New Strategies in Pleural Mesothelioma: BAP1 and NF2 as Novel Targets for Therapeutic Development and Risk Assessment

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Abstract

Malignant pleural mesothelioma (MPM) is a highly lethal cancer with limited therapeutic options. Recent work has identified frequent inactivation of two tumor suppressor genes in MPM—NF2 (Neurofibromatosis type 2) and BAP1 (BRCA associated protein 1). Additionally, germline mutations in BAP1 have been identified and an associated cancer syndrome which includes MPM, ocular melanoma and other cancers has been described. These recent advances may allow screening of high risk individuals and the development of new therapies that target key pathways in MPM.
Background

Malignant pleural mesothelioma (MPM) is an almost universally fatal disease striking approximately 3,000 individuals each year in the United States. Currently available therapies for MPM have limited effect on outcomes. Even with aggressive multimodality therapy including surgery for early stage disease, the median overall survival is 18 months from the time of diagnosis. For patients with advanced disease, first-line chemotherapy with pemetrexed and cisplatin improves median survival by a few months, but the benefit is limited and the utility of second line chemotherapy has not been established.

In this context, numerous clinical trials have been conducted in MPM to evaluate a variety of targeted agents. These include angiogenesis inhibitors such as bevacizumab and thalidomide, receptor kinase inhibitors such as erlotinib and sorafenib, and histone deacetylase inhibitors such as Vorinostat. Despite the activity of these drugs in other diseases, they have shown little activity in MPM. Ideally, treatments that more specifically target the unique biology of MPM should be more effective. Just as the identification of somatic mutations in other malignancies has led to the development of effective disease-specific therapeutics, the potential identification of similar, “targetable” somatic mutations in MPM would be important.

This review calls attention to emerging data on the frequent inactivation of two genes in MPM—Neurofibromatosis Type 2 (NF2) and BRCA1 Associated Protein 1 (BAP1)—that could help elucidate key pathways that drive tumorigenesis and which could subsequently be exploited as rational targets for drug development in this cancer.

NF2 Loss and mTOR Blockade

About 35-40% of MPMs carry inactivating mutations at the neurofibromatosis 2 (NF2) locus, which encodes for the FERM domain protein Merlin. The mechanisms by which Merlin suppresses tumorigenesis have remained poorly defined. Merlin is homologous to the ERM proteins Ezrin, Radixin and Moesin and, like these proteins, it associates with the cortical actin cytoskeleton. Since loss of Merlin causes activation of multiple mitogenic signaling pathways, such as HER1/2, mTOR, ERK, and FAK, it has been postulated that Merlin inhibits signaling by negatively regulating multiple cell surface receptors. However, Merlin lacks an actin-binding motif and its closed conformer, which is the only one active as a tumor suppressor, is not enriched at the cell membrane. Recent studies have revealed that the closed, active form of Merlin accumulates in the nucleus and interacts with DCAF1, the receptor component of the E3 ubiquitin ligase CRL4\(^{DCAF1}\). Notably, Merlin inhibits CRL4\(^{DCAF1}\), which in turn promotes a broad oncogenic gene expression program, presumably by ubiquitinating transcription factors, histones, or chromatin remodeling enzymes. This deregulation of nuclear ubiquitination events is an intriguing common thread with BAP1 inactivation as described below and the possible overlap between DCAF and BAP1 targets merits further investigation. Genetic epistasis experiments and an analysis of several Merlin missense mutations from NF2 patients support the hypothesis that the dephosphorylated form of Merlin suppresses tumorigenesis by inhibiting CRL4\(^{DCAF1}\). These results suggest that Merlin affects multiple mitogenic signaling pathways by controlling, through CRL4\(^{DCAF1}\), the expression of components of regulators of these pathways (Figure 1). It has also been proposed that...
Merlin loss in MPM, along with inactivating mutations in LAT52 in a small percentage of cases,\textsuperscript{10, 11} may also contribute to oncogenesis through activation of the Hippo signaling cascade.\textsuperscript{12} However, it remains unclear whether loss of Merlin inactivates the Hippo kinase in the cytosol, as genetic studies in the fly suggest, or it deregulates the oncoprotein and transcriptional coactivator YAP through activation of CRL4\textsuperscript{DCAF1}.\textsuperscript{9}

Merlin mediates contact-dependent inhibition of cell proliferation in normal cells, primarily through inhibition of mTOR in an AKT-independent manner.\textsuperscript{7} Without Merlin, mTOR activity is aberrantly upregulated, leading to increased cell proliferation.\textsuperscript{13} This mTOR activation has also been observed in other Merlin-deficient tumors.\textsuperscript{14} This increased proliferation can be reduced but not completely suppressed by mTOR inhibition. In the presence of Merlin, mTOR inhibition has little effect on growth. In MPM tumors and cell lines, there is an inverse relationship between phospho-mTOR expression and NF2 expression confirming the link between NF2 and the mTOR pathway and we have found that immunohistochemical staining for NF2 and phospho-mTOR could be useful in selecting MM patients for trials targeting mTOR pathway activation due to NF2 loss.\textsuperscript{15} These preclinical observations have provided the rationale for studying mTOR inhibitors in MPM patients. Everolimus, an oral derivative of the organ-transplant immunosuppression drug rapamycin, which has also been approved for the treatment of advanced renal cell carcinoma, is being investigated as second-line therapy in a Phase 2 trial run by the Southwest Oncology Group (SWOG). In addition, due to compensatory parallel pathway activation, mTOR inhibition alone may not be sufficient to suppress tumor growth. Indeed, we have found that MET, EGFR and IGF1R are variably activated after mTOR inhibition in MPM cell lines, contributing to AKT feedback activation.\textsuperscript{16}

Preclinical evidence indicates that isolated mTOR inhibition alleviates feedback inhibition on PI3K and thereby allows restoration of PI3K and downstream AKT signaling.\textsuperscript{17} To address this mechanism of mTOR resistance, numerous dual PI3K/mTOR inhibitors are in early development. Two compounds, BEZ 235 and GDC 0980, have shown promise in MPM. BEZ 235, a dual PI3K/mTOR inhibitor, is able to inhibit the growth of peritoneal mesothelioma cells.\textsuperscript{18} Additionally, in a Phase 1 trial of GDC 0980, 3 of 6 enrolled MPM patients had tumor shrinkage ranging from 23-28% and one patient had stable disease on this compound for more than one year.\textsuperscript{19} However, cases of grade 2 and 3 pneumonitis have been reported with GDC 0980, albeit at a dose above the maximum tolerated dose (MTD). It remains unclear if the pulmonary toxicity will pose a clinical obstacle to the further application of these agents in the treatment of MPM.

**BAP1 Somatic Mutations**

The frequent occurrence of NF2 gene loss in MPM, as well as the very high prevalence of p16/CDKN2A deletions,\textsuperscript{20-22} have been known since the mid-1990s. However, a recent integrated genomics analysis has discovered another frequently inactivated tumor suppressor gene in this cancer. By profiling genomic copy number alterations in 53 MPM tumor samples, Bott et al identified candidate regions for sequencing.\textsuperscript{10} The 3 most common deletions noted were at 22q (which includes NF2), 9p21 (which includes CDKN2A), and 3p21. Within these and other candidate regions, integration with gene expression data was used to prioritize genes for sequencing. The gene implicated by deletions in 3p21
was subsequently elucidated from sequencing of this region. Of the 25 genes selected for sequencing, the highest rate of non-synonymous somatic mutations was in the \textit{BAP1} gene at 3p21.1, occurring in 23\% of the samples. A high rate of somatic mutations was subsequently confirmed in 6/25 (24\%) MPM cell lines and 12/68 (18\%) of additional MPM tumor samples. In all, \textit{BAP1} loss, mutation, or both were identified in 42\% of cases. Thirty-two different \textit{BAP1} mutations were identified: 6 nonsense, 5 missense, 13 frameshifting, and 8 at or near splice sites. The majority of truncating mutations resulted in loss of the nuclear localization signal and/or the C-terminal protein binding domain (Figure 2). Several missense mutations were shown to affect the ubiquitin hydrolase activity of BAP1. Splice site mutations caused exon skipping leading to aberrant, out of frame transcripts. Immunohistochemistry for BAP1 confirmed the association between \textit{BAP1} mutation and an absence of BAP1 protein expression. Interestingly, 25\% of tumors without identified \textit{BAP1} mutations did not display any IHC staining for BAP1 suggesting the possibility of another subset of tumors with functional \textit{BAP1} loss arising by other mechanisms. A recent study has suggested that BAP1 inactivation may be more characteristic of epithelioid MPM\cite{10} although this was not observed in the original study.\cite{10}

\textit{BAP1} is a 729 amino acid nuclear ubiquitin hydrolase that has been implicated in numerous cellular processes such as cell proliferation and DNA repair (Figure 2) as well as chromatin-level control of gene expression (Figure 2).\cite{24} For example, BAP1 has been shown to enhance progression through the G1-S checkpoint and subsequently induce cell death by a process with similarities to both apoptosis and necrosis.\cite{24} Additionally, BAP1 regulates cell proliferation by deubiquitinating host cell factor-1 (HCF-1).\cite{25} a chromatin-associated protein believed to activate and repress transcription by linking appropriate histone-modifying enzymes to a subset of transcription factors, in particular of the E2F family.\cite{26} \textit{BAP1} knockdown using small interfering RNA (siRNA) in MPM cell lines inhibited cell growth, and resulted in inactivation of HCF1 and downregulation of downstream E2F-responsive genes.\cite{10} It has also been shown that BAP1 and HCF-1 can form a ternary complex with the YY1 transcription factor to regulate gene expression.\cite{27}

Inactivating somatic mutations of \textit{BAP1} have been identified in 47\% of uveal melanomas, primarily in the metastasizing subset, where the \textit{BAP1} mutation rate is close to 80\%.\cite{28} The possible involvement of BAP1 in modulating histone modifications prompted a study of histone deacetylase inhibitors in uveal melanoma cell lines which found that these agents appeared to counteract the expression profile of \textit{BAP1}-deficient uveal melanomas but the growth inhibitory effects on the cell lines were independent of BAP1 status.\cite{29} The relevance of these results to \textit{BAP1}-mutated MPM is unclear. We have not seen a significant effect of \textit{BAP1} knockdown on sensitivity of three \textit{BAP1} wild type MPM lines (0211H, H-Meso, H2373) to the histone deacetylase inhibitor suberoylanilide hydroxamic acid (vorinostat) (R. McMillan, M. Ladanyi, unpublished data). Furthermore, a recently completed Phase III trial of vorinostat in patients with advanced MPM observed few significant responses, well below what would be expected if \textit{BAP1}-mutated cases were preferentially sensitive.\cite{30} The relationship of \textit{BAP1}-related histone modifications to other previously reported epigenetic changes in MPM such as hypermethylation at gene promoters (such as WIF1, SFRP4, etc...)\cite{31-33} remains to be defined.
BAP1 Germline Mutations

Coming on the heels of the discovery of somatic BAP1 mutations is the recent discovery of germline BAP1 mutations in rare families predisposed to MPM. While the majority of MPM cases are linked to asbestos exposure with a 20-40 year latency, only a minority of those exposed to asbestos eventually develop malignant disease. For example, in the US, it is estimated that over 30 million workers were exposed to asbestos in the second half of the 20th century but the incidence of MPM has remained relatively stable and low in the US over the past 15 years at 1-15/100,000. Furthermore, numerous familial clusterings of MPM both with and without exposure to asbestos or erionite have been well described with an up to 50% incidence of MPM within such families. Taken together, these observations suggest that some individuals are more susceptible to the carcinogenic effects of asbestos and erionite and some are more susceptible to the development of MPM even in the absence of asbestos or erionite exposure.

Therefore, much work has focused on identifying genetic alterations that predispose individuals to MPM. Testa and colleagues performed array-comparative genomic hybridization (CGH) on two MPM tumors coming from two different familial clusters of MPM. Neither family had exposure to asbestos or erionite. Alterations were noted at or near the BAP1 locus at 3p21.1. Additionally, linkage studies were performed on germline DNA from each family, yielding a maximum lod score of 2.1 at 3p21-22. Given these observations and reports of frequent gene loss at 3p21.1 in sporadic MPM, germline BAP1 sequencing was performed in both families and revealed concordance between mutation status and linkage analysis. Furthermore, those with germline mutations manifested other malignancies in addition to MPM including uveal melanoma, breast cancer, renal cancer, and skin cancer. At the same time, Weisner et al. reported an association between germline mutations in BAP1 and familial melanocytic tumors ranging from epithelioid nevi to atypical melanocytic proliferations with features of cutaneous melanoma. Tumors were examined from two families and revealed biallelic somatic inactivation of BAP1. Each family had one person with uveal melanoma and one family had multiple members with cutaneous melanoma. Presently, with only few published pedigrees, the exact spectrum of this newly described hereditary cancer predisposition syndrome remains unclear. In fact, it is possible that two distinct cancer syndromes exist, both associated with uveal melanoma but one dominated by melanocytic tumors and another by MPM. Njauw and colleagues have recently published additional melanocytic tumor-rich pedigrees of families with germline BAP1 mutations. They have proposed for this phenotypic complex the acronym COMMON syndrome, which we would suggest to broaden as follows: cutaneous/ocular melanoma and atypical melanocytic proliferations, mesothelioma, and other internal neoplasms. Ultimately, the phenotype associated with germline BAP1 mutations will likely be complex and depend on a variety of factors such as the tissue in which the second BAP1 allele is inactivated, the mechanism of inactivation for the second BAP1 allele, the functional consequences of a particular BAP1 mutation, co-existent mutations, and environmental exposures.

On the Horizon

The recently published data reviewed above suggest that, together, a majority of MPM carry either NF2 or BAP1 mutations. Whereas BAP1 encodes for an ubiquitin C-terminal hydrolase (deubiquitinase, DUB)
and is therefore able to reverse the ubiquitin linkages formed by E3 ubiquitin ligases, NF2 encodes for a negative regulator of the E3 ubiquitin ligase CRL4\textsuperscript{DCAF1}. DUBs are thought to function by binding to the substrate receptor components of specific, partner E3 ligases and by reversing the linkage that the latter form. Interestingly, total loss-of-function mutations in NF2 and BAP1, as assessed by immunoblotting, occur in largely non-overlapping subsets of MPM patients (see immunoblotting of cell lines for Merlin\textsuperscript{13} and for BAP1\textsuperscript{10}). This raises the hypothesis that CRL4\textsuperscript{DCAF1} and BAP1 regulate in an opposing fashion a common subset of substrates, which in turn causes a pro-tumorigenic deregulation of gene expression in MPM. Alternatively, NF2 and BAP1 may suppress tumorigenesis by independent mechanisms. The therapeutic implications of distinguishing between these models will be significant.

If studies define a therapeutically accessible synthetic lethal target in the setting of BAP1 loss, this could eventually benefit the approximately 40% of MPM patients whose tumors have BAP1 loss or mutation. More speculatively, the same synthetic lethal target could be studied as chemoprevention drug targets in individuals (or, initially, mouse models) with germline BAP1 mutations that predispose to MPM development. More fundamentally, the biological insights into MPM pathogenesis emerging from further work on BAP1 could lead to novel, biologically rational treatment strategies for MPM. Finally, the impact of the work may also extend to other cancers with BAP1 mutations such as melanoma and, as recently reported, clear cell renal cell carcinoma\textsuperscript{42-44}. 

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Figure Legends

**Figure 1. Mechanisms of NF2(Merlin)-mediated inhibition of proliferation.** Merlin exists in an open, inactive form and a closed, active form. Matrix adhesion and the consequent activation of joint integrin-receptor tyrosine kinase signaling activate the serine-threonine kinase PAK. PAK in turn phosphorylates the C-terminus of Merlin, disrupting the intramolecular association that maintains the protein in a closed conformation. The resulting inactivation of Merlin removes a block to cell cycle progression in normal cells. Conversely, engagement of E-cadherin and the ensuing assembly of adherens junctions (AJs) inactivate PAK, leading to an accumulation of the closed, active form of Merlin, which migrates into the nucleus to inhibit CRL4<sup>DCAF1</sup>. When it is not inhibited by Merlin, CRL4<sup>DCAF1</sup> positively regulates a broad oncogenic program of gene expression, which includes mitogenic signaling components, anti-apoptotic proteins, and Hippo pathway target genes. CRL4, cullin-ring E3 ligase 4; DCAF1, DDB1- and CUL4-associated factor 1; PAK, p21-activated kinase.

**Figure 2. A. BAP1 functional domains and mapped interacting regions.** BAP1 is a nuclear deubiquitinating enzyme (DUB). DUBs catalyze the removal of single ubiquitin moieties from ubiquitin chains or cleavage of the isopeptide bond between ubiquitin and the substrate protein. BAP1 is composed of an N-terminal UCH domain (blue; a.a. 1-250), an HCF1-binding domain (HBM)-like motif (a.a. 363-366), a motif that shares conservation with UCH37 (ULD: UCH37-like domain; green, a.a. 634-693), and a bipartite nuclear localization signal (NLS, a.a.656-661, a.a.717-722). BAP1 has been reported to interact with BARD1 (a.a. 182-365), HCF1 (a.a. 365-385), BRCA1 (a.a. 596-721) and YY1 (a.a. 642-686).

**B. BAP1 functions: role of BAP1 in transcription factor regulation (left).** Host Cell factor 1 (HCF1) is detected as major binding partner of BAP1 by mass spectrum analysis. HCF1 interacts with specific transcription factors, including OCT1, E2F1, Kox20, Sp1 and GA binding protein (GABP). HCF1 also associates with several chromatin methyltransferases (Set1, MLL1, MLL5), chromatin acetyltransferases (tMOF) and deacetylases (HDAC1, HDAC2). HCF1 is known to recruit LSD1 to demethylate histone H4 lysine 9 and to promote the trimethylation of histone H3 lysine 4. YY1 is a transcriptional factor which binds to BAP1 and HCF1. BAP1 and HCF1 are recruited by YY1 to various promoters to upregulate gene expression. **Role of BAP1 in chromatin modifications (middle).** The trimethylation of lysine 27 of histone H3 (H3K27me3) is mediated the histone methyltransferase EZH1/2, a component of the PRC2 complex. This triggers recruitment of the PRC1 multiprotein complex through recognition of the H3K27me3 mark by the CBX subunit of the PRC1 complex. Another subunit of the PRC1 complex, the RING1 E3 ligase, then ubiquitylates lysine 119 of histone H2A (H2AK119ub1), which fixes chromatin in a repressed state, silencing gene expression. The H2AK119ub1 mark may be in a dynamic, continuously regulated state, as another part of the PRC1 complex, containing BAP1 and ASXL 1, displays an opposing, de-ubiquitylating activity (PR-DUB). A balance between ubiquitination and deubiquitination may be needed for appropriate PRC target gene repression. **Possible role of BAP1 in DNA repair (right).** Other binding partners of BAP1 include BRCA1 and its partner BARD1, which have important roles in the double strand DNA (dsDNA) repair process. Presently, the possible function of BAP1 in this process is not clear. The Rad51-dependent DNA repair pathway is highly regulated and includes many proteins, some of which may be potential substrates for BAP1-mediated ubiquitin hydrolysis.
References


Inhibition of PAK, nuclear translocation of merlin, and inhibition of CRL4δC

Engagement of cadherins
Loss of integrin-mediated adhesion
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