MOLECULAR & CELLULAR ONCOLOGY
2016, VOL. 3, NO. 2, e1083648 (3 pages)
http://dx.doi.org/10.1080/23723556.2015.1083648

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AUTHOR'S VIEW

OPEN ACCESS

hMENA^{11a}, a hMENA isoform sending survival signals

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ABSTRACT

Human MENA^{11a} (hMENA^{11a}), an epithelial-associated isoform of the actin binding protein enabled homolog (ENAH, also known as mammalian ENA [MENA]), is upregulated and phosphorylated following the activation of human epidermal growth factor receptor (HER) 1, HER2, and HER3. Here, we reveal a novel role of this isoform in sustaining cell survival and propose hMENA^{11a} as a marker of HER3 activation and resistance to phosphatidylinositol-3-kinase inhibition therapies.

Abbreviations: BIM, Bcl2-interacting mediator of cell death; CASP9, caspase 9; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ENAH, enabled homolog; ENA/VASP, enabled/vasodilator-stimulated phosphoprotein; ER, estrogen receptor; FOXO3a, Forkhead box O3a; HER, human epidermal growth factor receptor; MENA, mammalian enabled; PgR, progesterone receptor; NRG, neuregulin; PARP, poly (ADP-ribose) polymerase; PI3K, phosphatidylinositol-3-kinase; RPPA, reverse phase protein array

ARTICLE HISTORY
Received 7 August 2015
Revised 11 August 2015
Accepted 12 August 2015

KEYWORDS
Breast cancer; HER3; oncogenic signaling; PI3K; therapy resistance

Human enabled homolog (ENAH, also known as hMENA), an actin regulatory protein of the enabled/vasodilator-stimulated phosphoprotein (ENA/VASP) family, has a crucial role in cellular processes that rely on actin cytoskeleton dynamics.

hMENA is absent in normal breast but is overexpressed in breast tumors, where its expression correlates with the human epidermal growth factor receptor (HER) 2-positive, estrogen receptor/progesterone receptor (ER/PgR)-negative, and high-Ki67 phenotype. hMENA crosstalks with HER family signaling and its concomitant overexpression with HER2 identifies a subgroup of patients with the worst prognosis.[1]

The *hMENA* gene undergoes a splicing process that gives rise to multiple isoforms that are expressed in specific tissues and cell types.[2] Among these isoforms, our group has identified 2 alternatively expressed variants, one with the inclusion of exon 11a (hMENA^{11a}) and a proproliferative,[3] anti-invasive,[4] role in cancer, and the other with exclusion of exon 6 (hMENADv6) and an invasive role in breast and lung cancer.[4,5] Inclusion of the additional exon 11a is regulated by different splicing factors[6] and occurs in a site adjacent to the F-actin and G-actin binding sites in the enabled/vasodilator-stimulated phosphoprotein homology 2 (EVH2) domain. Three putative phosphorylation sites (serine 3, serine 18, and tyrosine 16) are located in the 21 amino acids encoded by the 11a exon although the corresponding kinases are still unknown.[3] hMENA^{11a}, together with hMENA (hMENA/hMENA^{11a}), is expressed in epithelial breast tumor cells. Epidermal growth factor (EGF) and neuregulin (NRG-1), as well as HER2 overexpression and activity, increase the expression levels of hMENA and hMENA^{11a} and phosphorylation of only hMENA^{11a}.[1,3] Conversely, trastuzumab decreases hMENA expression and reduces hMENA^{11a} phosphorylation.[1] Depletion of hMENA/hMENA^{11a} decreases HER3 phosphorylation, inhibits EGF- and NRG-mediated activation of epidermal growth factor receptor (EGFR, also known as HER1) and HER2, and counteracts growth factor-mediated cell proliferation.[1]

Actin binding proteins regulate different apoptosis pathways, and remodeling of the actin cytoskeleton favors evasion by tumor cells of normal apoptotic signaling. Currently no data are available on the role of hMENA^{11a} in signaling related to cell survival and apoptosis.

Recently, we highlighted a novel role for hMENA^{11a}.[7] We designed a reverse phase protein array (RPPA) assay to investigate whether hMENA^{11a} plays a role in oncogenic signaling linked to cell proliferation and survival (Fig. 1A). Notably, the heatmap generated by the RPPA analysis highlights how specific silencing of hMENA^{11a} dramatically switches off molecules relevant to cancer cell survival, such as survivin and, among the tyrosine kinases, EGFR, HER2, and HER3. Conversely, silencing of hMENA^{11a} switches on molecules linked to apoptosis such as cleaved forms of poly (ADP-ribose) polymerase (PARP) and caspase 9 (CASP9) (Fig. 1A). hMENA^{11a} silencing impaired the NRG-1-mediated activation of HER3 in HER2 overexpressing breast cancer cell lines, suggesting that NRG-1 was no longer able to activate HER3 in cells lacking hMENA^{11a}.

Thus, we reasoned that a correlation between hMENA^{11a} and activation of HER3 may also occur *in vivo*. Indeed, we found a significant ($p<0.0001$) correlation between P-HER3 and hMENA^{11a} in a cohort of primary HER2-positive breast tumors and showed that 95% of cases with a 2+ or 3+ P-HER3 score were also positive for hMENA^{11a}. Interestingly, strong and membranous staining of hMENA^{11a} was found in cells that were highly positive for P-HER3 whereas cells

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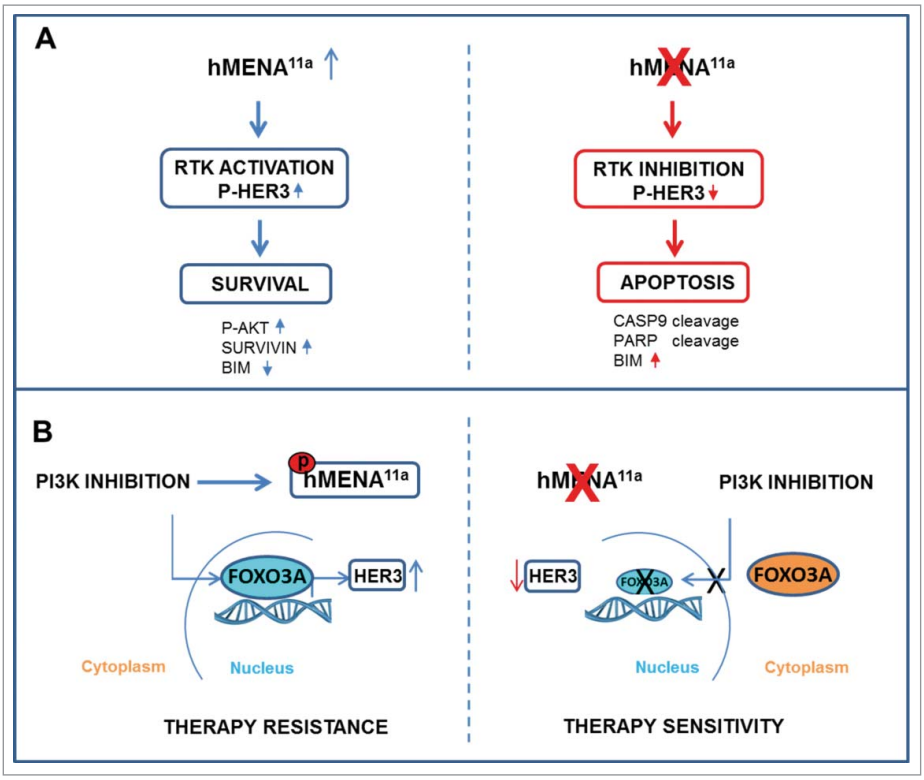

Figure 1. hMENA^{11a} delivers survival signals and promotes resistance to PI3K inhibition. (A) Overexpression of hMENA^{11a} participates in receptor tyrosine kinase (RTK) and survival pathway activation. Specific silencing of hMENA^{11a} inhibits RTK activation (i.e., P-HER3), favoring cell apoptosis. (B) Treatment of HER2+ breast cancer cell lines with PI3K inhibitors determines FOXO3a nuclear translocation and HER3 upregulation, a mechanism involved in therapy resistance. PI3K inhibitors induce phosphorylation of hMENA^{11a}. In cells that are specifically silenced for hMENA^{11a} and treated with PI3K inhibitors, FOXO3a is sequestered in the cytoplasm and does not shuttle into the nucleus and HER3 is not upregulated, rendering cells sensitive to therapy. BIM, Bcl2-interacting mediator of cell death; CASP9, caspase 9; FOXO3a, Forkhead box O3a; hMENA, human MENA; P-HER3, phospho-human epidermal growth factor receptor 3; RTK, receptor tyrosine kinase; P-AKT, phospho-protein kinase B; PARP, poly (ADP-ribose) polymerase; PI3K, phosphatidylinositol-3-kinase.

that scored low for P-HER3 showed a cytoplasmic localization of hMENA^{11a}, suggesting that when HER3 is activated hMENA^{11a} has a membranous localization. A correlation between HER3 and hMENA^{11a} expression was also found ($p = 0.008$).

Recently, the overexpression and activation of HER3 has been reported to be crucial for mechanisms of cell resistance to different therapies, including phosphatidylinositol-3-kinase (PI3K) inhibitors.[8] Thus, our study logically turned to investigate whether hMENA^{11a} has a role in HER3-based resistance mechanisms to PI3K inhibition in HER2 overexpressing breast cancer cell lines. Indeed, we found that HER3 upregulation and activation induced by the PI3K inhibitor BEZ235 were impaired in cells silenced for hMENA^{11a} at both protein and RNA levels. Surprisingly, nuclear accumulation of the transcription factor Forkhead box O3a (FOXO3a), which is responsible for HER3 transcription following PI3K inhibition,[9] was impaired after hMENA^{11a} silencing (Fig. 1B). Of note, the nuclear or cytoplasmic localization of FOXO3a depends on its binding to 14-3-3 scaffold proteins, which recognize phosphorylated domains and regulate subcellular protein localization.[10] Different predicted binding sites for 14-3-3 are present in exon 11a of hMENA^{11a}, suggesting that this isoform may have a role in the dynamics of FOXO3a. Nevertheless, BEZ235 treatment induced hMENA^{11a} phosphorylation (Fig. 1B) and movement of hMENA^{11a} toward membranous structures resembling focal adhesions, but did not affect hMENA^{11a} expression levels. PI3K inhibition leads to upregulation of a number of kinases that may render tumors insensitive to PI3K inhibition. Although at this point the kinases involved in hMENA^{11a} activation have yet to be identified, our data suggest that hMENA^{11a} overexpression and phosphorylation sustain HER3 expression and activation, in turn contributing to PI3K resistance mechanisms. Indeed, transfection of hMENA^{11a} into a breast cancer cell line lacking endogenous expression of any hMENA isoforms increased P-HER3 levels and determined cell resistance to BEZ235. At the functional level, hMENA^{11a} silencing followed by BEZ235 treatment strongly decreased the percentage of cells in S phase of the cell cycle and induced cell death in 3D cultured cells, and increased the expression levels of Bcl2-interacting mediator of cell death (BIM), a critical mediator of targeted therapy-induced apoptosis, indicating that hMENA^{11a} may participate in the balance of pro- and antiapoptotic proteins to favor survival signaling pathways.

Although further efforts are needed to unambiguously place hMENA^{11a} in druggable signaling networks, hMENA^{11a} is emerging as a key signaling hub that is able to intersect the axis connecting EGFR family proteins to downstream molecules such as PI3K. The critical contribution of hMENA^{11a} to cell resistance to PI3K inhibition highlights the need to shut down hMENA^{11a} signaling

when designing more efficacious breast cancer targeted therapies.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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