EXPRESSION OF MATRIX METALLOPROTEINASES (MMP-2) IN PROGNOSTICATION OF MENINGIOMA

DR. MARYAM AHMAD SHARIFUDDIN

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF PATHOLOGY
(ANATOMICAL PATHOLOGY)

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LIST OF ABBREVIATIONS

BMI	Body mass index
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
IHC	Immunohistchemistry
MMP	Matrix metalloproteinases
MVD	Microvascular density
NF	Neurofibromatosis
PCR	Polymerase chain reaction
TNF	Tumour necrosis factor
VEGF	Vascular endothelial growth factor
WHO	World Health organization

ABSTRAK

Latar belakang: Meningioma merupakan ketumbuhan otak yang biasa berlaku dikalangan orang dewasa iaitu sebanyak 20% daripada keseluruhan jenis ketumbuhan otak. Selain daripada kadar tisu ketumbuhan yang dibuang dan gred WHO, pembetukan salur darah juga merupakan antara faktor prognostik yang dipengaruhi oleh MMP-2. Kajian ini bertujuan untuk melihat kaitan antara gred dan kadar pembetukan salur darah dalam meningioma dengan ekpresi MMP-2.

Kaedah: Ini adalah satu kajian rentas yang dijalankan ke atas 99 kes meningioma yang berbeza gred dalam tempoh Januari 2008 hingga Disember 2017. Semua kes telah dilihat semula dan ujian antibody Ki67, MMP-2 dan CD34 telah dijalankan. Ujian 'Pearson chi square' dan 'Fischer exact' digunakan untuk melihat kaitan antara ekpressi MMP-2 dengan gred meningioma dan kuantiti pembetukan salur darah.

Keputusan: Sejumlah 99 kes meningioma terdiri daripada pesakit yang berumur 23 hingga 75 tahun. Kebanyakannya adalah daripada golongan wanita (74%). Kajian ini mempunyai sebanyak 85 kes gred rendah (Gred I) dan 14 kes gred tinggi (11 = Gred II, 3 = Gred III). 'Meningothelial', 'Transitional' dan 'Fibroblastic' adalah jenis meningioma yang paling kerap berlaku. Enam puluh dua daripada 85 gred rendah dan 10 daripada 14 kes gred tinggi mempunyai ekspresi MMP-2 yang tinggi, tetapi ia tidak signifikan, $p > 0.950$. Kebanyakan kes meningioma mempamerkan kuantiti pembetukan salur darah yang rendah iaitu skor 1+ (54/99) dan 2+ (33/99). Empat puluh dua daripada 54 kes yang mendapat skor 1+, mempunyai ekpresi MMP-2 yang tinggi, tetapi ia juga tidak signifikan.

Kesimpulan: Semua kes meningioma menunjukkan ekpresi MMP-2. Kebanyakan kes juga menunjukkan quantiti pembetukan salur darah yang rendah dan antara kes-kes ini, ada yang menunjukkan ekpresi MMP-2 yang tinggi. Tetapi semua ini tidak signifikan. Untuk kajian di masa hadapan, lebih banyak kes gred tinggi diperlukan dan jenis antibodi yang lebih spesifik untuk pembetukan salur darah supaya analisa lebih tepat.

ABSTRACT

Background: Meningioma is the most common intracranial tumour in adults which is 20% of all primary intracranial tumours. Besides extent of tumour surgical resection and WHO grading, angiogenesis is also one of the prognostic factors, which is influenced by MMP-2. Our study aimed to determine the association of these prognostic factors with expression of MMP-2 in meningioma.

Methodology: A cross sectional study was conducted on 99 samples of meningioma of different grades diagnosed from January 2008 until December 2017. All samples were re-reviewed and stained with Ki67, MMP-2 and CD34 immunohistochemistry markers. Pearson chi square and Fischer's exact test were used to examine the association between MMP-2 expression with WHO grades and Microvascular Density (MVD).

Results: A total of 99 cases consists of patients aged between 23 to 75 years old. Majority of patients were female (73.7%). This study consists of 85 cases of low-grade meningioma (Grade I) and 14 cases of high-grade meningioma (11 = Grade II, 3 = Grade III). The most common subtypes are meningothelial, transitional and fibroblastic. Sixty two out of 85 cases of low-grade and 10 out of 14 cases of high-grade shows high MMP-2 expression, however they were not significant, $p > 0.950$. Most of the cases in this study exhibits low level of angiogenesis with MVD score of 1+ (54/99) and 2+ (33/99). Out of the 54 cases that scored 1+, 42 expressed high MMP-2. However, this is not significant.

Conclusion: MMP-2 were expressed in all low- and high-grade meningiomas with varying intensity. Majority of cases exhibit low quantity of angiogenesis and among these cases, mostly expressed high MMP-2. However, these findings were not significant. In

future study, more higher grade meningioma is required to prevent bias in analyzing data. A more specific immunohistochemical marker should be used to evaluate angiogenesis to achieve accurate scoring.

Keywords: *Matrix metalloproteinase-2, meningioma, WHO grade, angiogenesis, prognosis.*

Chapter 1
Introduction

1.1 Overview of Meningioma

Meningioma is a benign, slow-growing tumour that occurs in adult and it is one of the most common tumour of the central nervous system (CNS). In the USA, it accounts for 36% of all brain tumours overall (1). In other parts of the world such as France, Italy, Australia and Japan, report showed an increased trend of primary brain tumours and in particular, meningioma, over the past decades (2). However, this increment is thought not to reflect the actual incidence due to several factors: 1) aging of the population 2) improvement of the health care facilities and 3) higher rate of histological conformation (2). The Global Cancer Statistic 2018 (3) revealed that brain and nervous system tumour contribute 1.6% of new cases from all cancer and 2.5% of death. However, the incidence according to specific brain tumour types are not available in this report.

In Malaysia, brain tumour is considered uncommon as compared to other tumours. The Malaysian National Cancer Registry Report for 2007 – 2011 (4) reported that the brain and nervous system tumour were ranked as 11th most common in males and 13th in females. The Chinese have the highest incidence rate and followed by Malay and Indian. Incidence according to different tumour subtypes were also not included in this report. There should be a proper data collection on the specific types of brain tumour in Malaysia which could accurately reflects the tumour burden. This can guide future research towards advancement in treatment and healthcare plan in Malaysia. This also should apply to other tumour besides brain tumour.

Yusof et al. (5) reported that the incidence of brain tumour in North East Malaysia is low (0.4 per 100,000 population) in 1996 and meningioma was the second most

common brain tumour after neuroglial tumour. One study conducted from 2011 to 2014 to determine the distribution pattern of brain tumour in Hospital Universiti Sains Malaysia (HUSM), Kelantan and revealed that meningioma is the most frequent tumour, which accounts about 36.4% from all types of brain tumour (6). The higher incidence of meningioma in Kelantan, particularly in USM is because it is the referral center for brain tumor for east coast region and thus, this study provides the descriptive distribution for Kelantan and Terengganu. The incidence of brain tumour in Sarawak is 4.6 per 100,000/year. Sarawak General Hospital reported about 35% of operated cases between 2000-2001 were meningioma and 32.3% between 2009-2012 (7).

There are several risk factors that contribute to development of meningioma. Ionizing radiation is one of the established environmental risk factors, with higher risk among people who were exposed in childhood than as adults (1). There is also higher incidence in female compare to male patient which suggest that female hormone could play a role via estrogen and progesterone receptor (8). Several studies reported a possible association between meningioma and breast cancer (9) (10). Both share the same common risk factors such as endogenous and exogenous hormones as well as shared genetic predisposition (11). Michaud et al. (12), found a positive association between risk of meningioma and body fatness which measures BMI, waist circumference and weight. The exact mechanism of this is unclear, but possible mediators could be involved which include hormonal factors, immunologic response and levels of insulin or insulin-like growth factors.

According to the WHO Classification of Tumours of the Central Nervous System Revised 4th edition 2016 (1), meningioma is divided into three grades, namely Grade I, Grade II and Grade III. Approximately 90% of meningiomas are benign (Grade I) and have slow growth, with incidence increasing with age (11). It is associated with relative

good outcome (13), however, a significant number of cases relapses occur among benign meningiomas (14). The remaining percentage of meningioma are atypical (Grade II) and anaplastic (Grade III), which represent a major challenge to neurosurgeons (15). Atypical (Grade II) meningiomas occupy an intermediate risk group between benign (Grade I) and anaplastic (Grade III) meningiomas. They carry a several-fold increased risk of recurrence, as well as an increased rate of mortality (16). As for meningioma Grade III, the prognosis is poor and complete or subtotal resection may prolong the patients' survival (17).

Each grade can be further divided into several subtypes (Table 1). The most common subtypes are meningothelial, fibrous and transitional meningioma (Grade 1) (1).

Table 1.1: 2016 WHO classification for meningioma.

Grade I	**Grade II**	**Grade III**
Meningothelial	Chordoid	Papillary
Fibrous	Clear cell	Rhabdoid
Transitional	Atypical	Anaplastic (malignant)
Psammomatous		
Angiomatous		
Microcystic		
Secretory		
Lymphoplasmacyte-rich		
Metaplastic		

The grading system is mainly based on histomorphology criteria and number of mitosis (1) (Table 1.2).

Table 1.2: Histomorphology criteria for grading of meningioma.

Grade I	**Grade II**	**Grade III**
i) less than 4 mitotic figures	i) 4 - 19 mitotic figures/10 HPF OR ii) Brain invasion OR iii) Three of these histologic features: a. Increased cellularity b. Small cells with high N/C ratio c. Large and prominent nucleoli d. Patternless or sheet-like growth e. Foci of "spontaneous" or geographic necrosis	i) Either 20 or more mitotic figures/10 HPF, or ii) Display frank sarcomatous or carcinomatous histology

In addition to histological grade, the other most powerful histological prognosticator for the outcome of meningioma is proliferation index (18). Many previous studies that have been carried out measuring Ki67 proliferation index in predicting behaviour of meningioma. Ki67 is one of the various immunohistochemical marker that is widely used to measure cell proliferation. Ki-67 antigen is a protein expressed only in proliferative phase of cell cycle, and can be detected on formalin fixed paraffin embedded tissue sections (19).

A literature review done by Abry et al. (20) showed positive correlations between Ki67/MIB-1 and tumor grade in human meningiomas in all 53 articles. However, the Ki67 labelling index varied considerably from one articles to another. This highlight several factors that contribute to these variations which includes block selection with representative tumor tissue for calculation, interobserver variability and non-standardization of Ki67 immunostaining procedure between laboratories.

There were conflicting reports in the literature about different histological subtype on Ki67 proliferation index. Some studies showed that there is positive correlation (19) (21) and others showed no correlation between subtypes of meningioma (20) (22). Other studies also had been done evaluating the correlation between Ki67 with brain invasion, recurrence, location of the tumor and other clinicoparameters (age, gender, progesterone receptor) (22) (23) (24) (25). Variable results were obtained from these studies. However, most studies agreed that Ki67 represent as a prognostic marker in human meningioma in term of histological grade and recurrence.

The most common chromosomal change is Monosomy 22 which is seen in 40-70% of all grades of meningioma (26). Other cytogenetic changes includes deletion of chromosome 1p in atypical meningioma that correlates with shorter progression-free survival (27), losses of chromosomes 6q, 9p, 10, 14q, and 18q (26) and gains of

chromosomes 1q, 9q, 12q, 15q, 17q, and 20q (1). Meningioma are also found to be closely related to tumor suppressor syndrome NF2 and 50–75% of patient with NF2 have risk to develop one or more meningioma in their lifetime (28). Currently, studies have identified several new recurrent mutations through next-generation sequencing approaches and is present in 40% of sporadic meningioma. These genes are pro-apoptotic E3 ubiquitin ligase TNF receptor-associated factor 7 (TRAF7), the pluripotency transcription factor Kruppel-like factor 4 (KLF4), the proto-oncogene v-Akt murine thymoma viral oncogene homolog 1 (AKT1), the Hedgehog pathway signaling member smoothened (SMO), and the oncogene PIK3CA (29) (30) (31). However, there are still around 20% of meningioma that have no identifiable oncogenic driver mutation (32). In future, these molecular advances will influence the classification and prognosis of meningioma, like in other Central Nervous Tumour such as medulloblastoma and glioblastoma. The management will also be changed due to the discovery of new therapeutic agents.

1.2 Matrix Metalloproteinase-2 (MMP-2)

Matrix metalloproteinases (MMPs) belong to a super family of endopeptidases which consists of over 20 enzymes. These enzymes are produced by various cells, including epithelial cells, fibroblasts, and inflammatory cells (33). MMPs are subdivided into 5 groups based on their structure and/or substrate specificities; matrilysins, collagenases, stromelysins, gelatinases and membrane-type MMPs (34). They are expressed only when needed for tissue remodelling. However, increased expression has been associated with many pathological conditions, such as rheumatoid arthritis and periodontitis (35). Many studies have been done on MMPs and the more recent advanced in technological development allows more understanding of the role of MMPs especially in the regulation of the tumor microenviroment (36).

MMPs contribute to the carcinogenesis at multiple stages; remodelling of extracellular matrix during early steps, followed by induction of angiogenic switch, further up-regulation of MMPs leads to degradation of the basement membrane and allows the tumour cells to invade into surrounding stroma and lymphovascular channels, and providing a favourable microenvironment at distant sites of metastasis **(**37). Clinical study from Jäälinojä et al. (38) suggest that MMP-2 is associated with histological malignancy such as anaplastic astrocytoma, glioblastoma and metastatic brain tumour and poor survival in brain tumour.

The two most widely studied MMPs are MMP-2 and MMP-9 (39). There are conflicting data between studies regarding the role of MMPs in the recurrence and invasiveness of meningioma, as well as the degree of expression in different grades and subtypes. Some studies showed evidence of high expression of both MMP-2 with recurrence and invasiveness (40), some studies only demonstrated only MMP-9 involvement, and not MMP-2 (41) (42). There is a conflicting findings with respect to this MMP-2 and MMP-9 and expressions of histological subtypes: positive correlation (43) and negative correlation (44), thus routine screening with these markers will not reveal any new diagnostically or prognostically relevant information.

1.3 Role of MMP-2 in angiogenesis

Angiogenesis is the process by which tumors induce the blood supply crucial for growth and progression. It is a complex process which involves multiple steps and is initiated by the release of proangiogenic factors (eg. VEGF, TNF-α) from inflammatory cells, mast cells, macrophages or tumour cells (45). High quantity of neoangiogenesis appears to be significantly associated with a higher proliferation index of meningiomas,

and it seems to represent a negative prognostic marker associated with shorter overall survival and higher recurrence risk in completely resected tumors (19).

MMPs are clearly implicated in angiogenesis especially MMP-9, which is one of the angiogenesis regulator in meningioma (19). The role of MMPs in angiogenesis is to degrade the basement membrane and other ECM components, allowing endothelial cells to detach and migrate into new tissue and releasing ECM-bound proangiogenic factors (eg vascular endothelial growth factor, VEGF; bFGF, basic fibroblast growth factor; TGFβ, transforming growth factor-β) (37). The role of MMP-2 in stimulation of angiogenesis has been in proven in several studies. For example; tumors expressing MMP-2 have more proliferating vasculature at the tumour-brain interface than MMP-2 negative tumours (46) and implantation of tumour cells into either MMP-2 or MMP-9 knockout mice shown to have reduced tumour-induced angiogenesis compared to implantation into wild type mice (34).

Current treatment for meningiomas involve surgery, radiation therapy, and stereotactic radiosurgery. However, with the new understanding of angiogenesis in meningioma, patients are amendable to antiangiogenic therapy, particularly in patients with high grade or recurrence after surgery. Many antiangiogenic drugs are currently being used in clinical trials, i.e, becacizumab, sunitinib, and vatalinib, and have shown potential efficacy in uncontrolled studies and should be investigated further (47). Studies are now directed towards identifying specific MMPs that mediate the angiogenic response for the purpose of targeted treatment, thus being able to disrupt tumour neovascularization and subsequent progression or recurrence.

1.4 Objective

- General objective

  To determine the expression of MMP-2 in meningioma in relation to its prognostic factors.

- Specific objectives

- To determine the expression of MMP-2 in different grades of meningioma.
- To determine the expression of MMP-2 with angiogenesis in meningioma.
- To determine the association between expression of MMP-2 with tumour grades and angiogenesis in meningioma.
- To determine the association between angiogenesis and different grades of meningioma.

1.5 Research hypothesis

- There is positive association between expression of MMP-2 with tumour grades and angiogenesis in meningioma.

Chapter 2
Study protocol

2.1 Study protocol

Title: Expression of matrix metalloproteinases (MMP-2) in prognostication of meningioma

Investigator: Dr Maryam Binti Ahmad Sharifuddin (MPATH (Histopathology) student from HUSM)

Supervisor: Professor Dr Hasnan Jaafar (Pathology department of HUSM)

Co-supervisor: Dr Sharifah Emilia Tuan Sharif (Pathology department of HUSM)

Introduction

Meningioma is the most common primary intracranial tumour (1) and the reported incidence is about 36% in the USA (2). In Malaysia, there is no specific data regarding meningioma reported in the Malaysian National Cancer Registry Report 2007-2011. However, there are studies that provide the descriptive data of brain tumour in Sarawak (2009-2012) and East Coast, Malaysia (2011-2014). Both regions reported meningioma being the most common brain tumour accounting for 32.3% and 36.4%, respectively (3) (4). Meningioma usually occurs in adults in 6th decades of life and the risk increases with age (5). It is uncommon in children with incidence rate of only 2.8% of all pediatric primary brain tumour (5).

Meningioma is diagnosed base on characteristic radiological features and histopathologic examination. The WHO Tumour Classification of the CNS (2) divided meningioma into 3 grades and 15 subtypes. The grading is based mainly on mitotic count and certain histological features. It has important implication on the patient management

and prognosis. Ninety percent of meningioma are benign and 10% are atypical (5-7%) and anaplastic (1-3%) meningiomas (6).

Literature review

The Matrix Metalloproteinases (MMPs) are a family of zinc-containing endopeptidases which consists of 20 members. They are divided into six groups based on several distinct domains; collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other non-classified MMPs (7). MMP-2, also known as Gelatinases A, belongs to the gelatinases group, together with MMP-9.

MMPs are not only involved in tissue remodeling, development and wound healing, but also contribute to tumorigenesis and progression of various tumour (8). They regulate the growth of tumour cells, which eventually leads to invasion and metastasis. High levels of certain MMPs are shown to have poorer prognosis (9), for example, MMP-2 overexpression in ovarian epithelial carcinoma (10), laryngeal cancer (11), gastric cancer (12), primary melanoma (13) and prostate cancer (14).

MMPs are also involved in angiogenesis. Angiogenesis is a complex process which is an important component in tumor development. It is regulated by many pro-angiogenic growth that is secreted by tumour cells. Many studies have proven that higher quantity of angiogenesis is related to higher tumour grade and poorer prognosis (15) (16) (17). Brain tumour are highly vascularized tumour, thus many anti-angiogenic treatments such as Neovastat and Suramin, were developed to target this process (18). Certain drugs such as VEGF inhibitor can serve as a possible safe treatment option (19), while others are still under clinical trials.

MMP-2 is the most studied for their role in angiogenesis. MMP-2 together with integrin $\alpha v\beta 3$ on the surface of vessels undergoes active remodelling in response to

angiogenic stimulus (18) (20). Previous studies have focused on establishing the role of MMP-2 in meningioma, in particular as it relates to histological grades, tumor recurrence, brain invasion, and peritumoral edema (1) (21) (22) (23). However, no study have been done specifically to evaluate the role of MMP-2 in relation to angiogenesis in meningioma.

Rationale of study

- Previous studies have focused on establishing the role of MMP-2 in meningioma, in particular as it relates to histological grades, tumour recurrence, brain invasion, and peritumoral oedema, however the data is conflicting.
- The relationship between MMP-2 expression and angiogenesis has been previously reported. However, no study has been done specifically to evaluate the role of MMP-2 in angiogenesis in meningioma.
- We hope this study can provide the evidence of positive correlation between MMP-2 and the prognostic factors of meningioma. Therefore, in future MMP-2 can be a useful and practical assessment tool for the pathologist and surgeon as it provides information on the prognostication and can predicts the outcome of treatment or recurrence in patient.
- As for the patient, MMP-2 also can be a potential therapeutic target and the use of their inhibitors may significantly improve the outcome after operation especially in high grade meningioma.

Research hypothesis

There is positive association between MMP-2 with tumour grades and angiogenesis.

Objective of the study

General objective

- To determine the expression of MMP-2 in meningioma in relation to its prognostic factors.

Specific objective

- To determine the expression of MMP-2 in different grades of meningioma.
- To determine the expression of MMP-2 with angiogenesis in meningioma.
- To determine the association between expression of MMP-2 with tumour grades and angiogenesis of meningioma.
- To determine the association between angiogenesis and different grades of meningioma.

Material and methodology

Study Design

This is a cross sectional (retrospective study).

Target population

All patient diagnosed with meningioma in Hospital Universiti Sains Malaysia from January 2008 until December 2017.

Sample size determination

Sample size is calculated based on Two proportion formula, using Sample Size Calculator version 3.1.2.

To associate the expression of MMP-2 with tumour grades.

P_0	= Proportion of high MMP-2 in patient with low tumour grades	= 0.16*
P_1	= Proportion of high MMP-2 in patient with high tumour grades	= 0.4
α	= Significance level	= 0.05
Power		= 0.8
Sample size calculated		= 62

**[Okada M, Miyake K, Matsumoto Y, Kawai N, Kunishio K, Nagao S. Matrix metalloproteinase-2 and matrix metalloproteinase-9 expressions correlate with the recurrence of intracranial meningiomas. J Neurooncol 2004;66:29-37.](24)*

To associate the expression of MMP-2 with angiogenesis in meningioma.

P0	= Proportion of high MMP-2 in patient with low angiogenesis in meningioma	= 0.19*
P1	= Proportion of high MMP-2 in patient with high angiogenesis in meningioma	= 0.4
α	= Significance level	= 0.05
Power		= 0.8
Sample size calculated		= 82

*[*Zhai L.L, Wu Y, Huang D.W, Tang Z.G. Increased matrix metalloproteinase-2 expression and reduced tissue factor pathway inhibitor-2 expression correlate with angiogenesis and early postoperative recurrence of pancreatic carcinoma. Am J Transl Res 2015;7(11):2412-2422.](25)*

Based on the calculation, it is decided that we are going to use n = 82 samples for the study. Sample size after including dropout of 10%, is n = 90 samples.

Sampling method

Convenient sampling method will be used. Cases will be retrieved from the computerized registry data (LIS and PATHOS system) of the Department of Pathology, Hospital Universiti Sains Malaysia, Kubang Kerian, Kelantan. All cases of meningioma diagnosed between January 2016 – December 2017 that fulfill the inclusion criteria will be included in the sampling. Paraffin tissue blocks for all cases and its respective Hematoxylin and Eosin (H&E) stained slides will be retrieve from the tissue archive. Based on the H&E slides, the slide with the most tumor tissue will determine the choice of tissue block for immunohistochemistry study. Only one representative tissue block from each case will be chosen for each case.

The inclusion and exclusion criteria are as follows:

- Inclusion criteria

  All cases diagnosed histologically as meningioma of various grades.

- Exclusion criteria

  Case excluded from this study were those with unavailable or missing paraffinized tissue blocks and paraffinized tissue block that are inadequate tissue or unsatisfactory for immunohistochemical study.

Research tools

- Paraffin blocks of patient will be used for sectioning and immunohistochemistry.
- Immunohistochemistry stains (monoclonal mouse anti-human CD34 Class III, rabbit monoclonal anti-Ki67 antibody and rabbit polyclonal anti-MMP2 antibody).
- Secondary antibody (labelled polymer-HRP, Dako Envision™ + Dual Link System-HRP, DAB+)

Immunohistochemistry (IHC) staining procedure

Tissue sections of 3-4µm thickness will be cut and transferred to poly-L-lysine precoated slides. The slides will undergo deparaffinization and rehydration process with xylene and four different alcohol solutions of descending concentration. This is followed by antigen retrieval process using heat-induced epitope retrieval method via pressure cooker (pressure cooker WMF Perfect) for 3 minutes. Antigen retrieval buffer (10mmol/L Tris buffer, 1mmol/L EDTA, pH 9.0) is used. Subsequently, peroxidase blocking is applied for 5 minutes. For MMP2 IHC test, the peroxidase blocking step was carried out after overnight incubation of the primary antibody. The slides will be incubated with the primary antibody (anti-Ki67; 1:200, anti-CD34; 1:100 and anti-MMP2 antibody; 1:500) for overnight at 4°C using Squenza Immunostainer (Shandon Sequenza). Then secondary antibody (labelled polymer-HRP, Dako Envision™ + Dual Link System-HRP, DAB+) will be applied and incubated for 30 minutes at room temperature, followed by incubation with 3,3'-diaminobenzidine (DAB) solution for 5 minutes. Finally, it is counterstained with Harris Hematoxylin for 5 seconds, followed by dehydration process. For the control tissue, neurofibroma will be used for anti-MMP2 whereas tonsil tissue will be used for anti-Ki67 and anti-CD34.

Immunohistochemistry (IHC) scoring

The slides will be examined under light microscope. For Ki67 scoring, it is recorded as percentage of positively stained tumor nuclei (brown satining) per 1000 tumor cells. The cell counts are performed in regions of maximum immunoreactivity (hot spot) under high power objective (x400). According to Perry et al. (26), Ki67 value is categorized into three grades;

- < 4% = WHO Grade I
- >4% = WHO Grade II
- >20% = WHO Grade III

The expression of MMP-2 will be evaluated by IHC scoring as the sum of the frequency and the intensity according to scale used by Strojnik et al. (27). Twenty representative fields were counted, and the IHC scores were determined. The total score includes the sum of the frequency of tumour cells that are stained and intensity of the cytoplasmic staining.

Frequency:

- Score 0 indicates no staining of tumour cells
- Score 1 for 0-29% positive tumour cells
- Score 2 for 30-60%
- Score 3 for 61-100%

Intensity

- Score 0 indicates no staining of tumour cells
- Score 1 for weak staining of tumour cells
- Score 2 for moderate staining of tumour cells
- Score 3 for strong staining of tumour cells

Total score of 4 and higher is considered ‘high expression’, whereas score of 3 or less is considered ‘low expression’.

Assessment for angiogenesis will be using CD34 immunohistochemistry stain. A brown staining of a single endothelial cells or clusters of endothelial cells, with or without a lumen is considered as an individual vessel. According to Weidner et al. (28), the total number of microvessels count in three fields (400x), will be calculated as mean value and recorded as the microvessels density (MVD). The MVD will be given a score as follows:

- Score 1+ for MVD value between 0 - 33
- Score 2+ for MVD value between 34 - 67
- Score 3+ for MVD value between 68-100
- Score 4+ for MVD value more than 100

Statistical analysis

All data will be entered and coded in SPSS version 26.

Descriptive data:

- Frequency and percentage will be presented for categorical data.
- Mean and standard deviation will be presented for numerical data.

Univariable:

- Pearson chi square test will be use to determine the association between MMP-2 expression with different grades of meningioma and also to determine the association between angiogenesis and tumour grades.
- Fisher’s exact test will be use to determine the association between MMP-2 expression with microvascular density (MVD).

Ethical consideration

Ethical approval will be obtained from Human Research Ethics Committee of Universiti Sains Malaysia. Approval will be obtained from Director of Hospital Universiti Sains Malaysia to access patient's folder and also from Head of Department Pathology to use paraffin block and histology slides. All the patient's clinicopathology data will be confidential.

Expected results

Table 2.1. Clinicopathological characteristic.

Variables	Frequency n (%)	Mean
Age		
Gender Female Male		
Race Malay Chinese Others		
Location		
Histological subtypes		
WHO Grade Grade I Grade II Grade III		
Recurrence Yes No		

Table 2.2. Association between MMP-2 expression and grade.

Variable	**Grade**		***p* value**
	Low **n (%)**	**High** **n (%)**	
MMP-2 Low High			

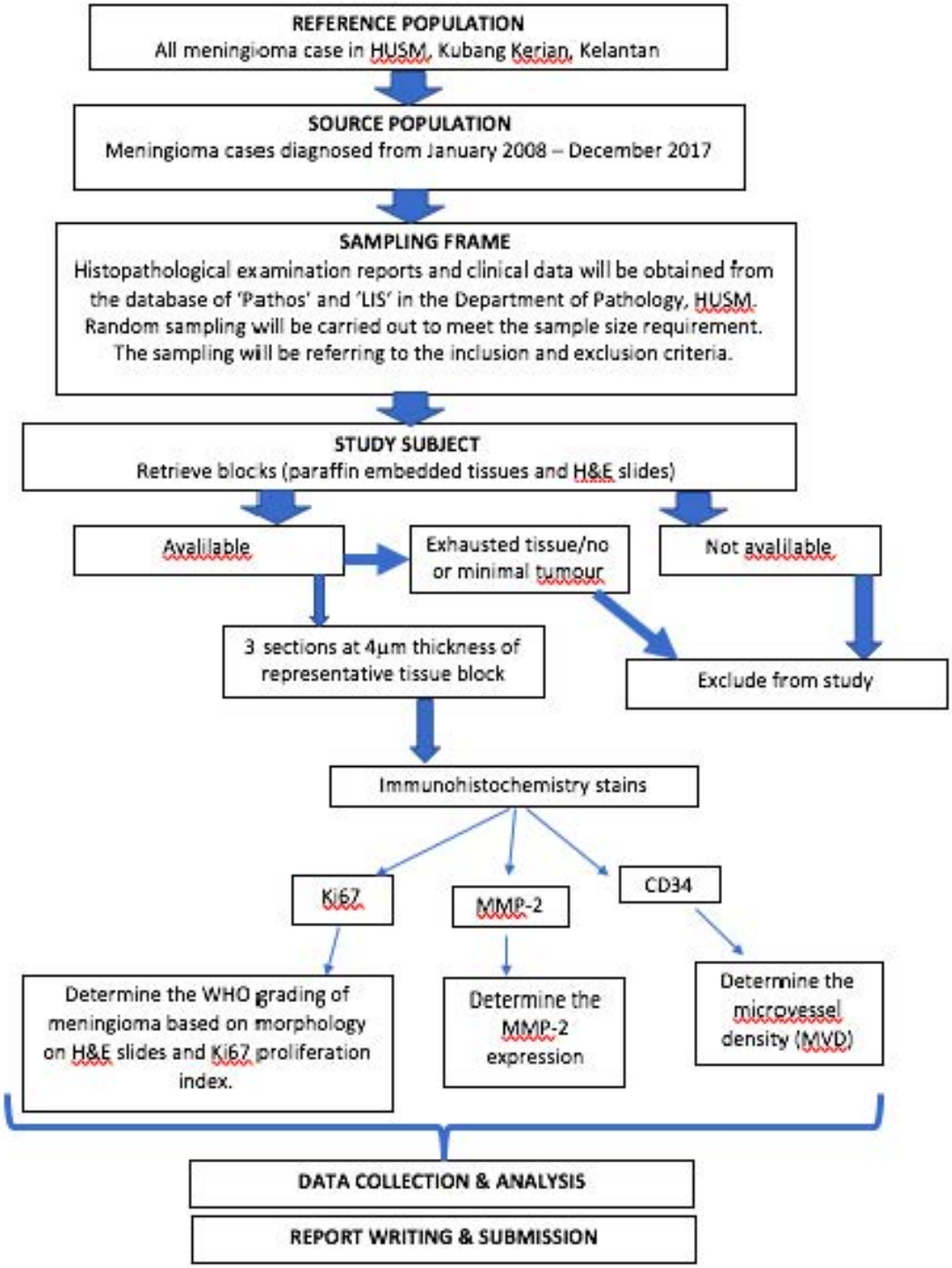
Table 2.3. Association between MMP-2 expression and Microvascular density (MVD).

Variable	**Angiogenesis score (MVD)**				***p* value**
	1+ **n (%)**	**2+** **n (%)**	**3+** **n (%)**	**4+** **n (%)**	
MMP-2 **Low** **High**					

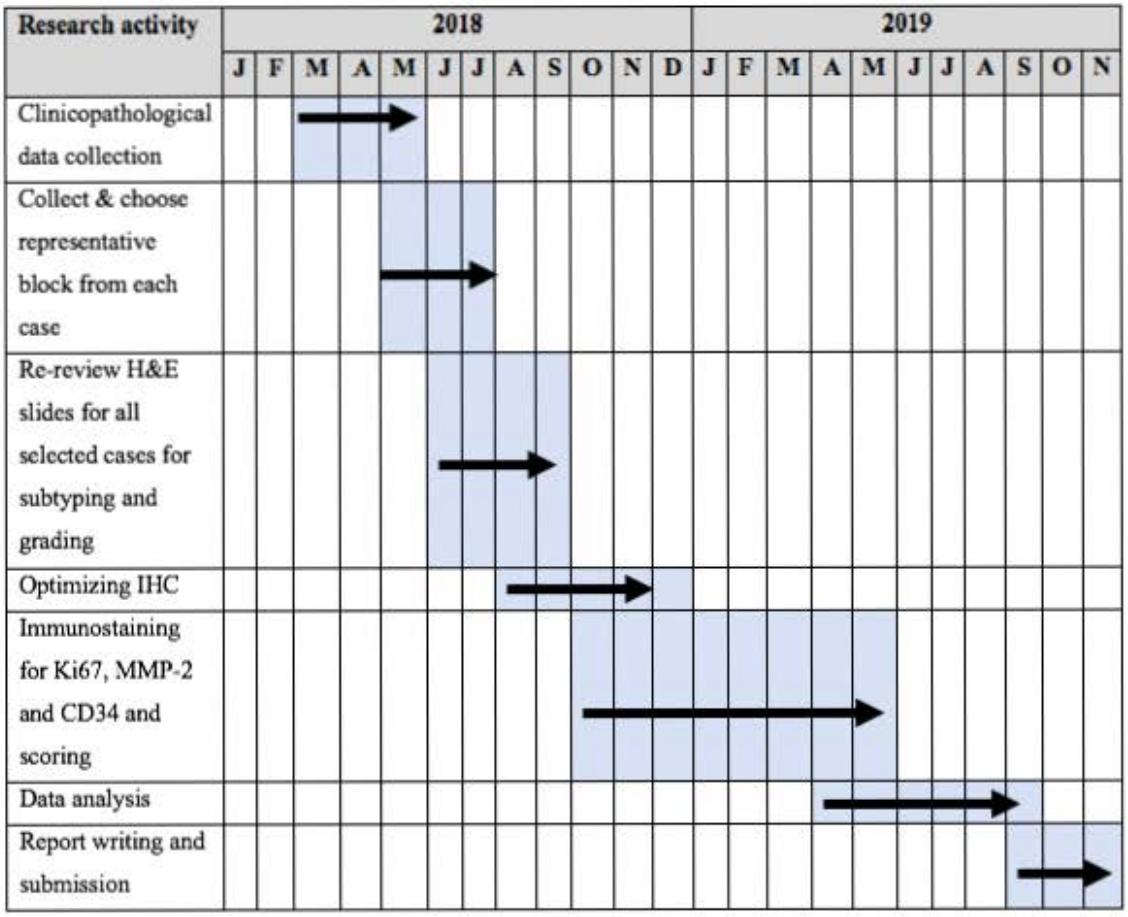
Table 2.4. Association between grade and Microvascular density (MVD).

Variable	**Angiogenesis score (MVD)**				***p* value**
	1+ **n (%)**	**2+** **n (%)**	**3+** **n (%)**	**4+** **n (%)**	
Grade **Low** **High**					

Flow chart


REFERENCE POPULATION
All meningioma case in HUSM, Kubang Kerian, Kelantan
SOURCE POPULATION
Meningioma cases diagnosed from January 2008 – December 2017
SAMPLING FRAME
Histopathological examination reports and clinical data will be obtained from the database of 'Pathos' and 'LIS' in the Department of Pathology, HUSM. Random sampling will be carried out to meet the sample size requirement. The sampling will be referring to the inclusion and exclusion criteria.
STUDY SUBJECT
Retrieve blocks (paraffin embedded tissues and H&E slides)
Avalilable
Exhausted tissue/no or minimal tumour
Not avalilable
3 sections at 4µm thickness of representative tissue block
Exclude from study
Immunohistochemistry stains
Ki67
MMP-2
CD34
Determine the WHO grading of meningioma based on morphology on H&E slides and Ki67 proliferation index.
Determine the MMP-2 expression
Determine the microvessel density (MVD)
DATA COLLECTION & ANALYSIS
REPORT WRITING & SUBMISSION

Gantt chart


Research activity
2018
2019
J F M A M J J A S O N D J F M A M J J A S O N
Clinicopathological data collection
Collect & choose representative block from each case
Re-review H&E slides for all selected cases for subtyping and grading
Optimizing IHC
Immunostaining for Ki67, MMP-2 and CD34 and scoring
Data analysis
Report writing and submission