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REVIEW

Circulating tumor cell clusters-associated gene *plakoglobin* and breast cancer survival

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Abstract Breast cancer recurrence is a major cause of the disease-specific death. Circulating tumor cells (CTCs) are negatively associated with breast cancer survival. Plakoglobin, a cell adhesion protein, was recently reported as a determinant of CTCs types, single or clustered ones. Here, we aim to summarize the studies on the roles of *plakoglobin* and evaluate the association of *plakoglobin* and breast cancer survival. Plakoglobin as a key component in both cell adhesion and the signaling pathways was briefly reviewed first. Then the double-edge functions of plakoglobin in tumors and its association with CTCs and breast cancer metastasis were introduced. Finally, based on an open-access database, the association between *plakoglobin* and breast cancer survival was investigated using univariate and multivariate survival analyses. Plakoglobin may be a molecule functioning as a double-edge sword. Loss of *plakoglobin* expression leads to increased motility of epithelial cells, thereby promoting epithelial–mesenchymal transition and further metastasis of cancer. However, studies also show that *plakoglobin* can function as an oncogene. High expression of *plakoglobin* results in clustered tumor cells in circulation with high metastatic potential in breast cancer and shortened patient survival. *Plakoglobin* may be a potential prognostic biomarker that can be exploited to develop as a therapeutic target for breast cancer.

Keywords Breast cancer metastasis · Circulating tumor cells (CTCs) · *plakoglobin* (*Junction plakoglobin*, *JUP*)

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Introduction

Breast cancer (BC) is the most common and the most deadly cancer worldwide in women. In 2015, it is estimated that additional 231,840 women will be diagnosed with breast cancer, and 40, 290 women will die of the disease in United States [1]. The recurrence of breast cancer is a major cause of the disease-specific death, which often occurs typically within 5 years or even up to 10–20 years after surgery, since the recurrence is usually more aggressive and untreatable [2–4]. Accumulating evidence has shown that cancer stem cells (CSCs) are a culprit of the recurrence, metastasis, and resistance to traditional chemotherapy in human cancer including breast, thereby shortening patient survival [5, 6]. Metastases can occur at the early stage of breast cancer [7, 8]. Disseminated breast CSCs either are in quiescent status living somewhere in the body or grow again and lead to recurrence once they meet favorable niches [9–11].

Circulating tumor cells (CTCs) are those tumor cells detaching from primary tumor tissues and circulating in the bloodstream after intravasation. Accumulating evidence shows that CTCs are linked to metastatic relapse and are

regarded as a prognostic marker for human cancer including breast, prostate, lung, and colorectal cancer [12–17]. CTCs also demonstrate the properties of CSCs that can generate the diverse tumor cells in immunodeficient mice [18]. Functional xenograft assays show that primary human luminal breast cancer-derived CTCs contain metastasis-initiating cells (MICs) with the phenotypes of $EPCAM^{low}$ MET^{high} $CD47^{high}$ $CD44^{high}$ [19]. The presence of CTCs clusters in the blood of patients with cancer has attracted attention, since CTCs clusters show more metastatic potential than single CTCs [20, 21]. It has been demonstrated that CTCs could aggregate with other types of cells that are present in the circulation, for example, platelets and leukocytes [22–24]. These accompanying components have either protective or cytotoxic effects on CTCs. On the other hand, tumor cells could also detach in clusters with either stromal or tumor cells from primary tumor tissues and enter into the circulation as partners to start their journey; the clustered cells traveling through the bloodstream facilitate the growth of metastatic loci at a distant site [20, 25].

Cell–cell adhesion is a determinant of CTCs in the form of either single or clustered cells, between which significant differences in the expression of *junction plakoglobin* (*JUP,* or *plakoglobin*) have been shown [20]. Plakoglobin is an important component of desmosomes (a junctional complex structure for cell–cell adhesion) and adherence junctions [20, 26]. Studies have shown that plakoglobin plays a key role in controlling the motility of epithelial cells [27–30]. The cells with upregulated levels of *plakoglobin* show lower motility, while those with low *plakoglobin* levels display high metastatic potential [27–30]. However, the association between *plakoglobin* and malignancies still remains controversial [31–33]. In this review, we summarized recent findings on the role of *plakoglobin* in breast cancer metastasis, as well as evaluated the association between *plakoglobin* and breast cancer survival using an open-access database of gene expression-based outcome for breast cancer.

Plakoglobin mediates cell adhesion

Junction plakoglobin (*JUP* or *plakoglobin*) gene is located on chromosome 17, neighboring breast cancer 1, early onset gene (*BRCA1*). *Plakoglobin* encodes an 83-kDa cell adhesion protein of γ-catenin (also known as plakoglobin), a homolog of β-catenin [34, 35]. The plakoglobin stability is associated with the status of threonine 14 in its amino acid sequence; the post-translational glycosylation of threonine 14 increases the stabilization of plakoglobin, which may prevent the access of proteasome for degradation [36]. Localization staining shows that plakoglobin is expressed in both desmosomes and the adherent junction [34]. Like β-catenin, plakoglobin can be a linker between E-cadherin (a calcium-dependent cell surface glycoprotein) and α-catenin in cell–cell adhesion (Fig. 1), stabilizing the localization of E-cadherin in cell surface [35, 37, 38]. Studies have shown that plakoglobin plays an important role in the formation of desmosomes, promoting the binding of desmoplakin proteins to intermediate cytoskeletal filaments, and recruiting plakophilin 3 to the membrane, where cadherin proteins are enriched [39, 40]. The positive associations of plakoglobin with both adherens junction and desmosome cadherin were observed in keratinocytes that overexpress UDP-*N*-acetylglucosamine-polypeptide β-*N*-acetylglucosaminyl transferase (*O*-GlcNAc transferase, OGT), which glycosylates plakoglobin protein [36]. Moreover, there is a dose-dependent correlation between plakoglobin levels and the function of cell adhesion, making cells functional coordination. For instance, $JUP^{-/-}$ mice displayed an embryonic lethal phenotype due to cell dissociation in the heart [41], whereas heterozygous $JUP^{+/-}$-deficient mice showed increased right ventricular volume with reduced right ventricular function, although the levels of β-catenin and N-cadherin did not change [42, 43]. This intercellular adhesion makes both epithelial and non-epithelial cells endure mechanical stress and maintain organ morphogenesis and cell polarity. Loss of the desmosomal assembly leads to the cytoskeletal reorganization and loss of polarity of epithelial cells, thereby increasing the capacity of cell migration and invasion with acquisition of metastatic seeding and stemness traits of epithelial–mesenchymal transition (EMT) [44–47]. A squamous cell carcinoma SCC9 cell line, which is insufficient in the expression of both *plakoglobin* and E-cadherin, exhibited a mesenchymal–epithelial transition (MET) upon the enforced *plakoglobin* expression [48, 49]. Another study demonstrated that ectopically expressed plakoglobin resulted in the decreases in metastatic potential by enhancing intercellular adhesive strength in prostate cancer [50]. A very recent study found that *plakoglobin* expression was downregulated by hepatitis C virus protein, which can induce EMT in human hepatocytes [51].

Plakoglobin is involved in cell signaling

Evidence that plakoglobin regulates the shuttle of different transcription factors to the nucleus suggests that it is also involved in cell signaling besides cell adhesion [52]. In consistence with the hypothesis of plakoglobin as cell signaling molecules, the interaction between plakoglobin and cytoplasmic domain of desmoglein could induce the alteration of downstream molecules, leading to the suppression of

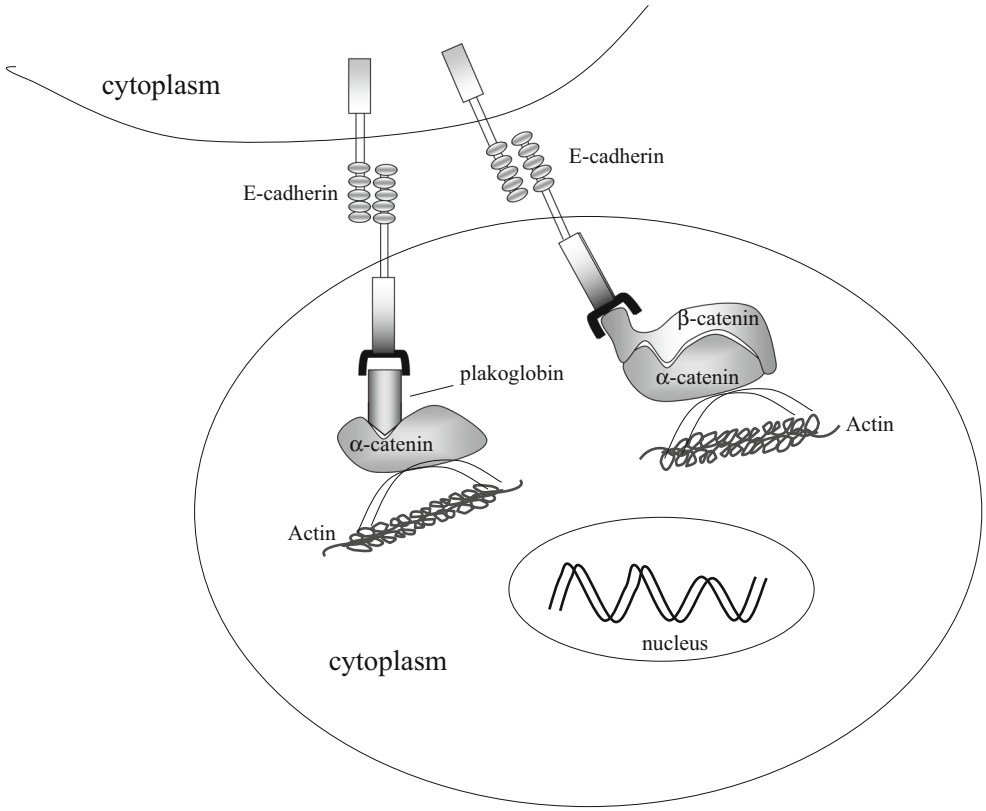


Fig. 1 Cell adhesion molecule of plakoglobin. Both plakoglobin and β-catenin can be a linker between E-cadherin (a transmembrane glycoprotein) and α-catenin that attaches to actin filaments (cytoskeleton elements), forming cell junction complex. The complex can enhance the endurance against mechanical stress and maintain cell polarity and organ morphogenesis

dorsalized gastrulation and anterior axis duplication in fertilized *Xenopus* embryos [53]. Recently, it has been reported that plakoglobin is involved in extracellular matrix (ECM)-Src (SRC proto-oncogene, non-receptor tyrosine kinase) and RhoGTPase-dependent pathways, controlling cell motility by inhibiting Src kinase [27], and keeping ECM protein vitronectin (CN) at a low level [50]. Moreover, evidence has exhibited that plakoglobin also participates in the Wnt signaling pathway [54], and it functions as an antagonist [55]. Further study has shown that the expressions of downstream genes in the Wnt signaling pathway increased in the *plakoglobin*-deficient zebrafish models, whereas *plakoglobin*$^{-/-}$-induced cardiac phenotype could be rescued by the expression of the Wnt inhibitor *Dkk1* [55]. In addition, Spindler et al. [56] demonstrated that plakoglobin was involved in the phosphorylation of p38 mitogen-activated protein kinase (p38MAPK). Knockdown of *plakoglobin* expression with the silencing RNA led to the activation of p38MAPK and reduced cell adhesion [56], thereby activating downstream effectors of mitogen-activated protein kinase-activated protein (MAPKAP) kinase 2 and heat shock protein 27 (HSP27) [57, 58]. Furthermore, plakoglobin can act as a transcription factor, regulating the expression of desmocollins 2 (*Dsc*2) or Dsc3 [59]. With the help of lymphoid enhancer factor 1 (Lef-1), plakoglobin translocates into cell nucleus and binds to the promoters of *Dsc*2 and *Dsc*3. However, this binding can be blocked by T cell factor (TCF)/Lef-1 complex [59, 60]. Similarly in tumor cells, reporter assays exhibited that plakoglobin was a key regulator of several genes, such as pituitary tumor transforming gene (*PTTG*) [61], special AT-rich sequence binding protein 1 (*SATB1*), and metastasis suppressor Nm23-H1 (*NME1*) [62]. Although plakoglobin can bind to the promoters of both *SATB1* and *NME1*, it showed differentiated regulatory roles; ectopic expression of *plakoglobin* results in decreases in the levels of *SATB1*, but not *NME1* [62].

Interestingly, plakoglobin may also be a regulator in glucose intolerance via the involvement of insulin signaling. In skeletal muscle, it has been shown that plakoglobin could bind to the insulin receptor and PI3K subunit p85, promoting PI3K-Akt-FoxO signaling, and enhancing glucose uptake to maintain glucose homeostasis [63]. The ubiquitin ligase tripartite motif-containing protein 32 (Trim32) could act as a suppressor of plakoglobin activity; overexpression of *Trim32* could induce glucose intolerance and cause muscle atrophy, whereas the inhibition of *Trim32* expression could release its suppressive role in plakoglobin-mediating insulin signaling pathway, making cells sensitize to insulin [63].

Plakoglobin: an oncogene or a tumor suppressor?

There are controversial reports on whether plakoglobin is an oncogene or tumor suppressor. Some studies indicate that plakoglobin has oncogenic activities. Kolligs et al. reported that enforced over-expression of *plakoglobin* in rat RK3E epithelial cells, in which considerable amounts of endogenous plakoglobin and β-catenin are expressed, promoted neoplastic transformation; the underlying molecular mechanism was that *plakoglobin* over-expression led to upregulation of c-Myc and activation of TCF/Lef signaling [31]. Similarly, ectopic over-expression of *plakoglobin* in HCT116 cells, a cell line carrying both wild-type adenomatous polyposis coli (*APC*) and *p53*, could result in the enhanced invasive capacity by decreasing Ecadherin and upregulating c-Myc [61]. Chen et al. showed that Desmoglein 3 (DSG3)/plakoglobin/TCF/Lef pathway facilitated cancer growth and invasion [64]. The authors found that knockdown of *DSG3* disrupted its association with plakoglobin and led to the down-regulated expression in the downstream target genes of c-*myc*, *cyclin D1*, and *MMP-7*, thereby inhibiting cell migration and invasion [64].

Plakoglobin also acts as a tumor suppressor, inhibiting tumor growth, migration, and invasion in some in vitro experiments [62]. In the *plakoglobin*-overexpressing cells, the BrdU incorporation is significantly decreased compared to their parental cells [62]. Loss of plakoglobin resulting from latent membrane protein 1 (LMP1) of Epstein–Barr virus (EBV) may also activate PI3 K/Akt/NF-*k*B signaling, thereby engaging in EBV-induced metastasis [28]. Restoration of plakoglobin could, however, inhibit LMP1-induced tumor invasion [28]. Moreover, plakoglobin could interact with the sex-determining region Y box 4 (SOX4) in response to the Wnt signaling in breast and prostate cancer cell lines, blocking the SOX4-DNA binding, and suppressing the Wnt-responsive transcription [65]. This blockade may reduce the metastatic potential and improve survival of breast cancer given that SOX4 is positively associated with distant metastases and death of the tumor [66, 67]. In addition, plakoglobin may also act as a tumor suppressor via enhancing the transcriptional activity of p53. It has been shown that plakoglobin could bind to the p53 consensus sequence in the promoter of *SFN* gene, inducing the expression of 14-3-3σ (also called stratifin, encoded by *SFN* gene) in MCF-7 cells [68].

Taken together, these findings suggest that plakoglobin functions as a two-edge sword as either an oncogene or tumor suppressor, depending on the cellular context and the activated downstream signaling pathways it regulates.

Plakoglobin, CTCs, and breast cancer metastasis

In *BRCA1*-associated breast cancer, loss of heterozygosity of *plakoglobin* is also common [69]. *Plakoglobin* mutation increases the risk of breast cancer [70]. Based on the datasets of breast cancer in The Oncomine® Platform (http://www.oncomine.org), *plakoglobin* was co-expressed in a strong correlation (correlation coefficients were 0.71–0.75) with epithelial cell adhesion molecule (EPCAM) [71, 72], a transmembrane glycoprotein that is involved in cell adhesion and cell signaling [73, 74]. This co-downregulation of plakoglobin and E-cadherin was also observed in other malignant cells [75]. Insufficient expression of *plakoglobin* could promote EMT, and loss of cell–cell adhesion is thought as the first necessary step for tumor cells to leave primary loci and enter the circulation. Studies have shown that low levels of *plakoglobin* expression are positively associated with high metastatic potential in breast cancer [76–78]. Axillary lymph node metastases showed a lower percentage of plakoglobin immunostatining than the regional metastases [79]. *Plakoglobin* silencing in vitro leads to the decrease in cell–cell contact and in vivo results in the increase of breast cancer dissemination [80]. In addition, studies also indicate the presence of an E2-box element in the promoter of *plakoglobin* gene. This element can be bound by the zinc finger transcription factor SLUG, which is highly expressed in triple-negative breast cancer [81, 82] and is a key regulator in EMT and stem cell phenotypes [83, 84]. Through recruiting co-repressor C-terminal binding protein 1 (CtBP1) and histone deacetylase 1 (HDAC1), SLUG inhibits *plakoglobin* expression [77, 85, 86].

Tumor metastasis, a major cause of cancer-specific mortality, is a complex process with a series of steps. First, tumor cells leave the primary disease loci, go through the extracellular matrix (ECM) and intravasate into circulation and lymphatic vessels. Then, CTCs survive all kinds of body defense systems, extravasate, and adapt to the new niches. Finally, as seeds, CTCs colonize and proliferate to form new tumor loci in new places [87]. Compared to via the blood systems, tumor spread via lymphatic vessels is still poorly understood [88]. CTCs in blood are present in different forms, single CTCs, clustered CTCs, and cloaked CTCs by platelets or coagulation factors [23, 89]. The diameters of all these types of CTCs are much larger than the bores of distal capillaries. Thus, most of CTCs are trapped and cleared out, resulting in the rare number of CTCs in the circulation [87, 88], which may be the subpopulation of CTCs with extremely small size and/or considerable flexibility to go through capillary beds, or those surviving through bypass tracts of capillary beds. Based on the currently available Chip-capturing detection methods, there were greater than 500 CTCs per 7.5 ml of blood in

approximately less than 1.5 % of patients with progressive breast cancer [19]. Clustered CTCs are most likely directly derived from the primary tumors, rather than the proliferation of single CTCs or the aggregation of circulating CTCs [20]. Although the clearance rate of clustered CTCs is higher than that of single CTCs, clustered CTCs have a higher metastatic potential to lung than single CTCs [20]. Clustered CTCs that account for only 2–5 % of all detectable CTCs in the circulation contribute to appropriately 50 % of all metastatic breast cancer loci in orthotopic breast cancer models [20]. Moreover, the clustered CTCs-derived lung metastases are more resistant to apoptosis than single CTCs-derived lung loci, and metastatic tumors expand more rapidly, thereby leading to shorter overall survival in mouse models [20]. In breast cancer patients, those with detectable clustered CTCs across more than three time points had significantly shorter progression-free survival than those with detectable clustered CTCs at less than 3 time points or with single CTCs [20]. The higher metastatic potential was also pronounced, leading to significantly shorter overall survival in prostate cancer patients who had detectable clustered CTCs during at least one time point than those with single CTCs only [20].

Transcriptome analyses using single-cell resolution next generation sequencing (NGS) showed that clustered CTCs consistently had a higher expression of *plakoglobin* than single CTCs, although there was no obvious difference at the global gene expression level between the two types, single and clustered CTCs [20]. As an important component of cell adherence complex, the heterogeneity of *plakoglobin* expression within primary tumors might lead to different types of CTCs, single or clustered. Plakoglobin protein staining was positive in multiple clustered CTCs, while matched single CTCs from the same breast cancer patient showed negative [20]. Interestingly, some mesenchymal markers, e.g., transforming growth factor (TGF)-β pathway components and the forhead box C1 (FOXC1) transcription factor, were also over-expressed in clustered CTCs [22, 23, 90], which may enhance the survival of clustered CTCs and interactions of cell–cell and cell–matrix during cancer spread. *In vitro* experiments showed that *plakoglobin* silencing resulted in the dissociation of cell–cell junctions in breast cancer cell lines, but not in non-transformed breast epithelial cells [20]. This finding suggested that cell–cell junctions of breast cancer cells might be more *plakoglobin*-dependent than normal epithelial cells. Similarly, the potential to form lung metastasis was reduced in in vivo animal models when *plakoglobin* was silenced in the breast cancer cell lines; orthotopic xenografts results showed that *plakoglobin* silencing significantly reduced both the number of clustered CTCs and metastatic loci in lung, despite neither the growth rate of xenografted primary tumors nor the number of single CTCs derived from the primary tumor were not affected [20].

Plakoglobin expression and breast cancer survival

In a cohort of 1,956 patients with either estrogen receptor (ER)-positive, HER2-positive, or triple-negative breast cancer, Kaplan–Meier survival curves analysis showed that patients with high *plakoglobin* in the primary tumors had a significantly worse distant metastasis-free survival compared to those with low expression ($p = 0.008$) [20]; the curves

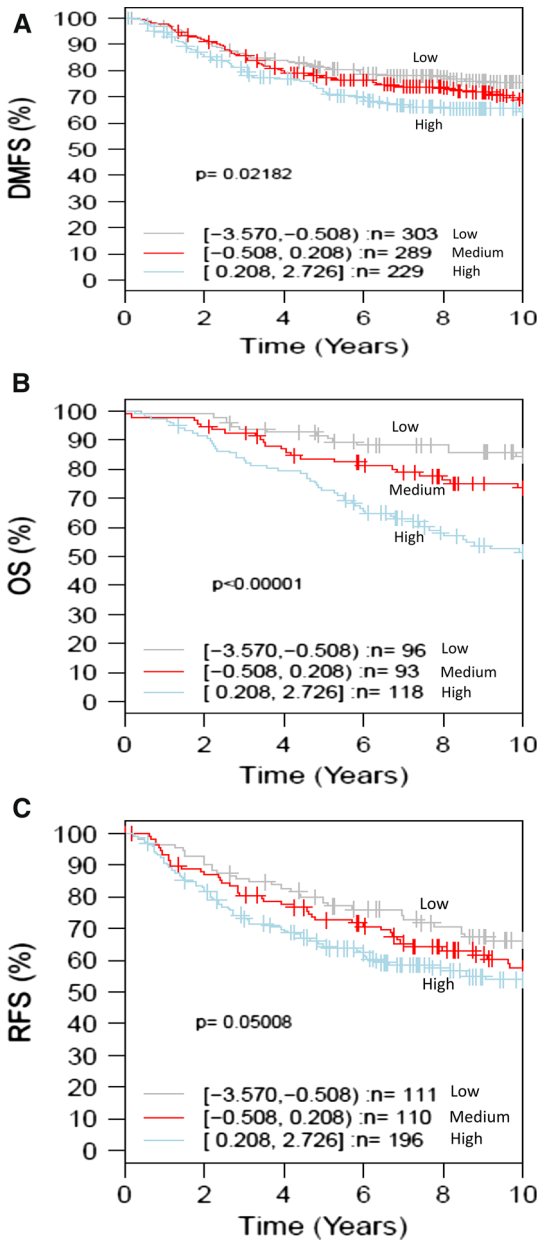
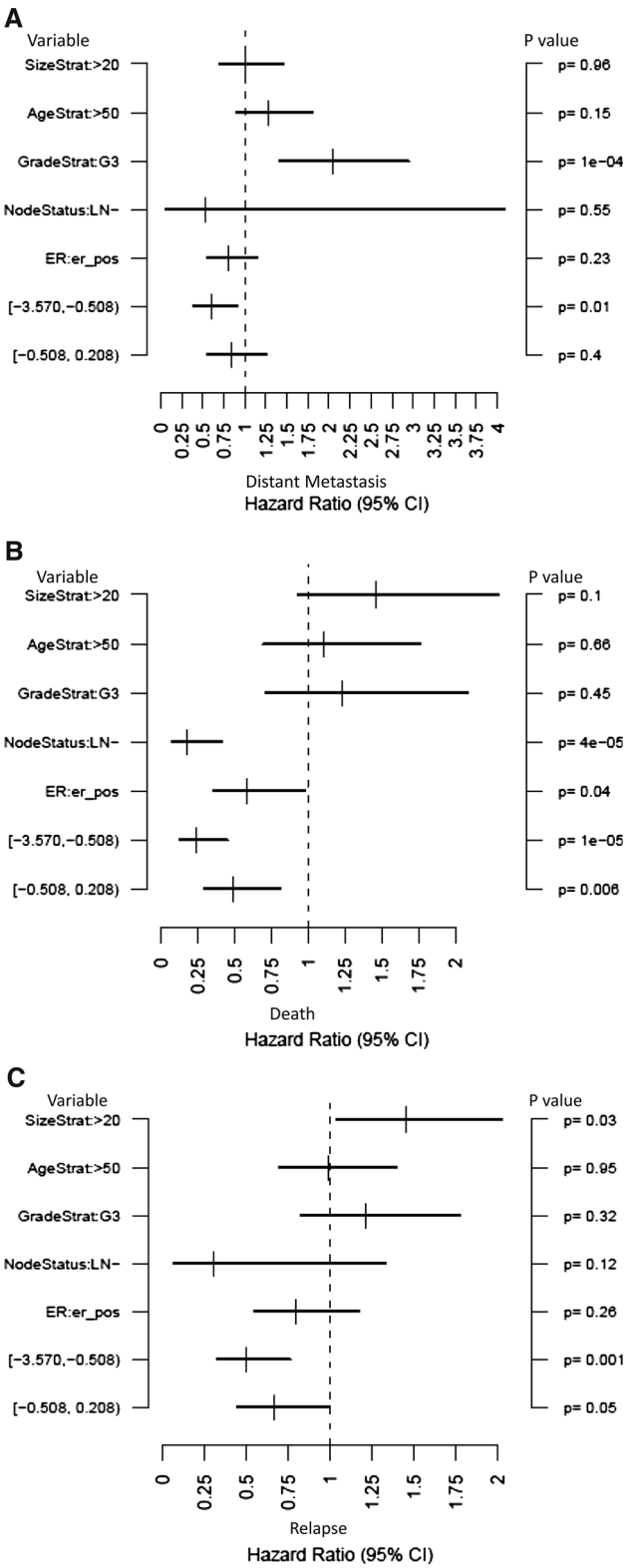


Fig. 2 Kaplan–Meier survival curves stratified by *plakoglobin* expression in breast cancer. Compared to those with high *plakoglobin* expression ($0.208 \leq$ *plakoglobin* ≤ 2.726), patients with low one ($-3.570 \leq$ *plakoglobin* < -0.508) had superior distant metastasis-free survival (DMFS) ($p = 0.02182$) (**a**), overall survival (OS) ($p < 0.00001$) (**b**), and relapse-free survival (RFS) ($p = 0.05008$) (**c**), respectively



◀**Fig. 3** Associations of *plakoglobin* expression and breast cancer patient outcome in multivariate Cox proportional hazard regression analyses. After the adjustment of estrogen status (ER), lymph node status (NodeStatus), tumor grade (GradeStrat), patient age (AgeStrat), and study (SizeStrat), patients with low *plakoglobin* expression ($-3.570 \leq$ *plakoglobin* < -0.508) and medium one ($-0.508 \leq$ *plakoglobin* < 0.208) had reduced distant metastasis risks compared to those with high one ($0.208 \leq$ *plakoglobin* ≤ 2.726); the adjusted hazard ratios (HRs) were significant ($p = 0.01$) for the low, but not statistically significant ($p = 0.4$) for the medium (**a**). Patients with low or medium *plakoglobin* expression had significantly reduced death risks compared to those with high one; the p values for their adjusted HRs were 1×10^{-5} and 0.006, respectively (**b**). Patients with low or medium one had reduced relapse risks compared to those with the high, the p values for their adjusted HRs were 0.001 and 0.05, respectively (**c**)

between low and high *plakoglobin* expression were not separated approximately until 3.5 years. Using a publicly available database on breast cancer (http://co.bmc.lu.se/gobo/) [91, 92], we analyzed the associations of *plakoglobin* expression and breast cancer 10-year survival using both univariate and multivariate analyses, in which patients were classified into three subgroups based on the tertile distribution of *plakoglobin* expression: low ($-3.570 \leq$ *plakoglobin* < -0.508), medium ($-0.508 \leq$ *plakoglobin* < 0.208), and high ($0.208 \leq$ *plakoglobin* ≤ 2.726). Kaplan–Meier survival curves showed that patients with low *plakoglobin* expression had significantly better distant metastasis-free survival (Fig. 2a, $p = 0.02182$) and overall survival (Fig. 2b, $p < 0.00001$), and borderline significantly superior relapse-free survival (Fig. 2c, $p = 0.05008$) compared to those with high one. After adjusting the potential confounding factors (which include estrogen status (ER), lymph node status (NodeStatus), tumor grade (GradeStrat), patient age (AgeStrat), and study (SizeStrat)) using multivariate Cox proportional hazard regression analyses, again, patients with low *plakoglobin* expression had significantly reduced risks of distant metastasis ($p = 0.01$), death ($p = 1 \times 10^{-5}$), and relapse ($p = 0.001$) compared to those with high one (Fig. 3). Patients with low *plakoglobin* expression had approximately 75 % reduced death risks, followed by 50 % reduced relapse risks and 40 % reduced distant metastatic risks, and that those with the medium one had approximately 50 % reduced death risks ($p = 0.006$), followed by 30 % reduced relapse risks ($p = 0.05$), 20 % reduced distant metastasis risks ($p = 0.4$). These findings suggest that *plakoglobin* expression is an independent prognostic factor in the patients with breast cancer; particularly for overall survival, those with low *plakoglobin* expression had superior survival than those with the high one. *Plakoglobin* may be a potential therapeutic target in the improvement of breast cancer survival and prevention of relapse and distant metastasis.

Conclusions

Plakoglobin is not only involved in cell adhesion, but can also be a regulator of signaling pathways. Both microenvironments and the activated signaling pathways determine its

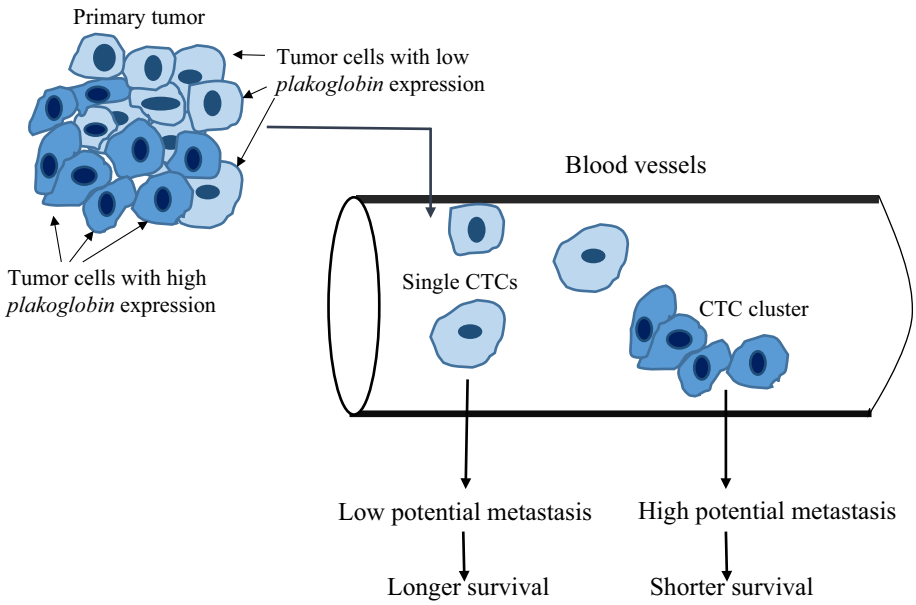


Fig. 4 A putative model for plakoglobin, types of circulating tumor cells (CTCs), and their metastasis capacities. Tumor cells with high *plakoglobin* expression escape from primary tumor masses, enter and circulate in clusters in the bloodstream. Such clusters promote their metastases to secondary sites, leading to worse survival of breast cancer patients

functions of plakoglobin as either an oncogene or tumor suppressor. The roles of plakoglobin in the development and progression of breast cancer seem to be phase-dependent. In the progression of breast cancer, *plakoglobin* expression was negatively associated with prognosis; high *plakoglobin* expression makes breast cancer cells move in clusters, which are more predisposed to form distant metastasis. In other words, the correlation between high *plakoglobin* expression and worse survival of breast cancer may have nothing to do with either oncogenic or tumor-suppressive function of plakoglobin. Instead, being an adhesion molecule, high *plakoglobin* expression enables tumor cells to stick together and move in clusters in the bloodstream, allowing more chances of metastasis, resulting in worse survival of breast cancer (Fig. 4). However, *plakoglobin* silencing only can reduce the clustered CTCs-associated metastasis, but not single CTCs-associated spread via blood. Thus, it is worthy of further investigations on (1) what factor(s) is involved in the single CTCs-associated metastasis; (2) what other factor(s) may be involved in clustered CTCs-associated metastasis besides *plakoglobin*; (3) what transcription factor(s) regulates the expression of *plakoglobin*; (4) what molecules are potentially involved in the aggregation of single CTCs to become clustered CTCs in the circulation; (5) whether or not and how the phenotypes of CTCs can be modified by the uptake of circulating exosomes (extracellular nanovesicles) that are released from other cells.

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Conflict of interest The authors declare that they have no conflict of interest.

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