UNDERSTANDING THE CELLULAR AND MOLECULAR MECHANISM OF ANTICANCER EFFECT OF TUALANG HONEY ON BREAST CANCER *in vivo* and *in vitro* MODEL

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by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

LIST OF ABBREVIATION

BC	Breast cancer
TH	Tualang Honey
MH	Manuka Honey
HER2	Human epidermal growth factor receptor 2
TNBC	Triple-negative breast cancer
MNU	N-methyl-N-nitrosourea
SD rats	Sprague Dawley Rats
H&E	Hematoxylin and eosin
NP	Natural products
MCF-7	Michigan Cancer Foundation-7
EMEM	Eagle's minimum essential medium
FBS	Fetal bovine serum
DMSO	Dimethyl sulfoxide
%	Percent
°C	Degree Celcius
MTT	[3-(4, 5-dimethyl thiazole-2-yl)-2, 5-diphenyltetrazolium bromide]
DOX	0.5 micromolar Doxorubicin
μM	Micromolar
FITC	Fluorescein isothiocyanate
PI	Propidium Iodide
FSC-A	Forward scatter parameter
SSC-A	Side scatter parameter
rpm	Revolutions per minute

RIN	RNA Integrity Number
TPM	Transcript per million
RC	Read count
TMM	Trimmed Mean of M-values
DE	Differential expression
miRNA	MicroRNA
DAVID	The Database for Annotation, Visualization and Integrated Discovery
BH	Benjamini-Hochberg
KEGG	Kyoto Encyclopedia of Genes and Genomes
GO	Gene Ontology

MEMAHAMI MEKANISME SELULAR DAN MOLEKULAR BAGI KESAN ANTIKANSER MADU TUALANG DALAM MODEL *IN VIVO* DAN *IN VITRO* KANSER PAYUDARA

ABSTRAK

Madu adalah produk semulajadi yang telah menjadi pilihan penting dalam rawatan kanser payudara. Ia terbukti mempunyai sifat antikanser, namun mekanisme tindakannya masih tidak jelas. Kajian ini dijalankan untuk meneroka kesan antikanser madu pada kanser payudara (BC) secara *in vivo* dan *in vitro* berhubung dengan keupayaannya untuk meningkatkan apoptosis, memodulasi perkembangan kitaran sel, dan mengawal ekspresi miRNA. Kesan madu tualang (TH) dan madu manuka terhadap histomorfologi BC tikus *Sprague Dawley* (SD) betina dorongan MNU dikaji di bawah mikroskop. Memandangkan terdapat potensi antikanser TH secara *in vitro*, analisis kemudiannya dilakukan pada sel BC MCF-7. Madu tualang dicairkan dalam kepekatan akhir 2%, 3%, 3.5% dan 4% (v/v). Sel MCF-7 telah dirawat dengan TH, manakala sel MCF-7 yang tidak dirawat berfungsi sebagai kawalan. Apoptosis dan kitaran sel dikaji oleh sitometri aliran. Penjujukan RNA kecil dilakukan untuk menganalisis kemungkinan modulasi ekspresi miRNA oleh TH berlaku. Kesan TH dibandingkan dengan ubat antikanser Doxorubicin (0.5 micromolar; DOX). Secara histomorfologi, rawatan madu pada BC dorongan MNU dalam model tikus menghasilkan gred histologi kanser yang lebih rendah dengan kehadiran vakuol dalam sitoplasma dan kurang nekrosis berbanding BC yang tidak diberikan rawatan madu. Rawatan madu tualang pada sel MCF-7 menghasilkan peningkatan pecahan sel dalam G2/M, pengurangan dalam fasa S, peningkatan sitotoksisiti yang bergantung kepada dos dan peningkatan apoptosis. Perubahan sitologi yang disebabkan oleh TH, proapoptotik dan kesan modulasi kitaran sel menunjukkan persamaan dengan rawatan DOX. Dalam 100

miRNA yang diekspresi secara berbeza (DE); 63 mengalami regulasi menaik manakala 37 regulasi menurun. Sembilan belas miRNA DE adalah biasa dalam tiga kepekatan TH. Kebanyakan DE miRNA rawatan DOX juga dinyatakan dengan rawatan TH. Peningkatan peraturan miR-129-5p, miR-139-5p, miR-215-5p, miR-184 & miR-574-5p; *downregulation miR-182-5p*, miR-103a-3p & miR-191-5p membuktikan antiproliferatif, modulasi kitaran sel, kesan proapoptotik TH. Analisis laluan KEGG menunjukkan bahawa pelbagai laluan yang berkaitan dengan apoptosis, percambahan sel, pertumbuhan sel, kemandirian dan perkembangan kitaran sel adalah laluan sasaran yang penting oleh miRNA termodulat TH. Maka, madu tualang boleh memberikan kesan antitumor dengan cara mengganggu miRNA dalam sel BC. Kajian ini telah mendedahkan peranan baru madu tualang dalam menghalang perkembangan BC iaitu dengan memodulasi miRNA, seterusnya memberi kesan kepada kitaran sel dan apoptosis.

UNDERSTANDING THE CELLULAR AND MOLECULAR MECHANISM OF ANTICANCER EFFECT OF TUALANG HONEY ON BREAST CANCER *IN VIVO* AND *IN VITRO* MODEL

ABSTRACT

Honey is a natural product (NP) that has become a significant option in breast cancer (BC) treatment. It has been shown to possess substantial anticancer properties, but the mechanism of action remains unclear. Therefore, this study was undertaken to explore honey's anticancer effect on BC in *vivo* and *in vitro* in relation to its ability to enhance apoptosis, modulate cell cycle progression, and regulate microRNA expression. Histomorphological effect of Tualang honey (TH) and Manuka honey on MNU-induced BC of female Sprague Dawley rats were studied by analysing archival routine histopathology slides with light microscope. Subsequently, considering the anticancer potentiality of TH *in vitro,* analysis was done on MCF-7 BC cells. TH was diluted in a final concentration of 2%, 3%, 3.5% and 4% (v/v). MCF-7 cells were treated with TH. Untreated MCF-7 cells served as a control. Apoptosis and cell cycle were studied by flow cytometry. Small RNA sequencing was done, using NGS Illumina platform to analyse possible modulation of miRNA expression by TH. The effect of TH was compared with that of the anticancer drug Doxorubicin (0.5micromolar; DOX). Histomorphologically, honey treatment on MNU-induced BC in SD rat models resulted in a lower histological grade, less necrosis but increased in cytoplasmic vacuolisation compared to non-treated; implicating positive anticancer efficacy of honey. TH treatment in MCF-7 cells resulted in increased cell fraction in G2/M with reduced cell fraction in the S phase, dose-dependent increased cytotoxicity, and enhanced apoptosis. TH-induced cytological changes, proapoptotic and cell cycle modulatory effect showed similarity with that of DOX treatment. One hundred

miRNAs were differentially expressed; 63 were upregulated (UR), and 37 were downregulated (DR). Nineteen DE miRNAs were common in three concentrations of TH. Most DE miRNAs of DOX treatment were also expressed with TH treatment. Upregulation of miR-129-5p, miR-139-5p, miR-215-5p, miR-184 & miR-574-5p; downregulation of miR-182-5p, miR-103a-3p & miR-191-5p attests to antiproliferative, cell cycle modulatory, the proapoptotic effect of TH. KEGG pathway analysis showed that multiple pathways related to apoptosis, cell proliferation, cell growth, survival and cell cycle progression are important targeted pathways by TH-modulated miRNAs. Honey may exert an antitumor effect by interfering with miRNAs in BC cells. The study reveals a novel role for honey in inhibiting BC progression by modulating miRNAs, thereby affecting the cell cycle and apoptosis.

CHAPTER 1

INTRODUCTION

1.1 Research Background

1.1.1 What is breast cancer?

Breast cancer (BC) is a malignant tumour of the breast. It is a highly heterogeneous group of genetically and epigenetically distinct diseases that exhibit diverse clinical features (Dai et al., 2017). Cancer in the breast commences in the terminal duct lobular unit (Figure 1.1) and progresses in a stepwise manner (Sinha et al., 2012).

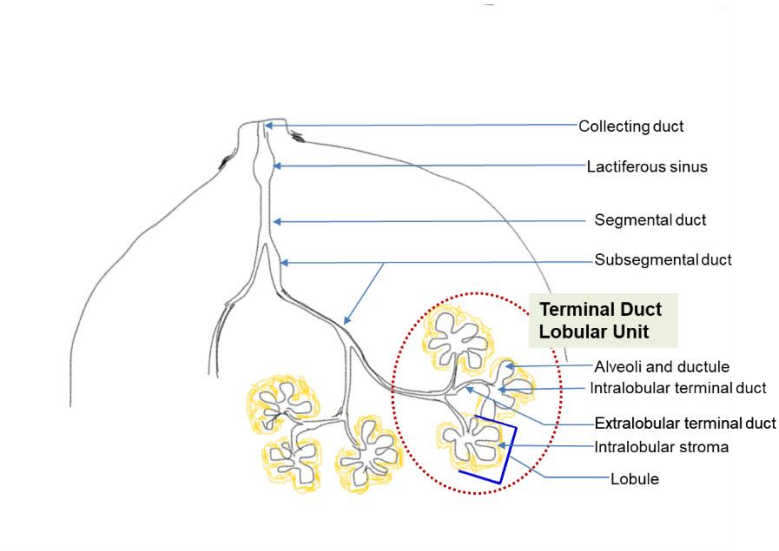

Figure 1.1: Schematic segment of breast lobe showing the lobules and the duct system. The morphofunctional unit of the breast is the terminal duct lobular unit (TDLU). TDLU is a grapelike cluster of small alveoli that comprises lobule and terminal duct. The terminal ducts drain into the subsegmental and segmental ducts, draining into the lactiferous and collecting duct (Banik et al., 2017).

According to traditional classification, primary breast adenocarcinoma is categorised as *in situ* and invasive. *In situ* cancers can be ductal (DCIS) or lobular (LCIS) and have an excellent prognosis. However, while 50–80% of the invasive cancers are invasive ductal carcinoma (IDC), only 25% of invasive BCs are ‘special

type' (invasive lobular carcinoma, invasive cribriform carcinoma, tubular carcinoma etc.) (Masood, 2016).

1.1.2 Breast cancer epidemiology

BC became the most commonly diagnosed cancer type in the world in the year 2020. In 2020, International Agency for Research on Cancer (IARC) estimated more than 2.26 million new cases of BC in both sexes (11.7%) (Figure 1.2). ("World Cancer Day: Breast cancer overtakes lung cancer in terms of new cancer cases worldwide. IARC showcases key research projects to address breast cancer – IARC," n.d.). Female breast cancer (BC) has exceeded lung cancer as the most commonly diagnosed cancer, and it was observed that 1 in every 8 cancers diagnosed in 2020 was BC (Arnold et al., 2022). More troublesome is that it has now become the fifth leading cause of overall cancer mortality worldwide, with 685,000 deaths recorded in the year 2020 worldwide (Arnold et al., 2022). As of the end of 2020, there were 7.8 million women alive who were diagnosed with BC within the past five years, making it the most prevalent cancer in the world (Sung et al., 2021).

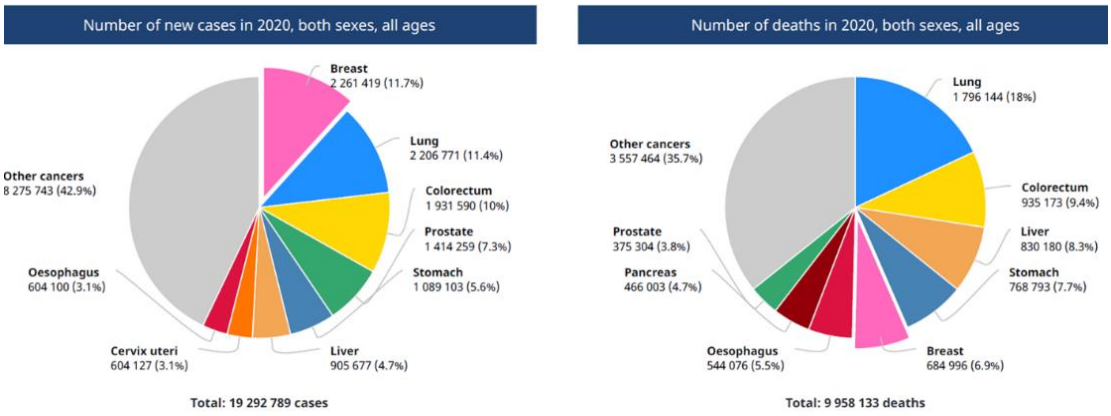

Figure 1.2: Estimated number of new cancer cases and deaths in 2020 among females of all ages, worldwide. Breast cancer is highlighted in pink. Source: https://www.uicc.org/news/globocan-2020-new-global-cancer-data

BC occurs in every country of the world in women at any age after puberty but with increasing rates in later life (Arnold et al., 2022). The global burden of BC mortality

is marked by inequality (Figure 1.3). Again, in high-income countries, where the prognosis for patients with BC is largely quite favourable, there are disparities in survival between different socioeconomic groups. In low- and middle-income countries, barriers to diagnosis and treatment are primary issues leading to the less likely survival of women from this cancer. In 2020, half a million women in low- and middle-income countries died of this cancer; approximately three-quarters of global deaths from the disease occurred in these countries (Arnold et al., 2022). To add to this global problem, there are more lost disability-adjusted life years by women to BC globally than any other type of cancer.

BC incidence in Malaysian multi-ethnic society varies from 1 in 22 Chinese women followed by 1 in 23 Indian women and 1 in 30 Malay women (Htay et al., 2021). Approximately 48% of BC cases in Malaysia are diagnosed late (Ministry of Health Malaysia, 2019). According to the National Cancer Registry, 2018 women who develop BC have an 81% (Stage II) to 88% (Stage I) chance of 5-year survival if their cancer is diagnosed early, whereas it is much lower for diagnosed cancer at Stage III (60%) or IV (23%). Therefore, implementing prevention measures, including screening, can potentially reduce the burden of BC, which is associated with late presentation (Al-Amri, 2005). According to GLOBOCON, in 2020., newly diagnosed cases were 8418 (17.3%) and ranked first among all cancer nationwide (Figure 1.3). GLOBOCON also reported that mortality from BC ranks second in Malaysia, and the country recorded 3503 deaths (11.9%) from BC in 2020.

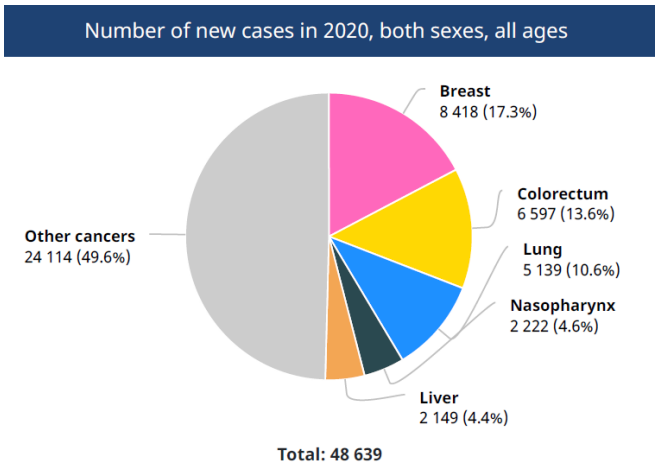

Figure1.3: Estimated number of new cancer cases in 2020 among females of all ages, in Malaysia. Breast cancer is highlighted in pink. Source: https://gco.iarc.fr/today/data/factsheets/populations/458-malaysia-fact-sheets.pdf)

The above statistics demonstrate the universal problem of the disease. Hence scientists and clinicians are evaluating and exploring various therapeutic modalities for BC for a long-drawn-out period. However, the disease has remained unconquered, and the search for a remedy continues.

1.1.3 *In vivo* and *in vitro* models for breast cancer experiments:

Current research on breast carcinomas is derived from *in vivo* and *in vitro* studies using BC cell lines and animal models. These in vitro and in vivo models can offer a limitless basis of homogenous self-replicating data by means of simple yet standard media and approaches.

Among the broad range of BC animal models, rat models have been valuable *in vivo* experimental model systems for the study of BC (Whittle et al., 2015). In rats, the mammary gland is the source of hormone-dependent neoplasms that are, in many ways, show likeness to the human BC (Fantozzi and Christofori, 2006; Korkmaz and Ustun, 2021). The predisposition of the rat breast to neoplasia has made this organ a unique target for testing the carcinogenic potential of specific compounds. These models have developed into premier tools for investigating the mechanisms and

genetic pathways in cancer progression and metastasis and for developing and evaluating clinical therapeutics. Tumours induced in rats by administration of chemical carcinogens such as 7,12-dimethylben z(a)anthracene (DMBA) and N-methyl-N-nitrosourea (MNU) create useful tools for studying the multistep process of carcinogenesis involving initiation, promotion, and progression (Barros et al., 2004; Zeng et al., 2020). Chemically induced mammary tumours are, in general, hormone-dependent adenocarcinomas. MNU-induced BC in SD rats is a commonly used cancer model to study the anticancer mechanism of NPs (Lu et al., 2013; Martinez et al., 2017). MNU can induce mouse BC similar to estrogen receptor-positive human BC (Kassayová et al., 2016). Histo-morphologically, the Russo and Russo classification scheme (Russo and Russo, 2000a) for the MNU-induced BC in SD rats are pretty similar to the traditional classification of human BC. The classification of breast tumours according to their histopathological type and benign or malignant nature is important because those characteristics have implications for interpreting the experimental data. The Russo and Russo recommendations provide a working framework for diagnosing the type of lesions found in the mammary glands of rats treated with chemical carcinogens (Russo and Russo, 2000b).

Cell culture is an extensively used *in vitro* tool for medical and biomedical science research, drug discovery, and development. However, only a handful of cell lines are primarily used for different in vitro BC studies. Cell lines like MCF7, T47D and MDAMB231 account for the major cell lines used in the different experimental studies (Lacroix et al., 2004). Many advantages vest BC cell lines and practicable cancer models for tumours of the same subtype. The molecular features of the cell lines usually remain the same with tissue tumours, with only a few exceptions.

Additionally, their genomic profiles stay invariant with the tumours. (Dai et al., 2017). MCF-7 is an estrogen-dependent BC cell line, a commonly used cell line for BC research for more than 40 years by multiple research groups (G. Chen et al., 2022; Comşa et al., 2015).

1.1.4 Natural product honey as a new therapeutic agent for breast cancer

Contemporary therapeutic options for BC are surgical resection, radiation, chemotherapy and immunotherapy (Arslan et al., 2014; Isakoff, 2010; Ishiba et al., 2015; Shi et al., 2016; Shin et al., 2016; van Rooijen et al., 2015). These are not only costly but may modify many normal gene functions also. The present-day approach to BC therapy and prevention is either combination of a number of drugs or a drug that modulates multiple targets. Nonetheless, it is still unknown how many BC targets there are. Again, how many targets must be confronted to control cancer growth is yet to be explored. BC is a heterogeneous disease sustained by interconnected and intricate signalling pathways (Singhal et al., 2016). Various genetic and epigenetic changes are critical to this carcinogenesis (Khan et al., 2012; Parise et al., 2009, pp. 1999–2004). Thus, aiming a single gene product or cell signalling pathway is unlikely to prevent or treat this cancer. Considering these facts, NPs are now becoming a significant option in BC prevention and treatment. The well-known uses in cancer treatment are due to their effectiveness, less side effect, relatively low cost and, notably, their ability to target various signalling pathways. Selected NPs, substances derived from living organisms like curcumin, green tea and EGCG, resveratrol, honokiol, quercetin, silibinin, genistein, and soy, promote apoptosis and inhibit metastasis and prevent cancer growth (Banik et al., 2017; Noel et al., 2020; Sinha et al., 2016a). As a result, these can potentially suppress BC progression, hence increasing patient survival rates and decreasing the number of BC-related deaths; in this regard, honey is gaining

attention as a potential anticancer agent (Waheed et al., 2019a). The anticancer effect of honey is attributable to its anti-proliferative and pro-apoptotic activities (Jaganathan et al., 2015). Furthermore, depending on concentration, honey can demonstrate either an oestrogenic or antiestrogenic mode of action (Jaganathan and Mandal, 2009a). Among the various Malaysian honeys, Tualang honey (TH) has been found to possess the highest anticancer potential due to the exceptional composition of polyphenols and antioxidants (Khalil et al., 2011).

1.1.4(a) Targeting cancer cell apoptosis and cell cycle by natural products in breast cancer as inventive therapeutic strategy

Various genetic and epigenetic alterations are critical to breast carcinogenesis. Like any other cancer, dysregulated cell proliferation and inhibition of apoptosis lie at the centre of breast carcinogenesis. As there are many mechanisms through which these two defects can occur, the efficiency of a targeted therapy rests a large part on the molecular analysis of each tumour to understand the primary pathological process (Evan and Vousden, 2001). Dysregulated apoptosis may lead to tumour formation or even the development of cancer cell drug resistance. Cancer cells evade apoptosis primarily by: i) imbalance of Bcl2 family members with overexpression of antiapoptotic proteins and downregulation of proapoptotic proteins; ii) loss of TP53 functions due to TP53 mutations and indirectly due to impairment of p53 function by amplification of MDM2 that encodes an inhibitor of p53 iii) downregulation of caspases iv) impairment of death receptor signalling v) overexpression of inhibitors of apoptosis proteins and hence binding and inactivating caspases 9 and 3 (Pistritto et al., 2016). Finding ways to tackle these issues can lead to restoring the apoptotic pathway in cancer cells and hence constitute a promising anticancer therapeutic approach. Studies also reported that many tumour promoter

proteins inhibit apoptosis by developing chemoresistance in the cancer cell. Thereby strategies to target proteins that manipulate the apoptotic programs are considered a prominent anticancer therapeutic approach, in which activation of apoptosis in cancer cells is the primary concern. In addition, targeting cell death receptors can be an appealing therapeutic strategy for cancer. Currently, many studies are focusing on the NPs that have been approved for clinical use in cancer treatment to find out their ability to inhibit the growth of cancer cells by inducing apoptosis through one or more than one mechanism. However, despite major efforts, the biological mechanisms involved in the various programmed cell death pathways are still not fully understood. Cell cycle deregulation is another major hallmark of cancer progression, and as such, induction cell cycle arrest is an important cause of inhibiting cancer growth and proliferation (Caglar and Biray Avci, 2020; Hsiao et al., 2012). The mechanisms regulating S phase progression in the cell cycle are essential for maintaining genome integrity and fidelity in any proliferating cell (Takeda and Dutta, 2005; Zhao et al., 2014). Studies show that there are many NPs, the growth inhibitory antiproliferative effect of which targets mainly the components of the cell signalling pathways related to cell cycle and apoptosis (Alghamdi et al., 2021, 2021; Anwar et al., 2018; Banik et al. 2017; Choi et al., 2021; Cui et al., 2020; I. El-Garawani et al., 2019; I. M. El-Garawani et al., 2019; Hosami et al., 2021, 2021; Jiang and Fan, 2020; Khan et al., 2020; Kizaibek et al., 2020; Lang et al., 2019; Lee et al., 2020; Lin et al., 2021; M Franco et al., 2019; Mansour et al., 2019; Mirza et al., 2018; Misir et al., 2020; Mohammed et al., 2018; Moosavi et al., 2021; Ngabire et al., 2018; Okon et al., 2020; Saleh et al., 2019; Virdis et al., 2020; M. Wang et al., 2019; Xie et al., 2019; Zhou et al., 2020). Hence NPs with proapoptotic activity and inhibitory effect on the cancer cell cycle can act as potential anticancer agents.

1.1.4(b) miRNAs can act as pivots in natural product-mediated modulation of apoptosis and cell cycle in breast cancer

One potential therapeutic target for BC is the miRNAs, and numerous studies show that regulation of miRNAs can be achieved by means of NPs. Owing to their significant and versatile roles, miRNAs are emerging as therapeutic tools for many cancers, including BC. However, several miRNAs are dysregulated in BC tissues compared to normal tissues (Elango et al., 2020). Alterations of miRNA expression are one of the most widely studied epigenetic deviations in cancer (Rahman et al., 2019). Deregulation of miRNA function is associated with numerous diseases like cancer (Bracken et al., 2016). These alterations are driven by the dysregulation of miRNA biogenesis and miRNA pool imbalance with up-regulation or down-regulation of miRNA-processing machinery components (Fridrichova and Zmetakova, 2019). Dysregulation of miRNAs is critical in breast carcinogenesis (Di Leva et al., 2014; Khan et al., 2019; Pouya et al., 2021; SANDHU et al., 2013; Søkilde et al., 2019). There is intricate involvement of miRNAs in BC tumorigenesis, progression, and metastasis by post-transcriptional regulation of target gene expression (Benedetti et al., 2021; Fridrichova and Zmetakova, 2019). Studies have demonstrated that miRNAs usually target multiple mRNAs and could act as oncomiR or tumour suppressors in various cancers (Shirjang et al., 2019). Either as a tumour suppressor or oncogene, miRNAs can coordinate multiple cellular processes related to two vital hallmarks of cancer: evading growth suppressors and resisting cell death. Studies show that the Bcl-2 family, TRAIL, Fas (Fas/APO-1/TNFRSF6) and p53 directly or indirectly via MDM2 are the major targets in the miRNA-mediated regulation of apoptosis in different cancers (Cai et al., 2015; Konno et al., 2014; Long et al., 2015; Patron et al., 2012; Shirjang et al., 2019; Tong et al., 2015; Zarogoulidis et al., 2015; T. Zhang et

al., 2016; Zhang et al., 2014). Reduction in anti-apoptotic miRNAs or induction in apoptomiRs might demonstrate the effectiveness of treatment or cancer eradication.

Again miRNAs are crucial transcriptional regulators of the cell cycle (Grolmusz et al., 2016). In cancer cells, microRNAs can control the levels of multiple cell cycle regulators and hence can control cell proliferation (Budakoti et al., 2021; Bueno and Malumbres, 2011; Di Leva et al., 2014; Mens and Ghanbari, 2018). The tumour suppressor miRNAs induce cell cycle arrest by downregulating multiple components of the cell cycle machinery. Recent data also suggest that miRNAs act co-ordinately with transcriptional factors involved in cell cycle regulation, such as c-MYC, E2F or p53 (Ali Syeda et al., 2020; Hill and Tran, 2021). These miRNAs can potentiate the function of these factors. They may also limit the excessive translation of cell cycle proteins upon mitogenic or oncogenic stimuli to protect cells from replicative stress (Ali Syeda et al., 2020).

Therefore, microRNAs can be a crucial pathophysiological component associated with BC progression and a therapeutic target for this cancer. Investigating the molecular regulatory mechanism of the common and drug-specific miRNAs will facilitate well understanding of the mechanism of action for different drugs as well as provide new insight into screening new drugs for BC treatment. Studies show cross-talk between phytochemicals, microRNAs and various cell signalling in the regulation of apoptosis, cellular proliferation, cell cycle regulation, and self-renewing cancer stem cell divisions (Bhardwaj and Mandal, 2019; Fix et al., 2010, p. 60; Kavitha et al., 2018a; Namima et al., 2020; Nwaeburu et al., 2017; Venkatadri et al., 2016; S.-M. Wang et al., 2021; W. Wang et al., 2019). Progressively more studies are needed to evaluate the effect of NP on miRNA modulation in BC. The potential of clinical applications involving miRNAs warrants continued cancer research in this area.

1.2 Problem statement: Present Study

Resistance, recurrence, metastasis and adverse effects are the major glitches in BC treatment and prognosis (Pashayan et al., 2020). Prolonged use of chemotherapeutics and radiotherapy against BC in many circumstances renders the therapy ineffective because of the development of resistance. Identifying alternative treatments is crucial to reduce the mortality rate related to BC. Thus, NPs are now the primary investigative molecules soaring in the hope of discovering new powerful classes of anticancer agents for BC.

Previous studies demonstrated the potentiality of Malaysian Tualang honey (TH) as an anticancer agent. It was found that standard compounds present in gamma-irradiated (25 kGy) TH are: catechin, p-coumaric acid, benzoic acid, naringenin and Trans-cinnamic acid as detected by HPLC analysis and contain the strongest antioxidant properties(Ahmed and Othman, 2013a; Khalil et al., 2011; Moniruzzaman et al., 2013a). Several studies point to polyphenols as the molecular components behind the anti-carcinogenic effects of TH. Such studies indicate that polyphenols induce apoptosis, cell cycle arrest and inhibition of angiogenesis (Ahmed and Othman, 2013a, 2017; Fauzi et al., 2011; Khalil et al., 2011; Mohd Kamal et al., 2021a).

In their study Ahmed et al, demonstrated that systemic administration of TH and MH (1.0 g/kg body weight/day) for 120 days in female SD rats with MNU induced BC, increased the expression of proapoptotic proteins (Apaf-1, Caspase-9, IFN-γ, IFNGR1, and p53) and decreased the expression of antiapoptotic proteins (TNF-α, COX-2, and Bcl-xL 1) (Ahmed et al., 2017a). In another study by Ahmed and Othman, TH in different concentration (0.2, 1.0 and 2.0 g/kg body weight/day) for 120 days was used in MNU induced BC in female SD rats and it was observed that TH alleviates breast carcinogenesis through modulation of hematologic, estrogenic and proapoptotic

activities (Ahmed and Othman, 2017). The tumours showed lower histologic grade. Thus, it was proposed that honey may be used as a natural 'cancer alleviating' agent or as a supplement to chemotherapeutic agents. However, advanced research is needed to develop an improved understanding of its anticancer effect and thus explore its potential health benefits in BC therapy.

1.2.1 Histomorphological evidence of the anti-cancer effect of honey against breast cancer

Post-treatment histopathological changes in tumour morphology plays a vital role in evaluating the therapeutic response. With increasingly accumulating data on distinct molecular-morphologic correlates, there is a resurgence of attention on the role of tissue evaluation in *vivo* cancer studies. In BC models, diverse histopathologic alterations are observed with the application of different therapeutic modalities. However, the histo-morphological effect of honey on in vivo BC model has not been studied in detail. Detailed analysis and comparison of the pattern of histological alterations in honey-treated MNU-induced BC in female SD rats with that of the non-treated ones are needed to correlate molecular and histological findings of honey-treated BC tissue in an animal model. This will pave the way towards a future anticancer study on honey and its derivatives.

1.2.2 Role of miRNA in the anticancer effect of honey in breast cancer

Till now, the molecular mechanisms underlying the antitumor activity of honey in BC is not well understood. Although anticancer studies of honey on BC primarily focus on the molecular mechanistic effect of honey (Jaganathan and Mandal, 2009b), its detailed mechanism of action is still unclear. Honey has been reported to induce apoptosis and cell cycle arrest in cancer cells. Among the various Malaysian honey, TH is emerging as a promising anticancer agent. TH has been shown to be

crucial in apoptosis induction in human cancer cells, including BC cells. Although the broad cellular impact of TH has been investigated, the molecular mechanisms behind such effects remain unclear. After that, considering the potentiality of TH as a possible anticancer agent, its apoptosis-inducing and cell cycle modulatory effect on BC cells needs more investigation. The growth inhibitory antiproliferative and anticancer effect of TH in BC needs more research and analysis. The link between TH-induced apoptosis, cell cycle modulation, and miRNA regulation related to BC has not been explored (Figure 1.4). Previous studies indicated that the expression levels of multiple genes associated with the proliferation of BC cells were altered in TH-treated BC cells. However, to the best of our knowledge, it remains to be investigated whether treatment with TH can regulate the expression of miRNAs in BC cells. Elucidating the effect of TH-mediated miRNA modulation during BC cell death and cell cycle progression will aid in a better understanding of the underlying mechanisms that have a critical anticancer role in BC.

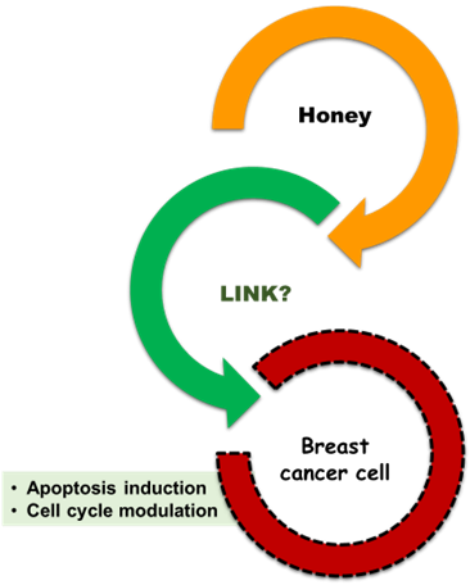

Figure 1.4: Schematic representation of the possible mechanistic path of tualang honey-mediated anticancer effect on breast cancer cells.

1.3 Research Questions

With the above background and coexisting gaps in the research of honey as a potential anticancer agent in BC, the current study was designed to answer the following research questions:

1. What are the histopathological alterations observed in the honey-treated MNU-induced BC model that implicates the positive efficacy of honey as an anticancer agent against breast cancer tissue?
2. How honey modulates the BC apoptosis and cancer cell cycle?
3. Are honey's apoptosis and cell cycle-modulating effects comparable to the anticancer drug Doxorubicin?
4. Does miRNA play any role in the anticancer effect of honey? Does it contribute to our understanding of the molecular events of breast carcinogenesis?

1.4 Objectives

1.4.1 **General objectives:** To explore the cellular and molecular mechanism of the anticancer effect of honey on breast cancer *in vivo* and *in vitro* models.

1.4.2 **Specific objectives**

Part1 *in vivo* study:

1. To explore the histopathological alterations observed in honey-treated MNU-induced BC in female Sprague Dawley rat model.

Part2 *in vitro* study:

1. To analyse the cytomorphological effect of Tualang honey (TH) on MCF-7 BC cells.
2. To investigate the effect of TH-mediated apoptosis in MCF-7 cells and compare it with Doxorubicin-treated MCF-7 cells.

3. To investigate the effect of TH on cell cycle phase distribution in MCF-7 BC cell line and compare it with that of Doxorubicin-treated MCF-7 cells
4. To explore whether the dose of TH affects apoptosis and cell cycle progression in MCF-7 BC cell
5. To analyse the effect of TH on known and putative novel microRNAs in MCF-7 BC cells and compare with that of Doxorubicin.
6. To correlate the effect of TH on miRNA expression with its effect on cell cycle and apoptosis in MCF-7 BC cells.

1.5 Hypothesis:

The anticancer mechanism of tualang honey in breast cancer is associated with distinct histo-cyto-morphological alterations and is related with its modulatory effect on cancer cell cycle, apoptosis and miRNA profile.

CHAPTER 2

LITERATURE REVIEW

2.1 How is breast cancer classified?

Breast cancer (BC) has several recognized histological and molecular subtypes with different aetiologies, risk factors profiles, treatment responses, and prognoses**.** Several different approaches have been used to subclassify BC into clinically meaningful subtypes.

BCs can be primary (originating in the breast) or secondary (metastatic). While more than 95% of the primary BCs are carcinoma being mostly adenocarcinoma (arising from tubules/ductules), the secondary cancers are commonly from a contralateral breast carcinoma, melanoma, carcinoma of the lung, ovary, kidney & stomach. Traditionally primary breast carcinoma is further classified into carcinoma in situ and invasive carcinoma. Carcinoma in situ is classified as DCIS or LCIS. The term "lobular" refers to invasive carcinomas that are biologically related to LCIS, and "ductal" is used more generally for adenocarcinomas that cannot be classified as a special histologic type (Figure 2.1). One-third of invasive/infiltrating carcinomas are of special types. Some of these are strongly associated with clinically pertinent biologic features. The remaining two third are grouped as "ductal" or no special type (NST). Previously known as Invasive Ductal Carcinoma, not otherwise specified (IDC, NOS). This NST group of invasive BCs encompasses all tumours without the specific differentiating features that characterize the other categories of BCs. The diagnosis is made by exclusion of recognized specific BC types (Luo et al., 2022; Sinn and Kreipe, 2013).

In the WHO classification, DCIS and lobular neoplasia are designated precursor breast cancer lesions. Lobular neoplasia is further subgrouped into classic lobular carcinoma in situ (LCIS) and pleomorphic lobular carcinoma in situ (PLCIS)(Sinn and Kreipe, 2013). These precursors possess different clinical behaviour and hence differences in therapeutic recommendations based on the disease biology. Therefore, these lesions need to be distinguished pathologically.

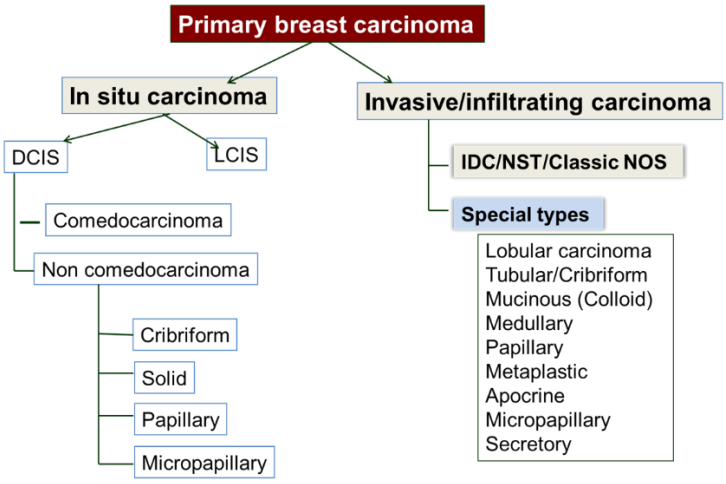

Figure 2.1: Traditional histological types of primary breast carcinoma. Modified from Robbins Pathologic Basis_10E_ 2020: Chapter 23 The Breast p. 1053.

The molecular classification of BC is based on the characteristic changes in DNA, mRNA, protein, and morphology. According to this classification, BCs can be segregated into three major groups distinguished by the expression of two proteins, ER (estrogen receptor) and HER2 (human epidermal growth factor receptor 2). The HER2 protein is a tyrosine kinase known as ERBB2 (Erb-b2 receptor tyrosine kinase 2). The three molecular subtypes correlate reasonably well with ER and HER2 protein expression and can be easily assessed by standard clinical assays like immunohistochemistry (Figure 2.2).

i) **Luminal (ER-positive/HER2-negative) cancers** are diverse, ranging from well-differentiated cancers with low proliferative rates and scarce chromosomal changes to poorly differentiated cancers with high proliferative rates and large numbers of chromosomal rearrangements. All these cancers express ER, which is an estrogen-dependent transcription factor. Highly expressing ER cancers usually express high levels of PR (progesterone receptor) as well, which is itself upregulated by estrogen and ER. These ER-positive/PR-positive cancers are usually slow growing and well-differentiated. On the other hand, carcinomas that express low ER and absent PR are typically poorly differentiated, having a high proliferative rate. Cancers detected by mammographic screening are usually small luminal cancers limited to the breast. Interestingly, miRNAs can cluster these cancer subtypes (Aure et al., 2017).

ii) **HER2-positive cancers** may be either ER-positive or negative, but when ER is present, levels are typically low. It is to be noted that HER2 positivity can be identified as an increase in HER2 gene copy number, an increase in HER2 mRNA, or an increase in HER2 protein.

iii) **Triple-negative breast cancers or TNBCs** (ER-negative/HER2-negative; this group largely overlaps with "basal-like" carcinomas defined by mRNA expression) are characterized by genomic instability, a high proliferative rate, and expression of many proteins typical of myoepithelial cells (e.g., basal keratins). These fail to express PR, as it is under the control of ER, often are associated with defects in DNA repair or genomic stability (e.g., due to silencing of BRCA1 or TP53 mutation), and have a relatively poor prognosis. Cytotoxic therapy combined with selectively active agents

against cancers with defective homologous recombination results in complete or almost complete responses in about a third of cases. The cancers that recur usually occur in the first 8 years after diagnosis. Patients who survive 10 years are likely cured, as late recurrences are unusual.

The mRNA expression profiling can also categorise BC into those above-mentioned three main groups. These three BC groups vary with regard to patient characteristics, pathologic features, therapeutic response, metastatic patterns, relapse time, and clinical outcome (Table 2.1).

Table 2.1 Molecular classification of invasive breast cancer

Defining Features	**Luminal (ER-Positive/HER2-Negative)**		**HER2 (HER2 Positive)**	**TNBC (ER-Negative/ HER2-Negative)a**
	Low to moderate proliferation	**High proliferation**		
Percent of breast cancers	~40%–55%	~10%	~20%	~15%
The most similar group defined by mRNA profiling	Luminal A	Luminal B	HER2-enriched (ER-negative), luminal B (ER-positive)	Basal-like
Most common gene mutations	*PIK3CA* (45%), *TP53* (12%)	*PIK3CA* (29%), *TP53* (29%)	*PIK3CA* (39%), *TP53* (70%–80%)	*PIK3CA* (9%), *TP53* (70%–80%)
Typical special histologic types	Tubular, grade 1 or 2 lobular, mucinous, papillary	Grade 3 lobular	Some apocrine, some micropapillary	Medullary features, metaplastic
Typical patient groups	Older women, men, cancers detected by mammographic screening	*BRCA2* mutation carriers	Young women, *TP53* mutation carriers (ER positive)	Young women, women of African heritage, *BRCA1* mutation carriers
A complete response to chemotherapy	<10%	~10%	ER-positive ~15%; ER	~30%

Table 2.1 Continued

			negative ~30%–60%	
Metastatic pattern	Bone (70%), more common than viscera (25%) or brain (<10%)	Bone (80%) is more common than viscera (30%) or brain (10%)	Bone (70%), viscera (45%), and brain (30%) are all common	Bone (40%), viscera (35%), and brain (25%) are all common
Relapse pattern	Low rate over many years, long survival possible with bone metastases	An early peak at <10 years, late recurrence possible	Bimodal with early and late (10 years) peaks	An early peak at <8 years, late recurrence rare, survival with metastases rare

a TNBC lacks expression of ER, progesterone receptor, and HER2.
b. The three major groups of cancer identified by protein expression or mRNA profiling largely overlap but are not identical. "Luminal B" can refer to ER-positive cancers with high proliferation with or without HER2 expression.

c Some rare special histologic types have a more favourable prognosis than this group as a whole (e.g., adenoid cystic carcinoma, secretory carcinoma, low-grade adenosquamous carcinoma).
ER, Estrogen receptor; mRNA, messenger RNA; TNBC, triple-negative breast cancer.

(Source: Robbins Pathologic Basis_10E_ 2020: Chapter 23 The Breast p. 1050)

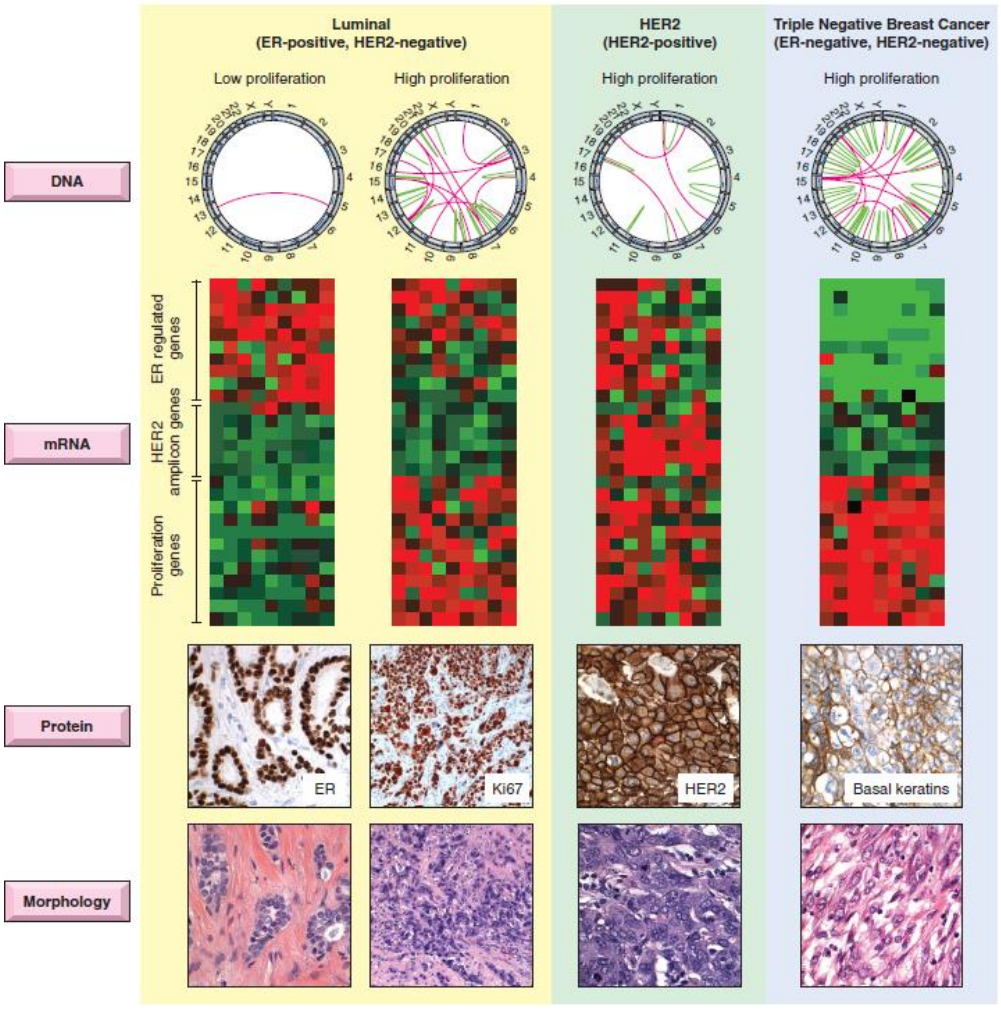

Figure 2.2: Molecular classification of invasive breast cancer. Circos plots show a snapshot of all of the genomic abnormalities within a particular tumour; these abnormalities are mapped onto the chromosomes, displayed at the periphery of a circle. Green loops show intrachromosomal rearrangements, while red loops show interchromosomal rearrangements. The mRNA profiling shows relative levels of mRNA expression. Red indicates a relative increase; green, a relative decrease; and black, no change in levels. Genes are arrayed from top to bottom, and tumours from left to right. Immunohistochemistry detects proteins using specific antibodies visualized with a brown chromogen. Cancer cell proliferation is estimated by counting mitoses or staining for cell cycle-specific proteins such as Ki-67. (Source: Robbins Pathologic Basis_10E_ 2020: Chapter 23 The Breast p. 1048).

2.2 Pathogenesis of breast cancer

Like other cancers, BCs arise through several pathways, including the step-by-step acquisition of driver mutations in breast epithelial cells (Figure 2.3). In addition, these cancers may develop in a hormonal background that enables mutagenesis and hence outgrowth of abnormal clones. Breast carcinomas associated

with germline mutations in cancer genes make up the minority of carcinomas (Heng et al., 2017). The most important risk factors for sporadic cancers in women are estrogenic stimulation and age.

2.2.1 Molecular carcinogenesis of familial breast cancer

One-quarter to one-third of BCs are familial and occur due to the inheritance of a susceptibility gene or genes. Single gene mutations with moderate to high penetrance account for 8% to 17% of breast carcinomas (Table 2.2). Likewise, inheritance has a critical part in an additional 15% to 20% of women based on a positive family history, defined as an affected first-degree relative (mother, sister, or daughter), cancer in multiple relatives, and early-onset cancers. In these cases, what happens is that inheritance of a single susceptibility gene with low penetrance or combinations of genes interact to increase risk. The essential high penetrance susceptibility genes for familial BC are tumour suppressors like p53, BRCA1, BRCA2 and CHEK2, regulating genomic stability or are involved in pro-growth signalling pathways. Normally, cells that incur DNA damage undergo cell cycle arrest and either repair their damaged DNA or die by apoptosis. *ATM* senses DNA damage and "activates" the guardian of the genome p53, thereby inducing cell cycle arrest and, if DNA repair is unsuccessful, apoptosis. *BRCA1, BRCA2,* and *CHEK2* all have important functions in the repair of double-stranded DNA breaks. Defect in any of these tumour suppressors increases the likelihood of permanent DNA damage, leading to potentially oncogenic mutations that will be passed to the daughter cells.

Table 2.2: Most common single gene mutations associated with hereditary susceptibility to breast cancer

Gene (Syndrome)	**% of Single Gene Cancers***	**Risk of Breast Cancer to Age 70#**	**Comments**

Table 2.2 Continued

High Penetrance Germline Mutations (>4-fold increased risk; 3%–7% of breast cancers)			
BRCA1 (familial breast and ovarian cancer)	~55%	~40%–90%, females; 1% males	The majority of cancers are TNBC
BRCA2 (familial breast and ovarian cancer)	~35%	~30%–60%, females; 6% males	The majority of cancers are ER positive. Biallelic mutations cause a form of Fanconi anaemia.
TP53 (Li-Fraumeni)	<1%	~50%–60%, females; <1%, males	The majority of cancers are ER and HER2 positive
PTEN (Cowden)	<1%	~20%–80%, females; <1%, males	Also associated with benign tumours
STK11 (Peutz-Jeghers)	<1%	~40%–60%, females	Also associated with benign colon polyps
CDH1 (hereditary diffuse gastric cancer)	<1%	~50%, females	The majority of cancers are lobular in type
PALPB2 (hereditary breast cancer)	<1%	~30%–60%, females; <1%, males	Biallelic mutations cause a form of Fanconi anaemia
Moderate Penetrance Germline Mutations (2- to 4-fold increased risk; 5% to 10% of breast cancers)			
ATM (ataxia-telangiectasia)	~5%	~15%–30%, females	Biallelic mutations cause ataxia-telangiectasia
CHEK2 (hereditary breast cancer)	~5%	~10%–30%, females	The majority of cancers are ER-positive
*The percentage of all breast cancers associated with a germline mutation conferring an increased risk of breast cancer. #Risk for specific patients can vary with the specific mutation and the presence of other gene mutations. ER, Estrogen receptor; TNBC, triple-negative breast cancer.			

(Source: Robbins Pathologic Basis_10E_ 2020: Chapter 23 The Breast p. 1049)

2.2.2 Molecular carcinogenesis of sporadic breast cancer

Breast carcinogenesis occurs via estrogen-positive and estrogen-negative pathways (Figure 2.3). The estrogen-positive pathway is the dominant pathway of breast carcinogenesis, and via this pathway, ***luminal cancers*** arise (Aure et al., 2017). Estrogen increases the local production of growth factors, such as transforming growth

factor α, platelet-derived growth factor, and fibroblast growth factor, and regulates the expression of dozens of genes in breast epithelial cells that may directly contribute to tumour growth and development. Estrogen exposure also stimulates the proliferation of breast epithelial cells during puberty, menstrual cycles, and pregnancy, thereby increasing the number of cells that are "at risk" for transformation (Ciriello et al., 2013). The DNA replication that attends cellular proliferation is conducive to the accumulation of mutations. The gap in cell division that occurs during the latter part of the menstrual cycle may permit time for defective DNA repair and mutations to become "fixed" in the genome. Repetition of this process during each cycle may underlie the association between the cumulative number of menstrual cycles a woman experiences and her risk of developing BC, as well as the strong association between luminal cancers and age. Some luminal cancers eventually escape from estrogen dependence through several mechanisms. These include the outgrowth of clones that lack ER expression, compensatory alterations in related growth factor signalling pathways, or acquisition of mutations in the ER gene *(ESR1)* that lead to estrogen-independent ER function.

The HER2-positive cancers arise through a pathway strongly associated with amplifying the HER2 gene on chromosome 17q and can develop via the estrogen-dependent and -independent pathway. In these cancers, HER2 acts as an oncogenic "driver." Clinically diagnosis can be made by detecting HER2 overexpression by immunohistochemistry or HER2 gene amplification by in situ hybridization (Marchiò et al., 2021).

The TNBCs arise through an estrogen-independent pathway that is not associated with HER2 gene amplification. A possible precursor lesion of morphologically normal cells that overexpress p53 has been identified (analogous to