RAF1 amplification: an exemplar of MAPK pathway activation in urothelial carcinoma

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Despite recent therapeutic gains in the treatment of advanced bladder cancer, the overall survival in patients with metastatic disease remains poor and further therapeutic discovery is needed. Advanced bladder cancer is a molecularly heterogeneous disease, and the identification of driver genetic alterations has led to effective targeted therapeutic agents, such as fibroblast growth factor receptor (FGFR) inhibitors. In this issue of the *JCI*, Bekele et al. identify a subtype of muscle-invasive bladder cancer (MIBC) that harbors *RAF1* amplification. The authors showed that RAF1 inhibition, with pan-RAF inhibitors, and the combination of RAF1 inhibition with MEK inhibition were efficacious in preclinical models harboring *RAF1* amplifications as well as in tumors with *HRAS* and *NRAS* mutations. This study highlights *RAF1* amplification as a driver event in bladder cancer and establishes the central role of the MAPK pathway in bladder tumorigenesis.

Bladder cancer and the MAPK pathway

In 2021, 83,730 cases of bladder cancer are expected, making it the sixth most common cause of cancer in the United States, which will result in an estimated 17,200 deaths (1). While there have been important advances in the treatment of advanced bladder cancer over the past decade, the mortality associated with metastatic disease remains particularly grim. Cisplatin-based regimens have long been the mainstay of first-line therapy in this setting. More recently, the development of other systemic therapies, including immunotherapy (specifically PD-1 axis inhibitors), antibody drug conjugates, and fibroblast growth factor receptor–targeted (FGFR-targeted) therapy have also proven effective (2). Nonetheless, overall survival with these regimens remains on the order of 10 to 14 months, highlighting the need for other effective therapeutic agents/combinations.

The MAPK pathway (in this case, RAF/MEK/ERK; Figure 1A) is a signaling cascade that links extracellular signals to processes, such as cell differentiation, proliferation, and growth. Dysregulation of the MAPK pathway through various means has been identified across a multitude of cancers, with the RAS and RAF proteins in particular having a high frequency of activating mutations (3). The RAS family of proteins, consisting of KRAS, NRAS, and HRAS, are guanine triphosphate–hydrolyzing (GTP-hydrolyzing) enzymes that cycle between an active GTP-bound and an inactive guanine diphosphate–bound (GDP-bound) state. RAS mutations impair GTP hydrolysis, promoting a GTP-bound state and downstream activation of the RAF, MEK, and ERK kinases (3). The RAF family of kinases consists of serine/threonine kinases with three known isoforms in humans (ARAF, BRAF, and RAF1, also known as CRAF). *BRAF* is the isoform most commonly mutated in cancer, most notably in the $BRAF^{V600E}$ mutation, which occurs in a number of different cancers, including melanoma and colorectal cancer, non–small cell lung cancer (NSCLC), and thyroid cancer (4). BRAF inhibitors are particularly effective in $BRAF^{V600E}$ mutated malignancies, especially when administered in combination with mitogen-activated protein kinase (MEK) inhibitors, and there are now multiple FDA-approved BRAF inhibitors and BRAF/MEK inhibitor combinations (4).

RAF1 is amplified in bladder cancers

Unlike *BRAF*, *RAF1* is rarely mutated in cancer, perhaps secondary to its low basal kinase activity (relative to BRAF) as well as the need for posttranslational modification for activation (4, 5). In this issue of the *JCI*, Bekele et al. note that 12% of muscle-invasive bladder cancers (MIBCs) harbor focal amplification of *RAF1*. Notably, an analysis across TCGA cancer types shows that MIBCs have the highest rate of *RAF1* amplification of all tumors, with the next most frequent tumor type being sarcoma (approximately 3%; ref. 6). The authors made a number of interesting genomic associations, including that *RAF1* amplification was enriched in the luminal unstable (LumU) consensus molecular subtype (35% of *RAF1*-amplified tumors are classified as LumU; refs. 6, 7) and that *RAF1*-amplified MIBCs had a significantly higher total mutation count than non-*RAF1*–amplified tumors. Additionally, *RAF1*-amplified tumors cooccurred with *PPARG* amplification (also on chromosome 3p) and *E2F3* amplification, and there was a trend toward cooccurrence with *TP53*

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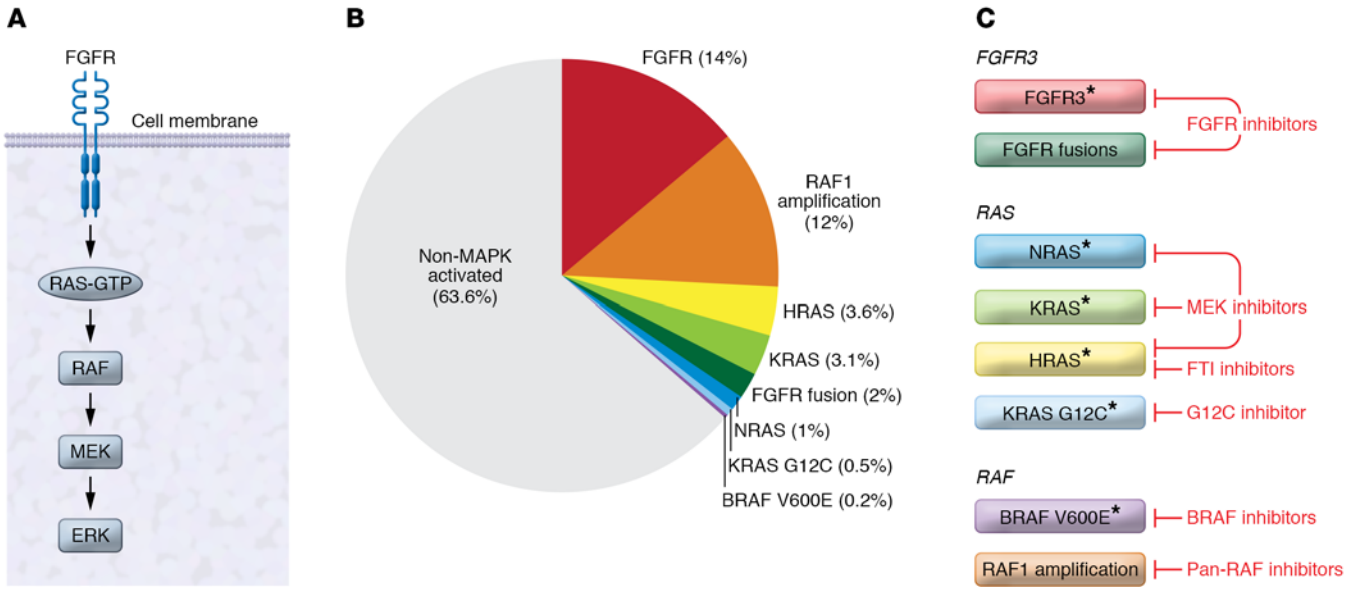

Figure 1. MAPK activation and targeting in bladder cancer. (**A**) The RAF/MEK/ERK signaling cascade links extracellular signals to cell differentiation, proliferation, and growth. (**B**) The Cancer Genome Atlas (TCGA) indicates that MAPK alterations make up 36.4 % of high-grade MIBCs. (**C**) Specific inhibitors may differentially target putative genomic alterations that activate the MAPK pathway to treat bladder tumors. Asterisks indicate mutations.

alterations. Conversely, the *RAF1*-amplified tumors and *FGFR3* mutations were mutually exclusive (6).

To investigate the functional relevance of *RAF1* amplification, Bekele et al. performed a series of elegant in vitro experiments and analyses of publicly available essentiality data sets. For example, siRNA of *RAF1* in bladder cancer cell lines with known *RAF1* amplification decreased MAPK signaling as well as in vitro proliferation. Query of the publicly available DepMap data showed that the *RAF1*-amplified 5637 cell line was highly sensitive to pharmacological RAF inhibition with the pan-RAF inhibitor RAF265 as well as the $BRAF^{V600E}$ inhibitor PLX4720. Importantly, bladder cancer cell lines without *RAF1* amplification showed no dependence upon RAF1. Given the known success of concurrent targeting of BRAF and MEK in MAPK-activated cancers, the authors showed that *RAF1*-amplified cell lines were highly sensitive to dual targeting of RAF1 and MEK by RAF265 and trametinib. Finally, the researchers validated their findings in two independent in vivo models: UMUC9 xenografts as well as a patient-derived xenograft, with known *RAF1* amplification. The combination of RAF265 and trametinib potently reduced tumor size and increased immunohistochemical staining for cleaved PARP consistent with an increase in cell death (6).

RAS mutations activate the MAPK pathway

While *RAS* mutations are uncommon in MIBCs, activating mutations in *KRAS*, *HRAS*, and *NRAS* do occur in a small percentage of patients (Figure 1B). Given the established importance of RAF and especially RAF1 in signaling downstream of RAS isoforms, Bekele et al. hypothesized that targeting RAF with or without MEK inhibition would effectively reduce *NRAS* and *HRAS* mutant tumors. They found that T24 cells (*HRAS* G12V mutant) and Ku-19-19 cells (*NRAS* Q61R mutant) were highly sensitive to RAF265 alone or in combination with trametinib (6). These studies, albeit in a single cell line, identify a further therapeutic role for dual RAF1 and MEK inhibition in *RAS* mutant bladder cancer. Nonetheless, these impressive preclinical results should be viewed cautiously, since the use of RAF and MEK inhibitors in *RAS* mutant cancers has shown variable clincial results. For example, the MEK inhibitor binimetinib demonstrated a progression-free survival benefit over dacarbazine in *NRAS* mutant melanoma (8), while trametinib did not show superiority over docetaxel in *KRAS* mutant NSCLC (9).

The MAPK pathway is targetable in bladder cancer

Bekele and colleagues describe *RAF1*-amplified bladder cancers as another subset of MIBC. Moreover, their studies also demonstrate that bladder tumors with *NRAS* and *HRAS* mutations are sensitive to dual RAF/MEK inhibition. Their work elegantly highlights a key role of the MAPK pathway, which is activated in a considerable proportion of bladder cancers (i.e., *RAF1* amplification, $BRAF^{V600E}$ mutation, and *RAS* [*KRAS, HRAS, NRAS*] mutations; Figure 1B and ref. 6). These findings are perhaps unsurprising, given the early recognition that *HRAS* mutations occur in bladder cancer cell lines (10–12), reports of low-frequency activating $BRAF^{V600E}$ mutations in high-grade bladder cancers (13), and the MEK/ERK pathway as the predominant signaling pathway downstream of FGFR3 in urothelial cells (14). Furthermore, the fact that *RAF1* amplification is mutually exclusive with *FGFR3* alterations and that *RAF1*-amplified tumors are enriched in the LumU consensus molecular subtype also underscores the importance of the RAF/MEK/ERK pathway in both bladder tumorigenesis and the signaling underlying development of the luminal molecular subtype (15, 16). Indeed, Bekele et al.'s work nicely emphasizes how we might invoke a more granular molecular stratification of driver events in MIBC (Figure 1). Despite the fact that these alterations all converge to activate RAF/MEK/ERK signaling, we propose that they each may be targeted differently given the development of therapies

such as farnesyl transferase inhibitors and RAS G12C inhibitors (Figure 1C).

Does *RAF1* amplification promote immune-checkpoint blockade resistance?

Notably, the highest frequency of *RAF1* amplification was seen in the consensus LumU subtype, which has considerable overlap with the genomically unstable (GU) subtype previously defined by the Lund classification (17). The Lund GU subtype responds best to the anti–PD-L1 antibody atezolizumab, with approximately 50% of patients with Lund GU tumors demonstrating a partial or complete response to atezolizumab (18). Given the evolving evidence that RAS/RAF/MEK pathway activation promotes immune evasion and subsequent resistance to PD-1/PD-L1 inhibitors (19, 20), one intriguing possibility is that the subset of Lund GU tumors that are nonresponsive to immune-checkpoint blockade (ICB) are those with RAS/RAF/MEK pathway activation. If true, ICB response could be augmented by combined MEK inhibition and PD-1 axis blockade.

Conclusions

In conclusion, the work by Bekele et al. underscores the importance of continued exploration into driver events underlying MIBC. The identification of this novel *RAF1*-amplified subtype that is highly dependent upon downstream MAPK signaling underscores the importance of this pathway in bladder tumorigenesis and perhaps development of the luminal molecular subtype. Further investigation will hopefully resolve questions such as whether *RAF1* amplification itself drives luminal differentiation in urothelial cells or whether this association is due to coamplification of *PPARG*, to what extent MAPK activation by these driver events (Figure 1B) fosters an immune-excluded phenotype that can be reversed by MEK inhibition, and the ultimate clinical efficacy of dual RAF/MEK inhibition in *RAF1*-amplified bladder cancers.

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