ELUCIDATING THE ROLE OF M2 TUMOR-ASSOCIATED MACROPHAGES AND ITS RELATION TO MICRORNA IN HUMAN INVASIVE BREAST CARCINOMA

SHARMILLA DEVI A/P JAYASINGAM

UNIVERSITI SAINS MALAYSIA

2025

ELUCIDATING THE ROLE OF M2 TUMOR-ASSOCIATED MACROPHAGES AND ITS RELATION TO MICRORNA IN HUMAN INVASIVE BREAST CARCINOMA

by

SHARMILLA DEVI A/P JAYASINGAM

Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

September 2025

ACKNOWLEDGEMENT

First and foremost, I would like to thank my supervisor, Associate Professor Dr Ch'ng Ewe Seng. Thank you Dr, for giving me the opportunity to work under your supervision. Thank you, for your countless advice and guidance throughout my journey. I could not ask for a more experienced, knowledgeable and inspiring supervisor. My deepest gratitude to my co-supervisors, Prof Tang Thean Hock and Associate Professor Dr Citartan Marimuthu. You both provided me with guidance and motivation throughout my study. To the rocking team in Unit Histopatologi; Foo, Sobri, Najwa, Ada and Kak Faizah. Thank you for your patience in guiding and helping me. You were all so nice and treated me as one of your colleagues. To my teammates, Anna, Sarminiyy, Sue, Dr Ang, Theven, Dr Siti and Chaoli, you made this possible. Thank you for sharing your vast knowledge and experience with me. I would also like to thank Universiti Sains Malaysia for providing the financial support for this project. This study was funded by the Research University Grant for Individual (RUI, Grant No: 1001/CIPPT/8012230).

My first-born, Roshyan. Thank you for the tender-age wisdom you share with me every time I felt low. Your constant love letters to amma, especially the one you wrote, 'Don't worry amma, you will be a 'dokter' soon was the soothing music that accompanied me in this journey. Sahanaa my sunshine, your antics and cheekiness were my daily dose of happiness. To my husband Akash, thank you for standing by me all these years. Your growth in your career has inspired me to do the same in mine. Let us both reach for the sky together. Lastly but never the least, my late grandma and Aunt Malar. Thank you for taking me in, clothing, feeding, educating and raising me up. I am standing here today with pride and confidence because of you.

TABLE OF CONTENTS

ACKNOWLEDGEMENT ..... **ii**

TABLE OF CONTENTS ..... **iii**

LIST OF TABLES ..... **vii**

LIST OF FIGURES ..... **viii**

LIST OF ABBREVIATIONS ..... **xii**

LIST OF APPENDICES ..... **xv**

ABSTRAK ..... **xvi**

ABSTRACT ..... **xviii**

CHAPTER 1 INTRODUCTION **1**

CHAPTER 2 LITERATURE REVIEW **4**

2.1 Breast cancer 4

2.2 Breast Tumor Classification: Grade, Stage and Subtype 6

2.3 Survival rate in breast cancer 9

2.4 Tumor-associated Macrophages (TAMs) 10

2.4.1 M2 Tumor-associated macrophages: The 'aggressor' of tumor 12

2.4.2 Evaluation of M2 Tumor-Associated Macrophages in tissue section 13

2.5 MicroRNAs: History and biogenesis 14

2.6 Transport of microRNAs 17

2.7 Mechanisms of action of microRNAs 18

2.8 Significance of microRNAs in human physiology 19

CHAPTER 3 TUMOR-ASSOCIATED MACROPHAGE- RELATED MIRNAS AS POTENTIAL PROGNOSTIC BIOMARKER IN BREAST CANCER PATIENTS **22**

3.1 Background 22

3.2 Materials and Methods 24

3.2.1 Literature Search for TAM-Related miRNAs 24

3.2.2 Data Mining of miRNAs, mRNAs and Clinicopathological Data 25

3.2.3 Prognostic TAM-Related miRNA Signature Construction 25

3.2.4 External Validation of the Prognostic Significance of TAM-Related miRNA Signature with the Gene Expression Omnibus (GEO) Dataset 26

3.2.5 Independent Prognostic Significance of the Risk Score Based on TAM-Related miRNAs Signature 27

3.2.6 Correlation between Prognostic Risk Score and Immune Infiltrate Populations 28

3.2.7 Differential Gene Expression and Enrichment Analysis 29

3.2.8 Relationship between miRNAs in the TRM Signature and the Differentially Expressed Genes of High- and Low-Risk Groups 29

3.3 Results 30

3.3.1 Construction of the Eleven TAM-related miRNA Signature 32

3.3.2 The Eleven-TAM-Related miRNA Signature Significantly Differentiated the High-Risk Group from the Low-Risk Group 37

3.3.3 Validation of Prognostic Significance of the Eleven-TAM-Related miRNA Signature in the GEO Dataset 40

3.3.4 Prognostic Significance of the Eleven-TAM-Related miRNA Signature Is Independent of Clinicopathological Parameters 41

3.3.5 Among the Different Immune Infiltrate Populations, TAMs Had the Highest Correlation with the Risk Score Responsible for Poor Prognosis 45

3.3.6 Analysis of the Differential Gene Expression and Gene Set Enrichment 48

3.3.7 Relationship between miRNAs in TRM Signature and Differentially Expressed Genes between High- and Low-Risk Groups 51

3.4 Discussion and Conclusion 53

CHAPTER 4 CHARACTERIZATION OF M2 TUMOR-ASSOCIATED MACROPHAGES IN BREAST CANCER 64

4.1 Background 64

4.2 Materials and Methods 67

4.2.1 Breast tissue Procurement 67

4.2.2 Sectioning of FFPE Tissue 68

4.2.3 Simultaneous Double Immunohistochemistry 68

4.2.4 Enumeration of $M_{non\text{-}M2}$ and M_2 TAMs 70

4.2.5 Statistical analysis 72

4.2.6 Construction of breast cancer metastases predictor 74

4.3 Results 74

4.3.1 Clinicopathological characteristics of patients 74

4.3.2 TAMs distribution in breast tumor 76

4.3.3 TAM population in tumor front were inter-related with the TAM population in the intratumoral region, but not within the tumor front itself 80

4.3.4 Correlation between different TAM parameters and histological features of breast cancer 82

4.3.5 Both tumor front and intratumoral region are significantly associated with breast intrinsic subtype, but while tumor front is associated with ER and PR status, intratumoral region plays a key role in HER2 status 85

4.3.6 Association of Clinicopathological Parameters with Lymph Node Staging 87

4.4 Discussion 90

CHAPTER 5 EVALUATING THE EXPRESSION OF MIR-21 AND MIR-146a IN BREAST TUMOR WITH HIGH AND LOW DENSTIY OF M2 TAMS 98

5.1 Background 98

5.2 Material and methods 99

5.2.1 Starting material 99

5.2.2 miRNA extraction and purification from FFPE breast tissue sections 100

5.2.3 Determination of RNA quality and concentration..... 102

5.2.4 RNA Gel Electrophoresis 102

5.2.5 Real-time RT-PCR detection of miRNAs 103

5.2.5(a) First-strand cDNA synthesis 103

5.2.5(b) Quantitative Real-Time PCR for miRNA 104

5.2.6 Data analysis..... 106

5.3 Results..... 108

5.3.1 FM2 macrophage count in the high and low FM2 groups 108

5.3.2 Confirmation of RNA quality and concentration 108

5.3.3 Gel Electrophoresis confirms the integrity of RNA 108

5.3.4 MiR-21 was highly expressed in all samples compared to miR-146a 110

5.3.5 MiR-21 expression was significantly higher in the high FM2 group compared to the low FM2 group 110

5.4 Discussion 113

CHAPTER 6 GENERAL CONCLUSION AND FUTURE DIRECTIONS..... 116

6.1 Conclusion 116

6.2 Future Directions 118

REFERENCES..... 120

APPENDICES

LIST OF PUBLICATIONS

LIST OF TABLES

Page

Table 2.1	Hormone receptor status and global prevalence of breast cancer subtypes	7
Table 3.1	List of TAM-related miRNAs from various cancers	33
Table 3.2	Pertinent clinicopathological parameters of the training, validation, and whole cohort of TCGA-BRCA	35
Table 3.3	Relationship between risk score and pertinent clinicopathological parameters in TCGA-BRCA cohort	42
Table 4.1	Primary antibodies and dilution used	70
Table 4.2	Definition of TAM terms	72
Table 4.3	Clinicopathological features of breast cancer included in statistical analysis	73
Table 4.4	Summary of demographic details	75
Table 4.5	TAM distribution in breast tumor	80
Table 4.6	Correlation between TAM parameters with breast clinicopathological features	82
Table 4.7	Comparison of macrophage parameters in relation to breast cancer tumor characteristics	83
Table 4.8	ANOVA analysis	84
Table 4.9	Multinomial logistic regression model	89
Table 4.10	Accuracy of the multinomial regression model constructed	90
Table 5.1	Reverse transcription reaction setup per sample	104
Table 5.2	Reverse-transcription reaction cycling protocol	104
Table 5.3	Reaction mix setup for qRT-PCR per sample	105
Table 5.4	PCR cycling conditions for miRNA PCR assays	106
Table 5.5	Mean and range of FM2 count in groups of high and low FM2	108

LIST OF FIGURES

Page

Figure 2.1 Illustration of how M1 and M2 TAMs are activated, their respective functions, and the common IHC markers used to distinguish the two phenotypes in human tissue samples. 12

Figure 2.2 Biogenesis and mechanism of action by microRNA [125] 17

Figure 3.1 Formula of the risk score constructed 26

Figure 3.2 The overall workflow describing the process involved in the construction of 11TAM-related miRNA signature. (A) Flow chart describing the process involved in developing and validating the prognostic significance of the 11TAM-related miRNA signature. (B) Flow chart showing the prognostic independence evaluation and downstream analysis of high-risk vs. low-risk group assigned by the TRM signature. 31

Figure 3.3 Cox regression analysis. (A) The 16 TAM-related miRNAs with p value < 0.15 and their hazard ratios from univariate Cox proportional hazards regression analysis. (B) Tuning parameter (λ) selection in the LASSO model used 5-fold cross validation for OS-relevant miRNAs. (C) The LASSO coefficient profile of the 16 miRNAs. The vertical line indicates the coefficient selected by LASSO. 36

Figure 3.4 Risk score distribution and TRM expression heat map in TCGA-BRCA dataset. (A) Risk score distribution where blue dot signifies low-risk group and red dot signifies high-risk group. Vertical dotted lines indicate the cut-off point for median risk score. (B) Survival time and status for all patients. (C) Heat map of the eleven selected TRM expression in the TRM signature. 37

Figure 3.5 Kaplan–Meier survival analysis and time dependent ROC curves of the risk groups based on TRM signature for training and validation cohorts. KM curves show that the low-risk group has significantly longer overall survival compared to the high-risk group in both the training (A) and validation (B) cohorts. The AUCs of time-dependent ROC curves at 5-year OS were 0.69 and 0.66 for the training (C) and validation (D) cohorts, respectively. 39

Figure 3.6 Kaplan-Meier survival analysis and time dependent ROC curves of the risk groups based on TRM signature for the whole cohort. (A) Patients of the high-risk group had significantly shorter OS (p value < 0.001). (B) AUCs of time-dependent ROC curves at 3-, 5- and 10-year OS were 0.68, 0.68 and 0.75 respectively. 40

Figure 3.7 Kaplan–Meier survival analysis and time-dependent ROC curves of the risk groups based on the TRM signature for the external GEO GSE22220 cohort. (A) Patients of the high-risk group had significantly shorter DRFS (*p* value < 0.001). (B) AUCs of time dependent ROC curves at 3-, 5- and 8-year DRFS were 0.54, 0.60 and 0.63, respectively. 41

Figure 3.8 Kaplan-Meier survival analysis of the breast cancer subtypes based on ER and HER2 status. High-risk group had significantly poorer prognosis as compared to that of low-risk group in the ER+HER2- and ER-HER2- cohorts (*p* value < 0.05), but not in the ER+/- HER2+ cohort. 43

Figure 3.9 Spearman correlation between risk scores and percentage of tumor infiltrating lymphocytes in TNBC cases. 43

Figure 3.10 Univariate and multivariate Cox proportional hazards regression analysis for pertinent clinicopathological parameters. (A) Higher pathological stages (stage III and IV), ER and HER2 negative status, higher age and high-risk groups were significant poor prognostic factors in univariate analysis. (B) High-risk group remains an independent poor prognostic factor (*p* value < 0.001) in the multivariate analysis. 44

Figure 3.11 (A) Nomogram for predicting 3-year, 5-year and 10-year survival of breast cancer patients incorporating independent prognostic factors from multivariate Cox proportional hazards regression. (B) AUCs of time-dependent ROC curves at 5-year OS for the independent prognostic factors. The nomogram incorporating all the independent prognostic factors exhibited the highest AUC at 0.79. 45

Figure 3.12 Correlation between risk score and immune infiltrate populations. M2 macrophages had the highest positive correlation with the risk score, while M1 had the third highest negative correlation, after CD8 T cells and CD4 T cells. 46

Figure 3.13 (A) Chord diagram displaying correlation between miRNA with positive coefficients in the TRM signature with immune cell type. (B) Chord diagram displaying correlation between miRNA with negative coefficients in the TRM signature with immune cell type 47

Figure 3.14 Volcano plot for the differential gene expression. Four genes were upregulated while 59 genes were downregulated in the high-risk group as compared to the low-risk group with the adjusted *p* value < 0.05 and |Log2(fold change) | > 1. 48

Figure 3.15 Overrepresentation analysis of the 59 downregulated genes by GO–biological process. The downregulated genes were mostly concentrated in the immune pathways, such as classical pathway of complement activation, adaptive immune response and humoral immune response. 49

Figure 3.16 KEGG pathway gene set enrichment analysis for the ranked genes. Size of dots represents the GeneRatio while the color represents the *p* value. (A) Proteosome, DNA replication, oxidative phosphorylation and base excision repair pathways were significantly activated in the high-risk group as compared to the low-risk group. (B) Immunity pathways such as Th1 and Th2 cell differentiation, JAK-STAT signaling pathway and Th17 cell differentiation were suppressed in the high-risk group as compared to the low-risk group. 50

Figure 3.17 Hallmark gene set analysis for the ranked genes. Size of dot represents the count while color indicates the *p* value. Activated gene sets in the high-risk groups as compared to the low-risk group included Myc Target V2, Oxidative Phosphorylation, and E2F Targets whereas suppressed gene sets included UV response and KRAS signaling. 51

Figure 3.18 (A,C) Venn diagrams displaying relationship between miRNAs in the TRM signature and regulatory miRNAs inferred from the differentially expressed genes. (B,D) Venn diagrams displaying relationship between differently expressed genes between the high- and low-risk group and predicted target mRNAs of the miRNAs in the TRM signature. 52

Figure 4.1 Layout of an IHC slide. Appendix section was placed below the breast section as a positive control…… 68

Figure 4.2 TAM distribution in a low macrophage density sample. Dashed line represents the border between tumor front and intratumoral. B: H&E slide of the low macrophage density sample (under 100X magnification). B1: immunohistochemistry staining under 100X magnification. B2,B3: tumor front and intratumoral region undrr 400X magnification. Black arrow indicates M2 TAMs while red arrow indicates non M2 TAMs…. ……..78

Figure 4.3 TAM distribution in a high macrophage density sample. Dashed line represents the border between tumor front and intratumoral. B: H&E slide of the low macrophage density sample (under

100X magnification). B1: immunohistochemistry staining under 100X magnification. B2,B3: tumor front and intratumoral region undrr 400X magnification. Black arrow indicates M2 TAMs while red arrow indicates non M2 TAMs..... 79

Figure 4.4 Pearson correlation illustrating significant associations between macrophage subsets and tumor compartments..... 81

Figure 4.5 A simplified illustration of the relationship among macrophage subpopulations in tumor front and intratumoral region 81

Figure 4.6 Mean of M non- M2 in tumor front (FM non-M2) according to subtypes. As the mean FM non-M2 increases, breast subtype gets more aggressive from Luminal A to TNBC 86

Figure 5.1 PCR plate layout used for qPCR..... 106

Figure 5.2 Formula used for relative expression of miRNA..... 107

Figure 5.3 RNA gel image of the selected five samples. The 28S rRNA band was not visible while the 18S rRNA bands appeared smeared due to RNA degradation. 109

Figure 5.4 DNA gel image of the same five samples after PCR amplification with miR- 21 (left) and miR-146a (right). All samples exhibited a band at 50 bp, which was the expected amplicon size. 110

Figure 5.5 Comparison of ΔCt values of miR-21 and miR-146a between high and low FM2 group. Expression was normalized relative to miR-16 expression. The horizontal lines represent the mean value for each group..... 112

LIST OF ABBREVIATIONS

AMDI	Advanced Medical and Dental Institute
ATM	Ataxia-telangiectasia mutated
AUC	Area under curve
BRCA	Breast cancer gene
BLBC	Basal-like breast cancer
BRIP1	*BRCA1* interacting protein
CDH1	Cadherin 1
CD 163	Cluster of differentiation 163
CHEK2	Checkpoint kinase 2
CLEC10A	C-type lectin domain family 10 member A
c-Maf	Cellular musculoaponeurotic fibrosarcoma oncogene homolog
CSF2	Colony stimulating factor 2
CXCL13	Chemokine (C-X-C motif) ligand 13
DNA	Deoxyribonucleic acid
DGCR8	DiGeorge syndrome critical region gene 8
DRFS	Distant-relapse-free survival
ER	Estrogen receptor
EOC	Epithelial ovarian cancer
GEO	Gene Expression Omnibus
GO	Gene Ontology
GP2	Glycoprotein 2
HuR	Human antigen R
HER2	Human epidermal growth factor receptor 2

H&E	Hematoxylin and eosin
IFNγ	Interferon gamma
IGKC	Immunoglobulin kappa constant
IL	Interleukin
JAK-STAT	Janus kinase/signal transducer and activator of transcription
KEGG	Kyoto Encyclopedia of Genes and Genomes
Ki-67	Kiel 67 marker of proliferation
LI	Lymphovascular invasion
LNM	Lymph node metastasis
LRRK2	Leucine-rich repeat kinase 2
miRNA	MicroRNA
mRNA	Messenger RNA
NFPA	Non-functional pituitary adenomas
OS	Overall survival
PALB2	Partner And Localizer of BRCA2
PD	Parkinson disease
PD-L1	Programmed death-ligand 1
PGE2	Prostaglandin E2
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
PTPRT	Protein tyrosine phosphatase receptor type T
PSCA	Prostate stem cell antigen
pSTAT	Phosphorylated Signal Transducer and Activator of Transcription
qPCR	Quantitative PCR
RNA	Ribonucleic acid

ROC	Receiver operating characteristic
ROS	Reactive oxygen species
SD	Standard deviation
STK11	Serine-threonine kinase 11
SELP	Selectin P
TAM	Tumor-associated macrophage
TCGA	The Cancer Genome Atlas Breast Cancer
TGFβ	Transforming growth factor beta
Th2	T helper 2
TIL	Tumor-infiltrating lymphocytes
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor alpha
TP53	Tumor Protein P53
TRBP	Transactivation response RNA binding protein
TRM	Tumor-associated macrophage-related microRNAs
USM	Universiti Sains Malaysia
UTR	Untranslated region
VEGF	Vascular epithelial growth factor

LIST OF APPENDICES

Appendix A Purity and concentration assessment of samples

Appendix B Mean Ct value for all samples

PENJELASAN PERANAN MAKROFAJ TERKAIT TUMOR M2 DAN HUBUNGANNYA DENGAN MIKRORNA DALAM KARSINOMA PAYUDARA INVASIF MANUSIA

ABSTRAK

Makrofaj berkaitan tumor (TAM) ialah makrofaj dalam persekitaran mikrotumor yang memainkan peranan penting dalam permulaan dan perkembangan kanser. Dalam kanser payudara, TAM terutamanya menunjukkan fenotip M2 yang dikaitkan dengan ciri-ciri anti-radang dan menggalakkan tumor. Pelbagai kajian telah menunjukkan nilai diagnostik dan prognostik tandatangan miRNA dalam pelbagai jenis kanser, namun tiada yang memberi tumpuan kepada miRNA berkaitan TAM (TRM). Selain itu, hubungan antara TAM M2 dengan parameter klinikopatologi kanser payudara masih belum diterokai sepenuhnya. Kajian ini bertujuan untuk menjelaskan peranan TRM dalam kanser payudara dan menentukan hubungan antara TAM dengan parameter klinikopatologi yang berkaitan dengan kanser payudara. TRM yang berpotensi telah disusun daripada carian literatur sebelum menilai nilai prognostik mereka dengan The Cancer Genome Atlas Breast Cancer dan dataset Gene Expression Omnibus GSE22220. TRM prognostik kemudiannya dianalisis dengan analisis pengayaan set gen manakala penyusupan imun dalam tisu tumor payudara disimpulkan menggunakan CIBERSORT. Sampel kanser payudara yang diproses menggunakan Formalin-fixed-paraffin-embedded (FFPE) dan data klinikal berkaitan juga diperoleh untuk menilai hubungan antara TAM M2 dan ciri klinikopatologi payudara. PCR kuantitatif kemudian dijalankan untuk menentukan dan membandingkan tahap ekspresi miR-21 antara sampel dengan TAM M2 yang tinggi dan rendah. Ujian signifikansi dilakukan menggunakan ujian t pelajar. Satu

tandatangan TRM terdiri daripada sebelas miRNA iaitu miR-21, miR-24-2, miR-125a, miR-221, miR-22, miR-501, miR-365b, miR-660, miR-146a, let-7b dan miR-31 telah dibina. Analisis pengayaan set gen mendedahkan bahawa gen yang diekspresikan secara berbeza terlibat terutamanya dalam laluan berkaitan imun yang dikaitkan dengan perkembangan kanser. Analisis CIBERSORT menunjukkan korelasi positif tertinggi antara TAM M2 dan skor risiko yang dibina daripada tandatangan TRM. Model regresi multinomial yang terdiri daripada TAM M2 dan pencerobohan limfovaskular telah berjaya dibina untuk meramalkan metastasis nodus limfa. Ujian T pelajar membuktikan bahawa tahap ekspresi miR-21 adalah berbeza dengan ketara antara kumpulan TAM M2 yang tinggi dan rendah. Kajian ini telah memberikan gambaran tentang hubungan antara TAM dan miRNA selain menggambarkan hubungan antara parameter klinikal kanser payudara dan TAM M2. Hasil kajian ini mencadangkan kerjasama antara TAM M2 dan miR-21 dalam membantu perkembangan kanser payudara dengan mempromosikan metastasis nodus limfa. Kesimpulannya, kajian ini telah menyediakan platform untuk rawatan sasaran yang berkesan bagi pesakit kanser payudara, dan wajar dikaji dalam kajian prospektif.

ELUCIDATING THE ROLE OF M2 TUMOR-ASSOCIATED MACROPHAGES AND ITS RELATION TO MICRORNA IN HUMAN INVASIVE BREAST CARCINOMA

ABSTRACT

Tumor-associated macrophages (TAMs) are macrophages within the tumor microenvironment that play an important role in cancer initiation and progression. In breast cancer, TAMs mainly demonstrate the M2 phenotype, which is associated with anti-inflammatory and tumor-promoting characteristics. Accumulating studies have demonstrated the diagnostic and prognostic value of miRNAs signature in a variety of cancers, but none has focused on TAM-related miRNAs (TRMs). Furthermore, the relationship between M2 TAMs with known clinicopathological parameters of breast cancer is still not fully explored. This study aims to elucidate the role of TRMs in breast cancer and to determine the relationship between TAMs and the relevant clinicopathological parameters of breast cancer. Potential TRMs were curated from literature search before evaluating their prognostic value with The Cancer Genome Atlas Breast Cancer and Gene Expression Omnibus GSE22220 datasets. Prognostic TRMs were then analyzed with gene sets enrichment analyses while immune infiltrates in breast tumor tissues were inferred using CIBERSORT. Formalin-fixed-paraffin-embedded (FFPE) breast cancer samples and their associated clinicopathological data were also retrieved to evaluate the relationship between M2 TAMs and breast clinicopathological features. Quantitative PCR was then performed to determine and compare the expression level of miR-21 and miR-146a between samples with high and low M2 TAMs at tumor front (FM2). Significance was tested using independent t-test. An eleven-TRM prognostic signature consisting of miR-21, miR-24-2, miR-125a,

miR-221, miR-22, miR-501, miR-365b, miR-660, miR-146a, let-7b and miR-31 was constructed. Gene sets enrichment analyses revealed that the differentially expressed genes were mainly involved in immune-related pathways associated with cancer progression. CIBERSORT analysis showed the highest positive correlation between M2 TAMs and risk score constructed from the TRM signature. A multinomial regression model comprising of M2 TAMs at tumor and lymphovascular invasion was successfully constructed to predict lymph node metastases. Independent t-test proved that miR-21 expression level was significantly different between the high and low FM2 groups. This study has shed some light into the relationship between TAMs and miRNAs besides illustrating the association between breast cancer clinicopathological parameters and M2 TAMs. Our results suggest a coalition between M2 TAMs and miR-21 in aiding breast cancer progression by promoting lymph node metastases. In conclusion, this study has provided a platform for effective targeted treatment for breast cancer patients, worthy of prospective studies.

CHAPTER 1

INTRODUCTION

Tumor-associated macrophages (TAMs) being immune cells within the tumor microenvironment have garnered much interest as basic science regarding their roles in tumor progression unfolds. Better understanding of their polarization into pro-tumoral M2 to promote tumor growth, tumor angiogenesis, immune evasion and tumor metastasis has prompted various studies to investigate their clinical significance as a biomarker of predictive and prognostic value across different cancer types, including breast cancer [1-3]. Mortality rate of breast cancer has reduced markedly compared to decades ago owing to the evolution in breast cancer diagnosis and treatment regime. Improved prognosis or survival in patients with breast cancer however, has remained low for many years [4-6].

MicorRNAs (miRNAs) are another integral part of cancer initiation and progression. Dysregulation of miRNAs has been known to play important roles in tumor development and progression. However, elucidating the involvement of miRNAs in regulating tumor-associated macrophages (TAMs) and how these TAM-related miRNAs (TRMs) modulate cancer progression is still in its infancy. MicroRNAs associated with tumor-associated macrophages (TAMs) have not been comprehensively investigated in prognostic studies, particularly in the context of breast cancer. Such investigations have the potential to reveal microRNAs that serve as independent prognostic indicators, as well as to clarify the roles of M2 TAMs and microRNAs *in vivo*, and to identify their interactions for improved discovery of biomarkers and therapeutic targets. This study aims to elucidate the involvement of

M2 TAMs and microRNAs in the progression of breast cancer. We postulate that M2 TAMs collaborate with miRNAs to facilitate and enhance tumor growth.

This study is divided into three main phases. In chapter 3 (phase 1), the roles of TAM-related miRNAs (TRMs) in breast cancer were explored. Potential TRMs were identified from the literature, and their prognostic value were validated using two separate breast cancer databases; The Cancer Genome Atlas Breast Cancer (TCGA) database and Gene Expression Omnibus (GSE22220) dataset. Gene sets enrichment analyses were then performed to gain insight into the biological functions of these TRMs.

Chapter 4 aims to understand the relationship between TAMs and breast cancer clinicopathological features, and to evaluate the prognostic capability of TAMs in breast cancer. A double immunohistochemistry detection method comprising of CD 163 and c-Maf was adopted to identify M2 TAMs. An analytical comparison was then done between TAMs in tumor front and TAMs in intratumoral region of local breast cancer samples to determine the region which is significantly associated with clinicopathological features of breast cancer. Considering the importance of metastases in disease staging and prognosis prediction, the relationship between lymph node metastasis with TAMs and relevant clinicopathological parameters were also analysed.

The final phase (Chapter 5) aimed to investigate and compare the expression levels of miR-21 and miR-146a between breast tumor samples with high and low M2 TAMs density. Quantification of miRNA was performed using real time RT-PCR whereby relative quantification method was applied to determine expression levels.

Independent t-test was then performed between the high FM2 and low FM2 cohort to determine the significance of miRNA expression.

Ultimately, this study demonstrates the potential of M2 TAMs and TAM-related miRNAs as prognostic biomarkers or targeted treatment options in breast cancer besides unravelling the dysregulated immune pathways in high-risk breast cancer.

CHAPTER 2

LITERATURE REVIEW

2.1 Breast cancer

Following lung cancer, breast cancer ranks as the second most commonly diagnosed cancer worldwide and is the main cause of cancer-related deaths among women [7]. In 2022, approximately 2.3 million women were diagnosed with breast cancer, resulting in 670,000 reported fatalities globally [8]. Closer to home, breast cancer remains as the most common cancer reported in Malaysia contributing 19% of total cancer cases from year 2012 to 2016 and 17.6 % from year 2017 to 2021 [9].

Breast cancer occurs when abnormal cells in the breast grow and divide uncontrollably to form a growth (tumor) [10]. The exact cause of breast cancer is not fully understood, but there are several risk factors which may contribute to the development of breast cancer. These risk factors could be genetic or non-genetic related. Age is one of the main non-genetic factors. Incidence of breast cancer is mainly observed in women above 50 years old [11] although a worrying increase in incidence rate is observed in women below 50 years old [12]. Other non- genetic factors include reproductive factor (e.g., early menarche or late menopause), lifestyle-related such as obesity and excessive alcohol consumption and also radioactive exposure [13-15].

The most known genetic risk factors associated with breast cancer are mutations in the tumor suppressor genes, *BRCA1* and *BRCA2*. Women with *BRCA1* or *BRCA2* gene mutations have an 85% lifetime risk of developing breast cancer [16]. Other highly penetrant breast cancer genes are the *TP53, CDH1, PTEN*, and *STK11* [17-19]. Carriers with these mutated genes are also susceptible to ovarian cancer. DNA

repair genes that interact with *BRCA* genes such as *ATM, PALB2, BRIP1*, or *CHEK2* have also been reported to induce breast carcinogenesis, although these genes are considered as lower penetrance [20-22].

Breast cancer is highly heterogeneous in nature at both histological and molecular level and thus, exhibits different clinical characteristic that would require different approach to treatment [23]. Cancer that starts at the linings of the ducts, which are tubes that deliver milk from glands to the nipple is known as ductal carcinoma while cancer that is formed in the lobules (milk producing glands) is known as lobular carcinoma. [24]. Breast cancer can be categorized mainly into non- invasive and invasive type. Non- invasive or *in situ* means that the cancer is contained and has not invaded or spread to adjacent tissues yet while invasive cancer indicates that it has spread into the surrounding tissues or organs [25]. Invasive breast cancer can be further differentiated by their morphological subtypes such as metaplastic, mucinuous, papillary and micropapillary carcinoma. The majority of the cases though, accounting for more than 70% are of the 'no special type' (NST) which like the name suggests, comprises of all breast tumors without specific differentiating features [26].

At molecular level, several biomarker signatures have been established to classify invasive breast cancer [24, 25, 27]. However, the most common and widely employed classification is from the immunohistochemical perspective. This classification is explained in detailed in subtopic 2.2 below.

2.2 Breast Tumor Classification: Grade, Stage and Subtype

Breast tumor classification is essential for an accurate diagnosis of the disease and prediction of tumor behaviour to guide oncologic decisions. The grade of a breast cancer is a prognostic indicator of how aggressive the tumor is, ranging from low to high grade. This grading is based on how abnormal the cancer cells look under the microscope. There are various scoring systems available to determine the grade of a breast cancer. The widely accepted system including in Malaysia is the Nottingham Histologic Score which is also known as the Elston-Ellis modification of the Scarff-Bloom-Richardson grading system[28, 29]. The three features considered in this system are **cell differentiation** which describes how well the tumor cells recreate normal glands, the **degree of nuclear pleomorphism** which describes how abnormal the tumor cells are and **mitotic rate**, which describes the amount of division or proliferation by the tumor cells [30]. Each feature is given a score from 1 to 3, where the scores are ultimately added to give a final total score ranging from 3 to 9. This final total score is then used to grade the tumor as below:

Grade I : total score of 3-5

Grade II : total score of 6-7

Grade III : total score of 8-9

The pathologic stage of breast cancer measures how advanced the tumor is and whether it has spread. Stage ranges from 0 which describes ductal carcinoma *in situ* to stage IV which is metastatic disease [31]. Patients of lower stage cancer (0 to II) usually have better survival outcome compared to patients with stage III or IV cancer. The three characteristics analyzed for stage determination are tumor size (T), lymph node status (N) and distant organ metastases (M). Similar to the grading system, these

three characteristics were scored individually before combining the scores for an overall pathology stage [32].

The immunophenotypic assessment of ER, PR and HER2 status is another main constituent of breast cancer classification. Estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) are prognostic markers that are important predictive factors of hormonal and anti-HER2-targeted therapy [23]. This assessment is further proven to be relevant when gene expression profiling studies classified breast cancer into five intrinsic subtypes, namely luminal A, luminal B, HER2-enriched, basal-like breast cancer (BLBC) and claudin-low breast cancer (outlined in Table 2.1) [33-37].

Table 2.1 Hormone receptor status and global prevalence of breast cancer subtypes

Subtype	**ER**	**PR**	**HER2**	**Ki-67**	**Prevalence (%)**
Luminal A	+	+	-	< 20%	40-50
		-			
Luminal B	+	+	+	>20%	15-20
		-			
HER2- enriched	-	-	+		10-15
Basal-like	-	-	-		20
Claudin-low	-	-	-		7-14

Luminal A is the most frequent intrinsic subtype representing 40- 50% of invasive breast cancer. This cancer is characterized by ER+, PR+/- and HER2- . Expression of the cell proliferation marker, Ki-67 is less than 20% [38]. Luminal A tumors are typically low grade, possess low proliferation rate and have the best prognosis among all the other subtypes. They can be treated with hormonal therapy, have favourable survival and respond positively to treatment [39].

The Luminal B subtype is characterized by reduced expression of estrogen receptor (ER)-related genes, elevated levels of Ki-67, and a variable expression of HER2-related genes when compared to the Luminal A subtype [38]. Patients with Luminal B typically present with higher tumor grades and poorer prognoses than those with Luminal A. While patients with Luminal A can often be treated effectively with hormonal therapy alone, those with Luminal B may require additional chemotherapy [23].

The HER2-enriched subtype constitutes 15% of all invasive breast cancers. They demonstrate over- expression of HER2 or HER2 signalling-associated genes [37]. HER2-overexpressing tumors are often high grade with negative ER and PR, and are more aggressive in nature. Nonetheless, they have high response rate to anti-HER2-targeted therapy, resulting in improved outcome [40].

Basal like breast cancers (BLBC) which are mostly triple negative breast cancers are associated with overexpression of proliferation-related genes but lack expression of ER, PR, and HER2- related gene. 20% of breast cancer cases are of BLBC. In addition, BLBC is mainly observed in *BRCA1* mutation carriers [41]. This subtype exhibits high tumor grade and high proliferation index [23]. BLBC patients have poor prognosis, high rates of recurrence and prone to metastasis. Due to its insensitivity to hormonal and targeted therapy, treatment options for BLBC remains limited [42]. Treatment currently ranged from chemotherapy, immunotherapy to surgery [43].

The claudin-low breast cancer discovered in 2007, like BLBC are mostly ER-negative, PR-negative, and HER2-negative, in addition to having low expression of genes involved in cell-cell adhesion, such as *claudins 3, 4,* and *7, occludin,* and *E-*

cadherin [44]. However, unlike BLBC, claudin-low subtype has low expression of proliferation genes and Ki-67. These tumors express high epithelial-mesenchymal transition (EMT) genes and stem cell-like gene expression patterns instead [45]. The overall prognosis for claudin-low tumor is poor, although a previous study has reported that overall survival rate at 3,5 and 10 years was higher than that of BLBC [44].

2.3 Survival rate in breast cancer

The survival rate in breast cancer patients is an important measurement of how effective the treatment programs are. Factors such as race, age and stage at diagnosis, tumor size, hormonal receptor status and treatment type can heavily influence the survival of a patient [46-49].

With the advent in breast cancer diagnosis and treatment, survival of patients has improved tremendously (74% and 59% for 5 years- and 10 years- survival respectively from 2010 to 2017) compared to decades ago where survival was only 55% and 40% for 5 years- and 10 years- survival respectively (from 1960 to 1969). The age-standardized 5-years survival rate currently varies from 80% in developed countries, 60% in developing countries and less than 40% in low-income countries [50].

However, recent trends showed a statistically significant decrease in breast cancer survival rates, especially in women below 50 years old or above 70. This trend is observed in every world region except in Europe Central Asia and North America [45, 51, 52]. Locally, the Malaysian Study on Cancer Survival reported a daunting 66.8% in the 5-year breast cancer survival rate [53]. This number is markedly lower than that reported globally (75%) [50] or in other Asian countries such as Japan (96.2%), Korea (92.6%) and Singapore (79.0%) [54].

Despite considerable advances in treatment and management of breast cancer over the years, the outcome in survival has not significantly improved. Treatment for breast cancer is complicated by the widely heterogenous nature of the cancer. This is further worsened by resistance to therapies introduced [55]. Hence, new and improved therapies are needed to help improve survival in breast cancer patients.

2.4 Tumor-associated Macrophages (TAMs)

Macrophages represent a category of specialized immune cells known for their phagocytic capabilities and distinct phenotypic traits. This diverse group of cells is influenced by microenvironmental factors, resulting in a heterogeneous population with varying properties and functions. Macrophages contribute to tissue homeostasis, immune defense, and the healing process of wounds. Additionally, they are implicated in a range of diseases, including autoimmune conditions, atherosclerosis, and tumor development [56-59].

Activated macrophages are often classified as proinflammatory M1 macrophages or anti-inflammatory M2 macrophages (Figure 2.1). The M1-like phenotype is induced by toll-like receptor ligands (bacterial lipopolysaccharide) or Th1 cytokines, such as tumor necrosis factor alpha (TNF-α), interferon gamma (IFNγ) and colony stimulating factor 2 (CSF2) [60, 61]. M1-like macrophages exert high antigen presenting capacity. They also secrete reactive oxygen species (ROS) and cytokines like IL-6, IL-12, IL-23, and TNF-α, associated with microbicidal and pro-inflammatory activities [62, 63]. Thus, they are termed as the “fight” macrophages and are associated with good prognosis in cancer context [64, 65].

M2-like macrophages, on the other hand, are polarized by Th2-derived cytokines such as IL4, IL10, IL13, transforming growth factor beta (TGFβ) or prostaglandin E2 (PGE2) [61]. They are known as the “repair” or “fix” macrophages as they promote tissue repair via immune tolerance, tissue remodelling, debris scavenging and immune modulation [66]. When it comes to cancer, M2-like macrophages support angiogenesis by secreting adrenomedullin and vascular epithelial growth factors (VEGFs) and express immunosuppressive molecules such as IL10, programmed death-ligand 1 (PD-L1), and TGFβ, favouring tumor growth [67].

It is noteworthy that the actual polarization state of macrophages is far more complex than the simple binary M1, M2 classification, which serves to define only macrophage functions. In fact, macrophages are highly plastic cells consisting of a spectrum of activation states, with M1 and M2 representing the extremes on each opposing end. Many of the subsets display mixed heterogeneity and some are yet to be discovered or fully characterized. Detailed explanation on macrophage polarization in the tumor microenvironment and the mechanisms involved has been described in numerous research articles [68-71].

A specific class of macrophages, tumor-associated macrophages (TAMs) are macrophages residing within the tumor microenvironment [72]. TAMs regulate metastasis by producing growth factors, cytokines, and other molecules to aid cancer progression. In recent years, researchers have been investigating TAMs as a therapeutic strategy to curb tumor progression and metastasis [73-75].

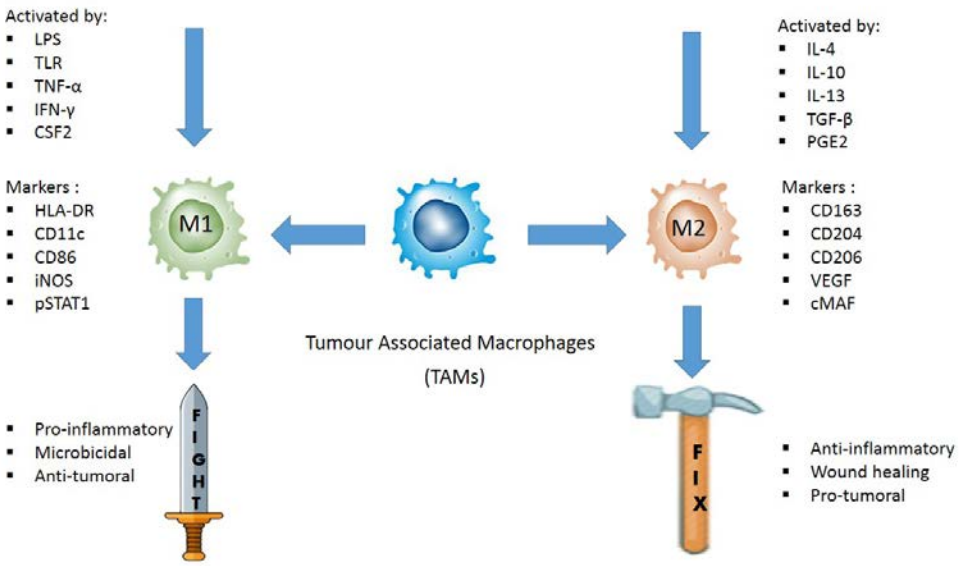

Figure 2.1 Illustration of how M1 and M2 TAMs are activated, their respective functions, and the common IHC markers used to distinguish the two phenotypes in human tissue samples.

TAMs play a crucial role in cancer progression as supported by many *in vitro* and *in vivo* studies [76-79]. In parallel to the growing insights into the roles of TAMs, many studies have been conducted to investigate the clinical significance of TAMs in tumors. Generally, higher densities of TAMs are often observed in more advanced tumor stages as evidenced in esophageal cancer [80], ovarian cancer [81], breast cancer [82] and pancreatic cancer [83]. Additionally, the negative impact of TAMs on patients' overall survival was also reflected in esophageal cancer [84], pancreatic cancer [83], breast cancer [85], lung cancer [86] and gastric cancer [87].

2.4.1 M2 Tumor-associated macrophages: The 'aggressor' of tumor

Many studies have demonstrated that TAMs in cancerous tissue mainly exhibit or are polarized to the M2 phenotype following interactions with molecules in tumor microenvironment [88-90]. Higher infiltration of the M2 TAMs is associated with a more aggressive tumor characteristic, reflected by tumor invasion, progression, and metastases. A study on non-functional pituitary adenomas (NFPAs) discovered that

the cultured M2 macrophages significantly enhanced the proliferation and invasion of the primary NFPA cultures as compared to the cultured M1 macrophages [91]. In hepatocellular carcinoma, higher M2 TAMs were strongly correlated with more tumor number and advanced stages [92]. Similarly, M2 infiltration in breast cancer was markedly correlated with larger tumor size, advanced stages, and angiogenesis. Polarization toward the M2 phenotype showed strong correlation with the aggressiveness of breast cancer characterized by higher histologic grade, higher Ki-67 proliferating index, and estrogen and progesterone receptor negativity [93, 94].

TAMs are also related to resistance towards cancer treatments. M2 macrophages can induce chemoresistance by secreting growth factors and inhibiting cell death signaling pathways in tumor cells, thus sheltering them from the chemotherapy effects [95, 96]. Clinical studies have demonstrated that a high number of M2 TAMs in tumor cause chemoresistance and radioprotective effects, leading to therapy failure and poor prognosis in patients [97, 98].

2.4.2 Evaluation of M2 Tumor-Associated Macrophages in tissue section

The field of immunology has several advancements in accessing TAMs in tissue biopsies. Among them are flow cytometry, gene expression profiling, and the recent high throughput single-cell sequencing [99-101]. These technologies are no doubt the advent in research field considering that they are more standardized and quantitative in nature. However, when it comes to routine clinical diagnosis where cost, feasibility and time are restricting factors, the immunohistochemistry is the preferred method.

What makes immunohistochemistry distinctive is the potential to assess a cell expression in its own microenvironment [102]. Visual details on protein distribution and localization can be procured without the need to destroy the histologic architecture of a tissue [103]. This is especially applicable in retrospective studies, where every tissue block is precious. Accordingly, studies focusing on TAM characterization in solid clinical samples often adopt immunohistochemistry in their methodologies till date [104-106].

In contrast, methods such as flow cytometry and gene expression are performed *in vitro* and although they do provide the general overview of the macrophage population, they could not reflect the distribution and complexity of the macrophages in and around solid tumors [107, 108]. Hence, when it comes to routine clinical practice, immunohistochemistry of histological section remains the best platform to study TAMs *in situ*.

2.5 MicroRNAs: History and biogenesis

MicroRNAs (miRNAs) are a large group of small, endogenous non-coding RNAs of 18–23 nucleotides in length. MiRNAs in general negatively regulate gene expression at the post-transcriptional level by mostly binding at the 3′ untranslated region (UTR) of target mRNAs to suppress their expression, although interaction of miRNAs with the 5′ UTR, protein coding sequence and gene promoters has also been reported [109]. Furthermore, miRNAs can positively regulate mRNA expression under certain circumstances, such as association with AU-rich elements at 3’ UTR to activate translation in serum starved cells and upregulation of gene expression via AGO2 and FXR in quiescent cells, like oocytes [110, 111]. Approximately 30% of the human protein coding genome is regulated by miRNAs [112].

MicroRNAs were first discovered in 1993 by Lee *et al* in the nematode *Caenorhabditis elegans.* The downregulation of LIN-14 protein, essential for the progress from the first larval stage (L1) to L2 was found to be dependent on the transcription of a second gene called *lin-4.* Surprisingly, the transcribed *lin-4* was not translated into a biologically active protein but instead, produced 2 small RNAs of approximately 21 and 61 nucleotides in length where the longer sequence formed a stem loop structure which served as a precursor for the shorter non-coding RNA [113].

This team later collaborated with Wightman *et al*, to discover that the shorter RNA had antisense complementary to multiple sites in the 3′ UTR of LIN-14 mRNA. When these complementary regions bind, they decreased the LIN-14 protein expression without any significant change in its mRNA levels. The base pairing between multiple lin-4 small RNAs to the complementary sites in the 3′ UTR of LIN-14 mRNA caused translational repression of LIN-14 and subsequent progression from L1 to L2 *during C. elegans* development [114].

Initially thought to be exclusive to *C. elegans*, it was uncovered in the early 2000s that another small RNA, let-7, was essential for the development of a late larval stage to adult in *C. elegans* [115]. This became the stepping stone in miRNA field and led to the discovery of hundreds of endogenous small RNAs that play a regulative role in many other organisms including humans [116-119].

The key players involved in miRNA biogenesis are Drosha, Dicer, exportin-5, and Argonaute family proteins. Most miRNAs are derived from intergenic regions, from introns of protein-coding genes or from exons of non-coding genes [120]. MiRNAs are encoded in the genome as long (several hundred nucleotides) primary

transcripts named pri-miRNA (Figure 2.2). This transcript contains a guanosine cap structure at the 5′end and is poly-adenylated at the 3′ tail. The synthesis of miRNA is initiated in the nucleus by the binding of RNA polymerase II/III to DNA genes encoding for pri-miRNA, either post- or co-transcriptionally. A stem-loop structure of pri-miRNA is transcribed, which is then cleaved by the enzyme Drosha together with DiGeorge syndrome critical region gene 8 protein, DGCR8 (a multiprotein complex called Microprocessor) into a hairpin structure of 60–110 nucleotides termed precursor miRNA (pre-miRNA). Pre-miRNA is then transported to the cytoplasm through the Exportin-5 complex [121-123].

In the cytoplasm, the pre-miRNA is cleft by the RNase III enzyme Dicer-1, and its binding partner, transactivation response RNA binding protein (TRBP), producing a short double-stranded miRNA duplex. This duplex is then unwound by a helicase into a mature miRNA of approximately 20 nt length, from which one strand (guide strand) is preferentially incorporated into an Argonaute protein to form a functional, multicomponent complex known as miRNA-induced silencing complex (miRISC) while the other strand (passenger strand) is usually degraded. Subsequently, miRISC is then directed to the target mRNA through sequence complementarity to perform its functional impact [124, 125]. Deep sequencing studies have demonstrated that a single pre-miRNAs can produce multiple mature miRNA species/sequences that can vary in length or sequence [122].

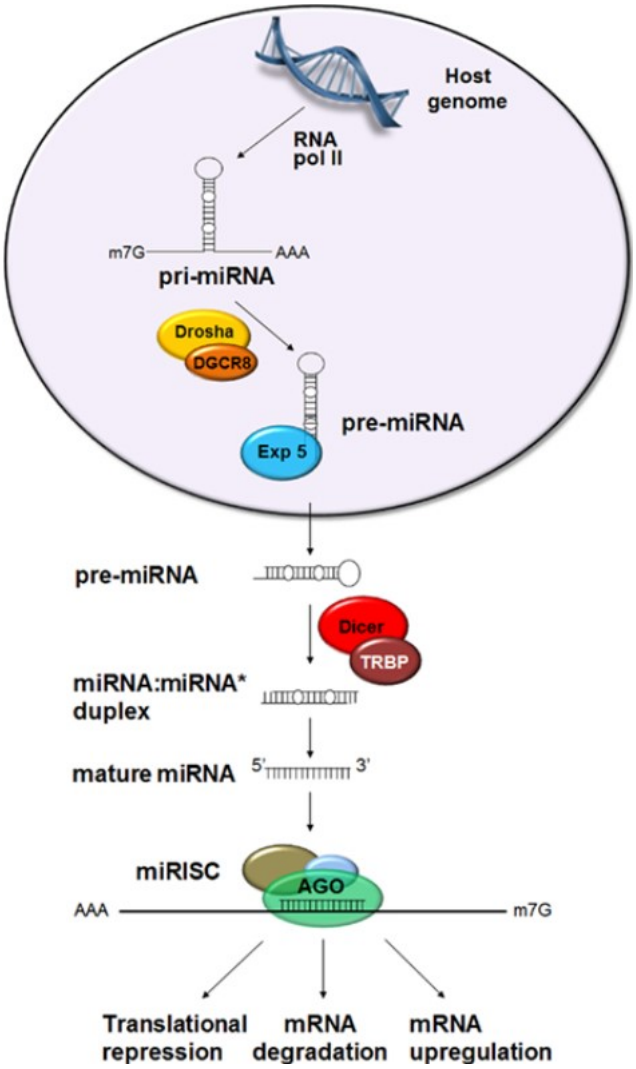

Figure 2.2 Biogenesis and mechanism of action by microRNA [126]

2.6 Transport of microRNAs

MiRNAs are susceptible to degradation by RNases in the extracellular environment. To prevent degradation by these enzymes they are bound to vesicles or a carrier protein. This provides a protective barrier and ensures targeted delivery of the miRNAs [127].

There are several mechanisms of how microRNAs are transported throughout the body. The specific mechanism of transport can depend on the type of miRNA, the

cell type releasing it, and the physiological context. Encapsulation within an extracellular vesicle is miRNAs' main mode of transport. MiRNAs are packaged into membrane-bound vesicles, like exosomes and microvesicles which are released by cells into extracellular space. These vesicles protect miRNAs from degradation by RNases in bodily fluids, such as blood, saliva and urine. They also carry specific surface proteins which facilitate targeted delivery to recipient cells [128]

Another mechanism of transport is via RNA-binding proteins, such as Argonaute 2 (Ago2) and via high-density lipoproteins (HDLs) [129]. MiRNAs associate with various proteins in the extracellular space, forming ribonucleoprotein complexes (RNPs). These proteins can protect miRNAs from degradation and facilitate their delivery to recipient cells.

2.7 Mechanisms of action of microRNAs

There are three main mechanisms in which miRNA negatively regulates or controls targeted gene expression. They are mRNA degradation, translational repression and mRNA deadenylation.

i. mRNA degradation

This mechanism is more common in plants while can occur in animals under certain conditions. Higher degree of complementarity between the miRNA complex and target mRNA can induce mRNA cleavage and degradation. This process is also known as RNA interference (RNAi) [130].

ii. Inhibition of translation

miRNAs with partial or less complementarity inhibit the translation of target mRNAs. This repression can occur at initiation, post-initiation or elongation step, preventing ribosomes from effectively translating the mRNA into protein [123].

iii. Deadenylation of mRNA

Binding of miRNAs leads to mRNA deadenylation. The poly(A) tail of the mRNA is shortened, which destabilizes the mRNA leading to decreased mRNA stability and consequently, accelerates their degradation [131].

Although miRNAs are primarily known for negatively regulating gene expression, they have been identified to positively regulate or activate translation of target mRNAs in certain conditions. They may act as activators of target mRNA transcription that play a role in maintaining quiescence during cell cycle, downregulate mRNAs that code for repressor proteins or modulate signalling pathways by targeting the inhibitor of the pathway, to promote gene expression [132-134].

On another interesting note, a single, tiny miRNA can target multiple mRNAs to regulate the expression of numerous genes simultaneously, while a single gene can be regulated by multiple miRNAs [135]. In addition, the effect of a specific miRNA can vary depending on the cellular context, including the presence of specific cellular conditions, cofactors or sequences [111].

2.8 Significance of microRNAs in human physiology

MicroRNAs have been reported to play a major role in various diseases. In cancer, miRNAs were reported to have altered or aberrant expression levels. They are

involved in many dysregulated pathways of cancer and can either act as an oncogene or tumor suppressor. Calin *et al* was the first to report the role of miRNA in cancer. They found that the genes for miR-15 and miR-16 were deleted or downregulated in more than 60% of B-cell human chronic lymphocytic leukemia (CLL), suggesting that these genes behave as tumor suppressors in CLL [121, 136].

MicroRNAs are also involved in cardiovascular diseases where the up or downregulation of miRNAs can influence angiogenesis, cardiac cell contractility, lipid metabolism, plaque formation, the arrangement of cardiac rhythm, and cardiac cell growth [137]. MiR-1 which is expressed specifically in cardiac and skeletal muscle precursor cells is reported to regulate ventricular cardiomyocytes. Overexpression of miR-1 leads to inhibition of cardiac cell proliferation [138]. In a recent study, miR-365 was revealed to regulate human cardiac action potential duration by targeting key factors of cardiac repolarization [139].

Modulation by miRNAs were also apparent in autoimmune diseases. MiR-203, which is expressed in keratinocytes was found to be upregulated in psoriasis-affected skin compared to healthy human skin [140]. As for rheumatoid arthritis (RA), miR-155 was found to be significantly elevated in RA patients compared to healthy control. Further investigation revealed that miR-155 promotes the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, leading to a cascade of inflammatory amplification. This would result in joint damage and disease progression [121, 141]. MiR-146a on the other hand plays a protective role in RA. MiR-146a deficiency was shown to cause aggravated inflammatory joint damage in a model of TNF-driven arthritis [142].

Altered expressions of miRNAs was also shown in neurogenerative diseases. MiR-29, MiR-15, MiR-107, MiR-146, MiR-9, MiR-101, MiR-106, and the cluster MiR-212/132 were consistently shown to be deregulated in experimental models and/or human samples of Alzheimer disease [143]. Likewise, MiR-133b and miR-205 were significantly reduced in patients with Parkinson disease (PD). MiR-133b is specifically expressed in the midbrain, where it regulates both the maturation and function of midbrain dopaminergic neurons. MiR-205 suppressed the expression of LRRK2 protein through a conserved-binding site at the 3′-untranslated region (UTR) of LRRK2 gene, the main genetic risk factor for PD [144, 145].

Since the discovery of miRNAs and their implications, our knowledge about microRNAs has expanded and application of miRNA in the medical field either as biomarkers or therapeutic targets has grown exponentially. Targeted modulation of specific miRNA function is currently actively being pursued as novel approaches for therapeutic intervention in many diseases.

CHAPTER 3

TUMOR-ASSOCIATED MACROPHAGE- RELATED MIRNAS AS POTENTIAL PROGNOSTIC BIOMARKER IN BREAST CANCER PATIENTS

3.1 Background

Tumor-associated macrophages (TAMs) are macrophages within the tumor microenvironment that play an important role in cancer initiation and progression. These macrophages could be polarized into two main phenotypes with distinct cytokine and chemokine profiles, the M1 phenotype which demonstrates proinflammatory and microbicidal/tumoricidal characteristics, and the M2 phenotype that shows anti-inflammatory and tumor-promoting characteristics [146] In breast cancer, TAMs mainly demonstrate the M2 phenotype [147]. It has been shown in many studies that the regulation of breast cancer by TAMs via microRNAs constitutes one of the crucial mechanisms, although the precise interaction requires further elucidation [148].

MicroRNAs (miRNAs) are a large group of small, endogenous non-coding RNAs of 18–23 nucleotides in length. MiRNAs are associated with immunomodulation in cancer progression and regression. Their expression patterns and implications, however, vary in different types of cancer. MiRNAs can either act as tumor suppressors or tumor promoters [149]. In addition, depending on the tumor context, miRNA which acts as a tumor suppressor in certain cancer types may act as a tumor promoter in others [150].

There are few distinct ways in how TAMs are associated with miRNAs. First, miRNAs can be transported from cancer cells to TAMs to regulate TAM polarization. Reciprocally, miRNAs derived from TAMs exert their effects on cancer cells to modulate tumor progression [151]. MicroRNAs can also be extrinsically derived from other cells in the TME, such as stromal or immune cells and delivered to TAMs via extracellular vesicles [152, 153]. To date, there are limited studies regarding miRNAs related to TAMs and how these TAM-related miRNAs (TRMs) modulate cancer progression, especially in breast cancer. Furthermore, the relationship between these TRMs with known clinicopathological parameters is yet to be explored. Accumulating studies have demonstrated the diagnostic and prognostic value of miRNA signature in a variety of cancers, including a few on breast cancer [154-158], but none has focused on specifically TAM-related miRNAs (TRMs). Elucidating the role of these TRMs in breast cancer can help establish a better understanding of the interplay between TAMs and miRNAs in breast cancer progression.

TCGA database is an online library that contains numerous genomic and gene expression profiles for various types of cancer. This huge database has helped many researchers in their gene and signaling pathway studies (5). TCGA provides an opportunity for researchers to conduct their work by replacing or minimizing laboratory-based research. Consequently, many studies have successfully uncovered clinically relevant cancer biomarkers by using TCGA (6). Although many molecular signatures to predict prognosis and treatment outcome have emerged, the results are often not reproducible *in vitro* (7), suggesting room for improvement.

This chapter is a novel effort at tumor-associated macrophages level to elucidate miRNAs' involvement in breast cancer in relation to M1/M2 polarization of TAMs. We hypothesize that miRNAs expressed by, or delivered to M2 TAMs

correlate with worse survival and in contrast, miRNAs expressed by or delivered to M1 TAMs correlate with better prognosis. By modelling the different miRNAs expression profiles related to TAMs, and integrating pertinent clinicopathological information, this chapter aims to provide a comprehensive analysis of TAM-related miRNAs and to construct a novel miRNA signature of prognostic value. This novel miRNA signature would aid in better prognostication of breast cancer, guide treatment and ultimately improve management in breast cancer patients.

The objectives of this chapter were:

i. To explore the roles of miRNAs in breast cancer in relation to TAMs with a focus on M1/M2 polarization

ii. To construct a novel TAM-related miRNA signature of prognostic value in breast cancer

iii. To understand the biological processes involved in TRMs regulation of breast cancer via various enrichment analyses

3.2 Materials and Methods

3.2.1 Literature Search for TAM-Related miRNAs

Literature search was performed with the keywords: “miRNA”, “tumor-associated macrophage”, “macrophage” and “cancer” from the year 2016 to 30 May 2021. The NCBI PubMed database was utilized to identify potential prognostic miRNAs that were either delivered to TAMs or derived from TAMs in various cancer types. The scope was broadened to include TAM-related miRNAs (TRMs) within