THE ROLE OF HIF-1α AND VGSCs IN INFLUENCING METASTASIS OF BREAST CANCER CELLS

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UNIVERSITI SAINS MALAYSIA

2018

THE ROLE OF HIF-1α AND VGSCs IN INFLUENCING METASTASIS OF BREAST CANCER CELLS

by

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Thesis submitted in fulfilment of the requirements

for the degree of

Doctor of Philosophy

September 2018

ACKNOWLEDGEMENT

First and foremost, I would like to praise God for giving me the strength to complete my PhD journey. It is indeed one of the most invaluable life experiences, full of good memories and also some life lessons which shaped me into a better person. Completion of this thesis would not been possible without the inspiration and support of a number of wonderful individuals especially my main supervisor, Dr. Noor Fatmawati Mokhtar for her continuous optimism, support and guidance throughout this project. I would never be able to complete this project without her! I would also like to express my warmest gratitude to my co-supervisors Prof. Nik Soriani Yaacob, whom I really look up as a role model in this field and Dr. Leow Chiuan Yee for his suggestions and motivation. I am forever thankful to all my friends for being a good support system and the wonderful times we shared in Kelantan. A very special gratitude goes out to all the administrative and laboratory staff of INFORMM for the assistance given. Also I would like to acknowledge MoE for the financial assistance (My Phd) provided and to USM RUI Grant (1001/CIPPM/813060) for funding the project. Finally, my deep and sincere gratitude to my beloved family especially my parents (Mr. Dewadas Samiveloo & Mrs. Paruvathy Munusamy) for their continuous and unparalleled love, help and support. I am also grateful to my siblings, uncles and aunties for always being there for me.

Thank you!

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LIST OF SYMBOLS AND ABBREVIATIONS

®	Registered
°c	Degree Celsius
µ	Micro
µg	Microgram
µL	Microliter
µM	Micro molar
A	Adenine
AP	Ammonium persulfate
ARNT	Aryl hydrocarbon receptor nuclear translocator
ASR	Age-standardised rate
ATCC	American Type Cell Culture
ATP	Adenosine 5'-Triphosphate
BD	Bromodomain
bFGF	Basic fibroblast growth factor
bHLH-PAS	Basic helix-loop-helix-Per-Arnt-Sim
BNIP-3	BCL2 Interacting Protein 3
bp	Base pair
BRCA	Breast cancer gene
BSA	Bovine Serum Albumin
C	Cytidine
CA9	Carbonic anhydrase IX
cAMP	Cyclic adenosine monophosphate
CBP	CREB-binding protein

cDNA	Complementary deoxyribonucleic acid
CHI-3	Cysteine and histidine-rich region 1-3
ChIP	Chromatin immunoprecipitation assay
CO_2	Carbon dioxide
$CoCl_2$	Cobalt chloride
CREB	cAMP response element-binding protein
C-TAD	C-terminal transactivation domain
CTC	Circulating tumour cell
C646	4-[4-[[5-(4,5-Dimethyl-2-nitrophenyl)-2-furanyl]methylene]-4,5-dihydro-3-methyl-5-oxo-1H-pyrazol-1-yl]benzoic acid
DCIS	Ductal carcinoma *in situ*
dH_2O	Distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E-cad	E-cadherin
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal Growth Factor
EMSA	Eelectrophoretic mobility shift assays
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
ET-1	Endothelin-1

FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
G	Guanine
gDNA	Genomic DNA
GLUT	Glucose transporters
HATs	Histone acetyltransferases
HCl	Hydrochloric acid
HDACs	Histone deacetylases
HDMs	Histone demethylases
HEK293	Human embryonic kidney cells 293
HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia-inducible factor
HMTs	Histone methyltranferases
hr	Hour
HRE	Hypoxia-response element
HRP	Horseradish peroxidase
IARC	International Agency for Research on Cancer
IC_{50}	Half maximal inhibitory concentration
IDT	Integrated DNA Technologies, Inc.
IL-8	Interleukin 8
i-NOS	Inducing nitric oxide synthase
IPAS	Inhibitory PAS domain protein
kb	Kilo base
kDa	Kilo dalton

L	Litre
LCIS	Lobular carcinoma *in situ*
LMIC	Low and middle income countries
mA	Milliampere
MCT	Monocarboxylic transporter
MET	Mesenchymal-epithelial transition
mg	Milligram
miRNA	MicroRNA
MITOMI	Mechanically induced trapping molecular interactions
mL	Milliliter
mmHg	Millimetre of mercury
MMP	Matrix metalloproteinase
MMP	Matrix metalloproteinases
MNCR	Malaysian National Cancer Registry
MoI	Motility index
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
mV	Millivolt
MW	Molecular weight
Na^+	Sodium ion
Nav	Voltage-gated sodium channel
NCBI	National Center for Biotechnology Information
NCR	National Cancer Registry
NHE	Na+/H+ exchanger

NIH	National Institutes of Health
nm	Nanometer
nNav1.5	neonatal Nav1.5
N-TAD	N-terminal transactivation domain
ODD	Oxygen-dependent dependent degradation
PBM	Protein binding microarray
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Tween-20
PCR	Polymerase chain reaction
PGK	Phosphoglycerate kinase
PI(3)K	Phosphatidylinositol-3-OH kinase
PTEN	Phosphatase and tensin homolog
pVHL	Protein von Hippel-Lindau
QP	Glutamine- and proline-rich domain
qPCR	Quantitative real-time PCR
Rcf	Relative centrifugal force
REST	RE1-silencing transcription factor
RID	Receptor-interacting domain
RIPA	Radioimmunoprecipitation
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minutes
rRNA	Ribosomal RNA
RT buffer	Reverse Transcription buffer
SCN5A	Sodium channel, Voltage-gated, Type V, Alpha subunit

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SELEX	Systematic evolution of ligands by exponential enrichment
SEM	Standard Error of the Mean
SID	Steroid receptor co-activator-1 interaction domain
siRNA	Small interfering RNA
SRC-1	Steroid receptor coactivator-1
T	Thymine
TAD	Transactivation domain
TAE buffer	Tris-acetate-EDTA buffer
Taq DNA polymerase	*Thermusaquaticus* DNA polymerase
TEMED	N, N, N', N'- tetramethylethylenediamme
TGF-β	Transforming growth factor beta
TNBC	Triple-negative breast cancer
TRANSFAC	Transcription Factor database
TTX	Tetrodotoxin
™	Trademark
UV	Ultraviolet
V	Voltage
VEGF	Vascular endothelial growth factor
VGPC	Voltage gated potassium channel
VGSC	Voltage gated sodium channel
VHL	Von Hippel-Lindau
WHO	World Health Organization

x g	Times gravity
ZEB1	Zinc finger E-box-binding homeobox 1
α	Alpha
β	Beta
β-actin	Beta actin

PERANAN HIF-1α DAN VGSCs DALAM MEMPENGARUHI METASTASIS SEL KANSER PAYUDARA

ABSTRAK

Peranan HIF-1α, CBP dan p300 dalam mengawal ungkapan VGSCs yang tinggi terutamanya jenis Nav1.5 dan nNav1.5 dalam sel kanser payudara agresif masih belum dikenalpasti. Kajian ini telah dibahagikan kepada tiga bab. Bab 1: Pencirian ungkapan Nav1.5, nNav1.5, HIF-1α, dan gen sasaran sepunya HIF-1α, CA9 dalam sel kanser payudara manusia dengan potensi metastatik berbeza; sel epitelium payudara bukan kanser (MCF-10A), sel kanser payudara metastatik lemah (MCF-7), dan sel kanser payudara agresif (MDA-MB-231). mRNA HIF-1α dikesan dalam semua sel yang mana apabila dinormalkan kepada MCF-10A, ungkapan tertinggi dikesan di dalam sel MDA-MB-231 (4.6-kali ganda, $P \leq 0.05$), diikuti oleh sel MCF-7 (1.6-kali ganda). mRNA CA9 juga dikesan lebih tinggi dalam sel MDA-MB-231 (12.9-kali ganda, $P \leq 0.05$) apabila dinormalkan kepada sel MCF-7. mRNA Nav1.5 dan nNav1.5 pula dikesan lebih tinggi dalam sel MDA-MB-321 berbanding dalam sel MCF-7 (masing-masing, 109.5-kali ganda, $P \leq 0.01$ dan 120.4-kali ganda, $P \leq 0.01$). Bab 2 adalah kajian peranan HIF-1α dalam mengawal ungkapan Nav1.5 dan nNav1.5 dan metastasis kanser payudara yang disebabkan oleh VGSC. Kajian bioinformatik menggunakan TRANSFAC® dan JASPAR^{2018} menemui 3 kemungkinan tapak pengikat bagi HIF-1α di dalam promoter Nav1.5. Penurunan ungkapan HIF-1α berjaya dicapai selepas rawatan siRNA-HIF-1α selama 24 jam apabila mRNA dan protein HIF-1α masing-masing menyusut sebanyak 86.3% dan 33%. siRNA HIF-1α juga disahkan dengan penurunan ketara mRNA CA9 sebanyak 74.7%, $P \leq 0.01$. siRNA

HIF-1α menyebabkan penurunan ketara migrasi sel (sebanyak 31%, $P \leq 0.0001$). Rawatan $CoCl_2$ (100 µM, 150 µM and 200 µM) pula berjaya meningkatkan protein HIF-1α (lebih daripada 600%) dan mRNA CA9 (6-11-kali ganda, $P \leq 0.05$). Ini diikuti dengan peningkatan mRNA Nav1.5 (1.3-1.6-kali ganda). Walaupun $CoCl_2$ tidak merencat pertumbuhan sel MCF-7, migrasi dan motiliti meningkat. Bab 3 adalah kajian ke atas pengawalan ungkapan Nav1.5/ nNav1.5 oleh HIF-1α dan pengaktif bersama, CBP dan p300. mRNA CBP dan p300 dikesan lebih tinggi di dalam sel MDA-MB-231 berbanding dengan sel MCF-7. siRNA HIF-1α dalam sel MDA-MB-231 menyebabkan penuruan ketara mRNA CBP (0.5-kali ganda, $P \leq 0.05$) manakala penstabilan HIF-1α oleh $CoCl_2$ menyebabkan mRNA p300 meningkat sebanyal 1.2-kali ganda, $P \leq 0.001$. C646 tidak mengubah HIF-1α tetapi menyebabkan penurunan ketara mRNA CBP dan p300 ($P \leq 0.001$). mRNA CA9, Nav1.5 dan nNav1.5 di dalam sel MDA-MB-231 menurun masing-masing sebanyak 60%, 50% dan 30% selepas rawatan C646 ($P \leq 0.05$). Keupayaan metastatik; motiliti dan migrasi juga didapati berkurangan masing-masing sebanyak 60% dan 53% di dalam sel yang dirawat dengan C646 ($P \leq 0.001$). Kesimpulannya, HIF-1α/CBP/p300 secara bersama mengawal ungkapan Nav1.5 dalam kanser payudara; sekatan ke atas HIF-1α/CBP/p300 menghalang keupayaan untuk metastasis di dalam sel kanser payudara. Bagi nNav1.5 pula, data kajian ini mendapati CBP/p300 sebenarnya dapat mengawal nNav1.5 walaupun pengawalan oleh HIF-1α/CBP/p300 belum dapat dipastikan. Namun, dengan menjadikan HIF-1α/CBP p300/Nav1.5 sebagai target dapat digunakan sebagai salah satu strategi bagi merencat metastasis kanser payudara yang disebabkan oleh Nav1.5.

THE ROLE OF HIF-1α AND VGSCs IN INFLUENCING METASTASIS OF BREAST CANCER CELLS

ABSTRACT

The role of HIF-1α as well as CBP and p300 in the regulation of VGSCs particularly the isoforms Nav1.5 and nNav1.5 which are highly expressed in aggressive breast cancer cells has yet to be explored. This study was designed to investigate the role of HIF-1α/CBP/p300 in the regulation of Nav1.5 and nNav1.5 in aggressive breast cancer. In doing so, this study was separated into three major chapters. The 1st Chapter: Characterisation of Nav1.5, nNav1.5, HIF-1α, and HIF-1α target gene, CA9 expressions in human breast cancer cell lines with different metastatic potential; the non-cancerous breast epithelial cell line (MCF-10A), the weakly metastatic breast cancer cell line (MCF-7), and the aggressive breast cancer cell line (MDA-MB-231). HIF-1α mRNA was detected in all three cell lines in which when normalised to MCF-10A, the highest expression was detected in MDA-MB-231 cells (4.6-fold, $P \leq 0.05$), followed by MCF-7 cells (1.6-fold). Consistently, CA9 mRNA expression was detected at a higher level in MDA-MB-231 cells (12.9-fold, $P \leq 0.05$) normalized to MCF-7 cells. Finally, expression level of Nav1.5 and nNav1.5 were also greater in MDA-MB-231 compared to MCF-7 cells (109.5-fold, $P \leq 0.01$ and 120.4-fold, $P \leq 0.01$ respectively). The 2nd Chapter: Elucidating the role of HIF-1α in regulating Nav1.5 and nNav1.5 expression and VGSC-mediated breast cancer metastasis. Using TRANSFAC and JASPAR[2018], 3 possible binding sites for HIF-1α in the promoter region of Nav1.5 was revealed. HIF-1α knock-downed was achieved after 24 hr treatment with siRNA; HIF-1α mRNA and protein expression was down-

regulated by 86.3% and 33%, respectively. The knock-downed was confirmed with down-regulation of CA9 mRNA expression level by 74.7%, $P \leq 0.01$. siRNA-HIF-1α treated cells also showed significant decrease (by 31%, $P \leq 0.0001$) in migration. $CoCl_2$ (100 µM, 150 µM and 200 µM) successfully increased HIF-1α protein (up to > 600%) and mRNA expression of CA9 (up to 6 – 11-fold, $P \leq 0.05$). This was followed by increased Nav1.5 mRNA expression (up to >1.3-1.6-fold). When growth of MCF-7 cells was not altered with $CoCl_2$, motility and migration remain intact. The 3rd Chapter: Investigating the transcriptional regulation of Nav1.5 and nNav1.5 by HIF-1α and its co-activators, CBP and p300. mRNA expression levels of CBP and p300 were detected at higher level in MDA-MB-231 compared to MCF-7 cells. HIF-1α knocked-down significantly reduced the mRNA of CBP by 0.5-fold ($P \leq 0.05$) in MDA-MB-231 cells whilst stabilisation of HIF-1α using $CoCl_2$ caused a significant increase in the mRNA expression level of p300 by 1.2-fold ($P \leq 0.001$). Treatment with C646 led to significant reduction in CBP and p300 mRNA ($P \leq 0.01$). Similarly, CA9, Nav1.5 and nNav1.5 mRNA expression level in MDA-MB-231 cells was also down-regulated by C646 (up 60%, 50% and 30%, respectively, $P \leq 0.05$). Finally, reduced metastatic abilities; motility and migration (up to 60% and 53%, respectively, $P \leq 0.001$) were observed in MDA-MB-231 cells treated with C646. As a conclusion, HIF-1α/CBP/p300 altogether regulates the expression of Nav1.5 in breast cancer; interruption of HIF-1α/CBP/p300 lead to metastasis suppression in breast cancer cells. As for nNav1.5, current findings indicate that CBP/p300 able to the regulate nNav1.5, although its regulation by HIF-1α/CBP/p300 is yet to be re-evaluated. Nevertheless, targeting HIF-1α/CBP/p300/Nav1.5 could still be useful as potential strategy to combat Nav1.5-mediated breast cancer metastasis.

CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer is a disease which occurs when a group of cells proliferate abnormally by overpassing the law of cell division. Commonly, cells are controlled by signals which instruct whether the cell must divide, segregate into daughter cells or undergo cell death. However, cancer cells over-rule these signals leading to uncontrolled proliferation and growth (Hejmadi, 2010; Chen *et al.*, 2014).

Exogenous and endogenous factors influence the formation of cancer cells. Exogenous factors consist of radiation, chemicals, tobacco and infectious organisms, and endogenous factors are mutations which can be inherited and acquired from the metabolism, hormones, immune conditions and epigenetic dysfunction have great influences on the initiation and progression of cancer cells (Tomasetti and Vogelstein, 2015; Tubbs and Nussenzweig, 2017). Both factors act simultaneously causing abnormality in uncontrollable cell proliferation and transformation. This results in rapid growth and expansion of cell mass known as tumour. Tumour can be benign or malignant in which malignant tumour (cancer) can invade adjacent tissues and spread to different parts of the body via bloodstream or lymphatic system (Hejmadi, 2010).

1.1.1 Cancer incidence worldwide and Malaysia

Based on surveys conducted worldwide, cancer is one of the root cause of high morbidity and mortality, causing more death than other diseases like stroke, coronary

heart disease, and chronic respiratory disease. According to worldwide statistic, 8.2 million cancer deaths out of 14.1 million cases are believed to have happened in 2012 (Ferlay *et al.*, 2015). Over the years, the growing overall demographic and epidemiologic shifts indicate an expansion in cancer burden in upcoming decades, primarily in the low and middle income countries (LMIC). Almost twenty million of additional cancer cases are predicted each year, earliest in 2025 (Cleary *et al.*, 2015).

The approximated total cases (**Table 1.1**) and deaths (**Table 1.2**) reported for several cancers based on gender were reviewed below. According to the worldwide data, lung cancer was recorded with highest number of new cases (1.8 million cases, 12.9% of total) and deaths (1.6 million deaths, 19.4% of total) due to high mortality. The second frequently diagnosed was breast cancer with 1.7 million cases (11.9% of total) but ranked 5th as cause of death with 522,000 number of death reported out of 6.4% total, followed by colorectal cancer, prostate cancer, stomach cancer and liver cancer based on the incidence rate (Ferlay *et al.*, 2015).

The frequency of cancer types diagnosed in men and women is different between both sexes. **Figure 1.1** shows the rank for fifteen most regular cancers among men and women globally based on the reported number of new cases and as well as deaths in more and less developed regions (Ferlay *et al.*, 2015). The highest occurring cancer was prostate cancer with 759,000 cases followed by lung cancer with 490,000 cases among men in more developed regions whilst cancers of the lung (751,000 cases, 682,000 deaths), liver (462,000 cases, 441,000 deaths) and stomach (456,000 cases, 362,000 deaths) are the three major cancers affecting males in less developed regions. They stand for 40% of the reported new cancer cases and 48% of the total reported

Table 1.1 Estimated new cancer cases (thousands) and cumulative risks to age 75 (percent) by sex and cancer site worldwide, 2012 (adapted from Ferlay et al., 2015).

Cancer site	Both sexes		Male		Female	
	Cases	(%)	Cases	(%)	Cases	(%)
Lip, oral cavity	300	2.1	199	2.7	101	1.5
Nasopharynx	87	0.6	61	0.8	26	0.4
Other pharynx	142	1.0	115	1.5	27	0.4
Oesophagus	456	3.2	323	4.3	133	2.0
Stomach	951	6.8	631	8.5	320	4.8
Colorectum	1360	9.7	746	10.0	614	9.2
Liver	782	5.6	554	7.5	228	3.4
Gallbladder	178	1.3	77	1.0	101	1.5
Pancreas	338	2.4	178	2.4	160	2.4
Larynx	157	1.1	138	1.9	19	0.3
Lung	1825	12.9	1242	16.7	583	8.7
Melanoma of skin	232	1.6	121	1.6	111	1.7
Kaposi sarcoma	44	0.3	29	0.4	15	0.2
Breast	1677	11.9	-	-	1677	25.2
Cervix uteri	528	3.7	-	-	528	7.9
Corpus uteri	320	2.3	-	-	320	4.8
Ovary	239	1.7	-	-	239	3.6
Prostate	1112	7.9	1112	15.0	-	-
Testis	55	0.4	55	0.7	-	-
Kidney	338	2.4	214	2.9	124	1.9
Bladder	429	3.1	330	4.4	99	1.5
Brain, nervous system	257	1.8	140	1.9	117	1.8
Thyroid	298	2.1	68	0.9	230	3.5
Hodgkin lymphoma	66	0.5	39	0.5	27	0.4
Non-Hodgkin lymphoma	386	2.7	218	2.9	168	2.5
Multiple myeloma	114	0.8	62	0.8	52	0.8
Leukaemia	352	2.5	201	2.7	151	2.3
All cancer excl. non-melanoma skin cancer	14090	100.0	7427	100.0	6663	100.0

Table 1.2 Estimated cancer deaths (thousands) and cumulative risks to age 75 (percent) by sex and cancer site worldwide, 2012 (adapted from Ferlay *et al*., 2015).

Cancer site	Both sexes		Male		Female	
	Deaths	(%)	Deaths	(%)	Deaths	(%)
Lip, oral cavity	145	1.8	98	2.1	47	1.3
Nasopharynx	51	0.6	36	0.8	15	0.4
Other pharynx	97	1.2	78	1.7	19	0.5
Oesophagus	400	4.9	281	6.0	119	3.4
Stomach	723	8.8	469	10.1	254	7.2
Colorectum	694	8.5	374	8.0	320	9.0
Liver	745	9.1	521	11.2	224	6.3
Gallbladder	142	1.7	60	1.3	82	2.3
Pancreas	331	4.0	174	3.7	157	4.4
Larynx	83	1.0	73	1.6	10	0.3
Lung	1590	19.4	1099	23.6	491	13.8
Melanoma of skin	55	0.7	31	0.7	24	0.7
Kaposi sarcoma	27	0.3	17	0.4	10	0.3
Breast	522	6.4	-	-	522	14.7
Cervix uteri	266	3.2	-	-	266	7.5
Corpus uteri	76	0.9	-	-	76	2.1
Ovary	152	1.9	-	-	152	4.3
Prostate	307	3.7	307	6.6	-	-
Testis	10	0.1	10	0.2	-	-
Kidney	144	1.7	91	2.0	53	1.5
Bladder	165	2.0	123	2.6	42	1.2
Brain, nervous system	189	2.3	106	2.3	83	2.3
Thyroid	40	0.5	13	0.3	27	0.8
Hodgkin lymphoma	25	0.3	15	0.3	10	0.3
Non-Hodgkin lymphoma	200	2.4	115	2.5	84	2.4
Multiple myeloma	80	1.0	43	0.9	17	1.0
Leukaemia	265	3.2	151	3.2	114	3.2
All cancer excl. non-melanoma skin cancer	8201	100.0	4653	100.0	3548	100.0

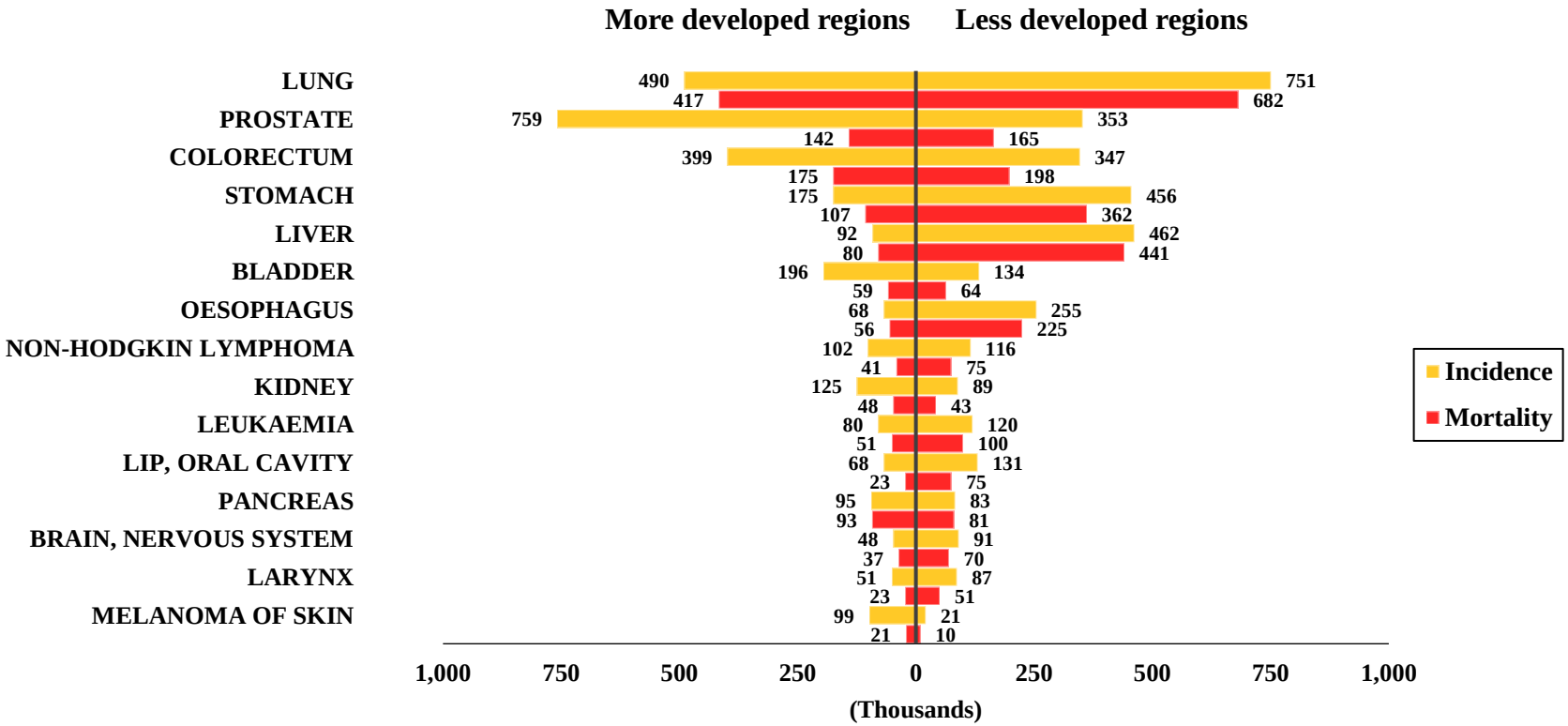

Figure 1.1 Estimated numbers (thousands) of new cancer cases (incidence) and deaths (mortality) in men in more developed and less developed regions of the world in 2012 (adapted from Ferlay *et al*., 2015).

cancer deaths. As for women, the most common cancer identified in more and less developed regions is breast cancer (**Figure 1.2)**. However, more breast cancer cases occurred in less developed regions (883,000 cases) compared to more developed regions (794,000), followed by cervical cancer being the second most prominent cancer in less developed regions with 445,000 cases and 11th in more developed regions with 83,000 cases. Lung cancer caused highest cancer death among women in more developed regions with 210,000 deaths followed by breast cancer with 198,000 deaths. However, in less developed regions, breast cancer causes more death (324,000 deaths) followed by lung cancer (281,000 deaths) and cervical cancer (230,000 deaths).

According to Ministry of Health in 2015, cancer constituted 13.56% of all deaths reported in Malaysia. Cancer ranked the third most common cause of death after death caused by diseases such as the circulatory system (22.77%) and respiratory system (18.54%). A report collecting cancer cases from 2007 till 2011 by Malaysian National Cancer Registry (MNCR) showed a total number of 103,507 new cancer cases diagnosed of which 46,794 (45.2%) was found in males and 56,713 (54.8%) in females. According to the report, the possibility for the males getting cancer was 1 in 10 and 1 in 9 for females. In males, cancers of the colorectum (16.3%), lung (15.8%) and nasopharynx (8.1%) are the top three common cancers. On the other hand, cancers of the breast (32.1%), colorectum (10.7%) and cervix uteri (7.7%) are the top three cancers in females (Azizah *et al.*, 2016).

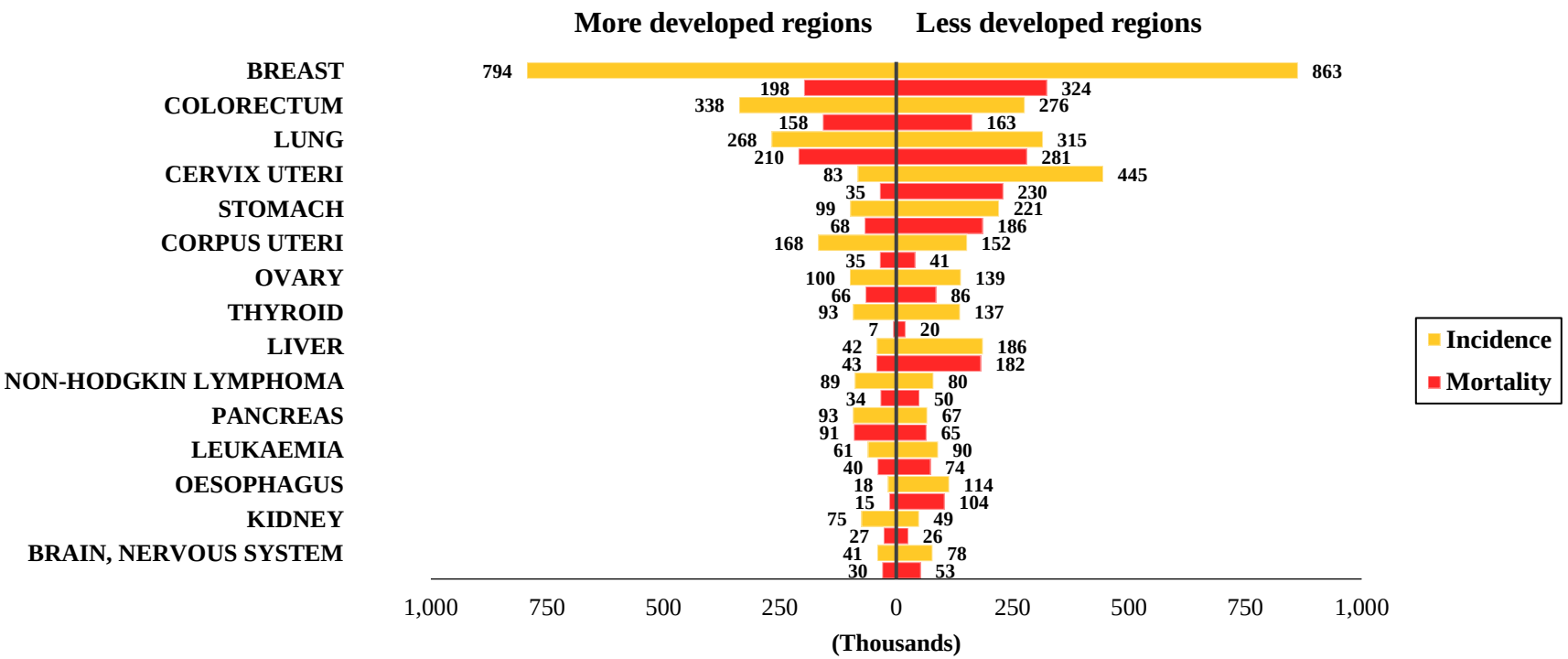

Figure 1.2 Estimated numbers (thousands) of new cancer cases (incidence) and deaths (mortality) in women in more developed and less developed regions of the world in 2012 (adapted from Ferlay *et al*., 2015).

1.2 Breast cancer

Breast cancer is the highest reported cancer affecting women with approximately 1.7 million new cases globally in 2012 with a ratio of one in four of all cancers among women (Torre *et al.*, 2015). Since 2008, the occurrence of breast cancer has increased globally with more than 13% mortality (Jemal *et al.*, 2011; Torre *et al.*, 2015).

According to MNCR statistic obtained from 2007- 2011, more than 18000 of breast cancer cases were reported with an average age-standardised incidence rate (ASR) of 31.08. Chinese has the highest cumulative risk with the possibility of developing breast cancer was 1 in 22, followed by Indian with 1 in 24 and the lowest cumulative risk was reported among Malay with lifetime risk was 1 in 35. The risk factors known to be correlated with breast cancer could be used as a guide to explain the dissimilarity in the incidence rate between different ethics in Malaysia (**Table 1.3**) (Yip *et al.*, 2006).

1.2.1 Breast cancer classifications

1.2.1(a) Breast Cancer Histology

There are several classifications used in breast cancer histology, dividing breast cancers into three distinct groups which are the non-invasive, invasive and miscellaneous. Non-invasive cancer comprises ductal carcinoma in situ (DCIS) or intraductal carcinoma which is a pre-invasive lesion for most women but if left untreated, it will eventually progress to an invasive ductal carcinoma. DCIS is characterised as cancerous epithelial cells which are distributed inside the mammary ducts and lobules. However, these cells exhibit no sign of invasion into the neighbouring breast tissue via basement membrane of the ducts/ lobules (Watson,

2001). Another form of non-invasive cancer is the lobular carcinoma *in situ* (LCIS) which serves as a marker for subsequent development of invasive cancer. LCIS has the ability to convert to the invasive ductal form of breast cancer, rather than the invasive lobular type (American Cancer Society, 2017).

On the other hand, the invasive category is divided into four types of breast cancer which consist of invasive ductal, invasive lobular, mucinous, and medullary cancers. The most common type of breast cancer diagnosed in 8 out of 10 invasive cases among women is the invasive ductal carcinoma, also well-known as infiltrating ductal carcinoma. This cancer starts from the duct of the breast and later develops the ability to invade and penetrate the basement membrane of the duct. It may be capable to metastasize via lymphatic system and bloodstream to the distant sites. Invasive lobular cancer begins from the lobular and terminal duct epithelium and is responsible for about 5-10% of all breast cancers (Watson, 2001). Similar to invasive ductal carcinoma, it can metastasize to different parts of the body. The presentation of this cancer tends to be a diffuse infiltrative process with no physical sign or identifiable mass through mammography which makes it even more difficult to be diagnosed in the initial stages especially among women with dense breasts. Mucinous carcinoma is an uncommon type of breast cancer. Like other types of invasive ductal carcinoma, mucinous carcinoma also arises in the milk duct of the breast before spreading to other tissues. It is distinguished by clusters of tumour cells found in the extracellular mucus and is classified as a low-grade form of invasive ductal carcinoma. This cancer is clinically presented as a discrete mass on clinical exam or on mammography. The last type of invasive breast cancer is the medullary carcinoma, which is extremely rare with less than 5% of reported breast cancers. Typically, the woman has a palpable mass,

Table 1.3 Risk factors for developing breast cancer among women (adapted from Yip *et al*., 2006).

Risk Factors
Increasing age
Family history
Reproductive factors
Early menarche less than 11 years
Late menopause more than 55 years
Nulliparity
Late first child-birth more than 30 years
Carcinoma of the uterus
Carcinoma of the ovary
Dietary factors – diet rich in animal fats
Exogenous hormones – Oral contraceptives
Hormone replacement therapy
Alcohol – more than 2 drinks per day
Postmenopausal obesity
Higher socio-economic group
Limited breastfeeding (for long periods is a protective factor)

but it can be mistaken for a benign condition such as cysts or fibroadenomas due to their benign appearance on mammography and ultrasound. However, in comparison to other type of breast cancers, the clinical prognosis for medullary carcinoma is better (Watson, 2001).

Miscellaneous breast cancer group includes inflammatory breast cancer and Paget's disease. Inflammatory breast cancer is an unusual and aggressive form of breast cancer as a result of cancer cells obstructing the lymph vessels found in the skin of the breast. This category of breast cancer typically looks red and swollen/ inflamed (National Cancer Institute, 2012). Paget's disease is also called as Paget disease of the nipple and mammary. It an uncommon type of breast cancer which appears as itching, scaling or skin excoriation around the nipple and areola, and does not subside with topical medication. Patient with Paget's disease commonly has more tumours in the same breast and they can be either invasive breast cancer or ductal carcinoma *in situ* (National Cancer Institute, 2012; İğci *et al.*, 2016).

1.2.1(b) Hormone receptors and HER2 status in breast cancer

Invasive breast cancer is often classified based on the presence of hormone receptors (which are the estrogen and progesterone receptors) and HER2/neu (human epidermal growth factor receptor 2). Basically:

i) Hormone receptor-positive – Breast cancer cells with either estrogen or progesterone receptors. This type of breast cancer is often treated with hormone therapy drugs which have the ability to decrease estrogen level or block estrogen receptors. Hormone receptor-positive breast cancer cells are more likely to grow

slowly than breast cancer cells with hormone receptor-negative (absence of estrogen or progesterone receptors) (American Cancer Society, 2017).

ii) Hormone receptor-negative – Breast cancer cells without estrogen and progesterone receptors. For these cancers, treatment with chemotherapy are better than hormone therapy drugs. Cancer cells with hormone receptor-negative are more likely to proliferate rapidly than the hormone receptor-positive breast cancer cells (American Cancer Society, 2017).

iii) HER2/neu positive – Breast cancer cells having excess HER2/neu protein or extra copies of HER2/neu gene are called HER2/neu positive. Treatments are carried out with drugs which target the HER2/neu (Iqbal and Iqbal, 2014).

iv) HER2/neu negative – Breast cancer cells having insufficient or with no HER2/neu protein or extra copies of HER2/neu gene are called HER2/neu negative. This type of cancer cells does not respond to treatment carried out with drugs targeting HER2/neu (Wilson *et al.*, 2002).

v) Triple-negative – Breast cancer cells with no receptors of estrogen and progesterone, and as well as HER2/neu. This type of cancer cells is more likely to grow and metastasize faster than other types of breast cancer. Cancer cells may respond to chemotherapy but will not respond to hormone therapy or drugs which target HER2/neu (Anders and Carey, 2009; Idil Cetin, 2014).

vi) Triple-positive – Breast cancer cells with the presence of estrogen and progesterone receptors, and as well as HER2/neu. Triple-positive cancer cells may respond to hormone drugs and drugs which target HER2/neu (Vici *et al.*, 2015).

1.2.2 Treatment for breast cancer

There are several methods established in treating breast cancer after considering the cancer cell type and stage. Local treatments which consist of surgery and radiation therapy are offered mainly to initial stage or less advanced cancers though it can also be used in some other stages depending on the purpose. This method of treatment is focused specifically to treat the tumour without affecting other parts of the body. The primary surgical options for management of the breast are mastectomy (removal of the entire breast tissue on the affected side) and breast conservation/ lumpectomy (tumour excision together with 1 cm macroscopic margin of normal tissue). Radiotherapy is carried out using high-energy x-rays or other particles to eradicate the breast cancer cells. It is essential in most patients undergoing wide excision of breast cancer to reduce local recurrence. Lymph nodes are also removed if cancer has spread into the lymph nodes (Maughan *et al.*, 2010; Anampa *et al.*, 2015; American Cancer Society, 2017).

Another method of treating breast cancers is through systemic treatments, which are conducted by using drugs given orally or directly into the bloodstream reaching cancer cells present anywhere in the body. Examples of systemic treatments are chemotherapy drugs (e.g. cyclophosphamide, methotrexate, 5-fluorouracil, epirubicin, doxorubicin, and docetaxel) to kill cancer cells rather than healthy cells, hormonal therapy (e.g. tamoxifen, aromatase inhibitors and fulvestrant) which blocks

the hormone receptors or reduce the production of the hormones and targeted therapy which aims proteins, specific genes, or the tissue environment which triggers cancer growth and survival. Cancer patients are usually suggested with more than one type of treatment depending on their state of disease for better prognosis (Anampa *et al.*, 2015; American Cancer Society, 2017).

1.3 Carcinogenesis

The supreme model of how cancers are formed is based on a succession of changes in the genome and epigenome of the cell, which are heritable changes in the programmes of gene expressions. Therefore, even a small subset of random occurring alterations may give rise to advantageous cell phenotypes resulting in clonal development of a group of cells expressing the phenotypes. Ultimately, the descendant of these cells will carry yet another advantageous alteration, causing once again in clonal expansion. This process is explained by Darwinian evolution theory with the condition that it occurs in the microcosm of a tissue rather than the wild. In the setting of cancer, every clonal expansion produces a cell population with larger neoplastic phenotypes. This results in highly aggressive populations of primary tumour or metastatic derivatives of the tumour (Nowell, 1976). The formation of a cancer is termed as carcinogenesis, where it involves multistep process in the revolution of normal/ healthy cells into cancerous cells. Carcinogenesis is characterised by the evolvement of deviations at the cellular and genetic level. The cause for these changes includes mutations and DNA repair deficiency (Anampa *et al.*, 2015), endogenous agents (e.g. hormones, genetic heritage) (Greaves, 2014) and exogenous agents (e.g. hazardous substances, radiation) (Dematteo *et al.*, 2013; Izzotti and Pulliero, 2014; Richardson *et al.*, 2015).

Previous studies have shown the significance of genetic aberrations in influencing the tumour characteristic in mouse models and human (Rasmuson *et al.*, 2012; Ben-David *et al.*, 2016; Kluzek *et al.*, 2017). Mutations in p53, Phosphatidylinositol-3-OH kinase (PI(3)K), MYC, PTEN, RAS, $p16^{Ink4a}$, BRCA and retinoblastoma protein are examples of oncogenes and tumour-suppressor proteins identified as main culprits for cancer development despite the difference in mutational profiles in different cancer types (Weinstein, 2002).

In eukaryotic cells, activity of DNA replication and partitioning of chromosomes are remarkably accurate processes. Each progenitor cell duplicates its genome and partitions the DNA equally producing two new daughter cells. In the process of division cycle, each daughter cells obtains an exact complement of genetic information. In the case of DNA damage, homeostatic equilibrium exists through multiple pathways for DNA repair. In normal cells, repair of DNA damage occurs without error. However, when error occurs and mutations are expressed, disease develops (**Figure 1.3**) where cancer is the prominent disease in human caused by mutations (Loeb *et al.*, 1974; Loeb, 2001).

In addition, cancer can develop from exposure to endogenous and exogenous agents. Endogenous carcinogens are carcinogenic molecules or metabolic intermediates produced by the organism as a result of respiration. It can as well be triggered through the food intake in people staying in a non-polluted safe atmosphere (Irigaray and Belpomme, 2010). Endogenous carcinogens include several reactive oxygen species (ROS) and their natural by-products (e.g. lipid peroxides), chemical DNA instability (e.g. depurination) and reactive chemicals (e.g. S-adenosylmethionine

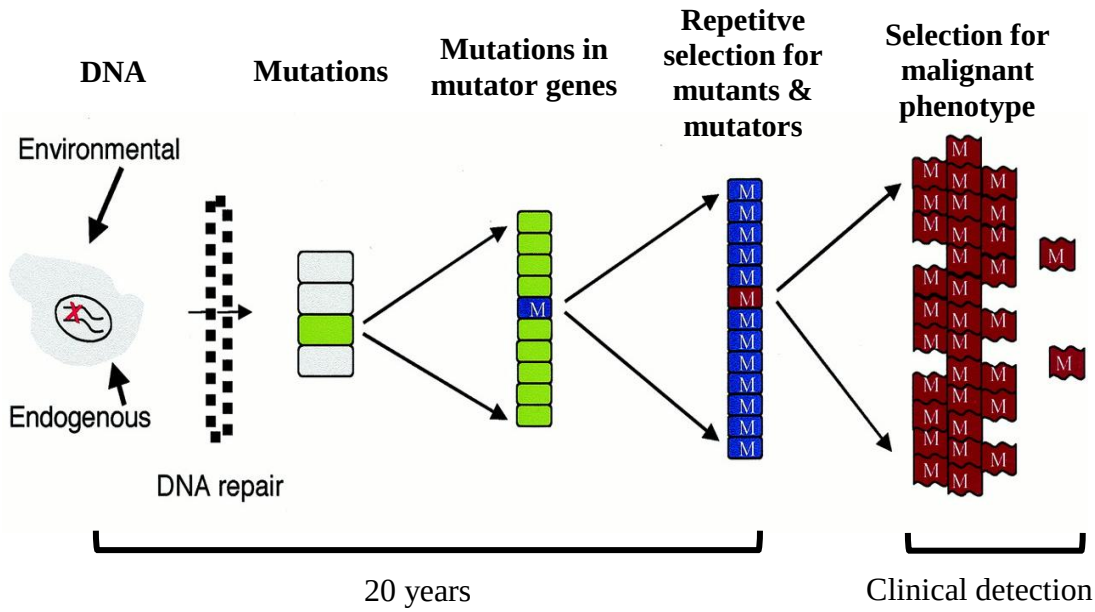

Figure 1.3 Mutation accumulation which leads to tumour progression.

When DNA damage surpasses the cell's capacity for error-free DNA repair, random mutations occurs. These random mutations cause clonal expansion and mutations in mutator genes. Repetitive rounds of selection for mutants produces further co-selection mutants in mutator genes and eventually produces malignant phenotype (adapted from Loeb, 2001).

and aldehydes) (Lutz *et al.*, 1996). When ROS level increases dramatically in the body, exceeding the natural antioxidant defence mechanisms, oxidative stress occurs resulting in impairment of macromolecules (e.g DNA, proteins and lipids) and inactivation of the antioxidant enzymes (Kono and Fridovich, 1982; Tabatabaie and Floyd, 1994; Birben *et al.*, 2012). Any changes occurring in the endogenous production of ROS or cellular antioxidants or even in the efficiency of DNA repair could trigger a corresponding modulation of the stable state levels of oxidative DNA modification. This increases the mutation rate and ultimately leading to initiation of cancer (Epe, 2002; De Bont and van Larebeke, 2004; Prasad *et al.*, 2016; Tubbs and Nussenzweig, 2017).

On the other hand, exogenous carcinogens are defined as agents that could include physical, chemicals and biological that initiate cancer upon penetration in the organism through the greatest surface exposure such as respiratory, skin, digestive or other potential contamination routes (Irigaray and Belpomme, 2010). Physical carcinogens include particle of hard or soft materials which are not soluble in water and exist as solid compounds such as fibres (e.g. asbestos, erionite and glass wool), particulate matters ranging from metallic cobalt, nickel and crystalline silica, and hard and soft materials including platinum, silver, steel and plastics. Chemical carcinogens comprise molecular substances which could be found in various compounds such as cigarette smoke and pesticides. Cancer causing biological agents include certain bacteria (*Helicobacter pylori*) and viruses (Hepatitis B virus, Hepatitis C virus, Human papilloma virus and Human T-cell lymphotropic virus type 1) (Correa and Piazuelo, 2011; de Flora and Bonanni, 2011; Morales-Sánchez and Fuentes-Pananá, 2014).

In the past decade, epigenetic have been extensively studied for its link to cancer. Epigenetic alteration on tumour suppressor genes may give rise to tumourigenesis (Chatterjee *et al.*, 2017). Epigenetics is related with heritable alterations in the gene expression due to modifications of DNA or chromatin proteins (e.g. methylation of DNA and modifications of the histone proteins) and not on the primary sequence of DNA (Feinberg, 2008; Sharma *et al.*, 2010). Epigenetic mechanisms are crucial for typical regulation and conservation of specific gene expression pattern found in different tissues. Any interruption in the epigenetic cycle may lead to altered function of the gene and eventually to cellular malignant transformation (Sharma *et al.*, 2010; Chatterjee *et al.*, 2017; Esteller, 2017). Even back in 1983, Feinberg and Vogelstein have reported the discovery of substantial hypomethylation in genes of cancer cells but not in normal counterparts suggesting epigenetics link to cancer (Chatterjee *et al.*, 2017).

1.4 Cancer metastasis

Invasive cancer cells can invade into neighbouring tissue and occupy it. Despite advance treatments for cancer patients, combating systemic metastatic disease is still one of the biggest clinical challenge (Kimbung *et al.*, 2015). Cancer metastasis involves multiple stages which starts when the cancer cells segregates from a primary tumour and eventually lose their epithelial polarity. They then further degrade and invade the basement membrane and extracellular matrix (ECM) to penetrate into the circulatory system in order to reach distinct sites for secondary tumour establishment (Engers and Gabbert, 2000; Wittekind and Neid, 2005). This is the most critical phase of cancer spread and is accountable for almost all death related to cancer. (Fidler *et al.*, 2007; Guan, 2015).

For cancer cells to survive outside the primary site, they need to find a location with the almost similar environment and adapt to it (Bhowmick, 2012). Some organs are much more susceptible than others for primary tumours to metastasize. Back in 1889, Stephen Paget was the very first person to explain the 'seed and soil' theory. The metastatic preference can be seen in several cancer where bones are the preferred site for prostate cancer, liver for colon cancer, and ovaries for stomach cancer which is then known as Krukenberg tumour. Malignant melanoma is likely to progress to the brain as it prefers melanocytes and nerves since both neural tissue and melanocytes derive from the similar cell line in the embryo (Paget, 1889). **Table 1.4** summarises the preferred choice of metastasis for several primary tumours.

As for breast cancer, the metastasis occurs when cancer cells detach from the breast tumour and enter the bloodstream in order to migrate to the distant organs. One of the preferential sites for breast metastasis is the bone where almost one-quarter of breast cancer cases are found to metastasize to the bone. In order to proliferate, breast cancer cells require calcium ions which can be obtained from the breast milk. Thus, bone is the preferential site of spread as calcium are abundant in them. Apart from bone, other usual sites of breast metastasis are the liver, lung, and brain. (Paget, 1889; Jena and Matic, 2016).

1.4.1 Mechanisms of metastasis

There are several steps involved for cells to metastasize as shown in **Figure 1.4**. The principal steps of metastasis are described as follows:

Table 1.4 Preferential metastatic sites for primary tumours (adapted from Paget, 1889; Jena and Matic, 2016).

Primary Tumour	**Regular distant site (s)**
Bladder carcinoma	Brain
Breast adenocarcinoma	Bone, brain
Kidney clear cell carcinoma	Bone, liver, thyroid
Lung small cell carcinoma	Bone, brain, liver
Neuroblastoma	Liver, adrenal
Prostate adenocarcinoma	Bone
Skin cutaneous melanoma	Brain, liver, bowel
Thyroid adenocarcinoma	Bone
Testis carcinoma	Liver

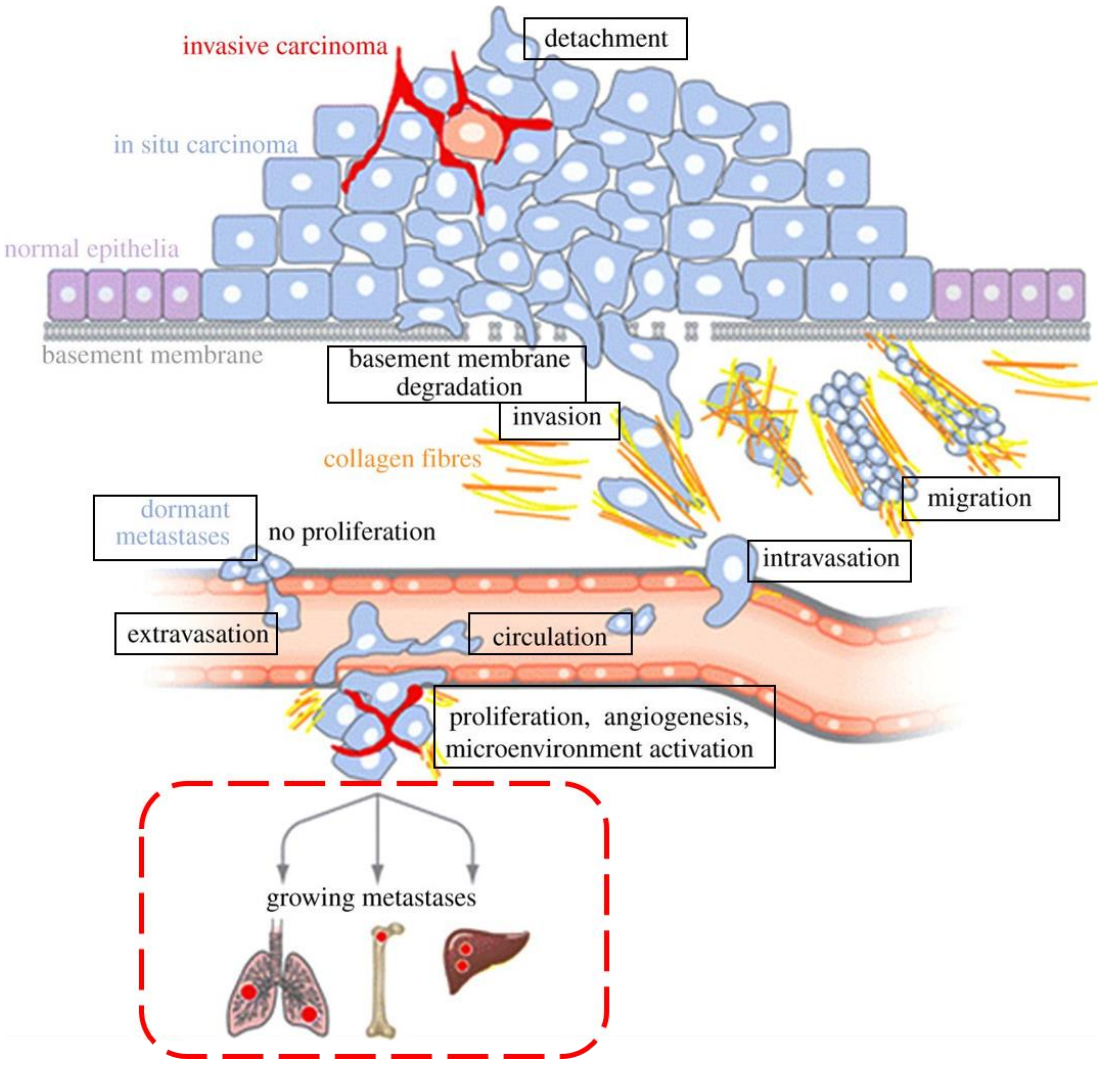

Figure 1.4 Mechanisms involve in cancer progression/ metastasis.

In situ carcinoma undergoes several transformation in becoming invasive carcinoma. Cells invade to the surrounding stroma through the degraded basement membrane. The cells then migrate and intravasate into the blood/lymph vessels reaching a distant site/organ (adapted from Bacac and Stamenkovic, 2008).

1.4.1(a) Primary tumour growth

Cancer starts to grow particularly at one site before spreading to other places of the body. This is defined as 'primary tumour/cancer' of that origin even after the cancer has spread to distant organs. For instance, breast cancer which already spread to the lung or any other parts of the body is still classified as breast cancer (Bacac and Stamenkovic, 2008; Maughan *et al.*, 2010).

1.4.1(b) Angiogenesis

Angiogenesis is referred to the formation of additional blood vessels which is extremely important for growing cancer cells for sufficient oxygen and nutrients supply and indirectly provide a pathway to other sites in the body. The angiogenetic phenotype of tumour cells is closely regulated by the activities of pro-angiogenic and anti-angiogenic factors. However, due to neovascularization, tumour cells express higher levels of proangiogenic proteins (e.g. VEGF) which bind and activate endothelial cells of a neighbouring blood vessel. The endothelial cells then start to proliferate and secrete proteases which help to penetrate the blood vessel wall while migrating towards the angiogenic stimuli. The proliferating endothelial cells soon enter into the new capillary tubes and through anastomosis process, the capillaries from the arterioles and venules adhere. This provides a consistent blood flow which aids in the tumour cell metabolism and eventually provides escaping routes for metastatic tumour cells. Proangiogenic protein expressions are induced in several conditions such as hypoxia, activation of oncogenes, or inactivation of tumour suppressor genes (Arbiser *et al.*, 1997; Wang and Keiser, 1998; Bergers *et al.*, 2000; Ravi *et al.*, 2000; Semenza, 2000; Pandya *et al.*, 2006; Gao *et al.*, 2014).

1.4.1(c) Epithelial-to-mesenchymal transition

To detach from the primary site, some tumour cells undergo epithelial-mesenchymal transition (EMT). However, once these cancer cells arrive at their 'new home', they return to epithelial form by initiating mesenchymal-epithelial transition (MET) (Thiery, 2002). EMT is a biological process involving concurrent down-regulation of epithelial proteins mainly E-cadherin and up-regulation of mesenchymal proteins especially N-cadherin and vimentin which eventually causes loss of cell-cell contacts and enhanced ability of cell motility (Steinestel *et al.*, 2014). Cell motility is the ability of tumour cells to move from original position to another site contributing to tumour invasion and metastasis (Wells, 2000). The 'loosened cells' are then ready to begin ECM invasion by communicating with the activated stroma cells and macrophages, which secrete a proteolytic enzyme known as matrix metalloproteinases (MMP). MMP causes the disruption of extracellular stroma creating a route for cancer invasion towards vasculature (Gupta *et al.*, 2007).

1.4.1(d) Invasion and intravasation

The initial step needed to achieve tumour dissemination for the cancer cells is to release itself out from the circle of a primary tumour. The cancer cells must obtain the capability to move and penetrate the basement membrane into the transport system which is either the vessels of the bloodstream or the lymphatic system. The activity of breaking through the wall of vessels and moving into the circulation is called intravasation (Reymond *et al.*, 2013). The degradation process of the basement membrane and invasion of the neoplastic cells are crucial in the progression of epithelial cancers. Numerous groups of proteolytic enzymes /proteases are engaged in the process of matrix degradation but the main key player controlling tumour invasion

and metastasis are the matrix metalloproteinase (MMP), a group zinc-dependent endopeptidases which consist of stromelysins, gelatinases, collagenases and membrane-type MMPs (Massova *et al.*, 1998; Curran and Murray, 1999; Bonnans *et al.*, 2014). The MMP had diverse functions other than structural protein breakdown including in cell proliferation, apoptosis, cell differentiation and autophagy through the activation of growth factors and their receptors or by releasing the cytokines from ECM (Liotta and Kohn, 2001; Sternlicht and Werb, 2001; Mannello *et al.*, 2005). Substantial evidence on the expressions of specific MMP on metastasis of cancer cells (e.g. colorectal, oesophageal and breast) causing poor prognosis had been documented for many years (Murray *et al.*, 1996; Murray *et al.*, 1998; Curran *et al.*, 2004).

1.4.1(e) Survival in circulation

Upon arriving in the circulation, the cancer cells need to survive the shear forces as well as the turbulent blood flow. On top of that, they also must escape from being detected by the immune system cells particularly the natural killer cells that would recognise cancer cells as abnormal and try to kill them. The cancerous cells interact with platelets which acts as a safeguard from the mechanical stress and immune cell lysis (Reymond *et al.*, 2013). One of the important molecules which works closely as a mediator is known as acute phase protein fibrinogen. Fibrinogens are not only secreted by megakaryocytes and hepatocytes but they can also originate from tumour cells (Rybarczyk and Simpson-Haidaris, 2000). Apart from acting as a plasma protein, fibrinogen participates in the activity of cancer cells as an ECM protein. Fibrinogen induces platelet adhesion by binding with thrombocyte receptor (Simpson-Haidaris and Rybarczyk, 2001; Venning *et al.*, 2015).