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Abstract

Breast cancer is the most common malignancy in women worldwide. Although important therapeutic progress was achieved over the past decade, this disease remains a public health problem. In the light of precision medicine, the identification of new prognostic biomarkers in breast cancer is urgently needed to stratify populations of patients with poor clinical outcome who may benefit from new personalized therapies. The microtubule cytoskeleton plays a pivotal role in essential cellular functions and is an interesting target for cancer therapy. Microtubule assembly and dynamics are regulated by a wide range of microtubule-associated proteins (MAPs), some of which have oncogenic or tumor suppressors effects in breast cancer. This review covers current knowledge on microtubule-associated tumor suppressors (MATS) in breast cancer and their potential value as prognostic biomarkers. We present recent studies showing that combinatorial expression of ATIP3 and EB1, two microtubule-associated biomarkers with tumor suppressor and oncogenic effects, respectively, improves breast cancer prognosis compared to each biomarker alone. These findings are discussed regarding the increasing complexity of protein networks composed of MAPs that coordinate microtubule dynamics and functions. Further studies are warranted to evaluate the prognostic value of combined expression of different MATS and their interacting partners in breast cancer.

Introduction

Breast cancer is a major cause of death in women worldwide. Clinical management of this heterogeneous disease relies on well-established clinico-pathological characteristics and molecular biomarkers, including estrogen and progesterone receptors as well as the HER2 oncogene [1]. The classification of breast tumors into distinct molecular subtypes (Luminal, HER2-positive and triple-negative) is of invaluable help in making clinical decisions. However, tumors from the same subtype still remain heterogeneous in terms of prognosis and response to therapy [2]. Over the past decade, the emergence of high-throughput techniques for molecular profiling and DNA sequencing of breast tumors has allowed extensive progress in the classification, prognosis and treatment of early and advanced breast cancer [3, 4]. In the light of precision medicine, the continuously increasing accumulation of big data opens new avenues of investigation [5]. The identification of novel biomarkers enabling personalized therapeutic strategies for selected breast tumors has become a major issue [6]. An important point to reach is the ability to identify prognostic biomarkers to stratify populations of breast cancer patients at high risk to relapse and develop metastasis. Selecting these patients is a prerequisite to the development of targeted therapies.

The microtubule cytoskeleton is an essential cellular component that plays a key role in various biological processes such as cell division, polarity and migration, all of which are altered in cancer. Microtubules are polarized protofilaments composed of □□-tubulin heterodimers that are organized head-to-tail and grow from the minus to the plus end. These are highly dynamic structures, constantly switching between phases of polymerization (growth) and depolymerization (shrinkage) at their plus ends, a process called dynamic instability. Dynamic instability allows the microtubule cytoskeleton to explore the cytosol and rapidly reorganize in response to external cues to ensure appropriate cell function. Coordinated regulation of microtubule dynamics during mitosis and interphase permits mitotic spindle formation and orientation, chromosomes segregation towards cell poles during anaphase, cytoskeleton and cell shape remodeling, intracellular trafficking and signaling as well as cell migration and polarization.

Microtubule assembly and dynamics are tightly regulated by a large number of microtubule-associated proteins (MAPs) that interact with tubulin or polymerized microtubules to either stabilize or destabilize the network. A subset of MAPs, known as kinesins, function as molecular motors to ensure intracellular transport of proteins and organelles. Some MAPs localize along the microtubule lattice (structural MAPs) whereas others preferentially bind to microtubule plus ends (+TIPs) or minus ends (-TIPs) [7]. At the plus end, End-Binding proteins (EB1, EB2, EB3) play a major role as scaffolds to recruit a wide range of plus-end tracking proteins that contribute to the regulation of microtubule dynamics and targeting of organelles [7, 8]. Minus ends are occupied by large complexes of MAPs, also including EB1, that cooperate to regulate microtubule nucleation and dynamics [9].

Because they regulate the organization and function of the microtubule cytoskeleton in interphase and mitosis, MAPs can be considered as the "guardians" of cellular integrity. Alterations in the sequence or expression levels of these proteins may lead to cytoskeletal defects with major consequences on cancer initiation or progression. Indeed, a number of MAPs have been described as oncogenes or tumor suppressors whose deregulation has a major impact on cancer aggressiveness and clinical outcome for the patients. In this review we will focus on microtubule-associated proteins with tumor suppressor effects (MATS) in breast cancer.

A family of microtubule-associated tumor suppressors (MATS) in breast cancer

A total of 10 MAPs with tumor suppressive functions have been identified in breast cancer. These proteins include Adenomatous Polyposis Coli (APC), Microtubule-associated tumor suppressor 1 (MTUS1) protein ATIP3, Breast Cancer 1 (BRCA1), Cylindromatosis tumor suppressor (CYLD), Fragile Histidine Triad (FHIT), Leucine Zipper putative Tumor Suppressor 1 (LZTS1), Neurofibromatosis 2 (NF2) protein Merlin, Navigator-3 (NAV3), RAS Association domain Family 1A (RASSF1A), and Von Hippel-Lindau (VHL) **(Table 1).**

BRCA1 is known as a master tumor suppressor in breast cancer. Interestingly, besides its key role in DNA repair, BRCA1 has also been shown to reduce microtubule dynamics [10]. BRCA1 was found to localize on the mitotic spindle and at the centrosome where it interacts with □-tubulin, a centrosomal protein crucial for microtubule nucleation [11, 12]. APC, another master tumor suppressor, also localizes

at the centrosome [13], a localization shared by other MATS such as ATIP3 [14], RASSF1A [15, 16] and VHL [17]. Of note, APC was the first protein shown to interact with microtubule end-binding protein EB1 [18] via a canonical SxIP motif [7, 19, 20]. APC was thus identified as a plus-end tracking protein [20], like NAV3 [21] and CYLD [22] that bind EB1 through SxIP and CAP-Gly motifs, respectively. In addition, most MATS were shown to decorate the microtubule lattice in interphase and/or the mitotic spindle during mitosis [14-17, 23-26] (**Table 1**).

The majority of MATS are microtubule stabilizers that regulate different parameters of microtubule dynamics. Indeed, ATIP3 [27], BRCA1 [10], Merlin [28] and RASSF1A [15, 16] decrease microtubule growth rate whereas APC [20] and VHL [17] protect MTs from depolymerization. FHIT [29] and LZTS1 [25] promote MT assembly. NAV3 [21] and CYLD [22] both stabilize microtubules and promote their assembly (**Table 1**). Considering that these proteins are often co-localized on microtubules and that they all contribute to the regulation of microtubule organization and dynamics, it will be of interest to evaluate whether different MATS may have compensatory effects - in which the loss of one of these proteins could be balanced by another - or cooperative effects - in which expression of two or more proteins is necessary for microtubule regulation.

In breast cancer, MATS have been shown to be downregulated in 30 to 80% of tumor samples compared to normal adjacent tissues [14, 21, 30-38] (**Table 2**). Except for BRCA1 and APC, MATS genes are not heavily mutated in primary breast tumors. Their reduced expression level in breast cancer results from promoter hypermethylation for APC, FHIT, LZTS1 and RASSF1A genes [36, 39-41] and loss of heterozygosity for APC, BRCA1 and FHIT [39, 40, 42]. Other mechanisms involving microRNA-mediated targeting of CYLD, LZTS1 and VHL genes in breast cancer have been more recently reported [43-45]. NF2 has been shown to be down-regulated at the post-translational level in advanced breast cancer by proteasome-dependent degradation [35]. The mechanisms responsible for down-regulation of MTUS1 and NAV3 genes in breast cancer still remain to be determined.

Interestingly, low levels of MTUS1/ATIP3 [14], CYLD [32], FHIT [40], NAV3 [21] and RASSF1A [36] are significantly associated with ER-negative, highly proliferative and/or high-grade breast tumors, indicating that loss of MATS in breast cancer is associated with tumor aggressiveness. Furthermore,

when considering MATS level and patient outcomes, studies have shown that for the majority of MATS, loss of expression is associated with reduced overall survival as well as reduced relapse free survival [21, 27, 32, 34, 37, 46-48] (**Table 2**). Thus, MATS may represent useful prognostic biomarkers in breast cancer.

Prognostic value of ATIP3/EB1 combination : two biomarkers are better than one

The ATIP3 protein is an interesting example of a MATS with prognostic value in breast cancer. ATIP3 belongs to a family of proteins encoded by alternative splicing of the *MTUS1* gene located at chromosome 8p22 [49, 50]. This protein is expressed in epithelial cells of the mammary gland in normal breast tissue and is markedly down-regulated both at the mRNA and protein level in half of invasive breast tumors [14]. Low levels of ATIP3 are associated with tumor aggressiveness, metastatic properties and reduced clinical outcome for breast cancer patients. Furthermore, re-expression of ATIP3 into breast cancer cell lines significantly reduces cell proliferation, polarity and migration, as well as tumor growth and metastasis in experimental mouse models [14, 27], highlighting ATIP3 as a prognostic biomarker and a potent anti-cancer and anti-metastasis protein.

As mentioned above, ATIP3 localizes at the centrosome and along the microtubule lattice in interphase and decorates the mitotic spindle and spindle poles during mitosis [14]. Proximity mapping and functional analysis of the human centrosome-cilium interactome also revealed that ATIP3/MTUS1 belongs to a centrosomal protein network that regulates centrosomal functions [51]. Like most other MATS (**Table 1**), ATIP3 stabilizes microtubules. Its depletion significantly increases microtubule dynamics at the plus end by increasing microtubule growth and growth rate and reducing the time spent in pause [27]. At the molecular level, ATIP3 directly interacts with end-binding protein EB1 via a proline-rich and basic SxIP-like motif (RPLPLP) [52]. ATIP3-EB1 interaction takes place in the cytosol, is independent of microtubules, and impairs EB1 binding and turnover at the plus ends [52]. Strikingly, while almost all SxIP-containing EB1-binding proteins are +TIPs that localize at the plus end [7, 8, 19], ATIP3 is a unique example of SxIP-containing protein that does not accumulate at the plus ends and rather acts as an endogenous antagonist of EB1 [53].

In breast cancer, EB1 levels were found elevated at the mRNA and protein levels compared to normal tissue [54, 55]. Furthermore, EB1 (but not EB2 or EB3) was identified as a prognostic biomarker whose high expression in breast tumors associates with tumor malignancy, high histological grade and reduced overall survival of the patients [55]. The observation that ATIP3 negatively regulates EB1 functions launched further studies to investigate the clinical relevance of ATIP3-EB1 interaction for breast cancer patients. Results showed that in high-EB1 expressing breast tumors, ATIP3 deficiency results in increased tumor aggressiveness, with subsequent consequences on patient prognosis (**Figure 1**). Furthermore, analyzing combinatorial expression of ATIP3 and EB1 in five independent cohorts of patients significantly improved breast cancer diagnosis and prognosis compared to each biomarker alone [55]. Importantly, these studies allowed to identify a population of breast cancer patients of worse clinical outcome expressing high EB1 and low-ATIP3 levels. This population of patients, that represents 30% of breast cancer cases, may be eligible to molecular therapies aimed at restoring or mimicking ATIP3-EB1 interaction. Integrative approaches have recently been described for the discovery of small molecule modulators of EB1 binding to SxIP motifs [56]. Such strategies may open interesting perspectives towards the development of personalized treatments to compensate for ATIP3 loss in a selected population of patients with high-EB1/low-ATIP3 expressing breast tumors.

Notably, other MATS such as APC, CYLD and NAV3 also interact with EB1 (**Table 1**). However, in contrast to ATIP3, these MATS are +TIPs that accumulate at the microtubule plus ends. Furthermore, APC and CYLD have been shown to cooperate with EB1 rather than antagonize its effects on cell migration [57-59]. These observations may suggest that loss of APC or CYLD in high-EB1 expressing breast tumors may impact tumor aggressiveness and clinical outcome for the patients. It will be of interest to investigate whether, similar to the ATIP3-EB1 couple, combinatorial expression of CYLD-EB1 and/or APC-EB1 may improve breast cancer prognosis.

Concluding remarks

In conclusion, among the large number of MAPs that control the microtubule network in interphase and mitosis, only few have been identified as *bona fide* microtubule-associated tumor suppressors (MATS) in breast cancer. Their deficiency in breast tumors is associated with poor prognosis of the patients,

highlighting their potential value as biomarkers. Pioneer studies of ATIP3, a MATS that binds to EB1 and antagonizes its oncogenic effects in breast cancer, provide the first proof-of-concept that combinatorial expression of two biomarkers with opposite actions on microtubules improves breast cancer prognosis compared to each biomarker alone.

Several MATS do co-localize inside the cell, and some of them have been shown to act in concert. This raises the possibility that combinatorial expression of those microtubule-associated tumor suppressors may have improved value as biomarkers for the stratification of breast cancer patients in the context of personalized medicine. These data are particularly important considering increasing evidence that large numbers of MAPs are engaged into protein networks whose coordinated actions allow fine tuning of microtubule dynamics and assembly. In this line, the concomitant loss of BRCA1 and FHIT in sporadic breast cancer has been associated with reduced patients survival, suggesting that combination of these two MATS may be used as a marker to identify a subpopulation of breast tumor with poor prognosis [60]. Further studies are warranted to evaluate the prognostic value of combined alterations of two or more MATS, together with other MAPs and their binding partners in breast cancer. The high complexity of MAPs networks suggests a novel paradigm for future discovery of a new generation of complex prognostic biomarkers in breast cancer.

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Conflict of interests :

Authors declare that they have no conflict of interests.

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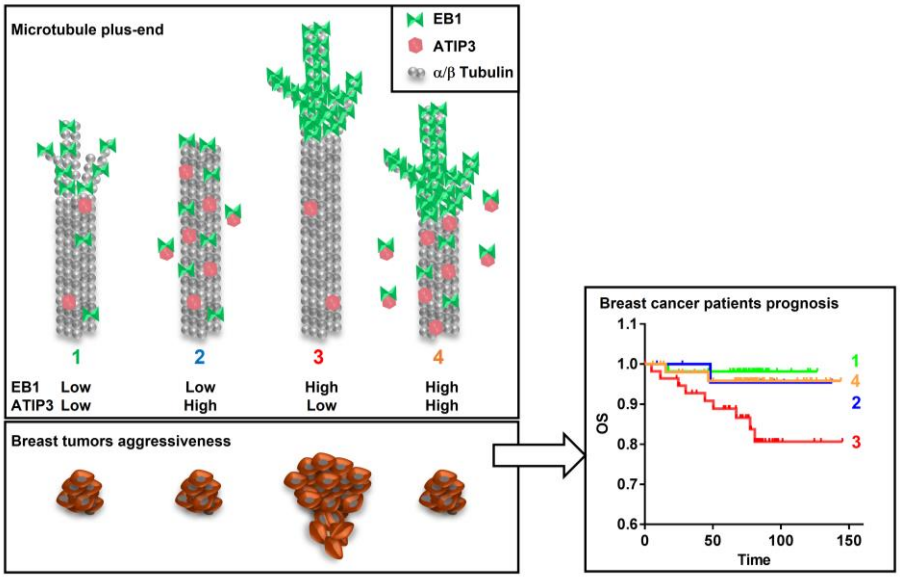

Figure 1

Legend to Figure 1.

Two biomarkers are better than one. Consequences of combinatorial expression of EB1 and ATIP3 on microtubule dynamics at the plus end (upper panel), breast cancer aggressiveness (lower panel) and overall survival of breast cancer patients (right panel). Tumors were classified according to combined EB1-ATIP3 expression as group 1 (EB1-low and ATIP3-low), group 2 (EB1-low and ATIP3-high), group 3 (EB1-high and ATIP3-low) and group 4 (EB1-high and ATIP3-high). Kaplan-Meier representation on the right indicates that breast tumors from group 3 are of worse prognosis compared to all other groups ($p<0.05$).

MATS	Name	Localization	Effect on MT	EB1 interaction
APC	Adenomatous Polyposis Coli	MT network, centrosome, MT plus ends [13, 23]	Stabilization [20]	SxIP [19, 20]
ATIP3	Microtubule associated tumor suppressor MTUS1 /ATIP3	MT network, centrosome, mitotic spindle [14, 27]	Stabilization [27]	RPLP [52]
BRCA1	Breast Cancer 1	Centrosome, mitotic spindle [11, 12]	Stabilization [10]	no
CYLD	Cylindromatosis tumor suppressor	MT network, MT plus end, midbody [22, 24]	Stabilization and assembly [22]	CAP-Gly [59]
FHIT	Fragile Histidine Triad	nd	Tubulin interaction, MT assembly [29]	nd
LZTS1	Leucine Zipper putative Tumor Suppressor 1	MT network [25]	MT assembly [25]	nd
Merlin/NF2	Neurofibromatosis 2 (NF2) protein Merlin	MT network, mitotic spindle [26]	Stabilization [28]	nd
NAV3	Navigator-3	MT plus end [21]	Stabilization [21]	SxIP [8]
RASSF1A	RAS Association domain Family 1A	MT network, centrosome, mitotic spindle [15, 16]	Stabilization [15, 16]	nd
VHL	Von Hippel-Lindau	MT network, centrosome, mitotic spindle [17]	Stabilization [17]	nd

Table 1. Microtubule-Associated Tumor Suppressors (MATS) properties related to microtubules. Protein localization, effect on microtubule assembly and stability, and ability to interact with EB1, are presented. nd : not determined

MATS	Reduced Expression in breast cancer	Inactivated by	Prognostic biomarker
APC	40,7% [30]	LOH - Promoter hypermethylation [39]	nd
ATIP3	48% [14]	nd	[27]
BRCA1	54% [31]	LOH [42]	[46]
CYLD	29,9% [32]	MicroRNA targeting [44]	[34]
FHIT	69% [33]	LOH - Promoter hypermethylation [40]	[47]
LZTS1	43,7% [34]	Promoter hypermetylation [41] MicroRNA targeting [45]	[34]
Merlin/NF2	75% [35]	*Proteasomal degradation [35]	nd
NAV3	37-79,3% [21]	nd	[21]
RASSF1A	53,3% [36]	Promoter hypermethylation [36]	[48]
VHL	nc [37, 38]	MicroRNA targeting [43]	[37]

Table 2. Microtubule-Associated Tumor Suppressors (MATS) in breast cancer.

The percentage of primary breast tumors with reduced expression of MATS, mechanisms of gene inactivation, and prognostic value of MATS in breast cancer patients, are presented. LOH: Loss Of Heterozygosity; nc : not calculated; nd : not determined. A star indicates down-regulation at the post-translational level.