

RASSF1A methylation, YAP1 activation and metastasis: a new role for an old foe in lung cancer

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Provenance: This is an invited Editorial commissioned by the Section Editor Chunlin Ou (Cancer Research Institute of Central South University, Changsha, China).

Comment on: Dubois F, Keller M, Calvayrac O, *et al.* RASSF1A suppresses the invasion and metastatic potential of human Non-Small Cell Lung Cancer Cells by Inhibiting YAP Activation through the GEF-H1/RhoB Pathway. *Cancer Res* 2016;76:1627-40.

Submitted Mar 26, 2017. Accepted for publication Mar 29, 2017.

doi: 10.21037/jtd.2017.04.16

View this article at: <http://dx.doi.org/10.21037/jtd.2017.04.16>

The majority of lung cancer patients die not from primary, but metastatic disease. While mutations/rearrangements in oncogenes such as *KRAS*, *EGFR* and *ALK*, occur early in tumorigenesis, experimental evidence suggests that additional alterations are required to promote tumor invasion and progression (1). Despite knowing a great deal about the molecular pathways associated with the metastatic phenotype, little is known about how these factors become deregulated to drive the spread of lung cancer cells. In a recent article in *Cancer Research*, Dubois and colleagues shed light on this process, demonstrating that a gene long known to be inactivated in lung cancer—*RASSF1A*—suppresses the invasive and metastatic potential of non-small lung cancer (NSCLC) (2). *RASSF1A*, which resides at 3p21.3, is silenced in up to 40% of NSCLC cases through promoter hypermethylation and impairs tumorigenicity when re-expressed in cell lines with inactivation (3), partly through regulating cell cycle and apoptotic signaling in response to Ras pathway activation (4). These findings have established *RASSF1A* as a tumor suppressor gene in NSCLC. The new findings from Dubois *et al.* suggest an additional role for this gene in inhibiting the hippo pathway transcription factor yes-associated protein 1 (YAP1), which when activated, can drive the spread of lung cancer cells. This provides critical insight linking a genetic/epigenetic alteration (*RASSF1A* methylation) to initiation of metastasis, which may drive disease progression in a large

fraction of NSCLC patients.

Using RNA interference in immortalized human bronchial epithelial cell (HBEC) and NSCLC cell lines, Dubois *et al.* demonstrate that *RASSF1A* depletion promotes epithelial-to-mesenchymal transition (EMT) and subsequently enhances metastatic capacity in cells. Upon *RASSF1A* suppression, the HBEC cells expressed several EMT markers, exhibited disorganized and reduced cell-cell contacts and developed long cytoplasmic extensions characteristics of invasive cells. Consistent with these findings, *RASSF1A* knockdown cells also exhibited increased cell motility and cell invasion as shown through migration and matrigel invasion assays. In subcutaneous xenograft mouse models using NCI-H1975 cells, a NSCLC line harboring activating *EGFR* mutations (L858R and T790M), they observed that stable knockdown of *RASSF1A* increased tumor growth and metastasis to the lungs, suggesting that the loss of *RASSF1A* can enhance tumorigenic and metastatic capacity.

To elucidate the mechanistic features of *RASSF1A* in EMT regulation, the authors investigated the relationship between *RASSF1A* and YAP1, which is a key transcriptional cofactor regulating this process (5). Silencing *RASSF1A* resulted in strong nuclear localization and activation of YAP1, which was confirmed by the increased transcription levels of its target genes, *ANKRD1* and *CTGF*. Additionally, co-transfection of siRNAs for both *RASSF1A* and YAP1

was able to restore EMT markers to normal levels, suggesting YAP1 as the key mediator of the phenotype induced by RASSF1A inactivation. Importantly, the authors also observed a significant reduction in the expression of genes that regulate cytoskeleton dynamics and motility in cells, such as cofilin, RhoB and mDia1. Most notably, increasing RhoB, which exerts anti-migratory effects on cells, in RASSF1A depleted cells made the cells resistant to the previously described effects of RASSF1A silencing on cell invasiveness in matrigel assays.

Similar effects were observed when key upstream activators of RhoB, such as its guanine nucleotide exchange factor GEF-H1 (also known as ARHGEF2), were increased in the RASSF1A knockdown line, providing further evidence to the relationship between RASSF1A and the RhoB pathway in regulating EMT. In addition, suppressing GEF-H1 expression in HBECS increased the cell invasiveness to levels equivalent to that of the RASSF1A knockdown, implicating GEF-H1 as a key downstream mediator of RASSF1A. Moreover, the authors were able to demonstrate that PP2A, which dephosphorylates and activates GEF-H1 (6), GEF-H1 and RhoB directly interact with each other in HBECS through a co-immunoprecipitation assays. This interaction is disrupted by suppressing RASSF1A in these cells, which indicated that RASSF1A plays an important role in maintaining the activity of this complex and its downstream effects.

Dubois *et al.* further elucidate how RhoB affects the nuclear localization of YAP1 and its activation. In the siRASSF1A HBECS, they show that expressing either RhoB (wild-type or constitutively active RhoBV14) or its upstream effectors, like GEFH-1, can disrupt the localization of the YAP1 protein in the nucleus and its subsequent transcription of *ANKRD1* and *CTGF*. Additionally, YAP1 and RhoB-GTP (activated form of RhoB), exhibit a reciprocal relationship as shown by YAP1 depletion resulting in increased RhoB-GTP levels in cells. Similar trends were observed in tumors obtained from transgenic mouse models expressing tetracycline-inducible EGFR^{L858R} and either intact or deficient RhoB. A higher number of tumors obtained from the EGFR^{L858R} mice with RhoB knockout exhibited strong nuclear staining of YAP1 compared to the mice with intact RhoB. Thus, the authors conclude that RASSF1A inhibits the migratory phenotype and EMT by stabilizing the activity of the PPA2/GEF-H1/RhoB complex, which in turn disrupts the nuclear localization of YAP1 and the subsequent transcription of genes that impart mesenchymal features on NSCLC cells.

While the experiments connecting RASSF1A

inactivation with GEF-H1/RhoB/YAP1 activity offer a new understanding of the metastatic process in lung cancer, additional questions remain. First, it will be important to understand the influences of histological and molecular diversity on the function of RASSF1A and whether these factors modify the ability of YAP1 to drive tumor spread in different contexts. For example, does the anti-apoptotic role of RASSF1A inactivation take priority in the context of KRAS activating mutations compared to those with other driver mutations? Does YAP1 require additional transcriptional inputs/cofactors in order to initiate EMT? With large genomic and epigenomic datasets being generated as a part of collaborative initiatives such as The Cancer Genome Atlas (7), it may be possible to assess associations to answer these and other questions in order to identify the subset of patients where RASSF1A may be driving the metastatic phenotype and identify potential routes for combination based therapies. Second, genetically engineered mouse models of RASSF1A inactivation will be needed to validate the cell model/xenograft experiments from the current study. While the authors used a transgenic lung cancer mouse model consisting of mutant EGFR expression with and without RhoB knockout, only YAP1 levels and not survival or metastatic spread were assessed in the resulting tumors. Creating RASSF1A knockout mice and crossing with established lung cancer models driven by conditional expression of oncogenes known to be associated with RASSF1A methylation in human tumors would provide the optimal system to determine the metastatic potential of this alteration in a physiologically relevant setting. Furthermore, using conditionally inactivated RASSF1A with Cre-Lox technology would allow researchers to assess whether the role of RASSF1A disruption shifts at different stages of tumor progression. Lastly, these same mouse models would serve as important preclinical systems to test the effects of YAP1 inhibitors in preventing tumor progression and metastasis in the setting of RASSF1A inactivation. Together, this exciting work by Dubois and colleagues provides molecular knowledge of a poorly understood aspect of lung cancer development that will open new avenues for therapeutic research and intervention aimed at reducing the mortality rates for patients affected by this devastating disease.

Acknowledgements

Funding: This work was funded by the Canadian Institutes of Health Research (CIHR, MOP-142313 and PJT-148725)

and a Terry Fox Research Institute New Investigator Award (11-364). WW Lockwood is a Michael Smith Foundation for Health Research Scholar and CIHR New Investigator.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Cite this article as: Oh MH, Lockwood WW. RASSF1A methylation, YAP1 activation and metastasis: a new role for an old foe in lung cancer. *J Thorac Dis* 2017;9(5):1165-1167. doi: 10.21037/jtd.2017.04.16