

Role of Novel Therapeutic Agents in Modulating Invadopodia Formation in Metastatic Breast Cancer

(Peranan Agen Terapi Baru dalam Memodulasikan Pembentukan Invadopodia dalam Kanser Payudara Metastatik)

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ABSTRACT

The ability to colonize distant organs which is lethal has made metastatic breast cancer become the top ten causes of mortality worldwide. Specialized actin-rich protrusions termed invadopodia were thought to be formed by highly invasive cells to degrade the extracellular matrix to drive cancer invasion and metastasis. Identification of compound(s) to hinder the formation of invadopodia is important to resist the metastasis of breast cancer as well as to yield anti-metastasis targeted therapy. The current review aims to provide new insights on cancer invasion and candidate compound(s) capable to disrupt invadopodia formation and invadopodia-related proteins.

Keywords: Cancer invasion; invadopodia; metastatic breast cancer

ABSTRAK

Keupayaan untuk merebak ke organ lain telah menjadikan kanser payudara metastatik antara puluhan penyebab kematian di seluruh dunia. Protusi khusus diperkaya-aktin ataupun invadopodia dikatakan terbentuk daripada sel-sel yang invasif sehingga mendegradasi matriks ekstrasel yang seterusnya mendorong kepada serangan kanser dan metastasis. Pengenalpastian kompaun untuk menghalang pembentukan invadopodia adalah penting untuk menentang kanser payudara metastatik serta mencari terapi sasaran anti-metastatik. Ulasan ini bertujuan untuk memberikan pandangan baru mengenai serangan kanser dan kompaun yang berkemungkinan menghalang pembentukan invadopodia dan protein berkaitan dengan invadopodia.

Kata kunci: Invadopodia; kanser payudara metastatik; serangan kanser

INTRODUCTION

Breast cancer incidences in Malaysia has recorded 31.1% cases from 2007-2011 which precede other cancer cases (Azizah et al. 2015). Breast cancer is widely recognized to disseminate to other parts of the body known as cancer metastasis (Scully et al. 2012) and it is also one of the hallmarks of cancer (Hanahan & Weinberg 2011). Triple negative breast cancer (TNBC) is characterized by negative expression of estrogen receptor, progesterone receptor and HER2 (human epidermal growth factor receptor 2). TNBC is often associated with poor prognosis and higher death rate (Anders & Carey 2008). Metastatic breast cancer patients frequently confront relapse after receiving the treatments indicating the failure of primary therapy (Dent et al. 2007). Since current treatments are also producing side effects and have promoted metastasis and recurrence, chemoprevention study by the examination of new compounds or drugs should be enthusiastically done (Weigelt et al. 2005). The first part of this review will discuss on the metastatic cascade and the proteins related to invadopodia and cancer invasion. The later part of the review will focus on the possible compound(s) that may

target invadopodia-related proteins to delay breast cancer metastasis.

METASTATIC CASCADES

Metastasis is one of the life-threatening events occurred among cancer patients (Steeg 2016). The concept of metastasis has been proposed since 1889 by Stephen Paget with the theory of seed and soil to define the journey of cancer dissemination (Paget 1889). Metastasis has been postulated that it does not occur by chance but the cancer cells ('seed') need to be well-matched with the organ of interest ('soil') then only the metastatic cancer cells can populate the new 'home' (Paget 1889). Study on cancer metastasis evolves with the findings that this process requires mechanical factors and the heterogeneity of the metastasis itself have caused the cancer to be difficult to be treated (Massagué et al. 2017). Cancer metastasis consists of several chronological steps which are interrelated to each other (Massagué et al. 2017). The tumor cells need to break away from the primary tumor to metastasize (Mendonsa et al. 2018). The acquisition of escaping from the primary tumor

happened due to the altered adhesion; loss of E-cadherin which initiate the tumor to detach from the primary site to proceed to malignancy (Riggi et al. 2018). The cells will then migrate and invade into the surrounding tissues which consist of dense meshwork of extracellular matrix (ECM) and adhere to the basement membrane of the lymphatic or blood vessels before degrading the membrane again to intravasate to the blood circulation and/or lymphatic system (Cheung & Ewald 2016). Still, to reside to the new microenvironment, the cancer cells needs to fight with the immune cells and cell death signals (Cheung & Ewald 2016). Only less than 0.01% of invasive cancer cells are able to survive the new microenvironment and metastasize after their long circulating journey (Langley & Fidler 2011).

MIGRATION AND INVASION

One of the hallmark of cancer metastasis is the spreading ability of tumor cells by the process of migration and invasion (Poste & Fidler 1980). Cell invasion involves three key steps which are the detachment of tumor cells from the primary tumor site, production of proteolytic enzymes to drive matrix dissolution and cell migration (Liotta et al. 1991). As stated previously, loss of cell-cell contact has initiated the migratory ability of the tumor cells with the release of proteases to aid the way to the blood vessels (Liotta et al. 1991). Cell migration involves the reorganization of the cytoskeleton to aid cell motility. The direction of the cells motion is pointed to the source of angiogenesis stimulus (Liotta et al. 1991). The cytoskeletal proteins implicated in cell motility are divided into three types which are microtubules, intermediate filaments, and actin filaments (also known as microfilaments) (Jiang et al. 2009). In breast cancer, the actin cytoskeleton has been found to regulate the dynamic cell motility (Pollard & Borisy 2003). The actin filaments are structured into three-dimensional (3D) networks that form either lamellipodia, filopodia and/or invadopodia in cancer cells (Chhabra & Higgs 2007). Lamellipodia are observed as protrusions at the leading-edge during cell movement, while filopodia are highly dynamic extension of the plasma membrane.

Invadopodia refers to the extension established by the F-actin that cross-linked into a network and used for invasion through the extracellular matrix (Chhabra & Higgs 2007). The family members of Rho GTPases including Rac, Rho and Cdc42 are the regulators that are well-studied for cell migration. Once the Rho GTPases are activated, these will lead to the assembly of actin-myosin contractile filaments into focal adhesion complexes which then promote the cell polarity and migration (Hall 1998). The migration of tumor cells has been studied in 2D culture systems and recently in three-dimensional (3D) culture systems (Doyle et al. 2013). Initially, the 2D method has been established to explain the concept of cell migration and their adhesion

onto the flat surface (Doyle et al. 2013). This method has guided the researchers to find out the molecular pathways involved. However, as the studies continued, the presence of ECM can also control and modulate the migration of the cancer cells which then lead the studies to use 3D culture system with addition of matrix to the model thus the invasive characteristics of cancer cells can also be investigated (Doyle et al. 2013). Moreover, the 3D model introduced to study cell migration is also mimicking the *in vivo* model thus, the molecular targets linked to cancer migration can also be scientifically investigated (Doyle et al. 2013).

Among the targets that are found by using the 3D experimental design are Cdc42, Rac1, and RhoA and the signaling proteins found are also associated with the presence of ECM (Doyle et al. 2013). Continuous studies have also reported tumor cells that migrate and invade the ECM form specialized protrusions which are called invadopodia (Artym et al. 2006; Bravo-Cordero et al. 2012; Gligorijevic et al. 2012). Migration and invasion are interrelated to each other as the cancer cells committed to both steps to detach from the origin of the tumor and continue to the subsequent growth at the new organ(s) (Sahai 2005). Application of therapies to prevent the migration and invasion are useful strategies to reduce the dissemination of tumor cells however, understanding the mechanisms and factors that promote the motility of the cancer cells should be prioritized (Sahai 2005).

INVADOPODIA

For the cancer cells to move into the distant parts of the body, researchers thought that the highly invasive cancer cells form 'invasive feet' known as invadopodia (Buccione et al. 2004). Invadopodia; specialized membrane protrusions which rich in actin cytoskeleton that extend into the ECM is thought to be the first step employed by the cancer cells to degrade the ECM and drive cancer invasion (Buccione et al. 2004). The cell-ECM contact structures has been first discovered in chicken embryonic fibroblasts transformed with the Rous Sarcoma Virus (RSV) oncogene v-Src (David-Pfeuty & Singer 1980). Later, these structures which contain actin-rich cores (Tarone et al. 1985) are described as podosomes. However, at the last stage of the discovery, when the cells are not only involved in cell-ECM contacts and adhesion but also having the potential to degrade the ECM to invade into surrounding tissues, the term invadopodia has been introduced (Chen 1989). Both podosomes and invadopodia are two terms used to characterize the protrusions that are being formed by the cells (Murphy & Courtneidge 2011). Meanwhile, podosomes have been shown to not only being formed in the cultured osteoclasts but also in other types of cells which include highly invasive normal cells; macrophages and dendritic cells, endothelial cells and vascular smooth

muscle cells (Zambonin et al. 1988). Invadopodia and podosomes require Src kinase and actin assembly as well as focal adhesion proteins, integrins, and proteases to form the protrusions and degrade ECM (Linder & Aepfelbacher 2003). In 2D gelatin degradation assay, invadopodia is recognized as actin-rich dots stained with fluorochrome dye which co-localized with black puncta or dots on gelatin or other extracellular matrix such as fibronectin and collagen which indicate the degradation area. In 3D experiment, the structure of invadopodia is more complex as the cells are growing comfortably like in a tumor microenvironment (Tolde et al. 2010; Wang et al. 2016). In a study by Tolde et al. (2010), the data showed that there is distinct characteristic of invadopodia formed in 2D culture and in the dermis-based 3D matrix. It has been shown that invadopodia which are formed in the 3D matrix have produced more prominent F-actin rich protrusions compared to invadopodia on thin matrix (2D culture). It is believed that cells cultured in 3D matrix are mimicking the *in vivo* condition and this would allow invadopodia to be introduced in a more physiological surrounding (Tolde et al. 2010). Meanwhile, A549 lung cancer cells cultured in 3D microfluidic device have also been demonstrated to appear more circular compared to 2D culture and the protrusions of invadopodia are more noticeably formed as the condition is physiologically mimicking the microenvironment of the tumor (Wang et al. 2013). There are several models of studying invadopodia *in vivo* such as by conducting the experiment in mouse tumor models, zebrafish, intestinal epithelia and *Caenorhabditis elegans* organogenesis (Lohmer et al. 2014). However, the challenges in studying invadopodia formation *in vivo* are the requirements for high-technology imaging systems to observe the 'feet' formation which are being used by the invasive cancer cells to invade into the surrounding tissues. Maybe with the findings of invadopodia in *in vivo* could be the kick-start to prove that these protrusions are to be a potential biomarker in cancer invasion in order to improve the prognosis in cancer patients (Lohmer et al. 2014).

INVADOPODIA AND THEIR COMPONENTS

Invadopodia are commonly found in highly invasive cancer cells and have been extensively studied in numerous cancer cell lines including lung (Wang et al. 2013), breast cancer and melanomas (Diaz et al. 2013; Md Hashim et al. 2013). There are plenty of studies being conducted in the past thirty years to study invadopodia associated protein driven, stimuli required for their formation, and applicability of *in vivo* study on invadopodia (Hoshino et al. 2013; Lohmer et al. 2014; Murphy & Courtneidge 2011; Tolde et al. 2010). The proteins that are found in invadopodia can be separated into several classes which are actin regulated proteins; cortactin, Neural-Wiskott Aldrich Syndrome

protein (N-WASP), actin-related proteins 2 and 3 (Arp 2/3) complex, WASP-interacting protein (WIP), cofilin, talin, fascin; signaling proteins; Cdc42, Nck1, Src, β -PIX; adhesion proteins; integrins, vinculin, paxillin, ezrin; proteases; matrix metalloproteinases-9 (MMP-9) and membrane type 1-MMP (MT1-MMP) (Hoshino et al. 2013).

Protein for actin regulations

F-actin is thought to be the biomarker for invadopodia and commonly stained in invadopodia formation study (Md Hashim et al. 2013). Cortactin, N-WASP, Arp 2/3 complex, WIP, cofilin, fascin, and talin are the proteins that are required for the regulation and stabilization of actin (Ridley 2015). Cortactin is an essential protein that promotes the nucleation of actin (Shen et al. 2015). Cortactin has been first recognized as Src kinase substrate which can be found at the sub-membranous zone (Schnoor et al. 2017). The genes encode cortactin is CTTN gene and this gene is situated in a chromosomal region 11q13 (Schnoor et al. 2017). Recent studies demonstrated that even one mutation occurred on the structure of cortactin can cause the inability of the invadopodia to be formed which indicates each structure on cortactin is necessary for the tumor cells to form invadopodia (Shen et al. 2015).

Signaling proteins

To date, numerous growth factors have been pointed out to be working on the establishment of invadopodia formation (Hoshino et al. 2013) such as colony stimulating factor-1 (CSF1), platelet product transforming growth factor β (TGF- β), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), heparin binding (HB)-EGF, hepatocyte growth factor/scatter factor (HGF) and stromal cell derived factor 1 α (SDF1 α) (Hoshino et al. 2013). These growth factors share common signaling hubs especially Src kinase, phosphoinositide 3-kinases (PI3Ks) and Rho family GTPases, which ultimately control invadopodia and podosomes (Murphy & Courtneidge 2011). Epidermal growth factor receptor (EGFR) has been proven to target Src kinase which later activates cortactin to drive invadopodia formation in breast cancer (Mader et al. 2011).

β -PIX is among the signaling protein that has been investigated to be associated in the formation of invadopodia as it is guanine nucleotide exchange factor for Rac and Cdc42 (Ridley 2015). The association of β -PIX and invadopodia formation has also been investigated in hypoxic background (Md Hashim et al. 2013). The β -PIX expression level has been up-regulated in the presence of hypoxia and β -PIX is shown to be required for invadopodia formation (Md Hashim et al. 2013). A study has also been conducted to check the relationship between β -PIX and also Src. The

results showed that the recruitment of β -PIX resulted in the activation of the Rac protein in invadopodia (Gujam et al. 2014). Besides, the regulation of cell adhesion and migration is set out to be implied with the β -PIX protein (Kuo et al. 2011).

PROTEASES

Proteases are recruited to invadopodia to degrade the matrix. Among matrix metalloproteinase (MMP) which have been commonly found at invadopodia are MMP-2, MMP-9, MT1-MMP, and ADAM12 (Clark et al. 2007; Díaz et al. 2013). Previous study has reported the role of cortactin in the production of invadopodia-associated MMPs: MMP-2, MMP-9, and MT1-MMP (Artym et al. 2006). Remarkably, cortactin has been shown to inhibit MMPs which then decrease the invadopodia actin puncta formation (Artym et al. 2006). A more recent study has been conducted to observe the synergistic effect on blocking cancer invasion using computer simulations (Ichikawa 2015). The data has shown that blocking MT1-MMPs alone; which are among the proteases that located at invadopodia is not enough but combination of several strategies to block MT1-MMPs could be possible ways to hinder invasion (Ichikawa 2015). Recently, a gene associated with the metastasis to bone in breast cancer which is Target of MYB1-like protein 1 (TOM1L1) (also known as Srcasm) has been recognized (Smid et al. 2006). This gene has been co-amplified with ERBB2 in breast cancer and is postulated to function as pro-oncogene (Orsetti et al. 2004). The TOM1L1 protein is an adaptor protein of the TOM1 family with post-Golgi trafficking and signaling functions (Wang et al. 2010). The TOM1L1 protein has been demonstrated to promote ERBB2-induced cell invasion by increasing the formation of invadopodia in favor of the MT1-MMP trafficking to the plasma membrane (Chevalier et al. 2016). Further investigation showed that TOM1L1 has regulated the MT1-MMP and the underlying mechanism of MT1-MMP trafficking in ERBB2-transformed cells involved the RAB-7 protein (Chevalier et al. 2016).

CANDIDATE COMPOUNDS TO INHIBIT INVADOPODIA

SAFFLOWER

A Chinese medicine named safflower has been tested on the formation of invadopodia in MDA-MB-231 breast cancer cells in which a reduction in invadopodia formation was observed and it is believed due to the restructuring of the cytoskeleton (Fu et al. 2016). In this study, the expression of matrix metalloproteinase-9 (MMP-9) and the phosphorylation of Src (p-Src) has been reduced after safflower treatment. It has been speculated that the reduction in the formation of invadopodia was partly due to the reduction of MMP-9 and p-Src (Fu et al. 2016).

CURCUMIN

Curcumin is an active compound in turmeric and was reported to exhibit anti-bacterial, anti-oxidant, anti-inflammation and anti-cancer activities (Gupta et al. 2012; Lee et al. 2016). Previous studies demonstrated that curcumin exerts anti-invasive effects in human breast cancer cells via downregulation of the MMP-2 and up-regulation of tissue inhibitor of metalloproteinase (TIMP-1) (Di et al. 2003; Hassan & Daghestani 2012; Shao et al. 2002). Besides, curcumin also inhibited the vascular endothelial growth factor (VEGF) and b-FGF (basic fibroblast growth factor) in ER-negative breast cancer cells thus reducing the tumor invasion (Shao et al. 2002). In MDA-MB-231 cells, treatment with curcumin inhibits cell proliferation and migration via NF- κ B pathway (Chiu & Su 2009). In addition, curcumin suppressed the migration and invasion of the LPA-induced MCF-7 breast cancer cells via downregulation of RhoA/ROCK/MMPs pathway (Sun et al. 2016).

The Cdc42 protein is overexpressed in many cancer cells and involved in the formation of invadopodia. The suppression of Cdc42 protein by curcumin is reported through the inhibition of reorganization of F-actin (Chen et al. 2012). Silencing of Cdc42 has also reduced the expression of p-cofilin and p-PAK-1 and increased the expression of E-cadherin (Chen et al. 2012). The Rac1 protein is another molecule associated with cytoskeleton rearrangement, cell adhesion, transcriptional activation and formation of invadopodia (Ward et al. 2015). Curcumin has been demonstrated to inhibit Rac1 in 801D lung cancer cells and reduce the migration and invasion of Rac1 overexpressed cells. The MMP-2 and -9 and also PAK1 expressions are inhibited in the Rac1 overexpressed cells suggesting that curcumin inhibit the 801D lung cancer migration and invasion via Rac1/PAK1 pathway (Chen et al. 2014). In spite of all the findings associated with curcumin, the bioavailability problem that curcumin displayed has been questioned and has encouraged the researchers to attempt on alternatives to overcome the issue by synthesizing curcumin analogue as nanoparticle (Krausz et al. 2015; Tham et al. 2010).

BHMC

BHMC is curcumin analogue, known as 2,6-bis-(4-hydroxyl-3-methoxybenzylidene) cyclohexanone. The presence of the β -diketone moiety in curcumin caused it to be rapidly metabolized in the liver by aldo-keto reductase, therefore limiting its therapeutic benefits on various types of diseases (Anand et al. 2007). The phenolic OH group is shown to be crucial for its anti-oxidative effects (Koo et al. 2015). The BHMC is synthesized based on the parental structure by replacing the unstable β -diketone moiety with conjugated

double bonds while preserving the phenolic OH group. As a curcuminoid analogue, BHMC has been studied in inflammation, sepsis and hyperalgesia (Ming-Tatt et al. 2013; Tham et al. 2010). Treatment with BHMC has shown to inhibit the pro-inflammatory cytokines and signaling pathways of inflammation as well as to protect lethal sepsis of the caecal-ligation puncture (CLP) model of severe sepsis (Tham et al. 2011). Besides, BHMC also exhibits anti-hyperalgesic effect in neuropathic pain model in mice (Ming-Tatt et al. 2013). Furthermore, BHMC treatment on murine 4T1 breast cancer cells (Razak et al. 2017) reduced the number of mitotic cells in 4T1 challenged mice demonstrating the anti-cancer effects by BHMC. Besides, the study also reported a reduction of metastatic 4T1 cells to the lung. The expression of MMP-9, TNF- α and NF- κ B genes have been reduced in the BHMC treated mice in comparison to the non-treated group (Razak et al. 2017). Recently, the effect of BHMC on the invadopodia formation in MDA-MB-231 cells was carried out and the findings showed that BHMC was able to reduce the number of cells forming invadopodia (Harun et al. 2018). Additionally, BHMC was able to suppress the expression of invadopodia-related proteins such as MT1-MMP, MMP-9 and β -PIX (Harun et al. 2018). Hence, it would

be interesting to evaluate the effect of BHMC on other possible targeted molecules that link invadopodia and BHMC in the search of a direct association of BHMC and the invasiveness of breast cancer.

RUTHENIUM COMPLEXES

Ruthenium (Ru) complexes, belong to the platinum-metal group have been shown to exhibit anticancer activities against breast cancer (Bergamo et al. 2010; Scolaro et al. 2005; Wu et al. 2014). In 2010, Bergamo et al. reported that treatment with Ru compound (RM175) for two weeks resulted in a reduction in breast tumor growth by 50% when compared to untreated control. This study also reported a reduction in secondary lung metastasis. The anti-metastatic effects of RM175 were shown to be mediated via the inhibition in MMP-2 production (Bergamo et al. 2010). RAWQ11 is a novel Ru complex agent found to inhibit invadopodia formation via inhibition in the AKT signaling pathway and upregulation of PTEN expression in MDA-MB-231 breast cancer cells (Wu et al. 2014). Treatment with RAWQ11 decreased the expression of MMP-2 and MMP-9 in MDA-MB-231 cells (Wu et al. 2014). The potential compounds targeting invadopodia-related proteins that we describe in this review are summarized in Table 1.

TABLE 1. Summary proteins involved in invadopodia formation and maturation including potential compounds being studied

Invadopodia-targeted proteins	Functions	Candidature compounds being investigated
phospho-Src (p-Src)	Promotes invadopodia formation (Balzer et al. 2010)	Safflower (Fu et al. 2016)
MMP-2	Involves in collagen and gelatin degradation and promotes angiogenesis (Egeblad & Werb 2002)	Curcumin (Shao et al. 2002) RAWQ11 (Wu et al. 2014)
MMP-9	Collagen degradation and promotes angiogenesis (Egeblad & Werb 2002)	Safflower (Fu et al. 2016) BHMC (Harun et al. 2018; Razak et al. 2017) RAWQ11 (Wu et al. 2014)
MT1-MMP	Promotes degradation of ECM (Miyazawa et al. 2013)	BHMC (Harun et al. 2018)
β -PIX	Involves in activation of Rac protein in invadopodia (Gujam et al. 2014)	BHMC (Harun et al. 2018)
Cdc42	Involves in invadosome formation (Di Martino et al. 2014)	Curcumin (Chen et al. 2012)
TNF- α	Promotes cell migration and invasion (Razak et al. 2017)	BHMC (Razak et al. 2017)
NF- κ B	Control cell survival and promotes tumor angiogenesis (Neil & Schiemann 2008; Tang et al. 2017)	Curcumin (Chiu & Su 2009) BHMC (Razak et al. 2017)
Rac1	Promotes invadopodia formation and actin reorganization (Revach et al. 2016)	Curcumin (Chen et al. 2014)

CONCLUSION

Invadopodia are believed to be the architect to drive cancer invasion. With the proteins related to invadopodia to be investigated thoroughly, we may also have a kick start to investigate the candidature compound(s) that may hit the invadopodia-targeted proteins as to hinder breast cancer invasion. The findings of the investigation might be valuable for the groundwork of anti- metastatic therapy in the future.

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