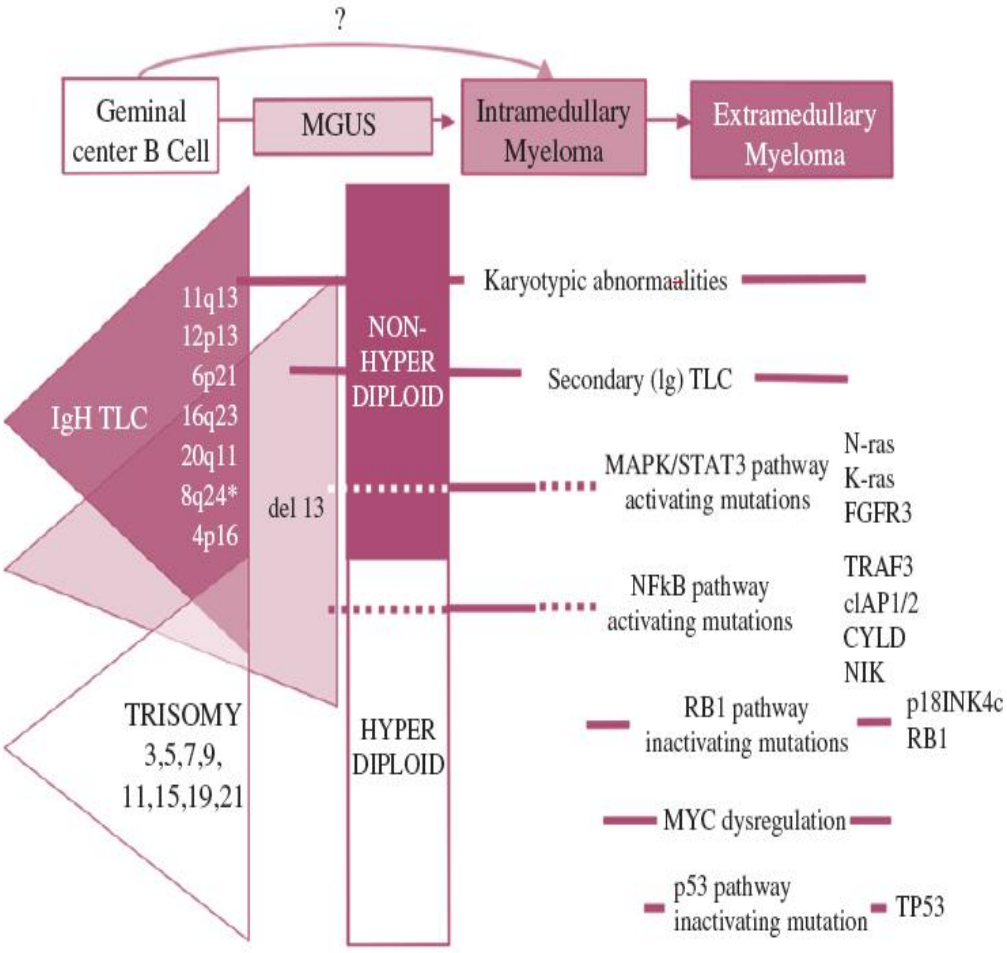


Figure 3: Disease stages and timing of oncogenic events. The earliest oncogenic changes are present in monoclonal gammopathy of undetermined significance (MGUS) and involve two minimally overlapping pathways, primary IgH translocations and multiple trisomies, each of which can include a del 13 pathway. Other karyotypic abnormalities, including secondary (Ig) TLC, and epigenetic changes can occur at all stages. Activating mutations of K- or N-RAS appear to mark, if not cause, the MGUS to multiple myeloma (MM) transition in some cases, but sometimes occur during subsequent progression of MM. Late oncogenic events that occur at a time when tumors are becoming more aggressive include MYC dysregulation by secondary (Ig) TLC, bi-allelic deletion of p18, inactivation of Rb, and loss or mutation of p53. *Adapted from Kuehl WM, Bergsagel PL: Early genetic events provide the basis for a clinical classification of multiple myeloma. Hematology Am Soc Hematol Educ Program 2005, :346-352 with modification.*

1.2 Monoclonal Gammopathy of Undetermined Significance

1.2.1 Definition

The term, monoclonal gammopathy of undetermined significance (MGUS), indicates the existence of a monoclonal protein (immunoglobulin or M-protein) in serum of individuals without the clinical features of primary amyloidosis (AL), Waldenström's macroglobulinemia (WM), and multiple myeloma (MM) or other plasma cell disorders. MGUS patients have a serum M- protein concentration of less than 3 g/dL, and the proportion of plasma cells in bone marrow less than 10%, with no clinical manifestations associated to their monoclonal gammopathy [18].

The term MGUS was first established in 1978 and replaced other terms such as benign or idiopathic monoclonal gammopathy and essential hyperglobulinemia. Monoclonal gammopathy cannot be considered as a benign disease, because a comprehensive study, which was done by Dr. Robert Kyle in 241 MGUS patients from the Mayo Clinic (Rochester, MN, USA) series in 1978, showed that a large number of these patients eventually progress to malignant disorders. Indeed, MGUS is an asymptomatic pre-malignant plasma cell disorder with high chance of progression to MM or other plasma cell dyscrasia with the passage of time [19].

1.2.2 Prevalence

The prevalence of MGUS is generally related to age, ethnic descent, and/ or race. The incidence of MGUS increases in the elderly, with the median age of 70 years at diagnostic time. Some studies showed that the prevalence rate is around 1% and 3% for individuals over the age of 50 and 70 years, respectively. In addition, the prevalence of MGUS has been reported to be two or three times higher in blacks compared with whites. For example, a study of four million white and African–

American (AA) people whom admitted to Veterans' Affairs hospitals in the USA showed that the incidence of MGUS was threefold higher in Blacks compared to whites. [20] As the incidence of MM is also twofold higher in Blacks, it can support the hypothesis that the high prevalence of MM in black population is probably due to an increased incidence of MGUS in blacks, rather than higher rate of MGUS transformation to MM [18].

Moreover, the prevalence of MGUS is low (2.4%) in Asia, which has been confirmed by a large study of atomic bomb survivors in Nagasaki of Japan. Table.4 shows the prevalence of MGUS across the world [21].

Table 4: Prevalence of MGUS across different countries

Study population	Subjects (n)	Age (years)	Prevalence (%)
Accra, Ghana	917	50-74	5.8
Olmsted county, MN, USA	21462	≥50	3.2
Provincial hospital, Italy	35005	11-75	2.9
Nagasaki city, Japan	62802	44-70	2.4
Finistere, France	30729	≥50	1.7
New York, NY, USA	73630	-	1.2
Varmland, Sweden	6995	≥25	0.9
Rangiora, New Zealand	2192	≥21	0.7
General hospital, Italy	102000	-	0.7

1.2.3 Diagnosis

MGUS is always diagnosed by a homogeneous spike in serum protein electrophoresis during routine blood test and it is an incidental finding through evaluation of other medical problems. As we mentioned, MGUS patients have serum M-protein level of less than 3 g/dL, less than 10% of bone marrow plasma cells, and also they do not have any clinical manifestations of plasma cell dyscrasia. Thus, their physical examinations are usually normal.

The most common diagnostic test is serum protein electrophoresis to determine a monoclonal protein. All MGUS patients with serum M-protein greater than 1.5 g/dL should do urine analysis as well. The urine electrophoresis is a useful method to measure the amount of M-protein in the urine, which is a guide of patient's mass and is helpful in monitoring the course of disease (Figure 4, 5).

In addition, serum or urine immunofixation is an essential, sensitive and rapid method to identify immunoglobulin class and light chain type in all monoclonal gammopathies (Figure 6) [22].

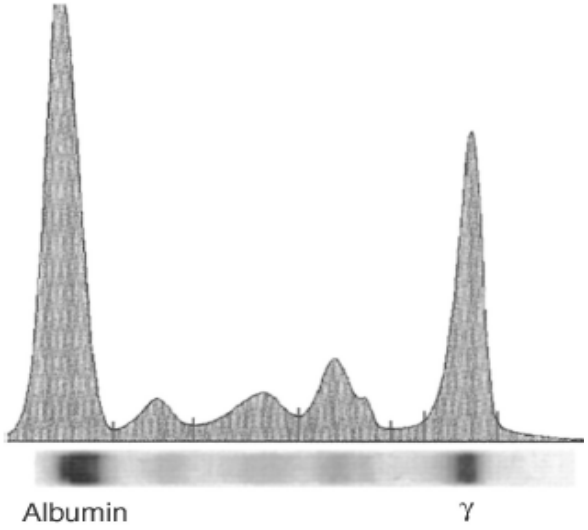


Figure 4: Pattern of serum monoclonal protein. Top) densitometer tracing after electrophoresis on agarose gel showing tall narrow-based peak of γ mobility. Bottom) electrophoresis of serum on agarose gel. Anode at left shows dense and the localized band in the right is the γ area. *Adapted from Kyle RA, Rajkumar SV: Monoclonal gammopathies of undetermined significance: a review. Immunol Rev 2003, 194:112-139 with modification*

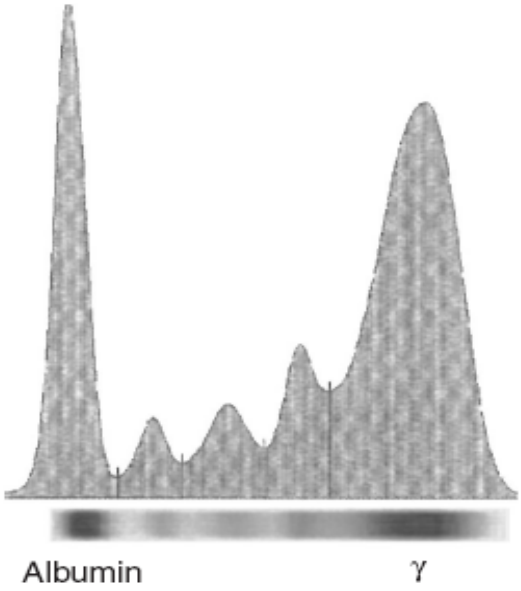


Figure 5: Polyclonal pattern from densitometer tracing of agarose gel, showing broad-based peak of γ mobility. Bottom) polyclonal pattern from electrophoresis of agarose gel (anode on left). Band on the right is broad and expand throughout the γ area. *Adapted from Kyle RA, Rajkumar SV: Monoclonal gammopathies of undetermined significance: a review. Immunol Rev 2003, 194:112-139 with modification*

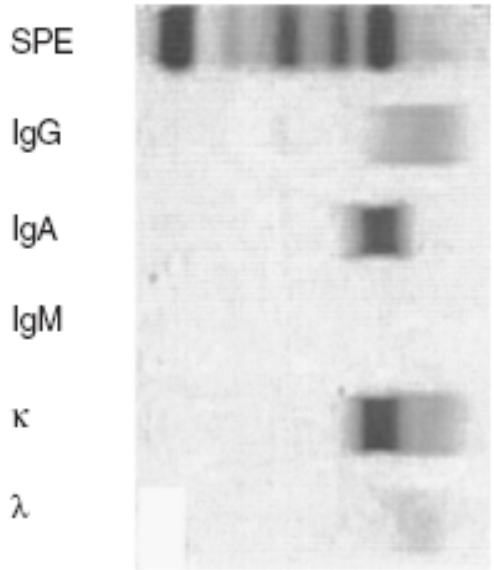


Figure 6: Immunofixation of serum with antisera to IgM, IgG, IgA, κ, and λ shows a dense band with IgA and κ, indicating an IgA- κ monoclonal protein. *Adapted from Kyle RA, Rajkumar SV: Monoclonal gammopathies of undetermined significance: a review. Immunol Rev 2003, 194:112-139 with modification*

1.2.4 Pathogenesis

MGUS are characterized by an increased level of monoclonal protein in serum or urine, which is caused by a rearrangement of immunoglobulin genes. It is important to know that a plasma cell clone has to be relatively large to produce a significant M spike at serum and urine electrophoresis. This large plasma cell clone generally remains stable for many years in MGUS patients. But, there is approximately 1% chance per year that this plasma cell clone develops into a systematic monoclonal gammopathy in patients, and then the MGUS state represents a pre-malignant phase in these patients. In fact, progression of MGUS to malignant plasma cell dyscrasia occurs by a plasma cell clone escaping from regulatory mechanisms that had restricted tumor growth for years [23].

The pathogenesis of MGUS is not known yet. However, it has been shown that half of the patients have primary IgH translocations that can be the early immortalizing events. Also, it is interesting that roughly all the MM genetic alternations such as chromosome 13q deletion, aneuploidy or translocation t(4;14) are present in many MGUS patients. Moreover, Brousseau M et al. found that MGUS and MM patients had an equivalent and high frequency of hyperdiploidy, whereas hypodiploidy was rare in MGUS. They also showed that deletion of chromosome 13q was always observed in hyperdiploid MGUS [24]. Tumor necrosis factor α and interleukin 6 (IL 6) have also been believed to be involved in pathogenesis of MGUS. On the other hand, these two cytokines were not related to progression of MGUS to malignant disorders.

Many above-mentioned genetic abnormalities observed in MGUS are early events in assembling of plasma cell clone and the pathogenesis, but they are not associated with progression of MGUS to MM. Indeed, the molecular basis of MGUS progression to

malignant gammopathies remains unknown. However, it has been recommended that one or more oncogenic events may promote genomic instability in MGUS and result in malignant transformation.

Based on recent studies, the bone marrow stromal cells might play an important role in transformation of MGUS to MM. In support of this hypothesis, it has been shown that bone marrow angiogenesis is significantly higher in symptomatic MM compared to MGUS, which may explain the role of angiogenesis in MGUS progression. Unfortunately, serial follow-up studies to show angiogenesis as a determinant factor in MGUS progression have not yet been accomplished. The immune-mediated conditions may also play an important role in progression and pathogenesis of MGUS and MM [25,26].

In conclusion, it is not clear what kind of cellular barriers control plasma cell growth in MGUS state for a long time and how these barriers break through progression to malignant plasma cell dyscrasia. Knowing the MGUS transformation events enhances understanding of MM pathogenesis, and perhaps results to develop the novel targeting therapies for treatment of MM patients as well as MGUS patients at high risk of progression.

1.2.5 Management

MGUS patients do not need any clinical interventions or treatments, but they should be followed up. Currently, there is no formal guideline to follow up the MGUS patients. However, physicians annually re-evaluate MGUS patients to determine Multiple Myeloma or other malignant gammopathies before complications. The follow up tests are including serum and urine protein electrophoresis, hemoglobin level as well as calcium concentration and serum creatinine.

The low risk MGUS patients (with M-protein level less than 1.5 g/dL, IgG type, and normal free light chain ratio) may not be followed up as the risk of malignant progression is very low. On the other hand, the high risk patients (M-protein greater than 1.5 g/dL, non-IgG type and an abnormal free light chain) should follow up every year, because they have nearly 60% chance to develop the malignant transformation during 20 years [18].

1.3 Immunohistochemistry (IHC)

The power and usefulness of immunohistochemistry (IHC) are measured by its continued successful use for years. The IHC protocols today are more or less the same as those protocols used 30 years ago. In this technique, a primary antibody is applied to tissue sections or cells to detect a target antigen by applying a detection system and also a visualization reagent (enzyme or fluorochrome). This antigen might be a protein, a lipoprotein, a glycoprotein, or even a carbohydrate.

Recently, IHC is being widely used as a diagnostic tool for detecting the presence or absence of specific proteins and certain carbohydrates in fixed and embedded tissue specimens. It is cheap, easy to perform and can help visualize cell types. The power of this technique originates from the great specificity of monoclonal antibodies for certain antigen epitopes. In general, this antibody detection system can provide valuable information based on location of normal and abnormal genes products (between different cell types or inside cellular compartments), and on the gene expression level of such products in malignant cells compared with normal cells. Indeed, IHC links the molecular biology of any cancer with its histological characteristics and behavior. In the light of abovementioned advantages, IHC has remained as the most popular method in the diagnosis of cancer.

Moreover, several reasons justify the efficacy of IHC in the field of surgical pathology. For example, this method facilitates the detection and localization of particular molecular components in cells. Furthermore, this technique has an excellent sensitivity and specificity and can be performed on chemically fixed cells, frozen cells, and archival tissues. In addition, routine IHC is quite easy to carry out in any laboratory and has been also automated. Other staining techniques do not have these advantages.

However, every method has its own limitations and IHC is not exempt from this fact. Many factors can influence staining results, which is causing a high level of interalaboratory variability in the IHC results. For example, it is applied to a large variety of tissues with different processing ways, involving some important steps with each step having potential to affect the final results. IHC is an operator-dependent method which is another limitation for this technique. Also, diverse results differed by identical antigens could be due to several factors including the use of various reagents, detection systems, and antigen retrieval methods. Moreover, the amount of antigen degradation or masking depends on the time of intraoperative anoxia, temperature and duration of fixation, the type of fixative, quantity and pH of fixative used, type of buffer, and penetration of fixative into entire tissue sample. To improve the reliability of the IHC technique, processing conditions should be optimized for each test.

During the evolution of IHC, a lot of efforts have been made to improve sensitivity for detection of antigens in the formalin-fixed and paraffin-embedded tissues. Several strategies are eventually being evaluated for increasing the sensitivity including use of direct peroxidise conjugates, avidin-biotin complex (ABC), peroxidise-anti-

peroxidase (PAP), labelled streptavidin-biotin (LSAB), and polymer based methods [27].

1.3.1 Antibodies

The key reagent common to all immunohistochemical methods is antibody. Antibodies belong to a group of proteins named immunoglobulins (Igs). The immunoglobulins have five major classes: IgM, IgA, IgE, IgG, and IgD. Each immunoglobulin has two identical heavy chains (H) and two identical light chains (L) L chains can be kappa or lambda; the H chains determine the class and subclass of Igs. In the five classes of immunolgobulins, IgG and IgM are the most frequently utilized antibodies in IHC.

Polyclonal and monoclonal antibodies are available, based on their interactions with epitopes. The polyclonal antibodies are produced by different plasma cells and are immunochemically divergent. They also react with different epitopes on the antigen against which they are raised. Rabbit is the animal that is mostly used to produce polyclonal antibodies. The polyclonal antibodies can be harvested from animal blood either in the form of stabilized antisera or immunoglobulin fractions. Precipitation by salts and ion exchange chromatography are applied to remove the bulk of other serum protein. Next, affinity chromatography can be applied to isolate the antigen-specific antibodies and free them of cross-reacting antibodies to other antigen.

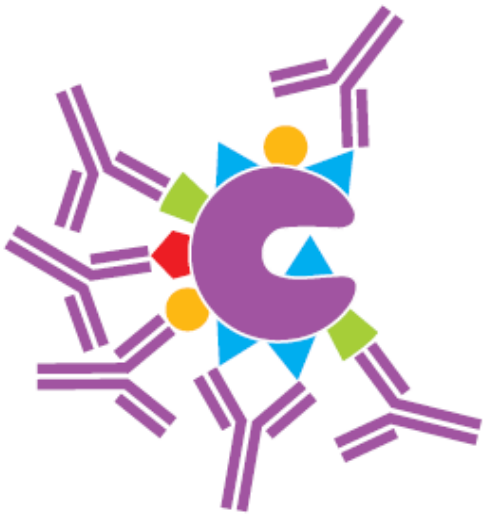


Figure 7: Polyclonal antibodies. *Adapted from* Boenisch T (Ed):*Handbook, immunochemical staining methods.* 2001.

Monoclonal antibodies are produced from an individual specific clone of plasma cells, which are identical and react with a particular epitope on the antigen against which they are raised. Mice are extensively used for production of monoclonal antibodies. When an immune response has been accomplished, the B lymphocytes of lymph nodes and spleen are collected and fused to non-secreting mouse myeloma cells to produce hybridoma cells, and then the hybridoma is cultured and tested for specific antibody reactivity. The targeted epitope of monoclonal antibodies must be unique to the certain antigen, as the specificity of a monoclonal antibody can be lost if the antibody is against an epitope shared between different antigens [28].

In addition, dilution of antibody, incubation time and its reaction temperature are important factors when optimizing the IHC technique and any changes in one factor will affect the others.

Figure 8: Monoclonal antibodies *Adapted from* Boenisch T (Ed):*Handbook, immunochemical staining methods.* 2001.

1.3.2 Fixation

The preservation of cells and tissues is an essential part of all histological and cytological techniques. Tissue blocks, sections and smear are immersed in fixative fluid. The fixative prevents autolysis by inactivating the lysosomal enzymes and inhibiting the bacterial growth. Moreover, fixatives stabilize the tissues and cells and protect them from damaged during sample processing and staining.

Fixation techniques are different and tailored according to the various techniques applied in visualizing the structure of tissues or cells. The majority proportion of immunostaining samples is embedded in paraffin and many fixatives have been formulated with this. There are several fixatives including formaldehyde-based fixative (the most common fixative), acetic acid-zinc chloride, periodate-lysine-paraformaldehyde (PLP), ethanol, and acetone [28].

1.3.3 Antigen Retrieval

The formalin-fixed paraffin-embedded (FFPE) tissues are the medium of choice in clinical and research studies, since it can generally preserve the morphology of cells

and tissues. However, fixation in formalin can mask many antigens of tissues and cause the loss of immunoreactivity. To improve the immunoreactivity of FFPE tissues, different antigen retrieval process emerged in history of IHC technique including the heating and enzymatic digestion methods (proteolytic induced epitope retrieval).

The principle of heating method depends on the time and temperature of heating in the FFPE tissues section. After deparaffinization and rehydration of tissue sections, the slides are immersed in retrieval solution (pH near 2, 6, 9, and 10). Following this step, the containers holding the slides are exposed to heat. This is the most critical step in antigen retrieval procedure. In addition, the duration and temperature of heating are important factors in antigen retrieval stage. Indeed, the majority of antigen retrieval methods used the temperature near boiling degree of water, also the optimal duration of heating varies from 10 to 60 minutes, for different fixation methods. The heating methods commonly used in IHC include the use of autoclaves, microwave oven, pressure cookers, steamers, and water baths. Consequently, each laboratory must identify the best antigen retrieval method for each antigen in specific tissues [28].

1.3.4 Enzyme Reaction

Immunoenzymatic staining techniques use enzyme-substrate reactions to change colorless chromogens into colored ones. The expressed enzymatic activity is dependent upon factors, such as enzyme and substrate concentrations, salt concentrations of the buffer milieu, pH, temperature and light exposure. Selection of the specific enzyme for each IHC application depends on different criteria:

1. The enzyme should be of high purity, but of reasonable cost.

2. Non-covalent binding and covalent binding to antibody and avidin respectively should not remove on attenuate the enzyme activity.
3. The products of reaction should be stable and easy to detect.
4. Active enzyme should be stable.

Calf intestine alkaline phosphatase and horseradish peroxidise have most of the above mentioned criteria. Therefore, they are suitable enzymes for IHC application.

Horseradishes peroxidise (HRP) enzyme is originated from the root of horseradish plant and its active site of haematin group generate its brown color when in solution. Like other enzymes, the activity of HRP can be stopped by excess substrate. There are several chromogens for the HRP enzyme including 3,3'-diaminobenzidine (DAB) with brown product, 3-amino-9-ethylcarbazole (AEC) with arose-red color end product, and 4-chloro-1-naphthol (CN) with blue product.

calf intestine alkaline phosphatase (AP) removes and transfers the phosphate group of organic esters by demolishing the phosphor-diester (P-O) bond. The key cationic activators for alkaline phosphatase are Mn++, Mg++, and Ca++. In the AP staining technique, the AP hydrolyzes its substrate to phenolic compounds and phosphates, and subsequently reaction of phenolic compounds to colorless chromogens generates colored azo dyes. There are several chromogens for AP staining, such as fast red TR with red color product and fast blue BB with blue color product, and new fuchsin with red end product [28].

1.3.5 Staining Methods

1.3.5.1 Direct staining technique

The direct method is the oldest technique, in which an enzyme-labeled primary antibody interacts with the antigen in the tissue and then substrate-chromogen is

added to reveal the reaction sequence. Since this direct method utilizes only one antibody, it is relatively fast and has limited non-specific reactions. On the other hand, because staining involves just one labelled antibody, the signal of amplification is weak and it does not have adequate sensitivity for today's demands.

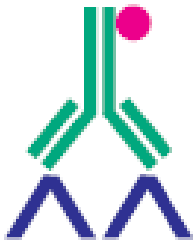


Figure 9: Direct method: enzyme labelled primary antibody reacts to tissue antigen. *Adapted from* Boenisch T (Ed):*Handbook, immunochemical staining methods.* 2001.

1.3.5.2 Two-step indirect technique

In this technique, a primary antibody binds to antigen, and then an enzyme-labeled secondary antibody is applied against the primary antibody which is now bound to antigen, followed by the last step, where in the substrate-chromogen solution is used to generate a colored product to enable detection these reactions. An important caveat is that the primary antibody is produced in mouse or rabbit, then the secondary antibody should also be raised against mouse or rabbit Igs, respectively. The indirect method is a more flexible and sensitive technique compared to direct staining.

Figure 10: Two-step indirect method: Enzyme labeled secondary antibody reacts with primary antibody bound to tissue antigen *Adapted from* Boenisch T (Ed):*Handbook, immunochemical staining methods.* 2001.

1.3.5.3 Avidin-biotin technology (ABC)

This is the most popular immunostaining method and it is based on high affinity of avidin (streptomyces avidin and chicken egg) for biotin. Both of these two avidins have four sites for biotin, but because of the molecular orientation of the binding sites, fewer than four biotins will bind to avidin. In this method, primary antibody binds to antigen, and then biotinylated secondary antibody is applied against primary antibody, followed by adding the avidin-biotin-enzyme complex (ABC) or by the enzyme-labeled streptavidin. [28]

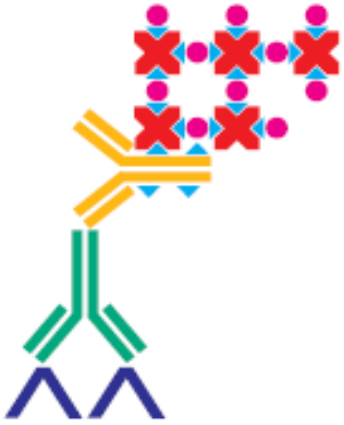


Figure 11: In the ABC technology: the streptoavidin-biotin-enzyme complex reacts with biotinylated secondary antibody *Adapted from* Boenisch T (Ed):*Handbook, immunochemical staining methods.* 2001.

1.3.5.4 Chain polymer-conjugated technology

There are two systems, namely, the DAKO EPOS and DAKO Envision systems, that used the Chain polymer-conjugated technology. This method employs an enzyme-labelled inert "spine" molecule of dextran and up to ten molecules of primary antibody can be attached to this spine molecule (polymer).

In the enhanced polymer one step (EPOS) method, one step of the immunestaining product is eliminated to prior conjugation of primary antibody to enzyme-labeled dextran. However, in the Envision system, the immunostaining is carried out first with incubation of primary antibody followed by polymer addition. Since this system avoid using the (strep) avidin and biotin staining, the non-specific staining of endogenous biotin is reduced.

The most important advantage of this technique is the reduced number of incubation steps for the staining protocol, which results in a more rapid procedure. On the other hand, increasing the incubation times of primary antibody and polymer can permit the dilution of antibodies several folds higher compared to what can be achieved in the standard ABC or LSAB protocols.

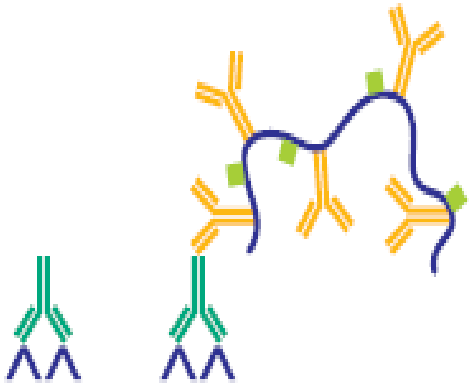


Figure 12: In the two-step EnVision System: the primary antibody is followed by a spine molecule which contains an average of 10 molecules of secondary antibody and 70 molecules of enzyme. *Adapted from* Boenisch T (Ed):*Handbook, immunochemical staining methods.* 2001.

1.3.5.5 Envision procedures for the simultaneous staining of several tissue markers

The concurrent staining of two or more antigens has a lot of limitations which make their use impractical. However, the chain polymer technology of Envision system makes this application practical. In this system, the secondary antibodies against mouse and rabbits are conjugated to peroxidase- and alkaline phosphatase-labelled polymers. A leaching step prior to the use of extra primary antibodies removes previous primary and link antibodies and leaves just the deposit of chromogens from last steps, therefore, reduce any potential carss-reactivity [29].

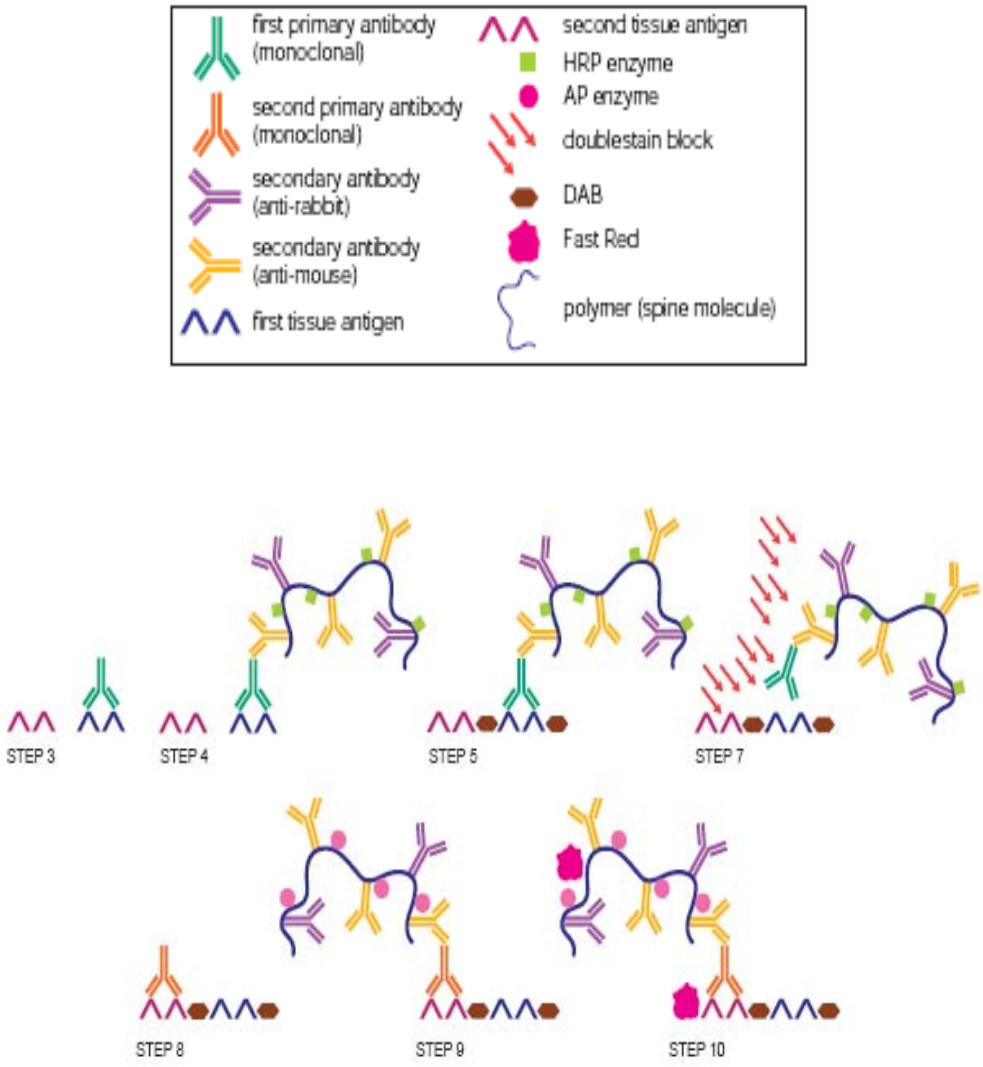


Figure 13: Double staining by the Envision System: the first primary antibody (step 3) is followed by the first spine molecule (step 4), use of a chromogen for the staining of the first antigen (step 5) is followed by a blocking reagent (step 7), application of the second primary antibody (step 8), the second spine molecule (step 9) and is concluded with the use of the second chromogen (step10). In this system a combination of rabbit polyclonal and/or mouse monoclonal primary antibodies, peroxidase and/or alkaline phosphatase, as well as chromogens such as DAB, Enhanced DAB, Fast Red, Fuchsin and BCIP/NBT can be used. (Peroxidase-block step and wash step were omitted in the figure. *Adapted from* Boenisch T (Ed):*Handbook, immunochemical staining methods.* 2001 *With modification.*

1.3.6 Automation in immunehistochemistry

In many histopathology laboratories, it is essential to develop an automation system, since manual efforts are not enough due to high demands for IHC as the most common diagnostic tool in pathology and hematology. There are several reasons that contribute to automate IHC, such as increasing output, improving reproducibility and quality, allowing better standardization and inter-laboratory comparison, and finally reducing the cost of labor and material.

The commercially available types of automated IHC stainers vary in a number of aspects, including the slide capacity per run (20-500), the technology for processing of slides (capillary gap method, liquid cover slip method), and flexibility of reagents (open versus closed system).

1.3.6.1 Bond-Max stainer

The Bond-Max system automates advanced staining processes including immunohistochemistry (IHC) and in-situ hybridization (ISH). The Bond-Max system consists of a central host computer and has 30 slide capacity. Three trays of up to ten slides each can be processed simultaneously, using different protocols if required, with each tray started separately to provide continuous processing.

Antigen retrieval procedures in Bond-Max include both enzyme and heating methods. There are two antigen retrieval solutions (ER1, ER2) for this heating system. Also, the temperature of ER solutions in this part would be around 100 C and the incubation time varies from 10 minutes to 30 minutes.

Bond-Max Stainer

1.3.6.2 Ventana BenchMark XT

This is a flexible automation stainer for IHC, ISH, FITC, and FISH methods. Optimization of protocols with different options such as extended incubation times and different temperatures are possible with this machine. Besides, there is a possibility to choose different primary antibodies from any vendor.

This system has two antigen unmasking techniques, which are heating (CC1) and enzyme (CC2) methods. CC1 is a Tris-based buffer with a slightly basic pH, which, at elevated temperatures, is capable of hydrolyzing the covalent bonds formed by formalin in tissue. Removing these bonds allows renaturation of protein molecules and increases antibody accessibility. Often these changes result in significant gains in antibody binding and improved signal-to-noise ratios. The automated slide stainer automatically heats the slide to the appropriate temperature and time as selected by the user. There are three CC1 condition: CC1 mild (30 min), CC1 standard (60 min), CC1 Extended (90 min).

Ventana Benchmark XT stainer

1.4 Background, Significance and Experimental Design

The main risk of genomic damage in mitotic cells is mutation. In addition, the host genome is always affected by many factors including the environment, products of oxidation metabolism, errors in DNA replication, etc. In the event of genetic damages by various extrinsic and/or intrinsic factors, the affected cells may attempt to repair these damages or may decide to sacrifice themselves. However, a few cells may accumulate the genetic instability and develop cancer.

Tumorgenesis is an evolutionary process in multicellular organisms that selects for epigenetic and genetic changes that are detrimental to the preservation of their survival. These changes prevent the anti-proliferative and cell death-inducing mechanisms that result in restricting the clonal growth of transformed somatic cells. The majority of tumors acquire genetic instability, but it is not clear when and how these early genetic instabilities persist, initiate, and promote cancer development. In

multicellular organisms, apoptosis and senescence are two cellular mechanisms that impede cell proliferation at the oncogenic transformation time.

In recent studies, the DNA Damage Response (DDR) and oncogene-induced senescence (OIS) have been proposed as two important in vivo barriers to tumor expansion [30-35]. These studies demonstrated the existence of DDR and OIS markers in a range of premalignant tumors, including those of the lung, skin, colon, prostate, and bladder, but more importantly, they also showed the absence of these markers upon transformation to malignant tumors.

An intricate balance between cell proliferation, cellular senescence and cell death construct a well-orchestrated harmony in cells. The widespread researches on tumor suppressor genes, oncogenes, cell cycle, and apoptosis regulatory genes, many of which are still ongoing, have shown how the DNA damage-sensing and -signalling pathways are associated with the DNA-damage response (DDR) network, cell-cycle arrest, cellular senescence and apoptosis.

Under conditions of stress, and even during normal cell cycle, checkpoint proteins play an important role in genome maintenance through the activation of DNA damage response (DDR). The DDR network is complex and involves a large number of proteins that sense the damage, transduce signals into cells, and complete cellular responses. DNA damages such as double-strand DNA breaks (DBS) are detected by different sensor proteins such as NBS1/Mre11/Rad50 in the nucleus of cells, which transmit the DNA damage signals via ATR (ATM and Rad3-related) or ATM (ataxia-telangiectasia, mutated) proteins to CHK1 (checkpoint kinase1) and CHK2 (checkpoint kinase2) proteins. Activation of these checkpoint kinases meditates activation of their downstream targets, including MDM2, p53, CDC25A, Rb, and

SMC1. Finally, the ATM and ATR activation results in G1 or G2 cell cycle arrest and triggers apoptosis or senescence (Figure 14) [36,37].

Activation of DDR checkpoints such as p-CHK2 (phospho-CHK2) and ATM were investigated in the early stages to advanced stages of bladder cancer by applying IHC, whereupon the strong positive staining was found in superficial lesions (Ta) and the early invasive stage (T1) of bladder carcinoma [30-35]. However, the more advanced stages (T2-T4) showed moderately lower staining in IHC. In addition, normal bladder tissues were completely negative for p-CHK2 staining. Analogous to its association with effects on bladder carcinoma, DDR activation was detectable in the majority of 244 invasive carcinomas of the colon, breast or lung. In contrast, 87 samples from normal, proliferative tissues, and also 26 inflammatory tissues showed negative DDR activation. Furthermore, the majority of pre-invasive carcinomas including lung hyperplasia, in situ lesions of breast cancer, and colorectal adenomas were strongly positive for DDR checkpoints. These results demonstrated that activation of DDR checkpoints occurs in premalignant tissues prior to progression to more advanced stages of cancers, presumably to prevent cancer formation in these tissues. Indeed, IHC staining of DDR checkpoints were strongly positive in premalignant tissues, but they were moderate or low in the corresponding carcinomas [30-35].

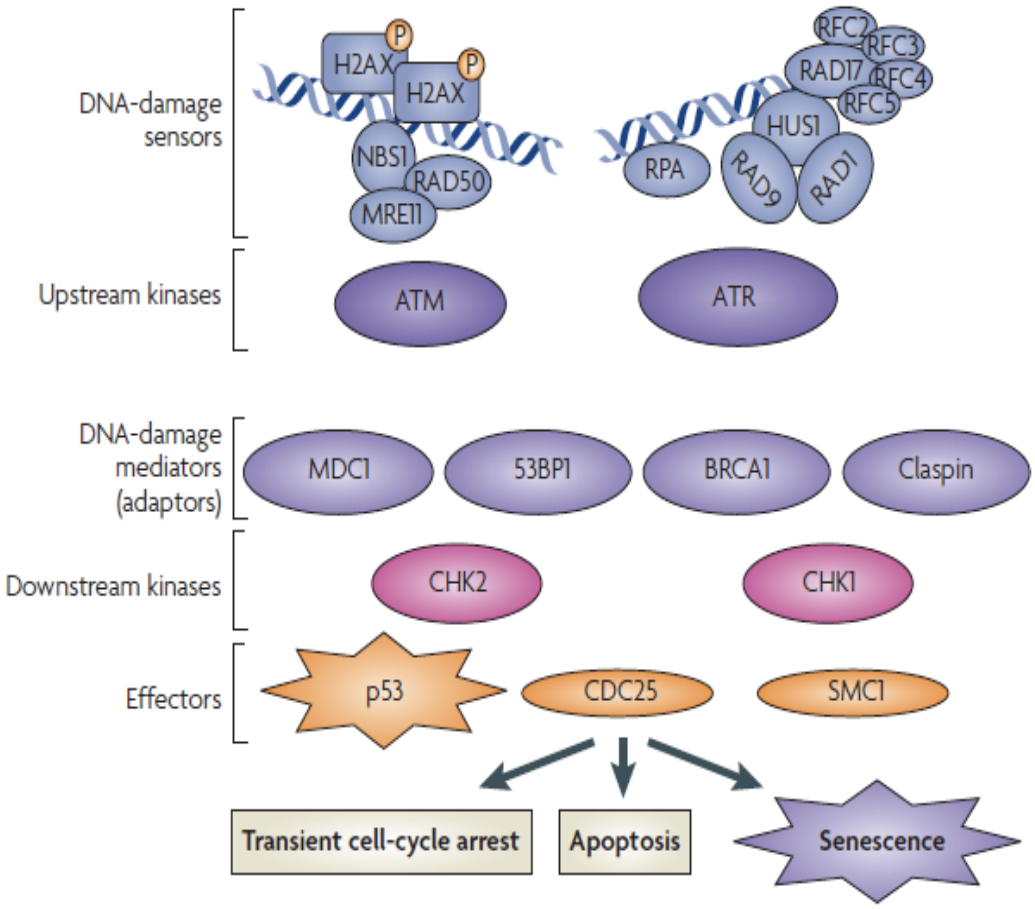
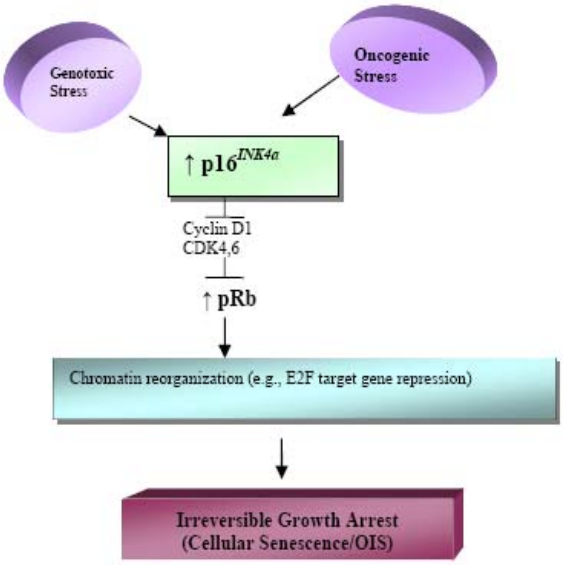


Figure 14: The DNA-damage response. *Adapted from Campisi J, d'Adda di Fagagna F: Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol 2007, 8(9):729-740*

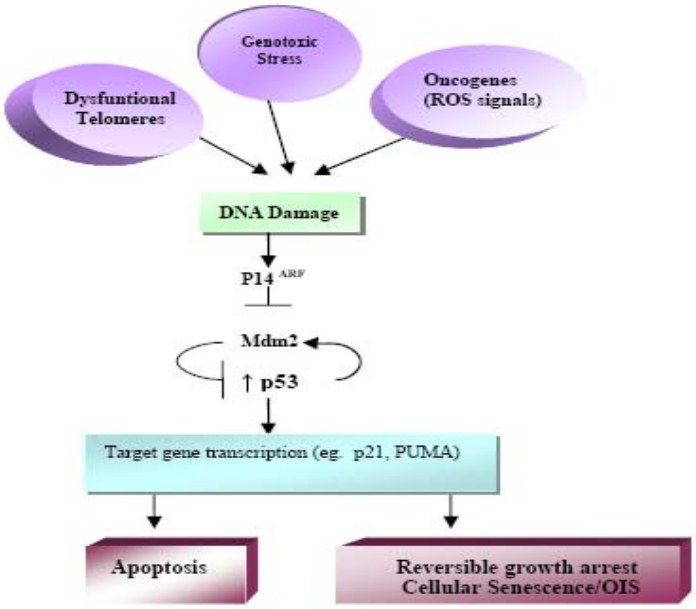
Cellular senescence was first described by Hayflick and colleagues in cultured cells. They showed that normal fibroblast cells had a limited capacity to proliferate in culture. Now, the senescence concept is generally applied to the irreversible growth arrest of cells due to various stress conditions, including telomere dysfunction, DNA damage, oncogenic activation, oxidative damage, and several chemotherapeutic drugs. There are several forms of senescence. The first is replicative senescence, the form stimulated by eroded telomeres, which relies on DDR pathways and activation of ATM and ChK2 proteins. A second type of senescence, which is induced by

cytotoxic agents, causes DNA double strand breaks (DBS) and also depends on ATM and CHK2 activations. Oncogene-induced senescence (OIS) is the third form of senescence that has been linked to activation of p16 and ARF rather than DNA damage response (DDR) or DNA DSB checkpoint pathway. Investigation of signaling pathways that are important for OIS has identified two main pathways, namely, the p16-Rb pathway and the ARF-p53 pathway (Figure 15). These two pathways are crucial for tumor suppression and are frequently altered, primarily due to mutated intermediates, in tumors. In fact, activation of OIS during tumorigenesis inhibits cancer formation. Thus, senescent cells are abundant in preneoplastic lesions compared to neoplastic tissues [32,38].

Moreover, recent studies demonstrated that OIS is connected to the presence of DNA damage and also the full activation of DDR pathways. [34] Therefore, efficient DDR activity is essential to initiate and drive OIS, and its loss allows damaged cells to proliferate. Indeed, the enforcement of DDR is both causative and necessary in the formation and maintenance of OIS. Furthermore, senescence is preceded by a "proliferation explosion", suggesting that OIS and DDR are interlinked with and reliant on DNA replication (Figure 16) [33,39].



(A)



(B)

Figure15: A) **p16INK4a-pRb senescence pathway**. **B) ARF-p53 senescence pathway**. *Adapted from Campo-Trapero J, Cano-Sánchez J, Palacios-Sánchez B, Llamas-Martínez S, Lo Muzio L, Bascones-Martínez A: Cellular senescence in oral cancer and precancer and treatment implications: a review. Acta Oncol 2008, 47(8):1464-1474 with modification.*

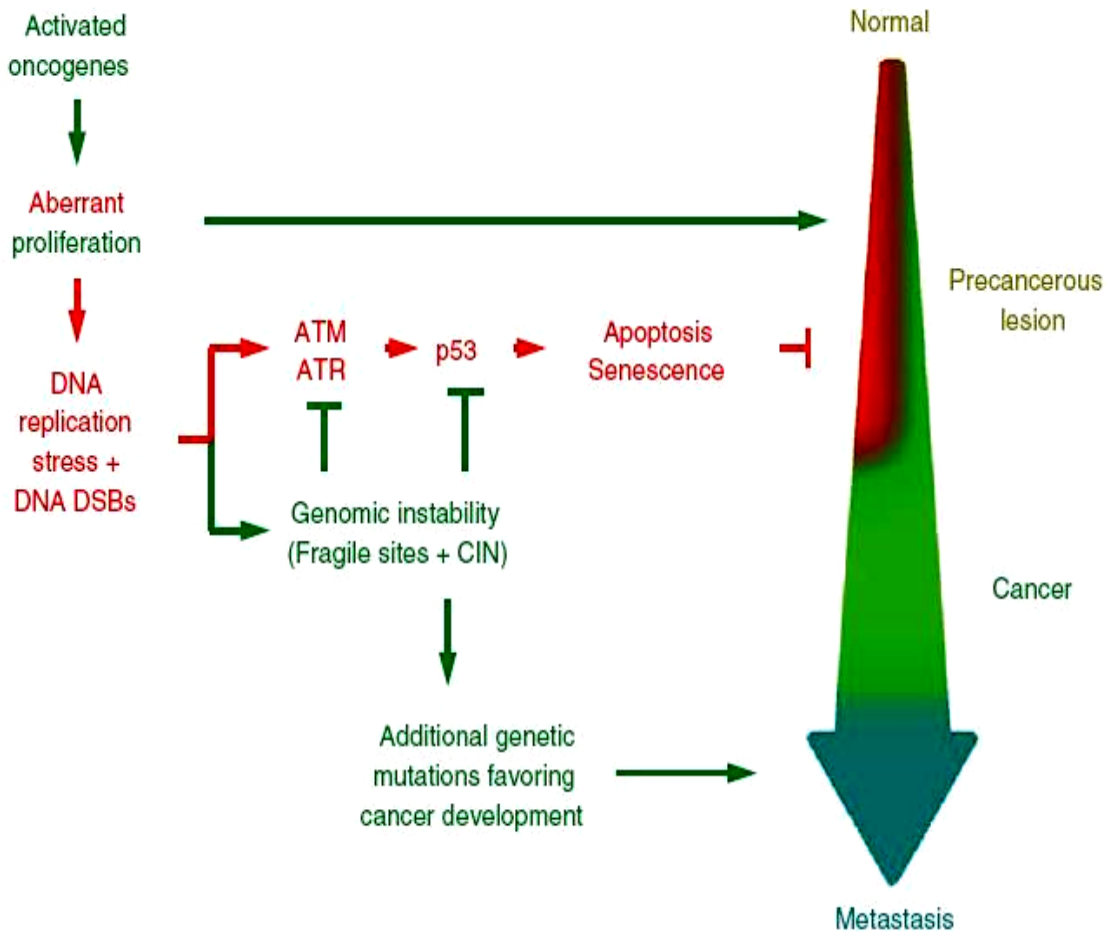


Figure 16: Oncogene-induced DNA damage model for cancer development and progression. Genomic instability and tumor suppression are direct outcomes of oncogene-induced DNA replication stress and are both present from the beginning of cancer development, before the transition from precancerous lesion to cancer. *Adapted from Halazonetis TD, Gorgoulis VG, Bartek J: An oncogene-induced DNA damage model for cancer development. Science 2008, 319(5868):1352-1355 with modification.*

The specific association of DDR and OIS with premalignant lesions might provide a unique opportunity to use the apoptotic and senescence markers as indicators of early stages of tumorigenesis events. It stands to reason that the markers that have been identified in previous studies of OIS and DDR could help in cancer staging and

treatment. In addition, chemotherapeutic drugs may induce senescence and DDR in tumor cells and thus monitoring of these markers will help to check the efficacy of therapy. Finally, recognition of additional markers of OIS and DDR and the evaluation of their values in diagnosis and prognosis offer valuable future prospects for fighting cancer.

In MGUS, oncogene activation by translocations involving the IgH (immunoglobulin heavy chains) locus, genomic instability with aneuploidy, and cell cycle deregulation by aberrant and universal D-type cyclins, are already well studied. Yet there is a long latency between MGUS and its progression to symptomatic myeloma. In addition, a few other transitional events have been implicated as contributory to transformation from MGUS to MM. One potential transition event is *ras* mutation, but it was found in just about 20% of patients at diagnosis and enriched in certain genetic subtypes such as: t(11;14) [40]. Also, a difference in hypermethylation of the promoter region of genes such as E-Cadherin and p16 between MGUS and MM was observed, but the functional effects of these changes, if any, are unknown [41]. In the case of p16, promoter hypermethylation did not appear to affect mRNA expression as the gene has, generally, a low expression in plasma cells [42]. Moreover, a new study investigated the methylation status of 6 tumor suppressor genes, including p14, p15, p16, hMLH1, MGMT, and DAPK and their association with loss of gene function [43]. Among these 6 genes, a loss of hMLH1 expression was observed in half of the MM cases compared to none of the MGUS or plasmacytoma cases. Thus, the loss of hMLH1 can plausibly play a role in the malignant transformation of plasma cells [43]. Another transition event is a breakdown in the immune surveillance by a reversible defect in NK T cells, which may contribute to transition from MGUS to MM [44]. A global genome study on MGUS and MM in Chinese patients has revealed that a

higher number of chromosomal imbalances and specific genetic alterations are involved in MGUS to MM transition (-6q,+ 3p, and+1p) and MM progression (+2p and +9q) [45]. In addition, Herve et al. showed that monosomy 13 is correlated with the transformation of MGUS to overt MM [46]. In contrast, Kaufmann et al. stated that IgH translocations and/or Del (13q) are early genetic events in monoclonal gammopathies and are not related to transformation events [47]. The summary of all studies which are directly or indirectly involved in transformation of MGUS to MM is shown in Table 5.

As was mentioned earlier, there is a long latency between MGUS and its progression to symptomatic myeloma. This suggests the existence of one or more tumorigenic barriers in MGUS prior to malignant transformation. Based on recent studies regarding activation of DDR and OIS in some premalignant tumors, we hypothesized that the absence of DDR checkpoint and senescence might be prime candidates in transformation of MGUS to MM. In support of our hypothesis, the presence of these tumorigenic barriers have been displayed in a mouse model of lymphoma, but they have not been demonstrated as yet in human hematological malignancies such as MGUS and MM [48].

In this study, we planned to collate and investigate the evidence and functional relevance of DDR and senescence in relation to cellular phenotype by using tumor samples from MM and MGUS patients. We would also look at the proliferation, pro-apoptotic and anti-apoptotic markers to evaluate the association of these markers with DDR and senescence markers. Our hypothesis model is displayed in Figure 17.

Table 5: Literature review for transformation of MGUS to MM

Author	Year	Focus of study	MGUS (%)	MM (%)	Type and size of samples	Methods
Cheng	2008	6q loss (-6q) 3p gain (+3p) 1p gain (+1p)	10.5% 10.5% 15.8%	41.5% 39% 43.9%	5 MM cell lines 19 MGUS and 69 MM BMA	CGH, CNAs cIg FISH
Paloma Martin	2008	Methylation p16 Methylation p15 Methylation hMLH1 Methylation MGMT Methylation DAPK	38% 15% 0% 8% 15%	50% 17% 10% 23% 30%	13MGUS, 29MM, 9 plasmacytoma (BMB and BMA)	Methylation specific PCR (MSP) IHC
Chng Wee Joo	2008	Ras Mutation	7%	23-25%	439 MM from ECOG14 MGUS, 88 MM from Mayo	Sensitive gel electrophoresis
Herve´ Avet-Loiseau	1999	Monosomy 13	20%	40%	19MGUS, 158 MM	FISH
Alessandra Tucci	2003	High level of IGF-1	–	–	1 MM derived from MGUS	
Seidl	2004	Methylation p16 Methylation p15 TIMP3 (Methy) ECAD (Methy) DAPK (Methy) Methylation p73 RASSF1A P14 MGMT RAR	28% 10% 35% 0% 17% 21% 14% 14% 7% 0%	36% 27% 29% 27% 22% 15% 15% 9% 4% 0%	29 MGUS, 123 MM, 7 PCL, 5 smoldering MM (BMA)	Methylation specific PCR
Dhodapkar	2003	loss of ligand-dependent IFN-γ and NKT dysfunction	–	–	6MGUS 11 MM 5 Healthy donor PB and BMA	Flow Cytometry, Cytolysis Assays, Single Cell ELISPOT,

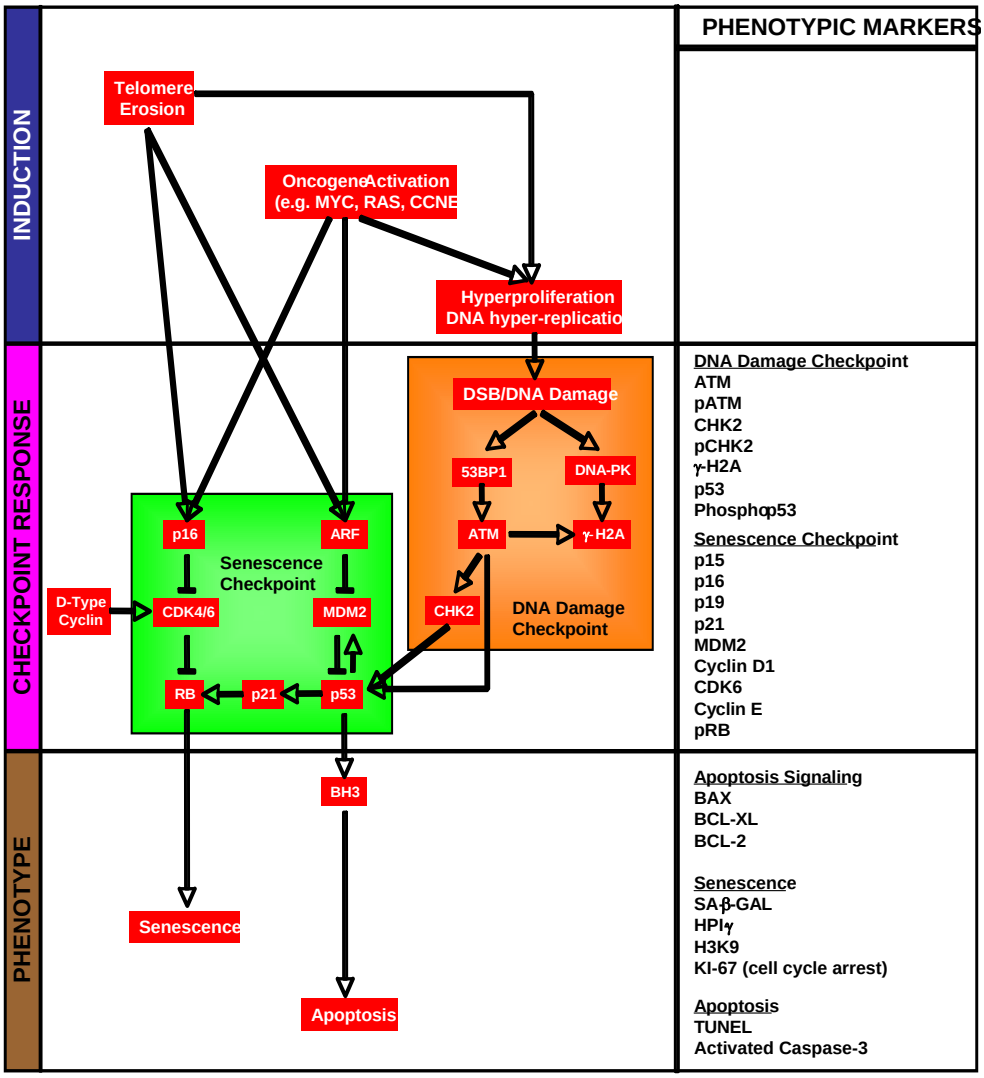


Figure 17: Model of the hypothesis for this study

2. Methods & Materials

2.1 MM Samples

Based on availability of clinical data and bone marrow biopsy (paraffin-embedded block), 92 myeloma patients were selected from NUH paraffin-embedded tissue archival. The 600 slides (a combination of H&E and different markers of IHC staining) from some of these patients were reviewed by a pathologist and a hematologist. In this review, patients with clinical diagnosis of MM and more than 15 percent plasma cells in bone marrow biopsy were selected as our MM samples. According to the first review of 92 MM patients, 10 patients were included, 14 patients excluded, and 78 patients were candidates for CD138 (marker of plasma cell) IHC and H& E staining.

In the next step, roughly 250 sections from 120 BMBs were cut and stained for H& E and CD138 (IHC) staining. In this second review, 17 patients were excluded and 47 included to our MM sample size. On the other hand, four cases were selected for repeating the CD138 and MUM1 (another marker of plasma cell). After repeating the CD138 and MUM1 IHC staining, 3 patients were excluded and 1 patient included to our cohort study of MM patients. In total, we had 58 MM patients with more than 15% plasma cells infiltration in the bone marrow biopsy of each patient. Approximately 25 to 30 sections of 3 to 4 micrometer thickness were cut for each patient and all 1500 sections stored in the minus 20 degree freezer.

Seventeen MGUS BMB samples were collected from NUH paraffin-embedded tissue archival. Roughly 500 sections were cut and stored in minus 20 freezer.

2.2 IHC single staining

Immunohistochemistry technique was selectively applied using a variety of different markers of senescence, apoptosis, and proliferation to the slides prepared as described above. Single staining IHC was optimized for CD138, MUM1, p53, Ki67, MDM2, p-CHK2, p-p53, CD20, and Bcl-2 markers and then all markers except MUM1 were tested for each MM sample individually. Finally, scoring of each marker was carried out by comparing the location of plasma cells in CD138 slide in every patient.

2.3 IHC double staining

Single staining technique was replaced by double staining method, since single IHC staining was not as accurate method to identify the plasma cells in different bone marrow cells and also was not able to evaluate the expression level of diverse markers in plasma cells precisely. In double staining IHC, one plasma marker (CD138 membrane staining or MUM1 nuclear staining) was combined with each of our interest markers such as p53, Ki67, Bcl-2, CD20. Indeed, by double staining method, all plasma cells were labeled by CD138 or MUM1, and then expression of second markers was evaluated in labeled cells (plasma cell).

The Bond-Max stainer was used for optimizing diverse combinations such as CD138/Ki67, CD138/p53, CD138/Bax, CD138/MDM2, MUM1/CD20, and MUM1/Bcl-2. When the optimizations in positive control tissues were completed, each combination of double staining was applied to all 58 MM samples (around 350 tests).

Scoring the double staining slides and comparing the result of double staining with those for single staining revealed an important problem in our Bond-Max staining system. Based on our effort to find out the problem, it was released that the AP red

detection Kit only worked randomly in each running. Another new AP red detection kit was tested and also had the same problem as the old kit. After a few months of evaluation work, it was clear that the reagent of Bond-Max stainer, AP red kit, was not suitable for double staining protocols in bone marrow.

The second double staining optimizations were carried out using the Ventana BenchMark XT stainer. Several combinations, including CD138/p53, CD138/Ki67, CD138/Bax, CD138/Bcl-1, CD138/Cleaved caspase 3, and CD138/p16 were optimized in this new machine. Finally, optimized protocols were applied to stain MM and MGUS sections and approximately 500 double staining tests were completed.

2.4 Single IHC Scoring

In each patient, five random fields of each marker were compared with the same fields of CD138 slide, which was viewed by 40X microscope objective. In the comparison of CD138 slide with any other markers, the location of plasma cells (positive CD138 cells) in CD138 slides were identified and then were correlated to the positive cells in slides of other markers. To get a ratio for expression level of each marker, the total numbers of positive plasma cells for each marker in all five fields were divided by the total numbers of CD138 cells and then this ratio were stated as percentage.

2.5 Double IHC Scoring

Scoring the double staining IHC slides is both accurate and easy to perform. In each slide, CD138 or MUM1 markers (plasma cell makers) were combined with another marker to evaluate expression level of the new marker in plasma cells (CD138 or

MUM1 positive cells). Five random fields were selected for any combination of double-staining slides. In the next step, the total numbers of double stained cells in five fields were divided by the total number of CD138 or MUM1 positive cells, and then this ratio were expressed as percentage for any markers.

2.6 Cut off determination for each marker

The cutoff point was different for each marker. A case was considered positive for Cyclin D1 or p53 if there was positive staining in ≥5% of plasma cells. In addition, positive staining in ≥ 1% of plasma cells was considered as cutoff point for Ki67, p16, and CC3. However, a case with Bax or Bcl-2 expression ≥50% of plasma cells was supposed as Bax and/or Bcl-2 overexpression case.

2.7 Dewaxing and hydration of tissue

Paraffin embedded sections were put on xylene solution 1 and 2 for five minutes each, and were rehydrated in graded concentration of ethanol (100%, 95%, 95% to 70%) and then were rinsed with distilled water.

2.8 Hematoxiline and Eosin Staining (H&E)

After deparaffinization and rehydration, sections were put into haematoxylin solution for 1 minute to stain nuclei, and then rinsed in running tap water. In addition, sections were dip in 0.3% acid alcohol one time and rinsed in running tap water. In the next step, sections were stained by Eosin for 2 minutes, rinsed by water, dehydrated by using 70%, 95%, 100%, 100% ethanol solutions and then cleared by xylene and mounted with DPX.

2.9 IHC staining

2.9.1 IHC protocol for CD138 (Single staining)

Paraffin sections were dewaxed in xylene and hydrated through the graded concentration of ethanol. Section was pressure cooked for 3 minutes at full pressure. Endogenous peroxidase activity in tissue was blocked with 3% hydrogen peroxide for 10 min. Incubate with CD138 diluted 1:100 and stain for 1 hour at room temperature (RT). CD138 was rinsed off with TBS and incubated with Detection reagent (DAKO ChemMate, Envision HRP kit). Freshly prepare DAB was applied for 6 min after rinsing off the reagent with TBS. Excess DAB was rinse off before counter-stain with hematoxylin. Sections were then dehydrated in graded concentration of ethanol, cleared in xylene and mounted with DPX.

2.9.2 IHC protocol for MUM 1 (Single staining)

Paraffin sections were dewaxed in xylene and hydrated through the graded concentration of ethanol. Sections were cooked with DAKO TRS buffer (pH 9.0) for 20 minutes in Microwave and washed in TBS. Endogenous peroxidase activity in tissue was blocked with 3% hydrogen peroxide for 10 min. Sections were incubated with MUM 1 diluted 1:50 and stains for 1 hour at RT. MUM1 was rinsed off with TBS and incubated with Envision HRP detection kit. Freshly prepare DAB was applied for 6 min after rinsing off the reagent with TBS. Excess DAB was rinsed off before counter-stain with hematoxylin. Sections were then dehydrated in graded concentration of ethanol, cleared in xylene and mounted with DPX.

2.9.3 IHC protocol for p53 (Single staining)

Paraffin sections were dewaxed in xylene and hydrated through the graded concentration of ethanol. Sections were cooked with DAKO TRS buffer (pH 6.0) for 20 minutes in Microwave and washed in TBS. Endogenous peroxidase activity in tissue was blocked with 3% hydrogen peroxide for 10 min. Sections were incubated with p53 diluted 1:100 and stained for 1 hour at RT. p53 was rinsed off with TBS and incubated with Envision HRP detection kit. Freshly prepare DAB was applied for 6 min after rinsing off the reagent with TBS. Excess DAB was rinse off before counter-stain with hematoxylin. Sections were then dehydrated in graded concentration of ethanol, cleared in xylene and mounted with DPX.

2.9.4 IHC protocol for Ki 67 (Single staining)

Paraffin sections were dewaxed in xylene and hydrated through the graded concentration of ethanol. Sections were cooked with DAKO TRS buffer (pH 6.0) for 20 minutes in Microwave and washed in TBS. Endogenous peroxidase activity in tissue was blocked with 3% hydrogen peroxide for 10 min. Sections were incubated with Ki-67 diluted 1:200 and stained for 1 hour at RT. Ki67 was rinsed off with TBS and incubated with Envision HRP detection kit. Freshly prepare DAB was applied for 6 min after rinsing off the reagent with TBS. Excess DAB was rinsed off before counter-stain with hematoxylin. Sections were then dehydrated in graded concentration of ethanol, cleared in xylene and mounted with DPX.

2.9.5 IHC protocol for Bcl-2 (Single staining)

Paraffin sections were dewaxed in xylene and hydrated through the graded concentration of ethanol. Sections were cooked with DAKO TRS buffer (pH 9.0) for

20 minutes in Microwave and washed in TBS. Endogenous peroxidase activity in tissue was blocked with 3% hydrogen peroxide for 10 min. Sections were incubated with Bcl2 diluted 1:100 and stained for 1 hour at RT. Bcl2 was rinsed off with TBS and incubated with Envision HRP detection kit. Freshly prepare DAB was applied for 6 min after rinsing off the reagent with TBS. Excess DAB was rinsed off before counter-stain with hematoxylin. Sections were then dehydrated in graded concentration of ethanol, cleared in xylene and mounted with DPX..

2.9.6 IHC protocol for CD 20 (Single staining)

Paraffin sections were dewaxed in xylene and hydrated through the graded concentration of ethanol. Sections were cooked with DAKO TRS buffer (pH 9.0) for 20 minutes in Microwave and washed in TBS. Endogenous peroxidase activity in tissue was blocked with 3% hydrogen peroxide for 10 min. Sections were incubated with CD 20 diluted 1:100 and stained for 1 hour at RT. CD 20 was rinsed off with TBS and incubated with Envision HRP detection kit. Freshly prepare DAB was applied for 6 min after rinsing off the reagent with TBS. Excess DAB was rinse off before counter-stain with hematoxylin. Sections were then dehydrated in graded concentration of ethanol, cleared in xylene and mounted with DPX.

2.9.7 IHC protocol for p-p53 (Single staining)

Paraffin sections were dewaxed in xylene and hydrated through the graded concentration of ethanol. Sections were cooked in 118 degree with DAKO TRS buffer (pH 6.0) for 20 minutes by pressure cooker and washed in TBS. Endogenous peroxidase activity in tissue was blocked with 3% hydrogen peroxide for 10 min. Incubate section with p-p53 diluted 1:100 and stain for 24 hour at four degree. p-p53

was rinsed off the with TBS and incubated with Envision HRP detection kit. Freshly prepare DAB was applied for 6 min after rinsing off the reagent with TBS. Excess DAB was rinse off before counter-stain with hematoxylin. Sections were then dehydrated in graded concentration of ethanol, cleared in xylene and mounted with DPX.

2.9.8 IHC protocol for p-CHK2 (Single staining)

Paraffin sections were dewaxed in xylene and hydrated through the graded concentration of ethanol. Sections were cooked with DAKO TRS buffer (pH 6.0) for 20 minutes in Microwave and washed in TBS. Endogenous peroxidase activity in tissue was blocked with 3% hydrogen peroxide for 10 min. Incubate section with p-CHK2 diluted 1:100 and stain for 1 hour RT. p-CHK2 was rinsed off with TBS and incubated with Envision HRP detection kit. Freshly prepare DAB was applied for 6 min after rinsing off the reagent with TBS. Excess DAB was rinse off before counter-stain with hematoxylin. Sections were then dehydrated in graded concentration of ethanol, cleared in xylene and mounted with DPX.

2.9.9 IHC protocol for MDM2 (Single staining)

Paraffin sections were dewaxed in xylene and hydrated through the graded concentration of ethanol. Sections were cooked in 118 degree with DAKO TRS buffer (pH 6.0) for 30 minutes by pressure cooker and washed in TBS. Endogenous peroxidase activity in tissue was blocked with 3% hydrogen peroxide for 10 min. Sections were incubated with MDM2 diluted 1:100 and stained for 1 hour at RT. MDM2 was rinsed off with TBS and incubated with Envision HRP detection kit. Freshly prepare DAB was applied for 6 min after rinsing off the reagent with TBS.

Excess DAB was rinse off before counter-stain with hematoxylin. Sections were then dehydrated in graded concentration of ethanol, cleared in xylene and mounted with DPX.

Table 6: Protocols of double staining IHC in Ventana BenchMark XT stainer

Combination of markers	Antigen retrieval condition	First marker staining protocol with DAB detection kit	Second marker staining protocol with AP red detection kit
CD138/p53	CC1-standard (60 minutes)	CD138 incubation 40 min with 37 C, dilution 1:100	p53 incubation 40 min with 37 C, dilution 1:100
CD138/Ki67	CC1-standard (60 minutes)	CD138 incubation 40 min with 37 C, dilution 1:100	Ki67 incubation 40 min with 37 C, dilution 1:100
CD138/Bax	CC1-extended (90 minutes)	CD138 incubation 40 min with 37 C, dilution 1:100	Bax incubation 40 min with 37 C, dilution 1:50
CD138/Bcl-1	CC1-extended (90 minutes)	CD138 incubation 32 min with 37 C, dilution 1:100	Bcl-1 incubation 32 min with 37 C, dilution 1:20
CD138/p16	CC1-standard (60 minutes)	CD138 incubation 32 min with 37 C, dilution 1:100	p16 incubation 32 min with 37 C, dilution 1:50
CD20/MUM1	CC1-standard (60 minutes)	CD138 incubation 40 min with 42 C, dilution 1:100	MUM1 incubation 40 min with 42 C, dilution 1:100
CD138/CC3	CC1-mild (30 minutes)	CD138 incubation 40 min with 42 C, dilution 1:100	CC3 incubation 60 min with 42 C, dilution 1:50

Table 7: Protocols of double staining IHC in Bond-Max stainer

Combination of markers	First marker staining protocol with bond polymer refine detection kit	Second marker staining protocol with bond polymer AP red detection kit
CD138/p53	CD138 incubation 30 min CD138 dilution 1:100 ER2 (Antigen retrieval) 20 min Detection kit (Bond polymer refine kit) 70 min	p53 incubation 30 min p53 dilution 1:100 ER1 (antigen retrieval) 20 min Detection kit (Bond polymer AP red) 75 min
CD138/Ki67	CD138 incubation 30 min CD138 dilution 1:100 ER2 (Antigen retrieval) 20 min Detection kit (Bond polymer refine kit) 70 min	Ki67 incubation 30 min Ki67 dilution 1:200 ER1 (antigen retrieval) 20 min Detection kit (Bond polymer AP red) 75 min
CD138/Bax	CD138 incubation 30 min CD138 dilution 1:100 ER2 (Antigen retrieval) 20 min Detection kit (Bond polymer refine kit) 70 min	Bax incubation 30 min Bax dilution 1:50 ER2 (antigen retrieval) 20 min Detection kit (Bond polymer AP red) 75 min
CD138/MDM2	CD138 incubation 30 min CD138 dilution 1:100 ER2 (Antigen retrieval) 20 min Detection kit (Bond polymer refine kit) 70 min	MDM2 incubation 30 min MDM2 dilution 1:50 ER1 (antigen retrieval) 20 min Detection kit (Bond polymer AP red) 75 min
CD20/MUM1	CD20 incubation 30 min CD20 dilution 1:1500 ER2 (Antigen retrieval) 20 min Detection kit (Bond polymer refine kit) 70 min	MUM1 incubation 30 min MUM1 dilution 1:100 ER1 (antigen retrieval) 20 min Detection kit (Bond polymer AP red) 75 min
Bcl-2/ MUM1	Bcl-2 incubation 30 min Bcl-2 dilution 1:100 ER2 (Antigen retrieval) 20 min Detection kit (Bond polymer refine kit) 70 min	MUM1 incubation 30 min MUM1 dilution 1:100 ER1 (antigen retrieval) 20 min Detection kit (Bond polymer AP red) 75 min

*ER1: antigen retrieval solution 1, *ER2: antigen retrieval solution 2

2.10 Double IHC staining in automated stainer

The two systems of automated stainer, Bond-Max and Ventana Bnchmark, have different programs for running the double staining IHC and also their antigen retrieval are particular for each stainer. Antigen retrieval procedures in Bond-Max include both enzyme and heating methods with two antigen retrieval solutions (ER1, ER2) for this heating system. In addition, the Ventana BenchMark applies two antigen unmasking techniques, including heating (CC1) and Enzyme (CC2). The CC1 that were used in this study is a Tris-based buffer with a slightly basic pH and has three conditions based on temperature and time of heating: CC1 mild (30 min), CC1 standard (60 min), CC1 Extended (90 min). All optimized double staining IHC protocols were summarized in Table 6, and 7.

2.11 Statistical analysis

Statistical data was computed utilizing SPSS version 17.0. Fisher exact test for two categorical variables was applied to identify the relationship between diagnosed group (MM and MGUS) and expression of each marker. In addition, Mann-Whitney U test applied to compare the Bcl-2/Bax ratio between MM and MGUS samples. Statistical significance was set at $p<0.05$.

The survival analysis of myeloma patients was done using Graphpad Prism software. The survival curves were constructed according to the Kaplan-Meier method and then the comparison between survival curves was done using the log-rank test.

2.12 Reagents for single staining IHC

Dako wash buffer (S3006)

Dako antibody diluents (S0809)

Dako proxidase blocking reagent (S2001)

Dako target retrieval solution (S1699)

Dako target retrieval solution high pH (S3307)

Dako Real ^{TM} (HRP Rabbit/ Mouse) (K5007)

Dako Real ^{TM} (Substrate buffer)

Dako Real ^{TM} (DAB + chromogen X^{50})

2.13 Leica Bond-Max reagents

Bond epitope retrieval solution 1 (1L AR9961)

Bond epitope retrieval solution 2 (1L AR9640)

Bond wash solution 10X (1L AR9590)

Bond primary antibody diluents (1L AR9362)

Bond dewax solution (4×1L AR9222)

Bond DAB enhancer (30mL AR9432)

Bond polymer AP red detection kit (DS9305)

Bond polymer refines detection kit (DS9800)

2.14Ventana BenchMark XT reagents

Ultraview Red Detection Kit (760-501)

Bluing Reagent (760-2037)

Haematoxylin II (790-2208)

Kit Pack, Ebar (US/Europe) (1418702)

CC1 (950-124)

Ultraview DAB Detection Kit (760-500)

Reaction buffer (950-30PC)

LCS (650-010)

EZ PREP solution (950-102)

Table 8: Characteristic of utilized antibodies

Antibody Name	Type	Company	Catalogue Number
Bcl-1 (SP4)	Rabbit Monoclonal	Termo scintific	RM-9104-R7
Bcl-2	Mouse monoclonal	Dako	M0887
Bax	Rabbit polyclonal	Dako	A3533
Dcr2	Rabbit polyclonal	abcam	Ab-2019
CD138	Mouse monoclonal	Dako	M7228
CD20	Mouse monoclonal	Dako	M0755
Cleaved Caspase3	Rabbit polyclonal	Cell signaling	9661s
Ki67	Mouse monoclonal	Dako	M7240
MUM1	Mouse monoclonal	Dako	M7259
p-p53	Rabbit polyclonal	Santa cruz Biotechnology	SC-101762
p-CHK-2	Rabbit polyclonal	Cell sinaling	2666
P16	Mouse monoclonal	Santa cruz Biotechnology	SC-56330
P53	Mouse monoclonal	DAKO	M7001
MDM2	Mouse monoclonal	Millipore	MAB3776

Table 9: Positive control tissues for each antibody

Name of Marker	Positive control tissue
CD138	Tonsil
MUM-1	Tonsil
Bcl-2	Tonsil
p53	Tonsil
p-p53	Breast carcinoma
MDM2	Tonsil
CD20	Tonsil
Bax	Hodgkin's lymphoma
P16	Tonsil, cervix
Cleaved caspase 3	Tonsil
p-CHK2	Breast carcinoma
Dcr2	Tonsil, breast carcinioma
Ki67	Tonsil

3. Results

3.1 Evaluation of p53 expression

3.1.1Staining pattern

The staining pattern of p53 expression is nuclear that is shown as red color (Figure 18). However, the CD138 (plasma cell marker) stains the membrane of plasma cells with brown color. Therefore, the positive plasma cells for p53 expression have a combination of red color in the nucleus and brown color on the membrane (Figure18). In this study, sample with more than 1 % positive p53 plasma cells was considered as positive case for p53 expression.

3.1.2 p53 expression in MGUS and MM

The results of p53 expression for MGUS and MM samples did not show any significant difference (p-value 0.109). All p53 positive cases (12%, 9 from 75 cases) had MM diagnosis and there were not positive p53 cases in MGUS patients (Table 10).

However, the survival analysis of the MM p53 positive patients indicated a significant association (log-rank p-value= 0.0003) between p53 expression and poor prognosis in MM patients. The median overall survival of p53 positive cases was 4.4 months while the median survival of p53 negative cases was 84 months (Figure 19). In addition, the median overall survival of the entire cohort in this study was 79.5 months.

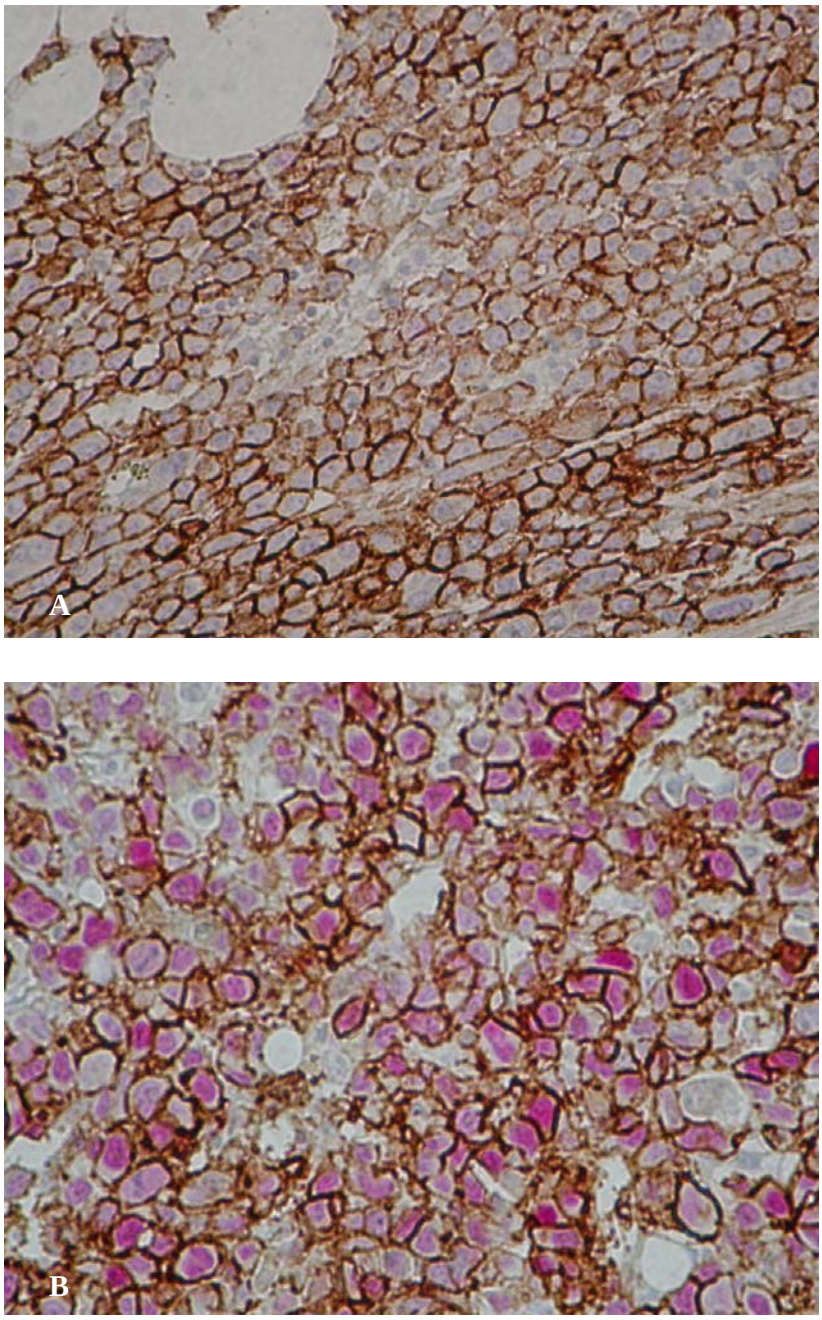


Figure18: A) Negative p53 expression in CD138/p53 double staining of MM case. **B)** CD138/p53 double staining with more than 90% expression of p53 in plasma cells.

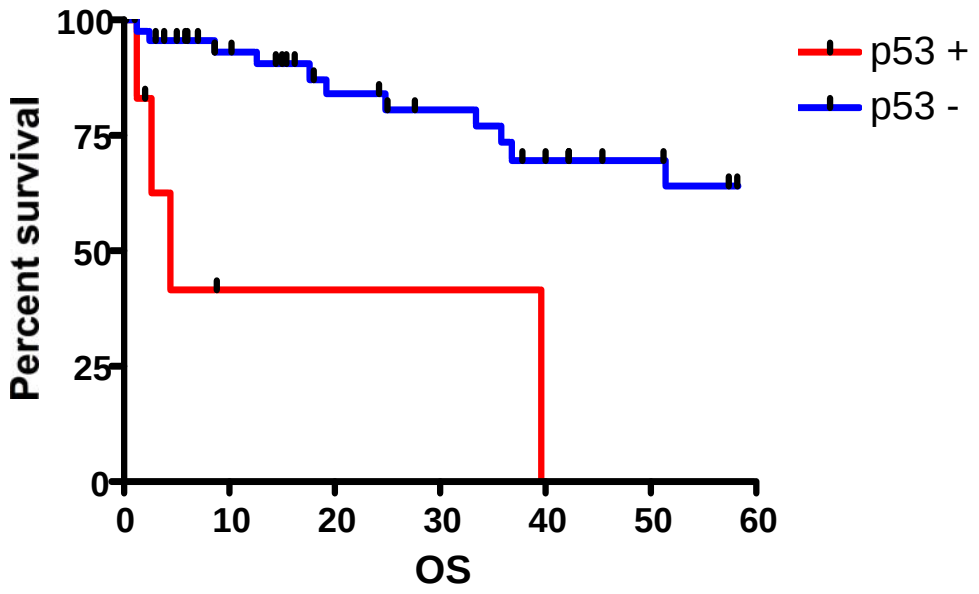


Figure 19: Overall survival of patients with positive p53 expression was significantly less than that of patients with negative p53% expression (log-rank p-value=0.0003)

3.2 Evaluation of Bax and Bcl-2 expression

3.2.1 Staining pattern of Bax

Bax expression has cytoplasmic staining that is shown as red color in Figure 20. Erythroid and myeloid precursors in bone marrow served as internal positive controls for Bax expression, whilst the CD138 (plasma cell marker) stains the membrane of plasma cells with brown color. Therefore, plasma cells that have positive Bax expression, have a combination of red color in the cytoplasm and brown color on the cell membrane (Figure 20). In this study, plasma cell samples with more than 49 % positive Bax staining were considered as positive cases with overexpression of Bax.

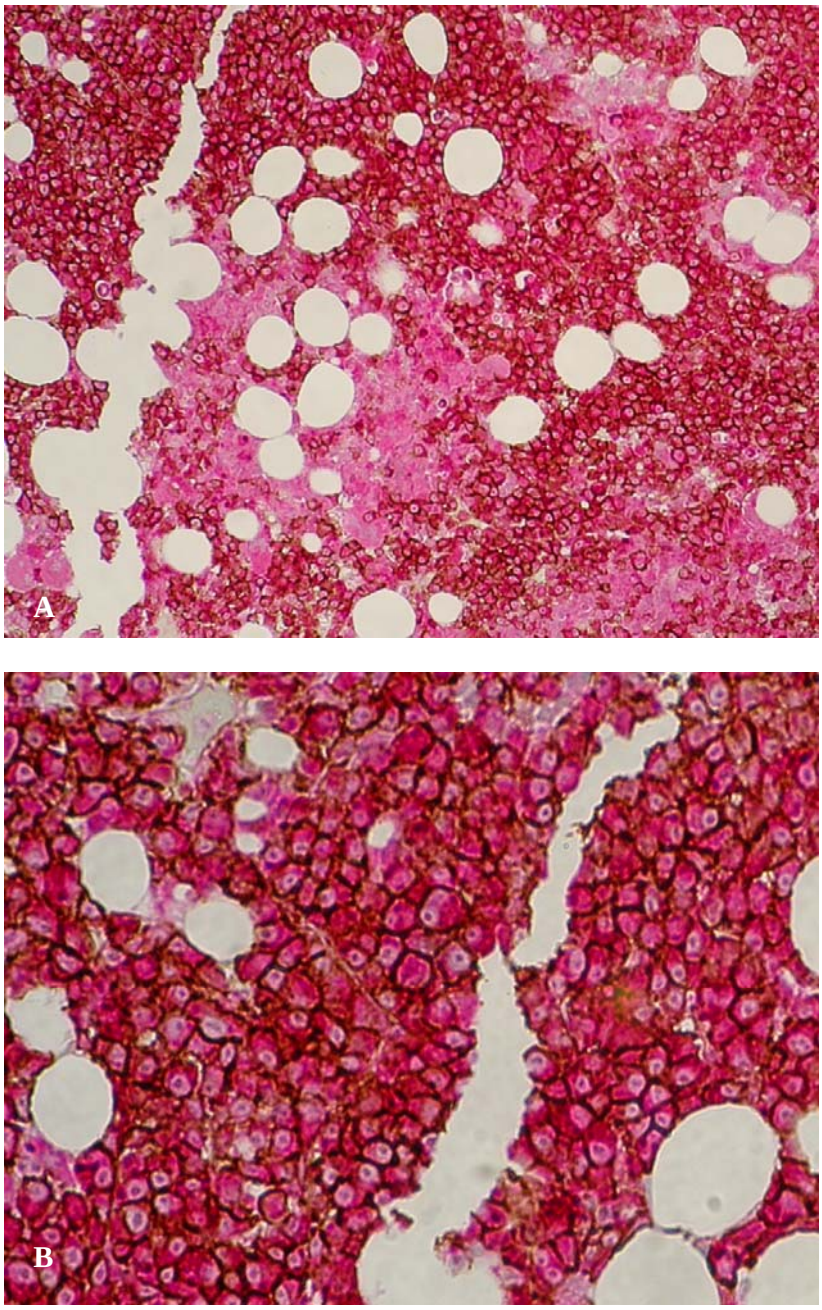


Figure 20: A) An MM case showing Bax expression >90% at 20X magnification. **B)** The same case at 40X magnification. *(continued)*

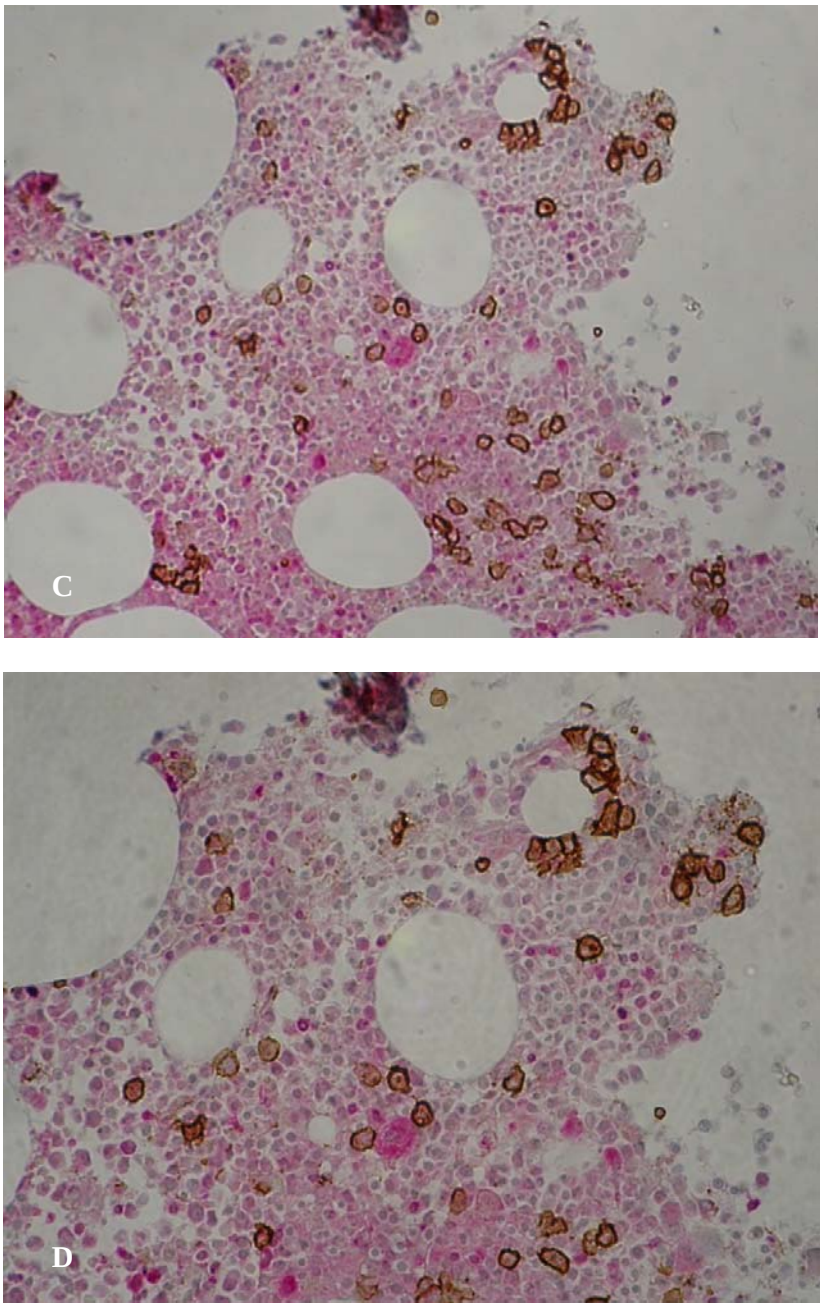


Figure 20: C) Low expression of Bax in MGUS case at 20X magnification. **D)** The same case of MGUS at 40X magnification. Bax has cytoplasmic staining in some cells such as erythroid cells which are internal positive controls.

3.2.2 Staining pattern of Bcl-2

Bcl-2 expression has cytoplasmic staining which is shown as a brown color in Figure 21. The internal positive Bcl-2 control in bone marrow is the lymphocyte. The morphology of plasma cells (small sized cells with prominent cytoplasm, and eccentric nucleus with dense chromatin) and also location of plasma cells in CD138 slides helped to differentiate plasma cells from lymphocyte in Bcl-2 immunohistochemistry staining. In this study, samples with more than 49 % Bcl-2 positive plasma cells were considered as positive cases for overexpression of Bcl-2.

Bcl-2 is the only single staining marker in this study, because all optimization protocols of CD138/ Bcl-2 double staining were not successful.

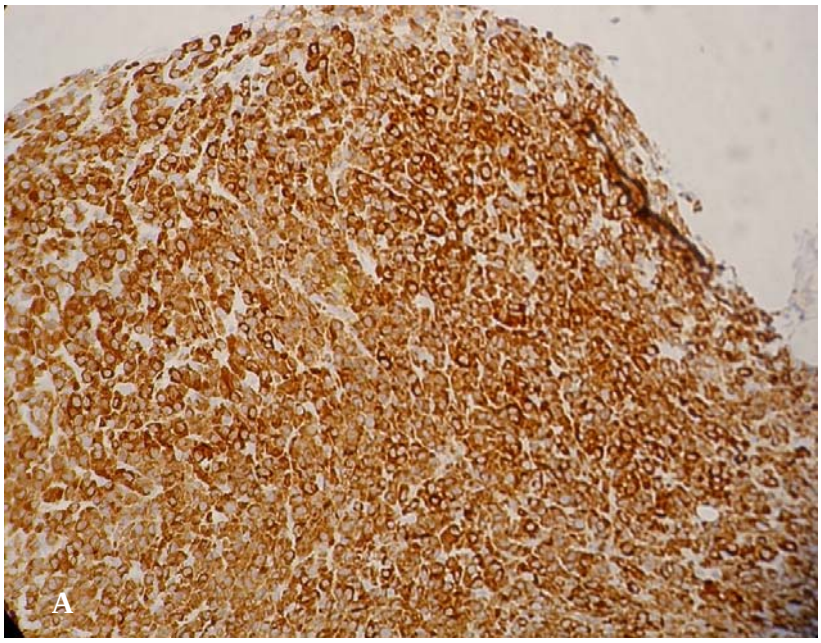


Figure 21: A) An MM case showing Cytoplasmic expression of Bcl-2 ≥ 90% in plasma cells infiltration≥90% in CD138 staing (20X magnification). *(continued)*

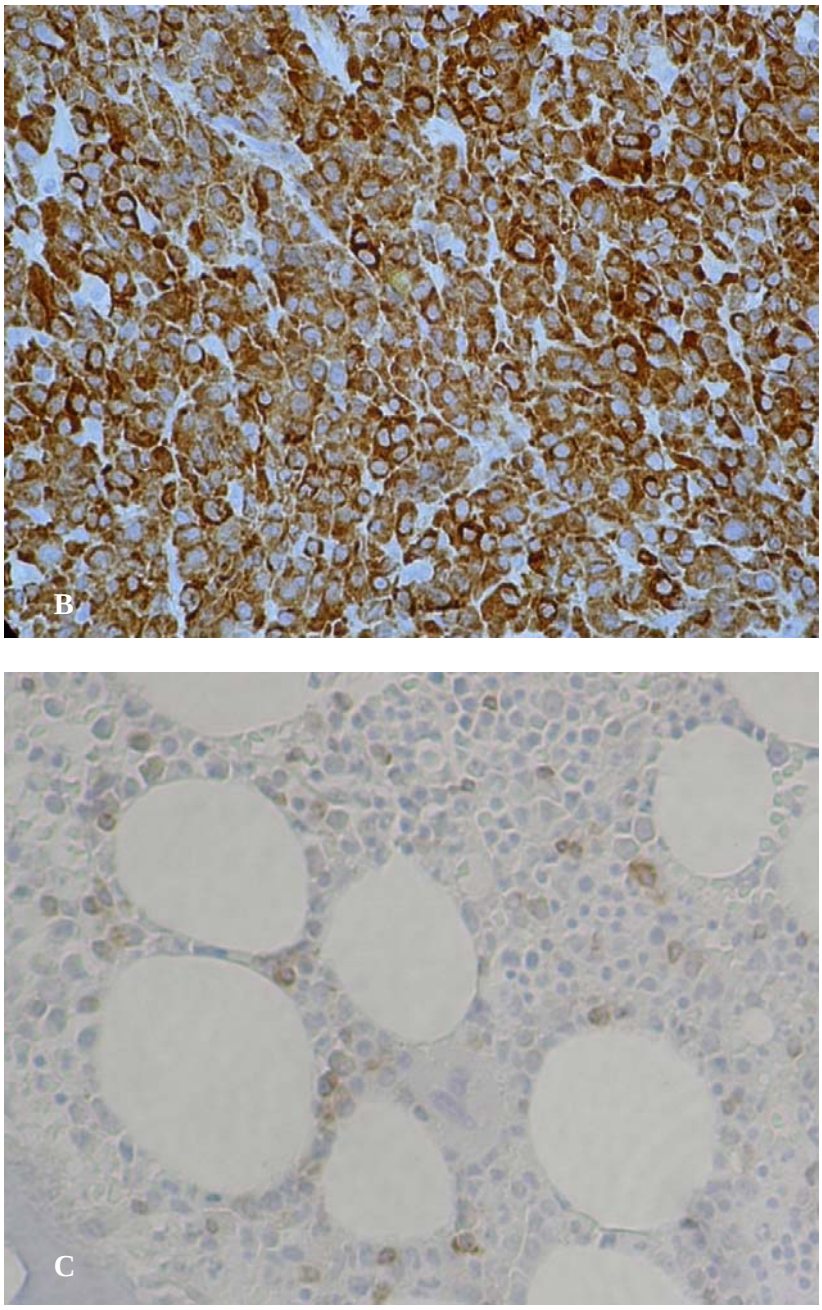


Figure 21: B) the same case of MM with 40X magnification **C)** low expression of Bcl-2 in an MGUS sample.

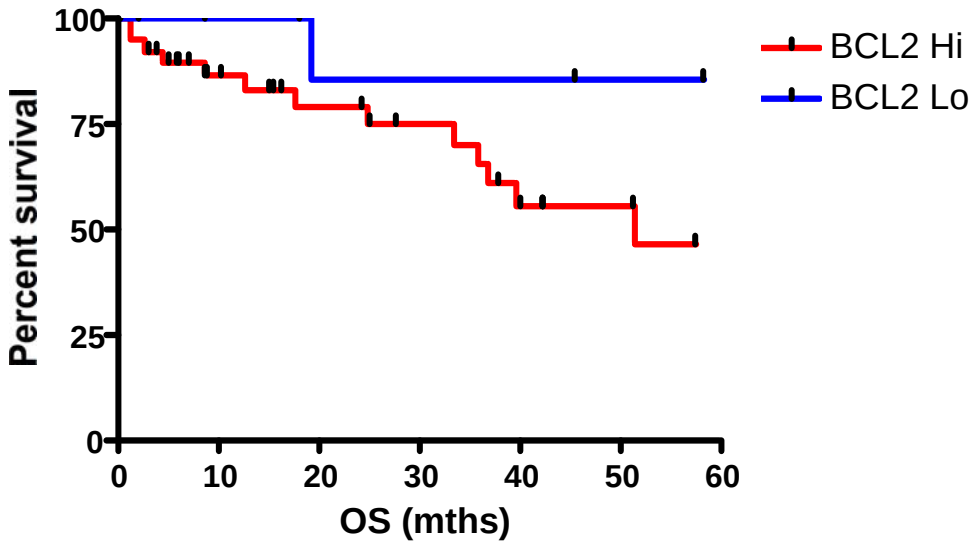
3.2.3 Bax and Bcl-2 expression in MGUS and MM patients

The results of Fisher exact test for Bax and Bcl-2 expression showed significant difference between MGUS and MM patients (p-value 0.001 and p-value< 0.001 respectively). As it is shown in Table 10, a large proportion of MM patients (65.5% and 76.4%) had over expression of Bax and Bcl-2 respetively, while a small fraction of MGUS patients showed overexpression of Bax (17.6%) and Bcl-2 (11.8%). However, the Mann-Withney U test for Bcl-2/Bax ratio did not reveal any significant association between MGUS and MM groups (p=0.209).

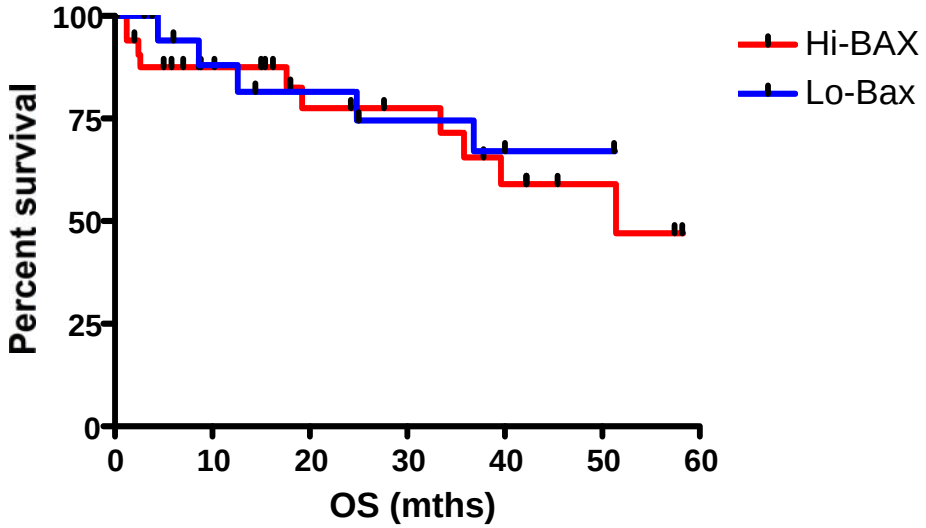
Interestingly, the survival analysis of MM patients indicated a significant association (log-rank p-value= 0.04) between Bcl-2 overexpression and poor prognosis in MM patients. The median overall survival of 51.5 months versus 84 months was obtained for cases with high Bcl-2 overexpression compared to those with low Bcl-2 expression (Figure 22.A). However, there was no association between Bax expression or Bcl-2/Bax ratio and MM prognosis (log-rank p-value= 0.46 and p=0.93) (Figure 22.B and Table 10)

Table 10: Result of Bax and Bcl-2 over expression in MGUS and MM samples.

Marker	MM	MGUS	P-Value	Log-rank p-value
Bcl-2 overexpression	**42/55 (76.4%)**	**2/17 (11.8%)**	**<0.001**	**0.04**
Bax overexpression	**38/58 (65.5%)**	**3/17 (17.6%)**	**0.001**	**0.46**
Bcl-2/Bax	**Mean 1.145**	**Mean 1.226**	**0.209**	**0.93**



(A)



(B)

Figure 22: A) Overall survival of MM patients with high expression of Bcl-2 was significantly less than patients with low expression of Bcl-2. **B)** There was no association between Bax expression level and overall survival of MM patients.

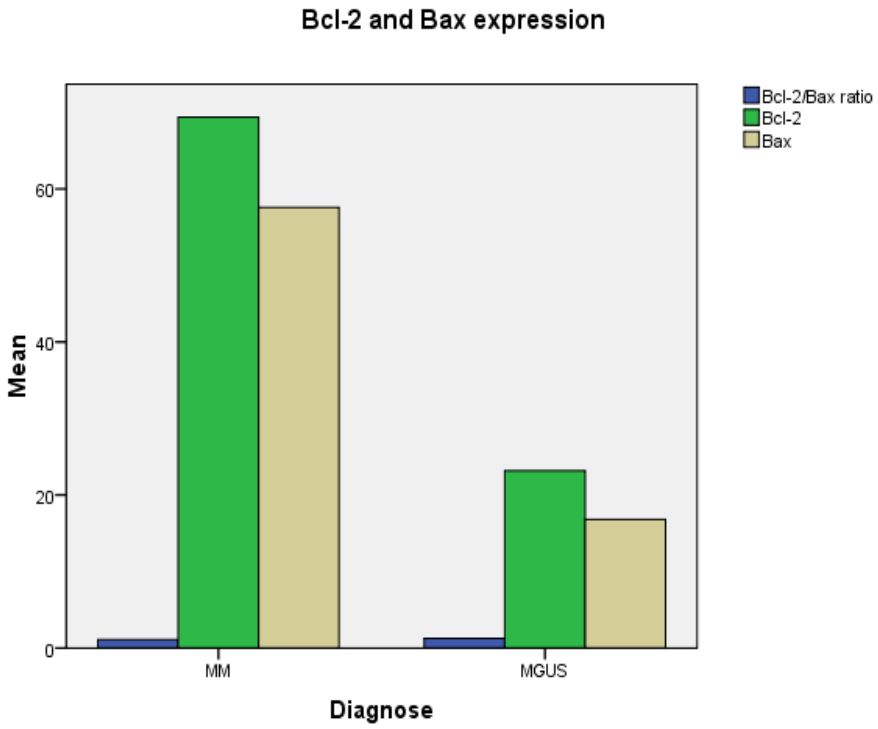


Figure 23: Overexpression of Bax and Bcl-2 has been shown in MM patients compared to MGUS patients. But, the Bcl-2/Bax ratio did not show any difference between these two group of patients.

3.3 Evaluation of Ki67 and Cycline D1 expression

3.3.1 Staining pattern of Ki67

The nuclear staining pattern of Ki67 expression detected in proliferative cells is shown in Figure 24 as red color. Whilst, the CD138 (plasma cell marker) stains the membrane of plasma cells with brown color in double-staining IHC. Thus, the proliferative plasma cells had a combination of red color in nucleus (Ki67) and brown color on the plasma cell membrane (CD138) (Figure 23). In this study, any sample with Ki67≥1 % was considered as positive case for Ki67 expression.

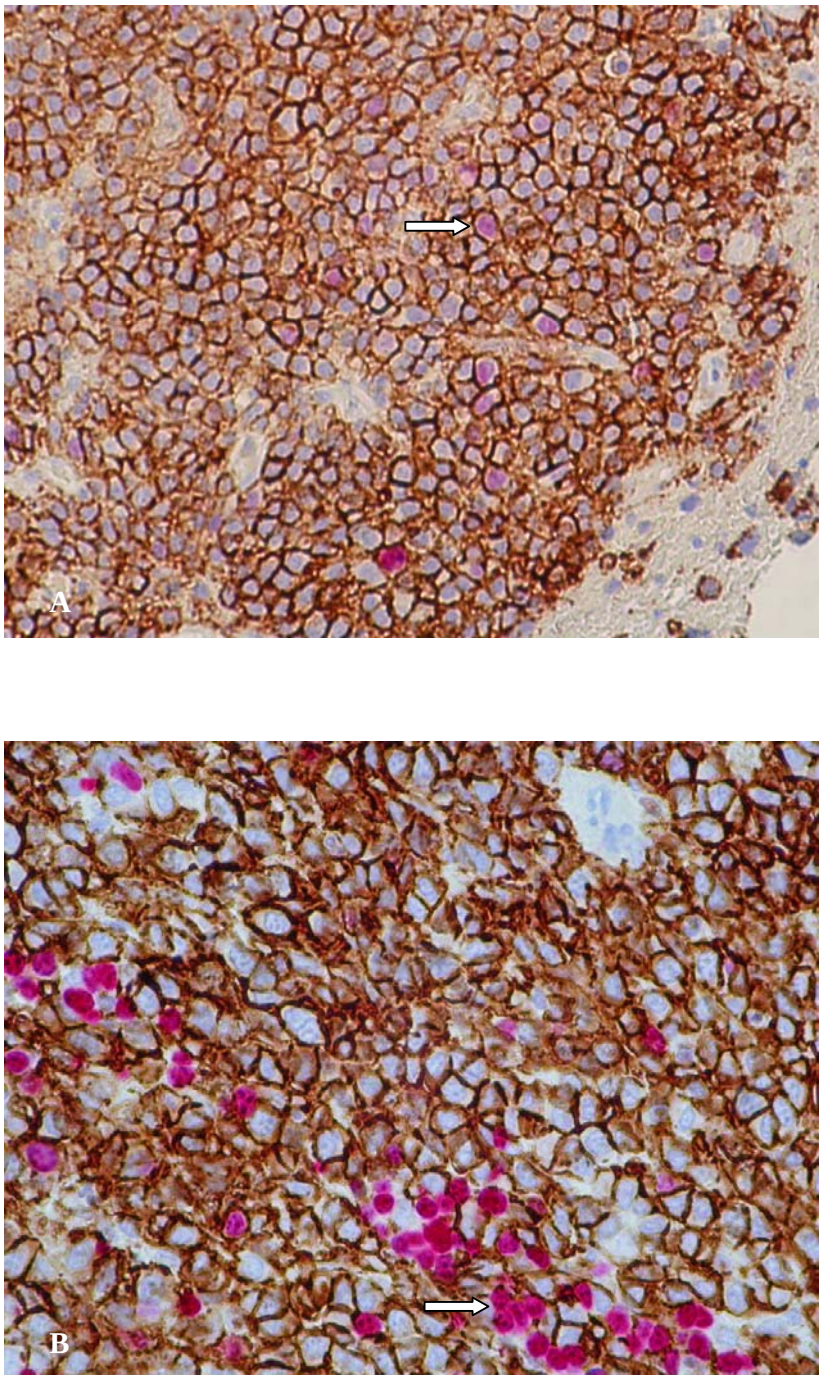


Figure 24: A) MM case with positive ki67 expression in plasma cells which is pointed (40X magnification) **B)** MM case with few positive ki67/CD138 double staining cells and a lot of other ki67 positive cells which pointed (40X magnification) *(continued)*

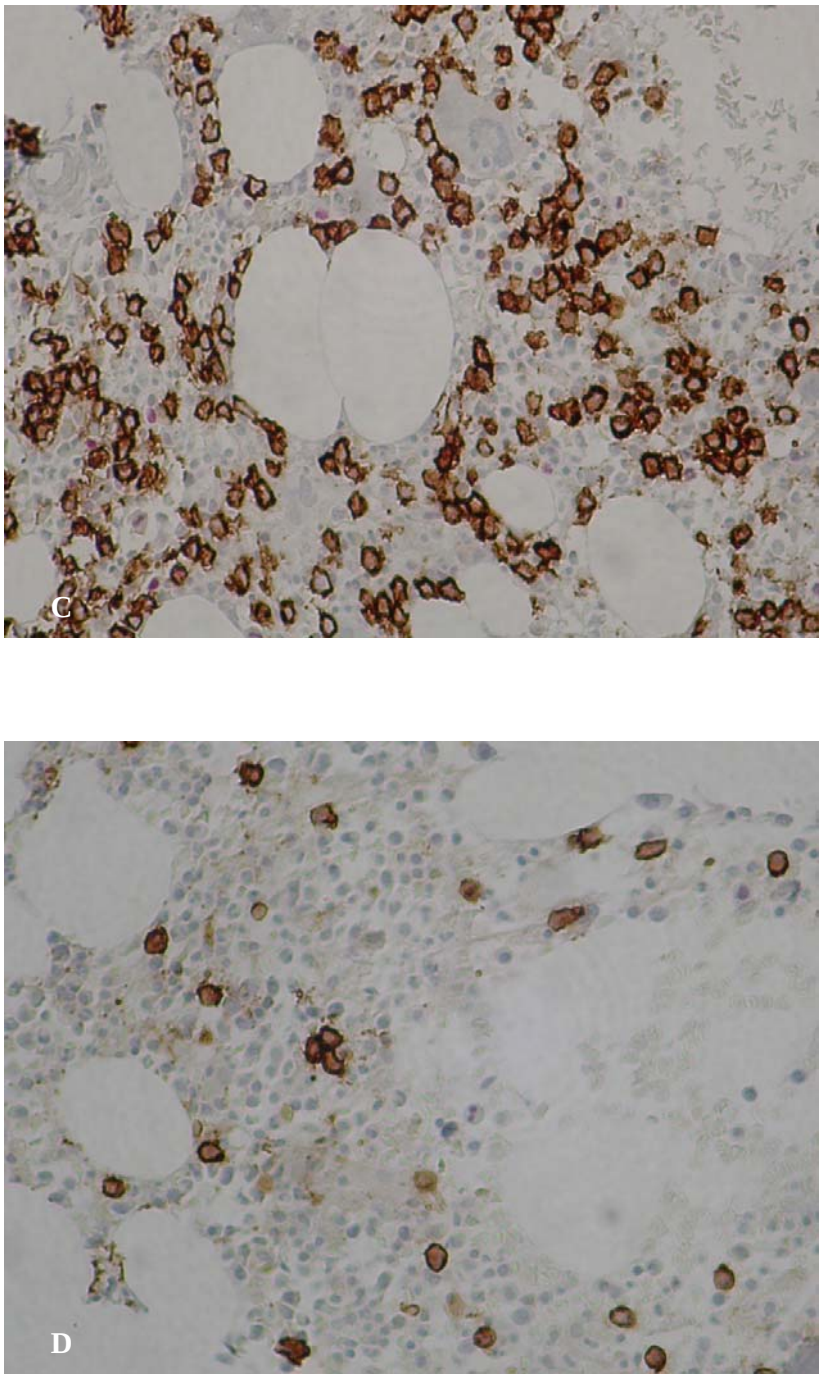


Figure 24: C) MM case with negative Ki67/CD138 staining **D)** MGUS case with negative Ki67/CD138 staining

3.3.2 Staining pattern of Cyclin D1

Staining pattern of Cyclin D1 expression shown as red color in Figure 25 is mainly nuclear and less cytoplasic. While, the CD138 (plasma cell marker) stains the membrane of plasma cells with brown color in double staining IHC. Therefore, the positive Cyclin D1 plasma cells had a combination of red color in nucleus and also cytoplasm (Cyclin D1) and brown color in membrane (CD138) (Figure 25). In this study, Cyclin D1 expression ≥5 % was considered as positive case.

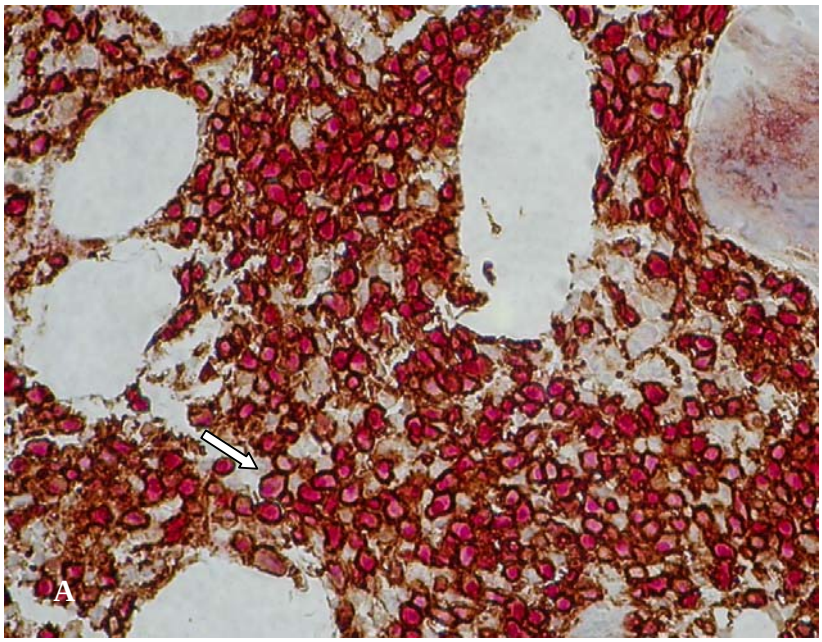


Figure 25: A) MM patient with Cyclin D1 expression≥ 90% *(continued).*

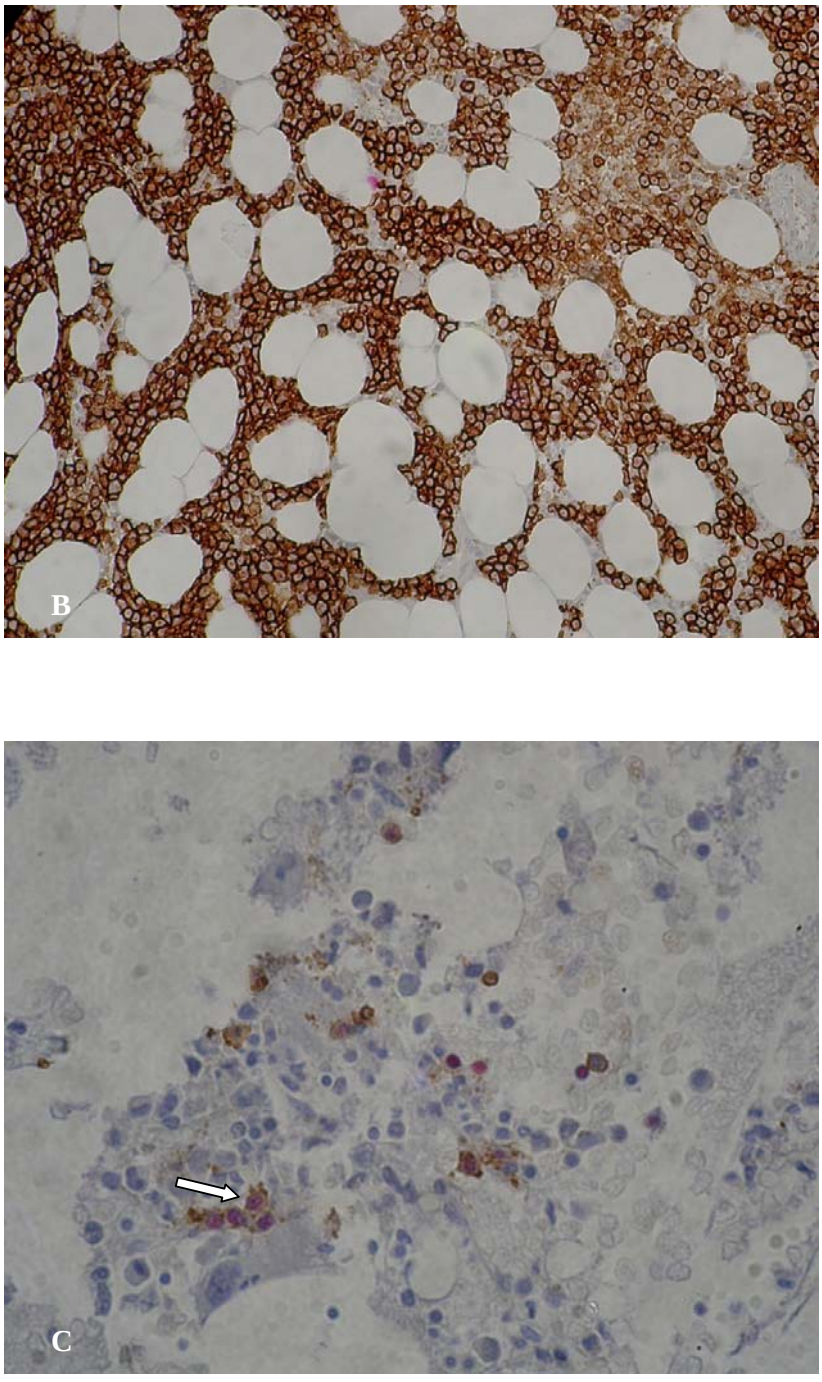


Figure 25: B) MM patient with negative expression of Cyclin D1 in plasma cells **C)** MGUS patient with positive Cyclin D1 expression in plasma cells. *(continued)*

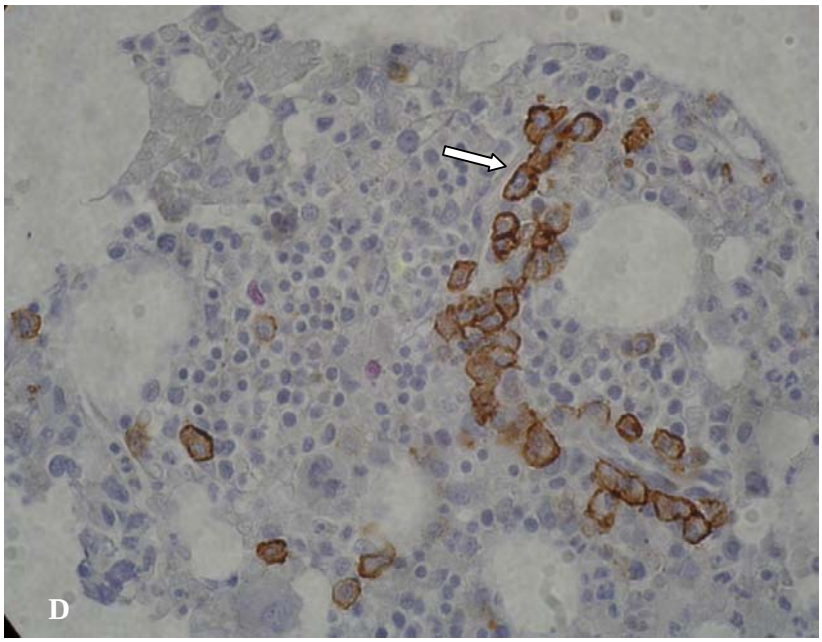


Figure 25: D) MGUS case with negative Cyclin D1 expression in plasma cells, pointed.

3.3.3 Cyclin D1 and Ki7 expression in MM and MGUS

Table 11 suggests that level of Cyclin D1 and Ki 67 expressions did not have any association with both MGUS and MM groups (p-value=1.000 and p-value=0.186). Cyclin D1 was positive in 22.4% of MM cases (13 out of 58) and 17.6% of MGUS (3 from 17). As shown in Table.11, 8 cases of MM (13.8%) were positive for Ki67staining while MGUS cases were entirely negative for Ki67staining.

The survival analysis in MM patients did not indicate any association between Cyclin D1 or Ki67 expression and MM prognosis (Cyclin D1 log-rank p-value=0.16 and Ki67 long rank p-value=0.55). Cyclin D1 positive cases had a median overall survival of 40 months versus 84 months in negative cases (Figure 26A). Moreover, the median overall survival of MM patients with high proliferation rate (Ki67≥1%) was not

reached, more than half of patients were died during the follow up, although the median overall survival for this entire cohort study was 79.5 months (Figure 26B).

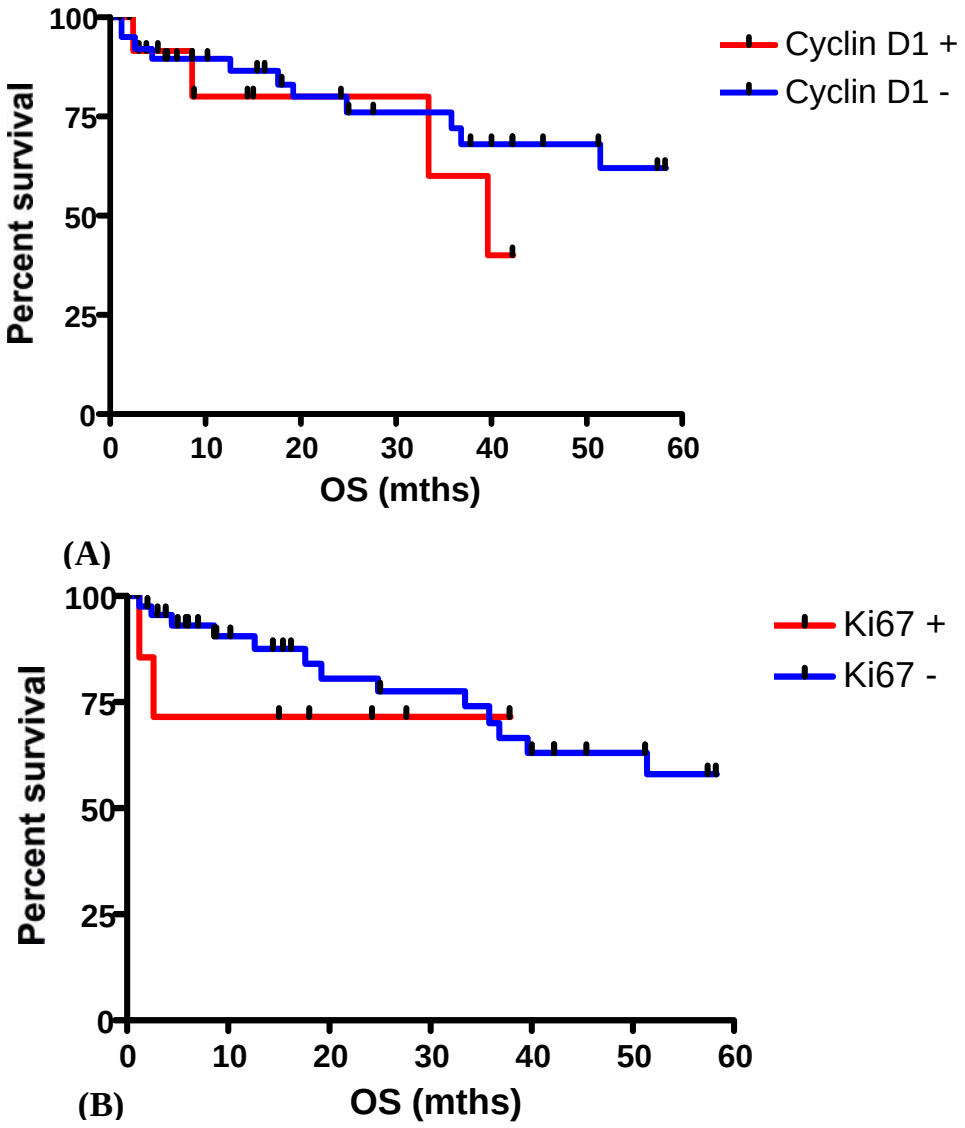


Figure 26 :A) Median overall survival was 40 months versus 84 months in Cyclin D1 positive MM cases **B)** Median overall survival of Ki67 positive patients was not reached compared to median overall survival of 79.5 months in this entire cohort study.

Table 11: The number of positive cases, p-value, and log–rank p-value (survival anaylsis) for different markers expressions in MGUS and MM groups.

Marker	MM	MGUS	Total case	P-Value	Log-rank P-value
Cyclin D1	13/58 (22.4%)	3/17 (17.6%)	16/75 (21.3%)	1.000	0.16
Ki67	8/58 (13.8%)	0/17 (0%)	8/75 (10.7%)	0.186	0.55
P16	15/58 (25.9%)	1/17 (5.9%)	16/75 (21.3%)	0.099	0.80
Cleaved Caspase 3	14/58 (24.1%)	0/17(0%)	14/75 (18.7%)	0.030	0.84
p53	9/58 (15.5%)	0/17 (0%)	9/75 (12%)	0.109	0.0003

3.4 Evaluation of p16 and cleaved caspase 3 (CC3) expression

3.4.1 Staining pattern

The cleaved caspase 3 (CC3) and p16 have nuclear and cytoplasm staining that is shown as red color in figure 26. On the other hand, brown color in the membrane of cells demonstrated the CD138 (plasma cell marker) staining (figure 27, 28). Therefore the positive plasma cells for p16 and CC3 had a combination of red color in either nucleus or cytoplasm (p16 or CC3) and brown color in membrane (CD138)(Figure 27,28). In addition, the endothelial cells are occasionally positive for p16 and were used as positive internal control. In this study, CC3 or p16≥1% was considered as positive staining for expression of these two markers.

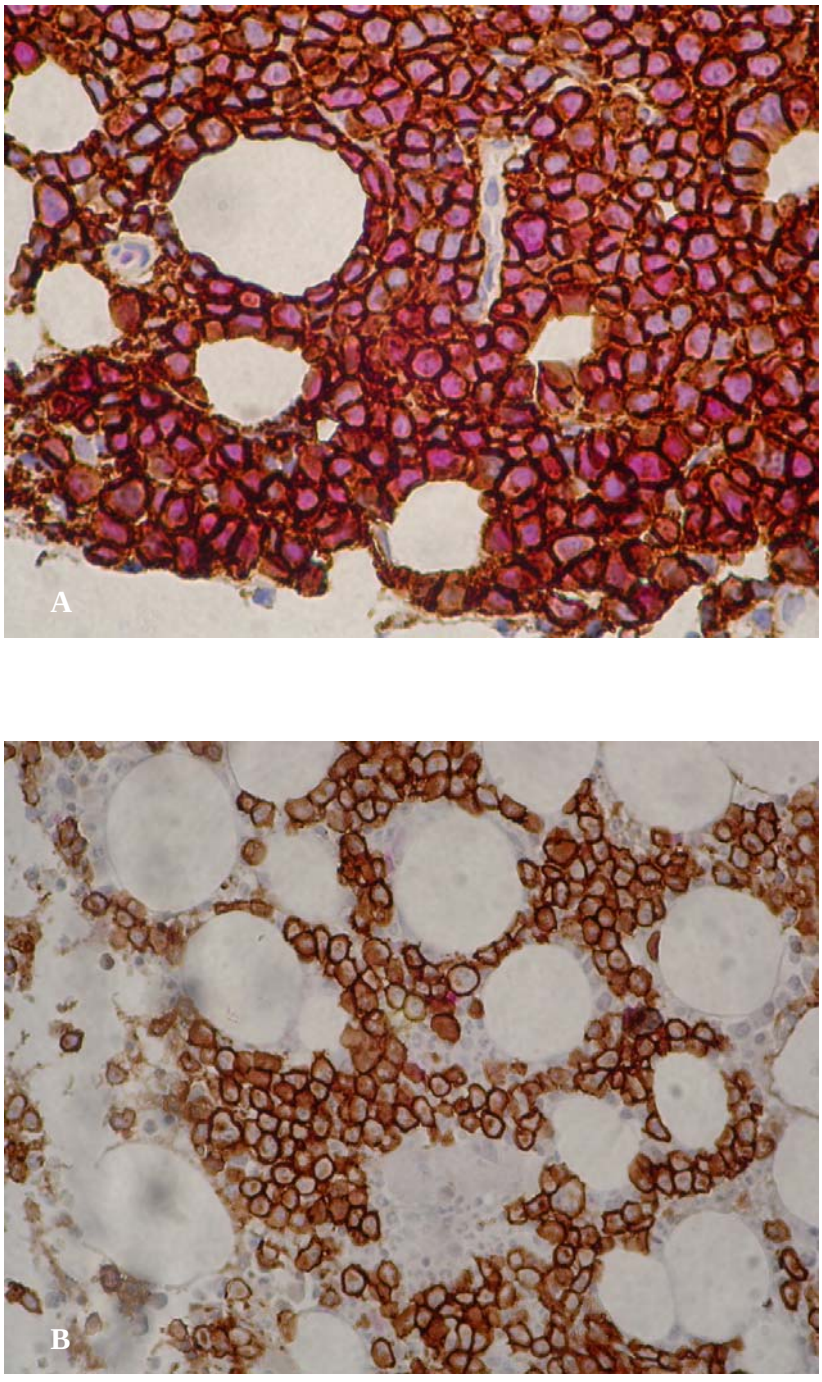


Figure 27: A) Positive p16 expression in MM case (40X manification) **B)** MM sample with negative p16 expression (40X magnification). *(continued)*

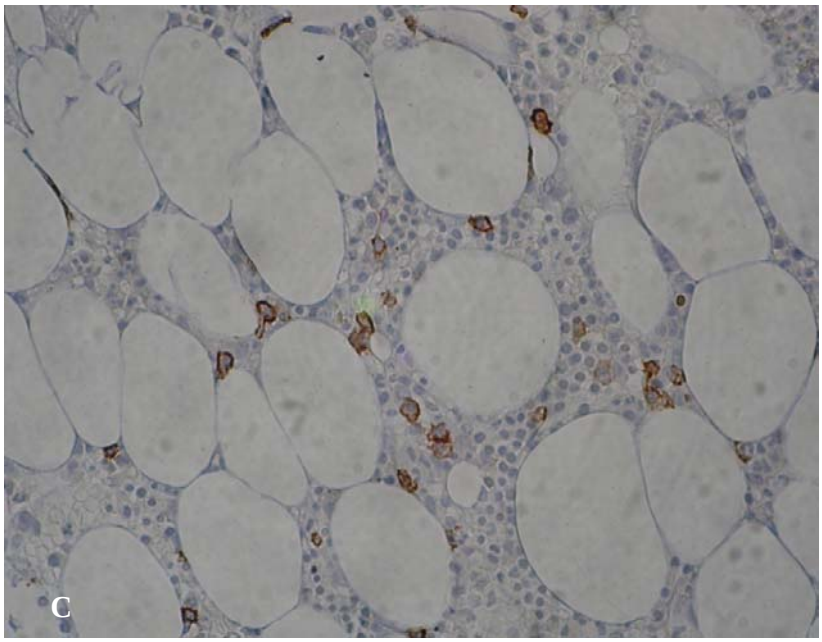


Figure 27: C) MGUS case with negative expression of p16

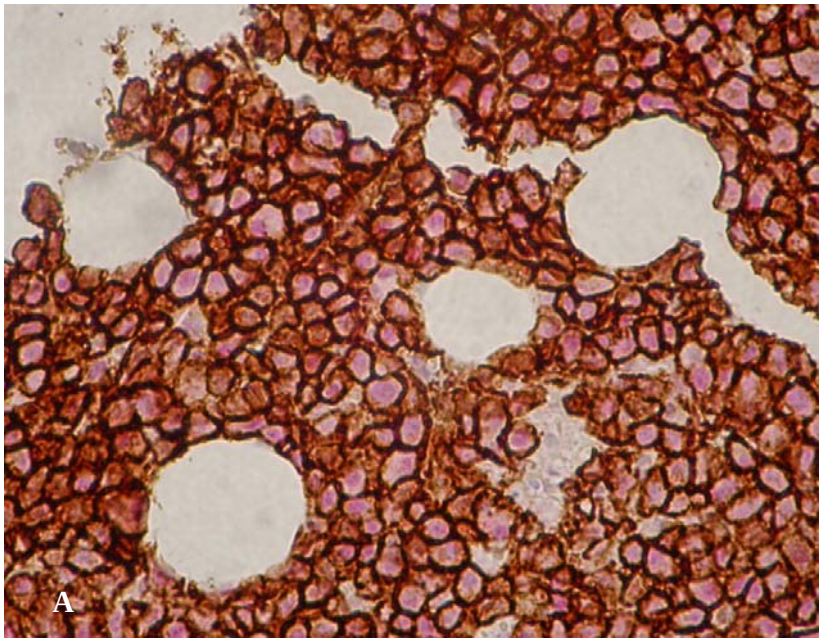
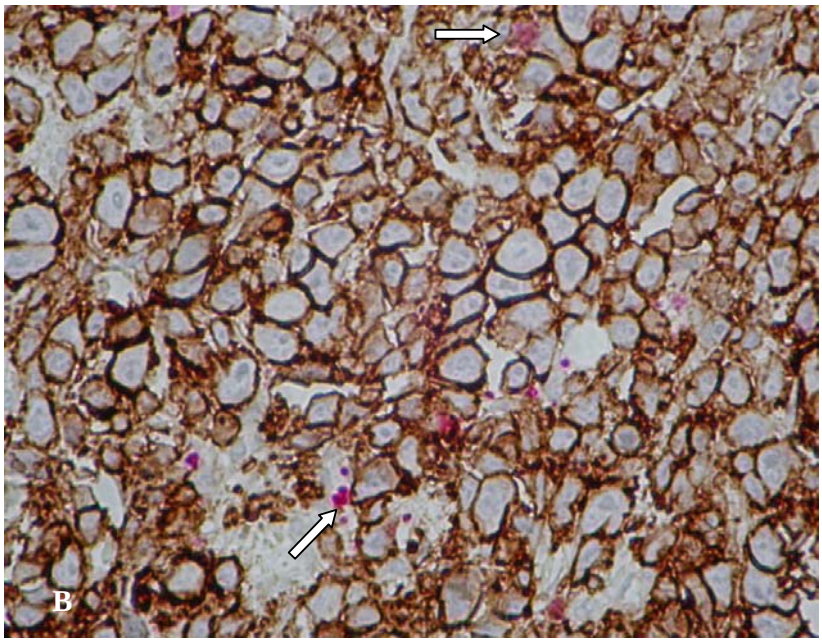


Figure 28: A) CC3 positive expression in nucleus and cytoplasm of MM sample.

(continued)



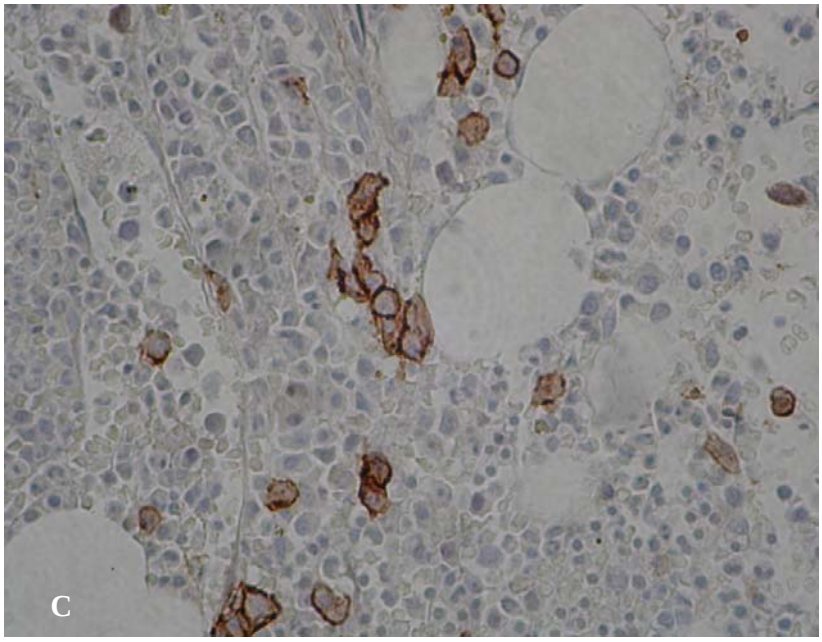


Figure 28: B) expression of CC3 in negative CC3 case of MM. There are a few apoptotic cells with apoptotic bodies which is pointed **C)** MGUS sample with negative CC3 expression.

3.4.2 p16 expression in MGUS and MM

The results were shown in Table 11 suggests that the p16 expression level was not significantly different in MM and MGUS patients (p-value=0.099). one quarter of MM patients (25.9%, 15 out of 58) expressed the p16 and one case of MGUS (5.9%) was p16 positive in this study. In addition, the survival analysis was not able to find any association between MM prognosis and p16 expression (log-rank p-value=0.80). The median overall survival for p16 positive cases was not reached, while the median overall survival of entire cohort was 79.5 months (Figure 29).

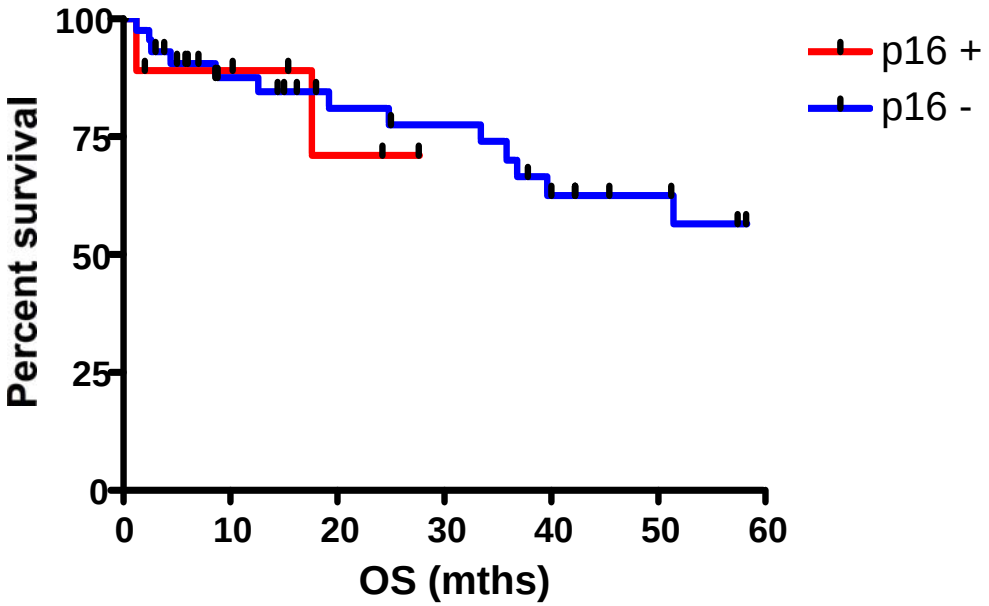


Figure 29: The overall survival of p16 positive cases was not reached versus 79.5 months.

3.4.3 Cleaved Caspase 3 expression in MM and MGUS

As it was demonstrated in table 11, expression of CC3 significantly and positively increased in MM group compare to MGUS group (p-value=0.030). All positive CC3 staining (18.7%, 14 from 75 cases) had MM diagnosis which was one quarter (24.1%)

of MM patients. In addition, MGUS samples were completely negative for CC3 staining (table 2). However, this study did not establish any association between CC3 expression and MM prognosis (log-rank p-value=0.84). The median overall survival for CC3 positive cases were 40 months versus 84 months in negative cases (Figure 30)

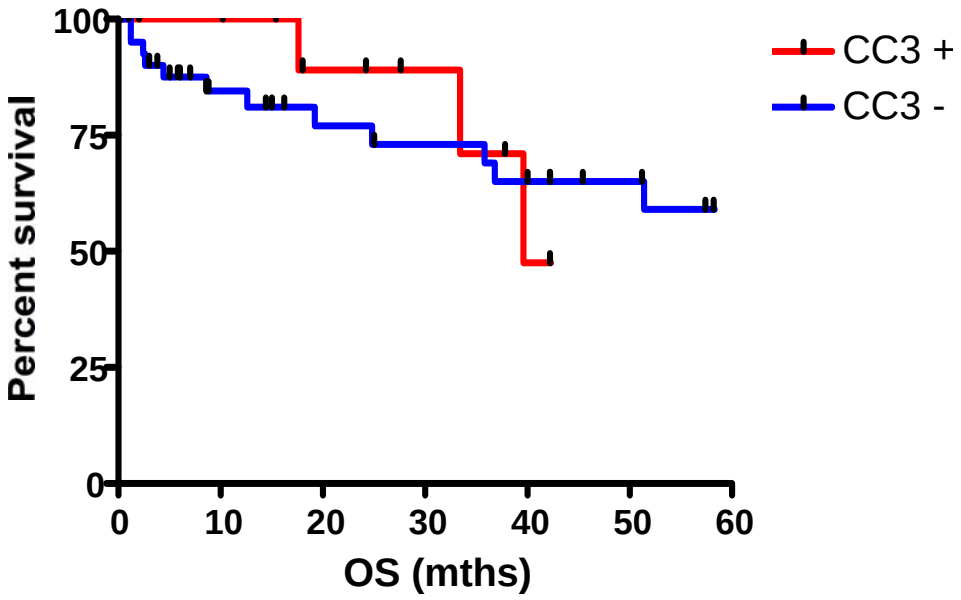


Figure 30: The median overall survival of CC3 positive cases was 40 months versus 84 months in negative cases.

4. Discussion

4.1 DDR and OIS barriers in MGUS and MM

DNA damage response (DDR) and oncogene-induced senescence (OIS) are two important tumorigenic barriers in some types of premalignant tissues such as lung, skin, bladder, prostate, colon, and they prevent formation of cancer in these types of tissues. In these studies, expression of DDR and OIS markers increased in premalignant tissues and then disappeared or decreased in malignant tissues[30-32]-31]-31,[34][33]33[35][34]-34,[49]. Therefore, some events that affect inactivation of OIS and DDR in premalignant tissues will accelerate a cancer formation. The role of these two barriers was not previously evaluated in monoclonal gammopathies, so we focused on these tumorgenesis barriers in MGUS as a premalignant state of plasma cell dyscrasia, and myeloma as malignant tumor.

Different types of cell stress can cause the DNA double strand breaks (DSB) that are able to activate checkpoint proteins such as ATM, ATR, CHK2, and CHK1. Activation of these checkpoints leads to the activation of downstream tumor suppressors such as p53, and Rb, which result in G1 or G2 arrest and then senescence or apoptosis. Also, activation of oncogenes in some tissues may result in senescence, known as oncogene-induced senescence (OIS) and activate the Rb and ARF proteins that arrest cell cycle in G1 or G2 phase. Thus, investigating the phenotypic markers of senescence (p16) or apoptosis (cleaved caspase 3, CC3) was the first step we took in studying the role of DDR or OIS in this study. We planned to examine the upstream markers of DDR or OIS to determine if we would find enough evidences for activation of apoptosis or senescence in MGUS samples as our premalignant tumor model compared to malignant myeloma.

To investigate the two tumorigenic barriers in MGUS and MM samples, phenotypic markers of apoptosis (CC3) and senescence (p16) were evaluated. CC3 was selected as a phenotypic marker of apoptosis, as it is a key downstream intermediate of intrinsic and extrinsic apoptosis pathways and for practical considerations, its antibody is widely available and applicable for IHC analysis on paraffin-embedded tissues. In addition, p16 was selected as a senescence marker because determination of β-galactosidase activity (phenotypic markers of senescence) in paraffin-embedded archival tissues is not possible and also p16 is a known senescence marker widely used in IHC studies. From our results, the CC3 expression was interestingly increased in MM samples compared to MGUS samples, but the p16 expression did not show any significant changes in these two groups. Based on our findings, apoptosis was more evident in myeloma samples than in MGUS samples, but senescence was not seen as an active phenomenon in MGUS samples. We were thus not able to find the primary evidence of DDR or OIS activation in the premalignant tumor of MGUS with inactivation of these barriers in malignant myeloma, and our hypothesis was not proven for this type of hematological malignancy.

Besides exploring CC3 and p16 expression, ki67, Cyclin D1, p53, Bax, and Bcl-2 expressions were also evaluated in our study. The p53 tumor suppressor is involved in both the DDR and OIS pathways which can trigger the apoptosis by activation of the BH3 family and can also trigger senescence by activation of p21. Based on our results, the p53 expression did not vary between MGUS and MM samples. This result also supports our previous results for CC3 and p16 expressions and demonstrated that the inactivation of the DDR and/or OIS barriers is not a transformation-linked event in MM tumorigenesis and development.

Moreover, Bax and Bcl-2 as pro-apoptotic and anti-apoptotic proteins were assessed in this project, given that activation of the DDR pathway will result in activation of the BH3 family and thereby increase apoptosis in premalignant samples. Interestingly, we found that both Bax and Bcl-2 expressions were low in MGUS samples; however, their expressions were significantly increased in MM samples. Overexpression of Bax is in accordance with our previous results for CC3 expression which also displayed an increased level of apoptosis in myeloma samples compared to MGUS. Indeed, Bax overexpression was in the malignant plasma cells of the myeloma samples and was not evident in the plasma cells of the MGUS samples in our study, lends further crapulence to the concept that the DDR pathway is not an important barrier in MGUS during the malignant transformation process.

The low expression of Ki67 (13.8%) that we found in the myeloma samples was not significantly different from that in the MGUS samples. Besides, our study suggests that Cyclin D1 was also not associated with transformation of MGUS to MM, because its expression level did not drastically change from MGUS to MM samples in double staining IHC. Cyclin D1 is involved in inactivation of Rb and progression of cell cycle from G1 to S phase. Thus, overexpression of Cyclin D1 might have prevented the cells from undergoing G1 arrest or senescence. However, whilst Cyclin D1 expression did not differ significantly between the MGUS and MM samples we analyzed, its expression level increased in both MGUS and MM samples, which can arguably be explained as the overexpression of Cyclin D1 being an early tumorigenic event. Furthermore, the observation of Cyclin D1 overexpression in some MGUS cases may be part of the reason why senescence is not stimulated in MGUS, since the Rb protein, a key molecule in senescence, might be inactivated by overexpression of Cyclin D1. Therefore, the Cyclin D1 expression finding can indirectly support

inactivation of senescence in MGUS samples and is consistent with our results for CC3, p16, p53 and Bax expression.

We found that DDR and OIS are not important tumorigenesis barriers in MGUS; so, other genetic abnormalities or dysfunction would be responsible for the transition of MGUS to MM. Our study suffers from several limitations. Our MGUS sample size was limited to 17 cases only and also we did not have serial bone marrow biopsies of patients with history of MGUS that progressed to myeloma during their follow up. Thus, we could not demonstrate the occurrence of inactivation of DDR and OIS in the MGUS premalignant tumors. Indeed, we would have to collect serial bone marrow biopsy of MGUS patients, who developed the MM disease during the time, and then examine OIS and DDR markers again in a much bigger study.

4.2 Individual marker evaluation in MM and MGUS

4.2.1 p53 expression

In this study, nine cases of MM (15.5%) were positive for p53 staining, showing a low rate of p53 mutation in myeloma. However, all MGUS cases were negative for p53 in double staining. The positive p53 staining in IHC indicates that p53 mutation occurred, since the mutated and nonfunctional p53 protein would accumulate in tissue and would be stained by IHC. Our p53 result in myeloma was consistent with a recent IHC report suggesting that p53 mutation is low in both newly diagnosed MM and relapse/refractory MM (4% and 20% respectively) [50]. In addition, our survival analysis of myeloma patients showed that there is significant association between short overall survival of myeloma patients (4.4 months) and p53 expression in bone marrow samples. Another study reported a low rate of p53 mutation (3%) in 268 myeloma patients by applying FISH and PCR methods and also described the poor

survival in patients with p53 mutation for the first time in multiple myeloma [51]. Our findings of low p53 expression in double staining IHC of myeloma samples and also short survival of these myeloma patients were consistent with the low p53 mutation and very poor survival of mutated p53 myeloma patients in the above-mentioned study by Chng WJ et al [51].

In conclusion, although p53 staining appears to be a rare occurrence in newly diagnosed myeloma patients, its presence gives a significantly worse overall survival in these patients compared to patients with negative p53 staining. So, the p53 expression in IHC is an independent poor prognostic factor in myeloma patients.

4.2.2 Bax and Bcl-2 expressions

Expression of Bax and Bcl-2 were remarkably increased in myeloma samples compared to MGUS group. Overexpression of Bax and Bcl-2 were found in 65.5% and 76% of MM patients respectively, whilst approximately 17% and 11% of MGUS patients showed overexpression of Bax and Bcl-2. In addition, there was a noteworthy association between short survival of MM patients and Bcl-2 overexpression in our study, but the level of Bax expression had no bearing on survival in myeloma patients. Bcl-2 is normally expressed in follicular lymphomas carrying the t(14;18) chromosomal translocation, but is also expressed in many lymphomas without Bcl-2 rearrangement. Cristina Largo et al. and Luigia Lombardi et al. reported the overexpression of Bcl-2 and amplification of this gene in myeloma cell lines by applying fluorescence in situ hybridization (FISH), quantitative real-time PCR (q-PCR) and comparative genomic hybridization (CGH) methods [52,53]. In addition, one study reported the Bcl-2 overexpression in MM compared to MGUS and reactive plasmacytoma, findings that are consistent with our results [54]. Indeed, the Bcl-2

overexpression is an early tumorigenic event, as Bcl-2 expression is increased from normal plasma cells to MGUS and also increased from MGUS to MM. Our Bcl-2 expression result might have a therapeutic application in the near future, as some Bcl-2 antagonists such as Genasense, TW-37, obatoclax and ABT-263 are now in clinical trial as new drugs in cancer treatment. However, ABT-737, a new antagonist of Bcl-2 family, has monotherapy toxicity to lymphoma and leukemia, and at higher concentrations, has been shown to induce apoptosis in multiple myeloma cell lines in *in vitro* studies. ABT-737 can mimic the BH3 domain of the BAD molecule and can bind to Bcl-2, Bcl-x_L, and Bcl-w, inhibiting the functions of these anti-apoptotic proteins [55].

The overexpression of Bax and Bcl-2 in our current study is consistent with the work of Renner S et al. that showed increased Bax and Bcl-2 expression in 58 MM patients compared to 29 MGUS/smoldering myeloma patients and also 17 reactive plasmacytosis [56]. Furthermore, they demonstrated that myeloma patients with extremely low Bax expression had significantly increased survival, but they were not able to find any correlation between Bcl-2 expression and survival. Our survival results for Bax and Bcl-2 overexpression were completely in contrast with their findings, as we did not observe any correlation between Bax and survival of myeloma patients and we found instead a significant correlation between Bcl-2 overexpression and short survival in myeloma patients. We believe the double staining IHC results we reported are probably more accurate than those obtained from single staining IHC. Bax staining has been shown for other normal bone marrow cells such as erythroid, megakaryocytic, and lymphocyte which can produce a false positive result of Bax expression in single staining and thus affect correlation of Bax expression with overall survival of patients. In addition, our contrasting results with those of the Renner et al

study [13] may be partly related to differences inherent in the samples that were from two diverse ethnic populations. Also, other confounder factors such as new chemotherapeutic treatments may prolong the survival of our cohort patients and then may affect the results. In conclusion, more studies are needed to investigate the association of Bax overexpression and survival in myeloma, because there is just one report beside our study regarding Bax overexpression and overall survival of myeloma patients.

" The role of Bax overexpression in myeloma is unknown, because it is a pro-apoptotic protein and should not be highly expressed in cancer. Recently, the role of Bax in plasma cell apoptosis was investigated by Pelletier et al [57]. They found that plasma cell apoptotic pathway is unique among other hematopoietic cells, as both the death receptor (extrinsic pathway) and the mitochondria (intrinsic pathway) do not play a central role. In fact, plasma cell apoptosis is started by the activation of Bax at the endoplasmic reticulum (ER) membrane and the subsequent activation of the ER-associated caspase-4 that occurs before release of apoptotic factors from the mitochondria [57]. The overexpression of Bax and increased level of the CC3 apoptotic marker in our cohort study may be consistent with activation of apoptosis by activation of Bax in the ER. The intrinsic pathway of apoptosis might be inhibited by overexpression of Bcl-2 in our myeloma samples, but overexpression of Bax in our cohort patients may also activate the ER apoptosis pathway and may then have increased the level of CC3 in these myeloma patients.

The correlation of Bcl-2 overexpression and survival of myeloma patients is not well established. In this current study, we reported the positive association of Bcl-2 overexpression and short survival of myeloma patients for the first time. However, three other studies were not able to show any relationship between Bcl-2

overexpression and survival of myeloma patients [54]-[56]. On the other hand, Ong F et al. reported a long survival in 27 myeloma patients with increased Bcl-2 expression that were in contrast to previously reported studies. A reason for the controversial result of Ong F et al. with our study might be related to their limited sample size (27 patients)[58].

Although the Bax and Bcl-2 expressions increased in MM compared to MGUS, the Bcl-2/Bax ratio did not vary from MGUS samples to MM samples. It is possible that increased expression of either Bax or Bcl-2 causes the increased expression of another one, because these two proteins have balanced expression in mitochondrial membrane. Moreover, it is possible that various factors independently influence the overexpression of Bax and Bcl-2, but the Bcl-2/Bax ratio may not reflect any changes.

4.2.3 Cleaved Caspase 3 expression

The expression of cleaved caspase3 (CC3) as a phenotypic marker of apoptosis was investigated in newly diagnosed myeloma and MGUS patients for the first time. While CC3 was not expressed in MGUS patients, one third of myeloma patients expressed this marker and showed significant difference between MM and MGUS. However, CC3 mean expression was low (9 ± 3) in myeloma, which can explain a low rate of apoptosis in malignant plasma cells. Moreover, overall survival of myeloma patients in the CC3-positive expression group did not significantly differ from that in the CC3-negative group.

Our study for CC3 is worthy of special mention for several reasons. Firstly, apoptosis was evaluated in newly diagnosed myeloma patients for the first time and also a low rate of apoptosis was found in malignant plasma cells. Secondly, there had been no previous reports of apoptosis and survival in myeloma patients and we were the first

to evaluate this issue for the first time. Thirdly, we used the double staining IHC, which is a powerful method to distinguish the expression of selected markers of interest in plasma cells by applying two antibodies and two colors in one slide that makes scoring of protein expression more accurate and precise.

In hyperproliferative cancers, the apoptosis index may be high due to several reasons. However, increased level of apoptosis in myeloma samples compared to MGUS may not be accounted by an increased level of proliferation, as MM is a hypoproliferative malignancy and 75% of positive CC3 cases did not express Ki67 in this study. In fact, the role of CC3 and Bax overexpression are not known in myeloma and further study are needed to determine the roles of these two proteins in MM pathogenesis.

Apoptosis in myeloma is executed via extrinsic and intrinsic pathways that lead to activation of caspases. When the apoptosis triggers either by the intrinsic or the extrinsic pathway, caspase 3 will be cleaved at Asp175 (cleaved caspase 3, CC3). The majority of chemotherapeutic drugs in MM act via a mitochondrial pathway that affects pro- and anti-apoptotic Bcl-2 family [59]. However, drug resistance in myeloma is more related to the extrinsic pathway and overexpression of TRAIL decoy receptors such as Apo-2L and DcR2. Agonist antibodies for TRAIL receptors have been reported to induce apoptosis in myeloma cells and were able to bypass the inhibition of TRAIL-induced apoptosis [60]. In addition, a recent study revealed that activation of Bax in the reticulum endoplasmic membrane initiates apoptosis and plays a central role in plasma cell apoptosis. The study also showed that death receptor and mitochondria did not have critical roles in initiation of PC apoptosis [57].

In conclusion, mechanisms of apoptosis in normal and malignant plasma cells are not well known and further studies are needed to investigate the underlying mechanisms of apoptosis in MM.

4.2.4 p16 expression

Most somatic cells, unlike germ cells and certain stem cells, stop dividing after restricted cell divisions in culture and go into a phase termed cellular senescence. However, recent studies suggested that the activation of cellular senescence takes place in vivo and plays an important role in tumor suppression, vascular disease, fibrosis, and aging. Activation of oncogenes can lead to oncogene-induced senescence and activate the p16/Rb pathway. In vivo evidence suggests that the self-renewing stem cells in higher eukaryotes on progressive ageing, become senescent due to p16 overexpression. In addition, Ito et al. showed that self-renewal of hematopoietic stem cells is harshly damaged because of increased ROS in ATM knockout mice, resulting in the up-regulation of p16 and increased senescence in hematopoietic stem cells[61]. Therefore, p16 is known as an in vivo senescent marker and down regulation of p16 is expected in a variety of cancers [62-65].

Based on our data, expression of p16 was not considerably altered from MGUS to MM samples. Also, one quarter of myeloma patients expressed p16 and the mean ratio (4.05 ± 1.8) was low in positive cases. However, one case of MGUS expressed p16. Our result is in agreement with the study of Urashima,M et al. that showed high expression of p16 in eight myeloma patients by immunoprecipitation and western immunobloting methods. While p16 methylation did not affect protein expression of p16, the majority of studies focused on comparing methylation of p16 in MGUS and MM patients in the last decade [42,66]. Our study is thus unique as the expression of p16 was not well established in both groups of MGUS and MM in previous reported studies. Besides, a recent study investigated the association of p16 expression and response to the Bortezomib therapy in 89 relapse/refractory myeloma by IHC and found that patients with p16 expression had a poor response to Bortezomib [67].

However, they used relapse/refractor myeloma patients and they did not evaluate p16 expression in MGUS or newly diagnosed MM patients.

Our survival analysis data suggested that there was no association between p16 and prolonged or short survival of myeloma patients. This is the first time that p16 expression as a prognostication indicator of myeloma patients was examined. Indeed, many studies had examined the correlation between p16 methylation and survival of myeloma patients [68-70]. But, there is no report about p16 protein expression and survival in previous studies. A comprehensive study on 561 monoclonal gammopathies had revealed that p16 methylation did not affect the p16 RNA expression and also did not associate with any poor or better prognosis in myeloma patients. The result of RNA expression and survival in the Gonzalez-paz-led study is consistent with our protein level of p16 result[42].

Although p16 expression did not significantly differ in both MGUS and MM, it was expressed in myeloma patients more than in MGUS patients. This result is in contrast with our expectation for down regulation of p16 as a tumour suppression gene in cancers and its increased level in premalignant tissues as a senescent marker [62-65]. In conclusion, further studies need to be done for understanding the exact role of p16 in multiple myeloma pathogenesis.

4.2.5 Cyclin D1 and Ki67 expressions

The major regulatory events of mammalian cells such as proliferation and differentiation occur in the G1 phase of the cell cycle. Deregulation of G1 cycling and Cyclin-dependent kinases may influence cell cycle control and then result in increased oncogenesis. Cyclin D1 as a major regulatory of G1 phase of cell cycle forms a complex with CDK4/CDK6, whose activities are required for G1/S transition and

results in cell proliferation. In addition, p16 as an inhibitor of CDKs prevents phosphorylation of Rb and then results in senescence or cell cycle arrest; however, overexpression of Cyclin D1 might overcome the role of p16 in cell cycle arrest and thus result in proliferation or differentiation of cells [71].

Cyclin D1 is expressed in wide variety of human tissues; however, it is not expressed in lymphoid cells and myeloid lineages. Overexpression of Cyclin D1 in malignant plasma cells has been investigated in many studies and ranges from 17%-50% when evaluated by different methods such as reverse transcription-PCR (RT-PCR), Northern blotting, and immunohistochemistry [72-76]. However, we chose to evaluate Cyclin D1 expression in MM and MGUS samples by applying the double staining IHC method, which is a precise technique to investigate two markers (such as CD138 and CyclinD1) in the same slide. Based on our data, overexpression of Cyclin D1 did not significantly differ between MGUS and MM, since Cyclin D1 was expressed in 22% of MM and 17% of MGUS patients. Our data is in agreement with other studies and suggest that deregulation of Cyclin D1 is an early pathogenesis event in monoclonal gammopathies and that this event alone is not enough to drive transformation of MGUS to symptomatic myeloma [50,77-80].

Furthermore, the prognostic significance of Cyclin D1 overexpression is still controversial. Several studies have reported that myeloma patients with either the t(11;14) or 11q abnormalities, and/or Cyclin D1 amplification, had a worse prognosis compared to the group without Cyclin D1 expression [71,72,81]. However, there are some studies reporting that patients carrying t(11;14) and treated with high-dose chemotherapy or conventional therapy had prolonged survival compared to patients without any genetic abnormalities [82,83]. In addition, Dawson et al. found that nucleus staining of Cyclin D1 in plasma cells was associated with a better response to

Bortezomib [67]. In contrast to prior studies, a recent study in 94 myeloma patients treated with Doxorubicin, Vincristine, Dexamethasone, and Thalidomide showed that Cyclin D1 negativity did not convey a poor overall survival or adverse progression-free. They also reported a similar incidence of Cyclin D1 expression between newly diagnosed patients and relapse/refractory myeloma patients by double staining IHC [50]. In our survival analysis, we were also not able to find any correlation between Cyclin D1 expression and overall survival of myeloma patients. Moreover, our result is in agreement of other published data that showed overexpression of Cyclin D1 in 27% of 59 myeloma patients and also they did not find any association between Cyclin D1 and overall survival of myeloma patients that is consistent with our result [78]. On the other hand, Cook JR et al. reported a longer overall survival in Cyclin D1 positive cases (15 out of 20, 75%) myeloma patients. Their results are in contrast with our data, which might be related to the technique used and their small sample size (20 cases) [84].

The proliferative activity of malignant plasma cells was measured with ki67 expression in our samples. Ki67 was not expressed in any MGUS samples; however, it was positive in 13% of MM. In addition, Ki67 expression did not significantly change from MGUS to MM samples. In fact, myeloma is a hypoproliferative disease and ki67 expression is low in this malignancy [85,86]. We could not reveal any association between ki67 and Cyclin D1positive cases in our study. Thus, Cyclin D1 overexpression does not influence higher proliferative activities in our study as well as other studies [76,87].

In conclusion, Cyclin D1 overexpression is an early pathogenesis event in plasma cell malignancies and it is not associated with improved or adverse prognosis in myeloma patients. Cyclin D1 overexpression in MGUS samples might yet be another

supportive evidence of inactivation OIS barrier in MGUS, since Cyclin D1 up-regulation may interfere to p16/ Rb senescence pathway and then inactivate it.

5. Conclusion

Recent studies proposed that the DDR and OIS are two tumorigenic barriers in several solid tumors. The researchers observed an increased level of DDR and OIS activations in premalignant tissues compared to malignant tissues. Activation of the OIS and DDR pathways in premalignant tissues can increase the level of senescence and apoptosis in these tissues. On the other hand, inactivation of these barriers will accelerate cancer formation and decrease the senescence and apoptosis in malignant tissues. Although several studies have focused on finding the transformation events in MGUS to MM, events or factors mediating this transformation are still unknown. In this study, we had planned to document by experimental evidence the existence of DDR and OIS as critical events in the transition of MGUS to MM, and to further investigate the specific markers involved, if their roles are verified.

The results of our study suggested that DDR and OIS are not important barriers of cancer development in multiple myeloma. In fact, the p16 expression as a phenotypic marker of senescence did not demonstrate significant changes between MGUS and MM samples. However, expression of CC3 as a phenotypic marker of apoptosis was unexpectedly increased in MM. In contradiction to our proposed hypothesis, the phenotypic markers of senescence and apoptosis as the outcome of DDR and OIS activations were not augmented in MGUS compared to MM.

Other pro- and anti-apoptotic markers as well as proliferation and cell cycle markers with their corresponding impacts on overall survival of MM were investigated in our study to find any association between senescence, apoptosis, and proliferation events in MGUS and MM. Bax and Bcl-2 overexpression were seen in MM samples compared to MGUS. Also, we demonstrated a positive association between BCl-2 overexpression and short survival of newly diagnosed MM patients for the first time.

However, we were not able to find any correlation between prolonged overall survival of myeloma patients and Bax overexpression. Moreover, Cyclin D1, Ki67, and p16 expressions did not significantly change when MGUS progressed to MM, and also their expressions were not associated with overall survival of MM patients. Despite the fact that p53 expression did not vary between MGUS and MM samples, the data from the survival analysis showed a significant correlation between p53 expression and poor overall survival in MM patients. In conclusion, increased levels of selective apoptotic markers were found in MM compared to MGUS patients; however, the senescence and proliferation were not statistically significantly varied between these two groups of patients.

Understanding of differential expression of various markers will provide significant insights into the biology of monoclonal gammopathies. More importantly, these insights may inform on chemo-preventive measures. Indeed, therapeutic targeting of various aspects of tumor suppressor genes such as reactivation of p53 or p16, and inhibition of anti-apoptotic Bcl-2, which have shown efficacy in variety of tumors, might also help treatment of MM patients.

6. Future work

To investigate the activation of DDR and OIS during the transformation of MGUS to MM, we would have to and indeed are planning to obtain serial samples of MGUS patients that progressed to MM during their follow up and examine DDR and OIS markers in all such serially collected samples. As noted throughout the Discussion chapter, additional MGUS bone marrow samples would be also be essential to arrive at a better understanding and conclusion of this critical transformation stage. In addition, more BM samples and clinical data of MM patients would strengthen the OS cure results.

We had to use p16 as a senescence marker, since this antibody was applicable for paraffin-embedded tissues and also many studies used it as a senescence marker. However, β-galactosidase is probably a better phenotypic marker of senescence and but the kit reagents for this marker was not applicable for paraffin-embedded tissues. In future, we plan to collect fresh plasma cells from bone marrow of MGUS and MM patients and measure the β-galactosidase activity in these samples. Furthermore, a future step would be to study the causes behind Bax overexpression in MM and find the role of this pro-apoptotic protein in the pathogenesis of myeloma. It is a notable finding of our study that CC3 expression was significantly increased in MM samples, which suggest that the apoptotic index in MM patients is higher than that of MGUS samples. However, we did not investigate which apoptotic pathways (intrinsic or extrinsic) were exactly involved in the demonstrated increased level of CC3. A logical next step would be to find events that mediate apoptosis in malignant plasma cells.

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Appendix

The IHC technique generally includes the following steps:

1. De-paraffinization of tissue sections mounted on coated slides.
2. Antigen retrieval with methods available.
3. Blocking of endogenous enzymes (which otherwise would react with IHC reagents giving false-positive results). Peroxidases, alkaline phosphatases, and biotin are examples of endogenous enzymes. This step is usually done by 3% hydrogenperoxide or with free avidin.
4. Binding with primary antibody.
5. Binding with secondary antibody.
6. Applying detection methods such as avidin-biotin conjugates, peroxidase-antiperoxidase methods, peroxidases complexes, or the more recently used polymer-labeling two-step method.
7. Adding the chromogen substrate, usually DAB (3.3'-diaminobenzidine).
8. Counterstaining, dehydrating, clearing, and cover-slipping the slide.

A brief introduction of staining methods, antigen retrieval procedures, enzyme reaction, fixation of tissue and type of antibodies in IHC would help to better understanding of IHC concept.

General double staining procedure

1. Quench for endogenous peroxidase activity.

2. Rinse and incubate for 3–5 minutes in wash buffer. (Incubate for 10* minutes with each reagent in Steps 3, 4, 8 and 9; repeats Step 2 after each step)

3. First primary antibody incubation.

4. First link antibody/peroxidase-conjugated polymer.

5. First substrate-chromogen incubate for 5–10 minutes.

6. Rinse with distilled water.

7. Incubate in double stain block reagent for 3 minutes.

8. Second primary antibody incubation.

9. Second link antibody/alkaline phosphatase conjugated polymer.

10. Second substrate-chromogen incubates for 5–10 minutes.

11. Rinse with distilled water.

12. Counterstain, clear and coverslip.