Chapter

Evidence of BK_{Ca} Channelopathy-Driven Breast Cancer Metastasis to Brain

Divya Khaitan and Nagendra Ningaraj

Abstract

KCNMA1 encodes the a-subunit of the large conductance, voltage and Ca^{2+}-activated and Voltage-dependent potassium channel (BK_{Ca}) and was shown by others and us to be a potential drug target gene in several cancers, including breast cancer. In addition, we studied the role of alternative pre-mRNA splicing events of *KCNMA1* in migration, invasion, proliferation and dispersal of breast cancer cells. It is conceivable that by targeting gene variants we can attenuate processes such as distant metastasis and angiogenesis. Here we reviewed literature on the alternative splicing events specific to breast cancer metastasis to brain, its microenvironment, the biological activity of most alternatively spliced isoforms. We conclude that based on our and others' work *KCNMA1* and other such gene variants contribute to breast cancer dispersion, invasion, growth, and progression in the tumor microenvironment. Thus *KCNMA1*/BK_{Ca} channels and their variants are opportunistic diagnostic, prognostic and treatment targets in breast cancer.

Keywords: *KCNMA1* pre-mRNA splicing, BK_{Ca} channelopathy, breast cancer-dispersion, invasion, growth, angiogenesis, progression, treatment target

1. Introduction

1.1 Metastatic breast cancer etiology

Breast cancer is the most common type of cancer affecting women. Despite great advances in primary breast cancer treatment a significant number of women develop metastases in different organs of the body, especially brain [1], possibly as a result of the emergence of targeted and aggressive systemic cancer therapy. The actual incidence of brain metastases is not precisely known; however, studies suggest that 6–16% of patients with metastatic breast cancer develop brain metastases during their lifetime. Furthermore, autopsy studies have reported brain metastases in 18–30% of patients dying of breast cancer [2]. The majority of women who develop brain metastases have undergone aggressive treatment for stage IV disease [3–5]. Although brain metastasis is the leading cause of breast cancer death, its pathogenesis is poorly understood and the predictors of breast metastasis to brain are yet to be characterized. Albeit recent studies found genes that mediate breast cancer metastasis to brain [6, 7]. Targeting metastatic breast cancer cells in brain is

extremely difficult as brain provides a "safe haven" for cancer cells. Gene expression profiling has been used to predict metastatic gene-expression signature that is present in a subset of primary breast tumors [8]. However, a reliable profile has not yet been identified that specifically predicts brain metastases. Therefore, it is extremely important to study the genetic changes in breast cancer cells that metastasize to brain and develop specific targeted therapeutic molecular agents.

2. Channelopathy promote breast cancer metastasis

Cancer research is not only focusing on understanding the possible role of transmembrane-BK_{Ca} channels in cancer development and progression but also on development of BK_{Ca} channel modulator drugs to attenuate cancer growth. Several researchers, including us have shown that brain tumor cells express BK_{Ca} and ATP-sensitive potassium (K_{ATP}) channels that are highly responsive to minute changes in intracellular Ca^{2+} and ATP levels. This allows the brain tumor cells to develop pseudopodia for migration through constricted spaces in the brain parenchyma, as depicted in **Figure 1**. Several articles have described the efficacy of BK_{Ca} channel-inhibiting drugs or molecules in reducing tumors in preclinical mouse tumor models. A recent study has shown the role of intracellular BK_{Ca} channels (mitoBK_{Ca}) in cancer cell biology [9, 10].

2.1 Ion channels in breast cancer metastasis

Even now the metastatic breast cancers are incurable. Extensive research has shown that breast cancer metastasis to other organs, including brain is a complicated process. It is widely believed that breast cancer cells escape the primary site

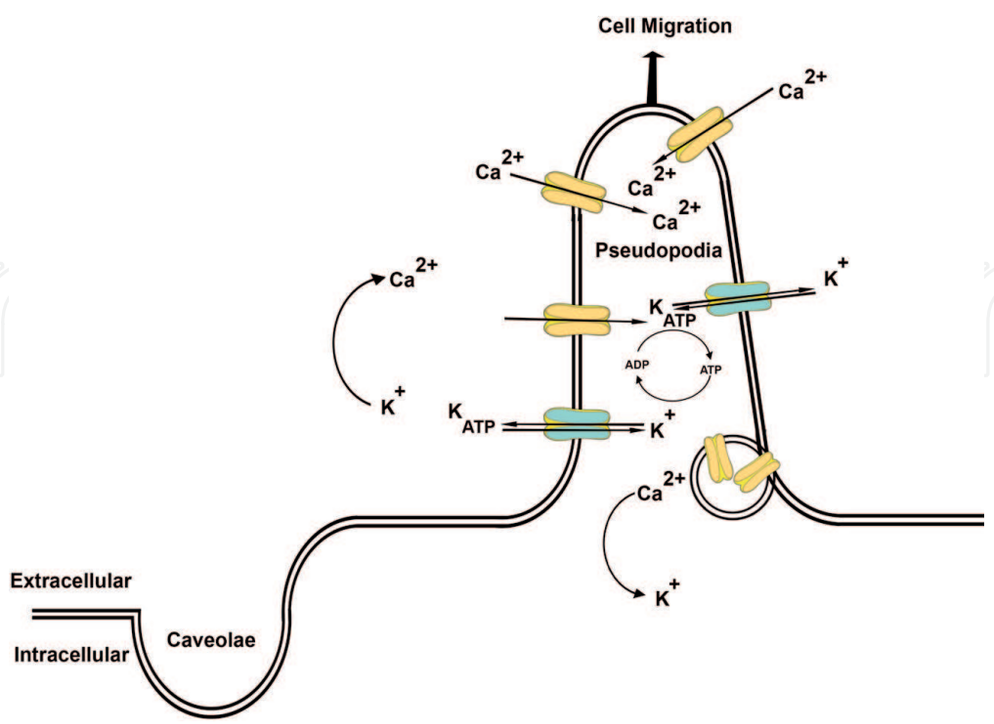

Figure 1.
Anticipated role of BK_{Ca} and K_{ATP} channels in breast cancer cells that seek brain and colonize in brain parenchyma. The potassium ion channels expressed in breast cancer cells are extremely sensitive to minute surge of extracellular and intracellular Ca^{2+} and cause K^+ efflux through BK_{Ca} channels. Similarly, slight imbalance in ADP-ATP levels in the cell causes K^+ efflux through K_{ATP} channels. Then the ion imbalance triggers the Ca^{2+} entry, which promotes cancer cell migration though pseudopodia.

and migrate by lymphatic route to lymph nodes and vascular route to colonize in other organs including brain [11, 12]. Gene-expression profiling studies of breast cancer cells indicate that specific molecular pathways are associated with dissemination of primary tumor cells through a vascular route and not by lymphatic dissemination [12]. There is much interest in studying how and when the cancer cells initiate the metastatic cascade so that a therapeutic intervention can be developed to stop or delay the metastasis. Some cancer researchers [13] believe that targeted treatment of breast cancer with ER/PR modulators (Aromatase inhibitors) and targeted biologics such as Herceptin (Her-2 neu inhibitor) [14] and bevacizumab [15] (anti-vascular). Others argue that the metastasis of cancer cells is triggered by a dysregulated cellular Ca^{2+} homeostasis and altered Ca^{2+} signaling caused by imbalanced fluxes through ion channels and transporters [10, 16]. The BK_{Ca} channels are more sensitive to Ca^{2+} ions in cancer cells. In this regard, we studied whether the increased sensitivity of potentially new BK_{Ca} channel variant protein encoded by splice variants (**Figure 2**) *KCNMA1ΔE2* and *KCNMA1vE22* to intra and extra cellular Ca^{2+} in breast cancer [17]. In fact, a recent evidence indicates that KCa-Ca^{2+} channel complexes were found in cancer cells and contribute to cancer-associated functions such as cell proliferation, cell migration and the capacity to develop metastases [10]. The BK_{Ca} channels are unique since its activity is triggered by depolarization and enhanced by an increase in μM range of intracellular calcium [$Ca^{2+}{}_i$]. In this regard, we recently showed that BK_{Ca} channel variant encoded by a new splice variant *KCNMA1vE22* is highly sensitive to [$Ca^{2+}{}_i$] and causes glioma progression to high grade glioblastoma multiforme (GBM) [18]. We also discovered a new splice variant *KCNMA1vE22* in breast cancer cells that contributes to breast cancer metastasis to brain (to be published). Epigenetics play an important role in cancer initiation, growth and progression. Understanding the precise mechanism helps us in developing diagnosis, prognosis and treatment strategies for affected cancer patients. For example, overexpression of Ezh2 plays a role in many cancers,

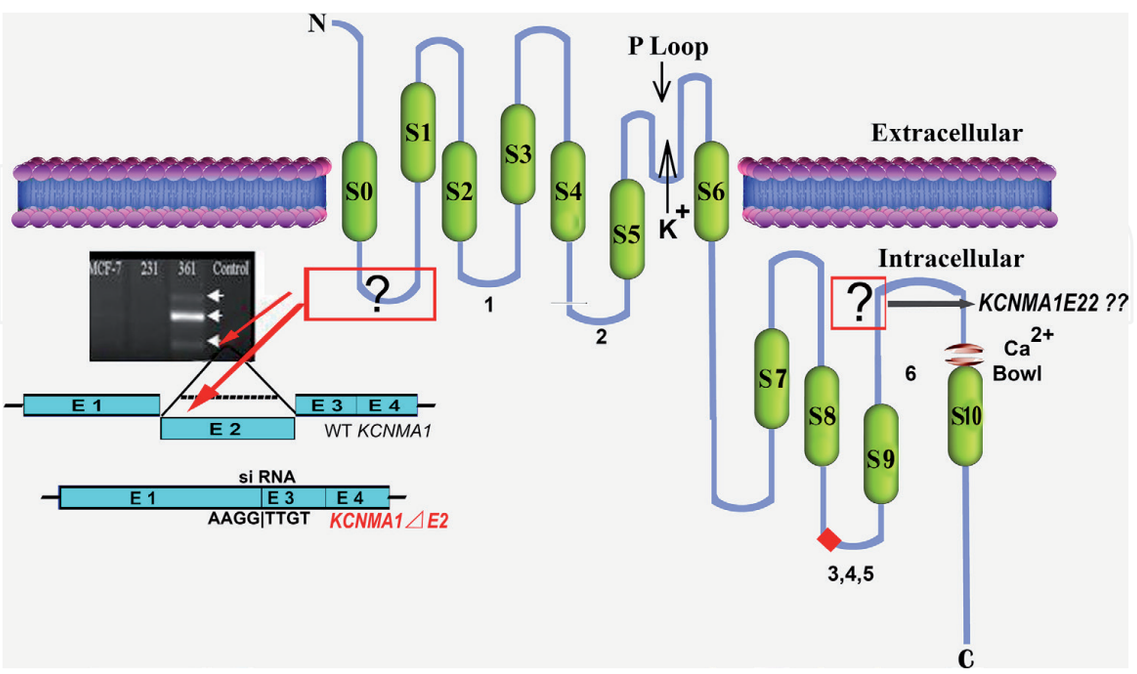

Figure 2.
BK_{Ca} channel is a 7-transmembrane tetramer of four monomeric pore-forming alpha-subunits encoded by KCNMA1. The cytoplasmic C-terminal domain has RCK1 and RCK2 (with calcium bowl) segments. We identified KCNMA1ΔE2 and KCNMA1E22 in human brain-specific metastatic breast cancer cells. Using relevant siRNA designs, we showed that these splice variants are formed by the deletion of exon 2 (E2) and 108 base pair deletion in exon 22 (E22), respectively.

including breast cancer and brain tumors. H3K27M serves as an oncohistone and, if mutated it contributes to tumor development as Ezh2 is no longer able to methylate the histone and gene expression is aberrantly upregulated.

Furthermore, a recent computational analysis of human genomic sequence identified mutations that cause pathogenic splicing abnormalities in breast cancer susceptibility genes, BRCA1, BRCA2 and other genes [19]. Several investigations have reported that voltage gated ion channels are expressed in several cancers and contribute significantly to cell signaling, cell cycle progression and cell volume regulation, cancer cell proliferation, as well as metastasis. Hence, there is a great deal of interest in possible therapeutic potential of voltage gated ion channels as pharmacological targets [20, 21].

2.2 Metastatic breast cancer in brain microenvironment

Cancer cells have the innate ability to "exploit" the "chaotic" environmental challenges surrounding them and grow uninterrupted by manipulating transportome that regulate proliferation, apoptosis, metabolism, growth factor signaling, migration and invasion. Ion channels and transporters are some of the key modulators of cancer progression in hostile tumor microenvironment that includes hypoxia. It has been suggested that modulation of ion channels by the hypoxic environment may contribute to the aggressive phenotype observed in GBM cells residing in a hypoxic environment [22]. In hostile microenvironment such as hypoxia, BK_{Ca} channels are modulated to aid cancer cell invasion and neovascularization. Affymetrix Array analyses of brain tumor cell lines where KCNMA1 was either overexpressed or suppressed showed significant changes in genes involved in cell proliferation, angiogenesis, cell cycle, and invasion [18].

2.3 *KCNMA1*/BK_{Ca} channel splice variants in breast cancer

During the past decade, a number of genes associated with breast cancer have been cloned and identified. Gene expression levels alone cannot fully explain gene function as alternative splicing produce multiple mRNAs and protein isoforms. New molecular insights indicate that the metastatic capacity of breast tumors is an inherent feature, and not necessarily a late, acquired phenotype [23, 24]. Breast cancer cells show alternative mRNA splicing and have prognostic and therapeutic value [21]. Although there are many reports of alternative splicing events specific to breast cancer [25, 26], the biological activity of majority of alternatively spliced isoforms, and specifically their contribution to metastatic breast cancer biology, remains to be investigated. As many researchers are focusing on "Understanding and Preventing Brain Tumor Dispersal", we recently reported on a novel *KCNMA1* mRNA splice variant with a deletion of 108 base pairs (KCNMA1v) mostly overexpressed in high-grade gliomas [18]. In order to understand the role of alternative pre-mRNA splicing events of *KCNMA1/* BK_{Ca} channels, we employed specific inhibitors. We showed that the modulation of *KCNMA1*/BK_{Ca} channels in brain specific metastatic breast cancer cells (MDA-MB361) resulted in attenuation of migration, invasion [11, 17]. Further, we identified a hitherto unknown *KCNMA1* variant *KCNMA1vE22* (to be published) with a deletion of 108 base pairs of nucleotides and deletion of the entire exon-2 (*KCNMA1ΔE2*) expressed only in metastatic breast tumor cells seeking brain (**Figure 2**). However, biological function of *KCNMA1ΔE2* under different tumor microenvironment is yet to be elucidated. The *KCNMA1* splicing effects and the potential role of *KCNMA1ΔE2* as a critical posttranscriptional regulator of BK_{Ca} channel isoform resulting in diversified channel functions merit further investigation.

Cell line	Proliferation (at 72 h)	Invasion	Trans-endothelial migration	Functional activity	Tumor volume at the 5th week
Untransfected	1 ± 0.09	1 ± 0.10	1 ± 0.17	1 ± 0.11	1 ± 0.21
Vector-transfected	0.98 ± 0.04	1.01 ± 0.12	0.95 ± 0.13	1.2 ± 0.15	1 ± 0.27
KCNMA1vE22-transfected	1.4 ± 0.12	1.9 ± 0.15	1.5 ± 0.19	1.6 ± 0.21	3.8 ± 0.42

Data shown are in SEM of n = 3 in triplicates. Biological function assays [11] and functional activity of BK_{Ca} channels were measured by membrane potential assay using FlexStation and in vivo mouse brain tumor models as described by us earlier.

Table 1.
Effect of KCNMA1vE22 expression on biological functions of MDA-MB-231BR cell line.

Identifying the most optimal and novel biomarker(s) for breast cancer metastasis to brain is ideal [27, 28] yet challenging because of the multi-factorial nature of the disease. The roles of roles of different ion channels in the development of cancer have been reported [29]. The identification of a potential new biomarker has relied heavily on an increase or decrease in gene expression, but these changes may not always result in altered protein expression. Growing evidence indicates that alternative or aberrant pre-mRNA splicing resulting in protein isoforms with diverse functions occurs during the development, progression, and metastasis of breast cancer [29]. Earlier, we have reported that the BK_{Ca} channels play a role in human breast tumor progression, cell proliferation, invasion, and micro-metastases [11, 17]. Nevertheless, the precise role of *KCNMA1* and its splice variants in modification of BK_{Ca} channel functions in promoting breast cancer metastasis to brain is still unclear. Therefore, to understand the role of BK_{Ca} channels in breast cancer metastasis to brain the we showed that the relative messenger RNA levels in MDA-MB-361 cells derived from human metastatic breast tumor in brain were higher than metastatic breast cancer cells (MDA-MB-231) that prefer other organs and primary breast cancer (MCF-7) cells. In addition, using GeneChip Exon array (Affymetrix) we probed the presence of alternative splicing of *KCNMA1* in MDA-MB-361, MDA-MB-231 and MCF-7. The array data showed that *KCNMA1* splicing pattern is different among cell lines with three different phenotypes (to be published).

The PCR results validated the findings of Exon array study. Two distinct splice variants expressed in breast cell line (MDA-MB-361) metastatic to brain were identified (i) deletion of exon 2 (*KCNMA1ΔE2*) between S0-S1 protein subunit (**Figure 2**) corresponding to the cytoplasmic potential domain of BK_{Ca} channel α-subunit and (ii) deletion of 108 bp in exon 22 (*KCNMA1vE22*) between the S9 and S10 protein subunit (C-terminus). However, the biological function of these alternative splice variants in breast cancer remains to be investigated. To our knowledge we believe that our laboratory is the first to report the presence of these variants in metastatic breast cancers. We established that *KCNMA1vE22* plays a key role in several biological functions of MDA-MB-231BR cell line as represented in **Table 1**.

3. BK_{Ca} channels and neovascularization

Altered ion channels could play a pivotal role in physiological angiogenesis in including cancer [30, 31]. BK_{Ca} channel inhibitor modulated the tumorigenic ability of hormone-independent breast cancer cells via the Wnt pathway [32].

Our work shows an association between the BK_{Ca} channel isoform expression and VEGF secretion by breast tumor cells, which might be exacerbated under hypoxia that has implications for vascular permeability and anticancer drug delivery (to be published). Understanding the underlying mechanism and splicing patterns of *KCNMA1* and expression of the splice variant *KCNMA1ΔE2* under normoxia and hypoxia alone and in coculture with brain endothelial cells will shed light on the role of *KCNMA1* alternative splicing in metastatic breast tumor biology. Perhaps the discovery and validation of brain specific metastasis-associated *KCNMA1* alternate splice variants will serve as new tools for the diagnosis and classification of breast tumor patients with high risk of brain metastasis. In fact, splice variations in a number of genes have already been shown to correlate with malignancy and their occurrence could precede clinical cancer diagnosis [33]. To date, however brain-specific alternate *KCNMA1* splice variants in breast cancer have not been reported. The variant *KCNMA1ΔE2* that we have discovered potentially may fill the gap to serve as a biomarker of breast cancer metastasis to brain. Undoubtedly, the research on the putative association between *KCNMA1* splice variants and breast cancer metastases to brain will prove to be an extremely productive exercise for the identification of a new generation of biomarkers. *KCNAM1*/BK_{Ca} channels are hypothesized to be involved in VEGF secretion and neovascularization in brain tumors. We tested this hypothesis by activation and suppression of *KCNMA1* in brain tumor cells and constructed a potential VEGF signaling pathway adapted from KEGG VEGF signaling pathway (**Figure 3**).

We rationalize that *KCNMA1ΔE2* is expressed specifically in metastatic breast tumors in brain, and this requires validation to confirm its role as a potential transformation biomarker of breast cancer metastasis to brain. In metastatic breast tumor cells seeking brain there is an upregulation and constitutive activation of *KCNMA1*, which correlates with increased malignancy [11]. In this context,

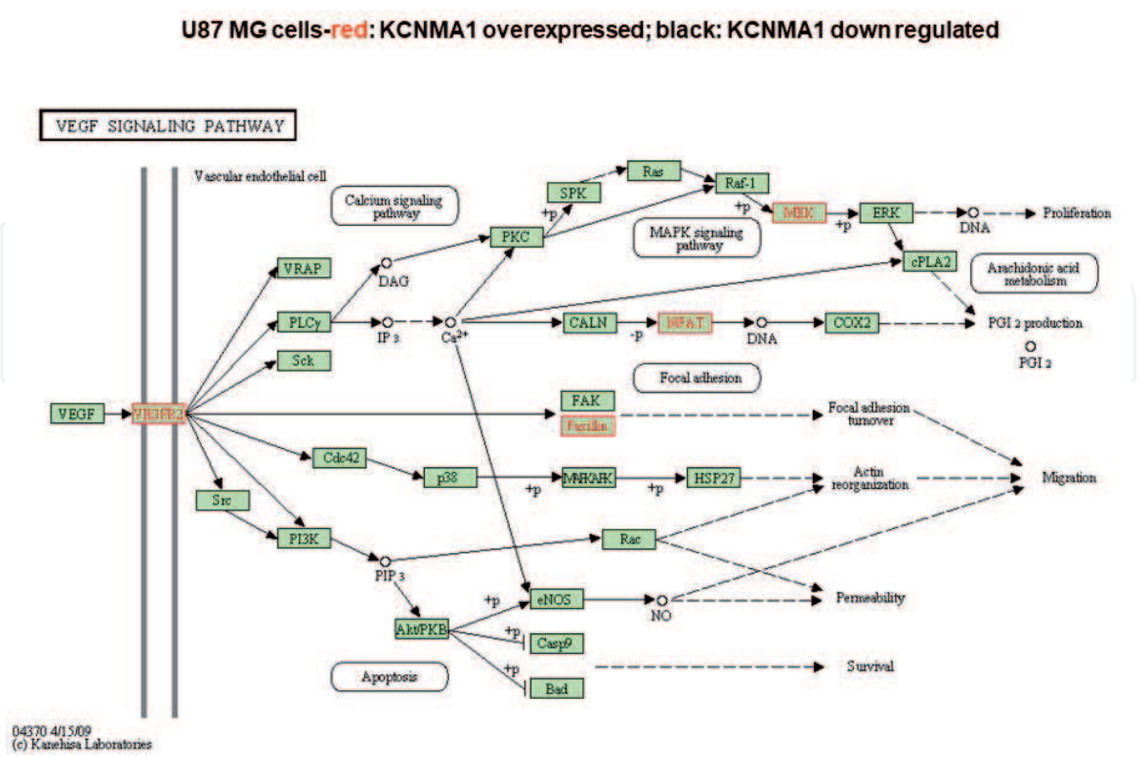

Figure 3.
Adapted from KEGG-VEGF signaling pathway: we activated and suppressed KCNMA1 in brain tumor cells and constructed a probable VEGF signaling pathway affected by modified KCNAMA1 expression. The genes in rectangular boxes—red represents genes overexpressed by KCNMA1 overexpression and black represents genes downregulated by KCNMA1 inhibition in U-87 (glioma cells).

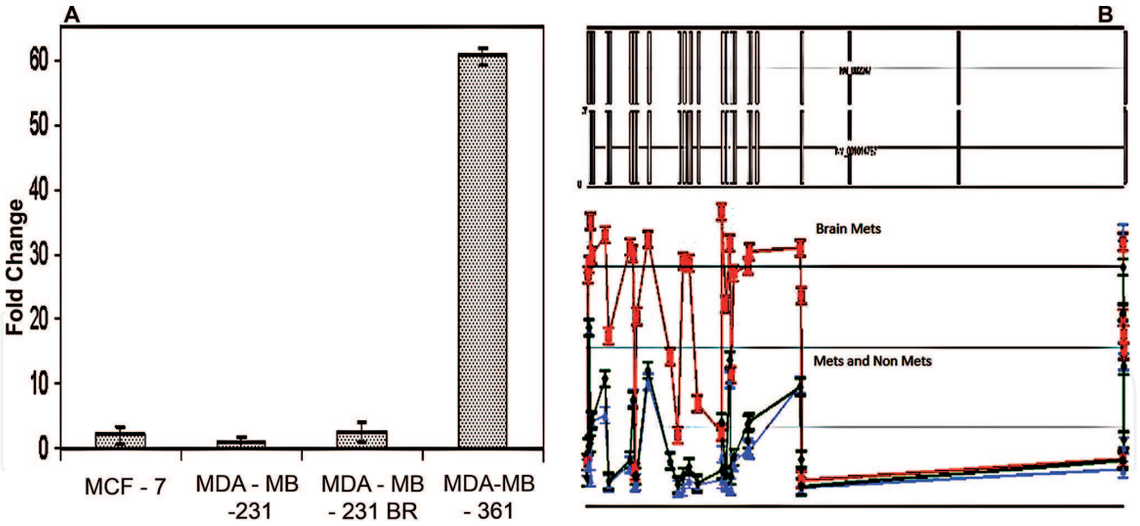

Figure 4.
BK_{Ca} channels in breast cancer biology-expression of KCNMA1 by qPCR (A) and alternate splice variants (B) using Affymetrix Genechip Exon Array in MCF-7 (non Mets), MDA-MB-231 (Mets) and MDA-MB-361 (brain Mets) cell lines.

we showed (**Figure 4**) that the *KCNMA1* is overexpressed in breast cancer cells metastatic to brain (MDA-MB-361) and exhibit differences in expression levels in other non-metastatic (MCF-7) and metastatic to other organs (MDA-MB-231). MDA-MB-231 BR was established from the triple negative MDA-MB-231 cells, which are highly metastatic but have no organ specificity. The MDA-MB 231-BR cell line was derived from MDA-MB-231 cells following sequential rounds of implantation, resection from the brain, and re-injection into mice. Eventually a subline with selectivity for the brain was isolated [34], and exhibit higher KCNMA1 level than parental MDA-MB-231 cells, however the expression was far lower than the naturally-selected MDA-MB-361 cells (**Figure 4**).

In addition, alternate splicing of *KCNMA1* [17] including *KCNMA1ΔE2* may provide a mechanism to generate a physiologically diverse complement of functionally and structurally diverse BK_{Ca} channel isoform that might affect cell proliferation, cell cycle, migration and micrometastases in brain. Future studies will validate the role of *KCNMA1ΔE2* in brain-specific metastatic process. Inhibiting *KCNMA1ΔE2 in in vitro* and *in vivo* models with shRNA or the variant BK_{Ca} channel using specific inhibitor like Iberiotoxin to attenuate breast tumor metastasis to brain using human metastatic breast tumor xenograft mouse models will be very stimulating.

4. Discussion

4.1 Splicing in health and disease

Many human diseases are implicated to errors in mRNA splicing. These aberrant splicing also provides an opportunity to develop targeted treatment to correct the faulty gene in some genetic disorders, or target aberrant protein encoded by these gene variants in human cancers. Breast cancer-specific biomarkers might generate specific epitopes that offer targets for developing diagnostic, prognostic and immunotherapy [35]. Articles on alternative pre-mRNA splicing regulation in cancer [36] and misregulation of mRNA splicing in cancer [29] highlights the important roles in promoting aberrant splicing, which in turn contributes to all aspects of tumor biology.

4.2 BK_{Ca} channels as target to attenuate breast cancer metastasis

The BK_{Ca} channels are known to function as oncogenes in certain cancers. These channels besides being sensitive to $[Ca^{2+}{}_i]$ are highly dependent on amounts of outward K^+ currents, which modulate the transmembrane potential of a cell. The BK_{Ca} channels are overexpressed in many types of cancers via gene amplification, alternative splicing or increased protein half-life. A recent study showed that by inhibiting BK_{Ca} channels with Iberiotoxin in breast cancer cells, tumorigenicity was reduced by downregulation of β-catenin and (phospho)Akt and HER-2/neu protein levels [37]. Evidence presented above clearly show that over expression of wild type BK_{Ca} channels or the presence of BK_{Ca} channel variant support breast cancer metastasis to brain. Understanding the mechanism of its action in brain metastasis will provide a unique opportunity to identify and differentiate between low grade breast cancers that are at high risk for metastasis from those at low risk for metastasis. This distinction would in turn allow for the appropriate and efficient application of effective diagnosis, prognosis and treatments while sparing patients with low risk for metastasis from the toxic side effects of chemotherapy. Activation of BK_{Ca} channels was shown to be a novel molecular pathway involved in zoledronic acid-induced apoptosis of MDA-MB-231 cells *in vitro* [32]. Du et al. [8] showed that BK_{Ca} promotes growth and metastasis of prostate cancer through facilitating the coupling between αvβ3 integrin and FAK. BK_{Ca} channels are shown to support cancer cell migration, invasion and tumorigenesis [11, 17, 18]. Hence it is extremely interesting to explore BK_{Ca} channels as putative targets for anti-breast cancer therapies.

4.3 Alternate splicing of BK_{Ca} channels in diagnosis and prognosis

Several articles have highlighted the use of alternative splicing as a promising source for new diagnostic, prognostic, predictive, and therapeutic tools [38–40]. The diversity of RNA species detected through RNA-seq holds the potential of extracellular RNAs as non-invasive diagnostic indicators of disease [41–44]. We recently reported that targeting the KCNMA1 variants may be a clinically beneficial strategy to prevent or at least slow down glioma transformation to GBM [18]. In both human and mouse lymphoma models, researchers have shown that MYC directly induced the transcription of genes encoding core splicing machinery components. They also showed that PRMT5 is involved in MYC-driven tumorigenesis in mice with lymphoma and discovered that tumor development was delayed [44]. Now due to high-throughput New Generation Sequencing (NGS) technologies the splicing diagnostic methodologies have improved. Hence NGS can be utilized in clinical diagnostics of splice variants in diagnosis, prognosis and treatment of breast cancers.

4.4 Perspective of *KCNMA1* splice variants

We believe that future therapies for metastatic breast cancer depend on further investigation into the mechanisms and cellular events caused by oncogene splicing such as *KCNMA1*. Such studies should lead to the development of future therapies for this deadly type of cancer. *KCNMA1* splice variants that are identified in breast tumor patients with brain metastasis will pave for accurate diagnosis and prognostication. Furthermore, they provide potential targets for anticancer drug development. Clinical outcome of *KCNMA1vE22* expression in breast metastasis is expected to reveal the variants' clinical importance. Quantifying the levels of *KCNMA1vE22* could be useful to identify biological process that increases the malignancy and affect prognosis of patients with breast cancer metastasis in the brain.

5. Conclusions

Perhaps the discovery and validation of brain specific metastasis-associated *KCNMA1* alternate splice variants will serve as new tools for the diagnosis and classification of breast tumor patients with high risk of brain metastasis. In fact, splice variations in a number of genes have already been shown to correlate with malignancy and their occurrence could precede clinical cancer diagnosis. To date, however brain-specific alternate *KCNMA1* splice variants in breast cancer have not been reported. The variant *KCNMA1ΔE2* and *KCNMA1E22* that we have recently discovered potentially may fill the gap to serve as a biomarker of breast cancer metastasis to brain. Undoubtedly, the research on the putative association between *KCNMA1* splice variants and breast cancer metastases to brain will prove to be an extremely productive research to identify new generation of biomarkers for early detection and therapeutic intervention in breast cancer patients with high risk for brain metastases.

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Author details

Divya Khaitan[1] and Nagendra Ningaraj[1,2]*

1 Department of Molecular Oncology, Scintilla Academy for Applied Sciences' Education and Research, Bangalore, India

2 Molecular Diagnostics Labs, Scintilla Bio-MARC, Bangalore, India

*Address all correspondence to: sainagendra50@gmail.com

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