SMALL GTPASES
2016, VOL. 8, NO. 3, 187–192
https://doi.org/10.1080/21541248.2016.1212689

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COMMENTARY

What makes oncogenes mutually exclusive?

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ABSTRACT
Cancer is driven by mutations in genes whose products participate in major signaling pathways that fuel cell proliferation and survival. It is easy to assume that the more of these so-called driver mutations a tumor accumulates, the faster it progresses. However, this does not appear to be the case: Data from large-scale genome sequencing studies indicate that mutations in driver oncogenes often are mutually exclusive. The mechanisms underlying the mutual exclusivity of oncogenes are not completely understood, but recent reports suggest that the mechanisms may depend on the tumor type, and the nature of interacting oncogenes. Here we discuss our recent findings that the oncogenes $KRAS^{G12D}$ and $BRAF^{V600E}$ are mutually exclusive in lung cancer in mouse models because their coexpression leads to oncogene-induced senescence.

ARTICLE HISTORY
Received 22 June 2016
Accepted 11 July 2016

KEYWORDS
mutual exclusivity; oncogene; RAF; RAS; senescence

Major driving oncogenes are commonly mutually exclusive

Tumor development is driven by mutations that stimulate intracellular pathways that regulate cell proliferation, survival, and invasion. Additional mutations that synergistically increase tumor growth are retained; but mutations that counteract each other, are selected against in tumor evolution. Data from cancer genome sequencing efforts have revealed that mutations in major cancer driving oncogenes (*e.g.*, RAS, RAF, and EGFR) are often mutually exclusive, especially if the oncogenes participate in the same signal transduction pathway.[1-10] Why should 2 activating mutations in the same pathway that exert similar effects be disadvantageous for a tumor cell? One potential and frequently cited explanation is that 2 activating mutations do not occur in the same tumor cell because they are functionally redundant; *i.e.*, that their coexistence does not provide an additional benefit to the cell.[11-13] If that was the case, their coexpression should not bring any negative consequences to the cell. Another potential explanation is that, coexpression of 2 oncogenes is harmful and causes cell cycle exit, senescence, or death (Fig. 1). Until recently, those 2 possibilities have not been addressed experimentally under physiological conditions *in vivo*.

Coexpression of mutant forms of KRAS and BRAF induces senescence

A significant body of evidence pointed to induction of a permanent cell cycle arrest, often referred to as senescence, after activation of more than one oncogene.[14,15] Senescence is a stress response of a cell to different insults, including suboptimal growth conditions, toxins, reactive oxygen species, and radiation. Oncogene-induced senescence (OIS) results from oncogene activation that leads to the generation of unusually high number of origins of replication and subsequent DNA damage response (DDR) and cell cycle arrest. A common denominator of diverse pathways leading to senescence is expression of cell cycle inhibitory proteins (CDK inhibitors).[16] Among them, $p16^{Ink4a}$ and $p19^{Arf}$ play a pivotal role, and together with $p15^{Ink4b}$ are encoded by the *INK4* locus.[17,18]

Could senescence explain the mutual exclusive pattern of oncogenic *RAS* and *RAF* mutations? In melanoma, the most frequently mutated driving oncogenes, $NRAS^{Q61R}$ and $BRAF^{V600E}$, activate the mitogen-activated protein kinase (MAPK) pathway.[19] Those *NRAS* and *BRAF* mutations are mutually exclusive to a point that even when both mutations are found in the same tumor, they can be traced to different clones, each with a single mutation.[20] Consistent with the senescence

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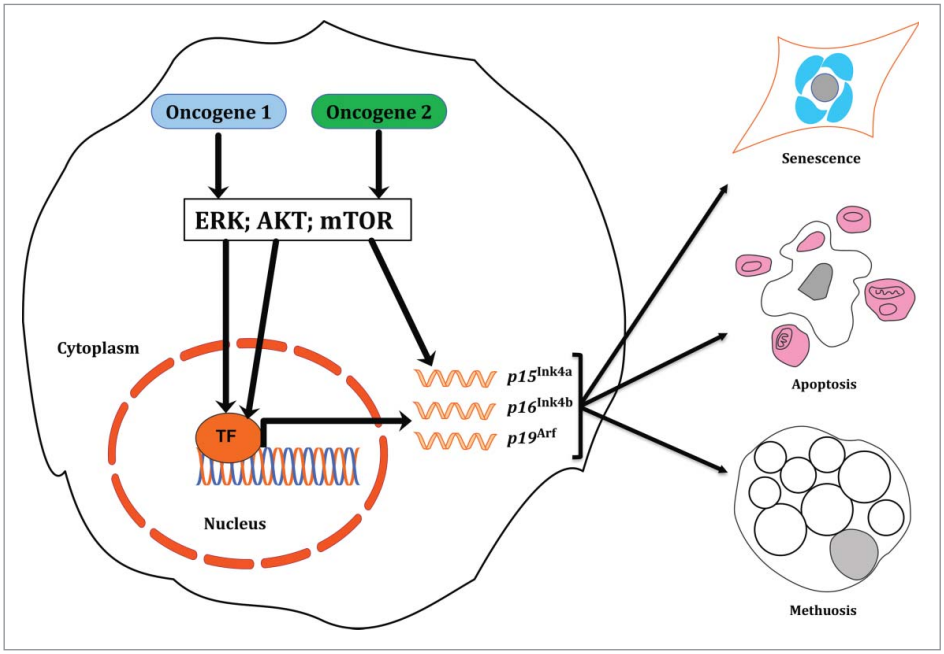

Figure 1. Co-expression of 2 potent oncogenes in the same cell may result in different outcomes. Expression of 2 strong oncogenes (KRAS and BRAF; or KRAS and EGFR) hyperactivates their downstream signaling pathways (ERK, AKT, mTOR etc.), and leads to transcriptional activation of target proteins. The most frequently reported suit of proteins encompasses cyclin-dependent kinase inhibitors, $p15^{Ink4b}$,$p16^{Ink4a}$, and $p19^{Arf}$. Those proteins may, in turn, drive cells toward senescence, or death through either apoptosis, or the recently described form of cell death, methuosis. In our recent paper[22] we suggest that induction of senescence underlies the mutual exclusivity of $KRAS^{G12D}$ and $BRAF^{V600E}$ in lung cancer.

hypothesis of mutual exclusivity, forced expression of $NRAS^{Q61R}$ in an endogenous $BRAF^{V600E}$-expressing melanoma cell line resulted in a flattened cell morphology, accumulation of cells in G_1/G_0 phase of the cell cycle, and increased levels of senescence-associated (SA)-β-galactosidase (SA-β-gal) activity.[21] Those changes were accompanied, and potentially induced by, hyperactivation of the MAPK pathway as judged by high levels of phosphorylated (p)-MEK and pERK. However, those studies did not address whether the crucial CDK inhibitors $p16^{INK4a}$ and $p14^{ARF}$, and also p53, were involved. Moreover, $NRAS^{Q61R}$ was ectopically overexpressed rather than expressed at physiologic levels from the endogenous promoter; it is well known that overexpression of oncogenes can cause senescence.

We recently studied the mutual exclusive nature of $KRAS^{G12D}$ and $BRAF^{V600E}$ – 2 potent oncogenes and activators of MAPK signaling, in mouse models of lung cancer.[22] In these models, inhalation of a *Cre*-adenovirus induces expression of $KRAS^{G12D}$ or $BRAF^{V600E}$ from the endogenous promoters which results in physiological levels of oncogene expression in the lung.[23,24] Under these conditions, $BRAF^{V600E}$ produced more tumors and an overall higher tumor burden than $KRAS^{G12D}$. On the other hand, even though $KRAS^{G12D}$ produced fewer tumors, the tumors were larger and of a more advanced grade, suggesting a faster progression to established advanced tumors. Those results are consistent with previous studies showing that $BRAF^{V600E}$ has a stronger tumor-initiating capacity than $KRAS^{G12D}$, whereas $KRAS^{G12D}$ can generate bigger and more advanced lesions.[25-27] Indeed, the cell proliferation index was higher in $KRAS^{G12D}$ than in $BRAF^{V600E}$ tumors.

We then determined the impact of expressing both oncogenes simultaneously. To approach this issue, we intercrossed $BRAF^{V600E}$ and $KRAS^{G12D}$ mice to produce offspring where both oncogenes could be induced in the lung following *Cre*-adenovirus inhalation. Strikingly, tumor burden, number, and individual tumor area were markedly lower in double-mutant mice than in $BRAF^{V600E}$ mice, indicating that activation of $KRAS^{G12D}$ expression in the setting of $BRAF^{V600E}$ expression is disadvantageous and reduces tumor formation. This result suggests that functional redundancy of $BRAF^{V600E}$ and $KRAS^{G12D}$ is not a likely explanation for their mutual exclusivity: If the mutations were functionally redundant, we should not have observed reduced tumor burden caused by $BRAF^{V600E}$. More importantly, the number of proliferating cells was lower in double mutant tumors, raising the possibility of growth arrest due to senescence induction. To investigate the molecular mechanisms behind the oncogene mutual exclusivity more closely, we isolated mouse embryonic fibroblasts (MEFs) from 12.5-day-old $BRAF^{V600E}$, $KRAS^{G12D}$, and double-mutant

embryos. We first evaluated proliferation of MEFs after *in vitro* transduction with the *Cre*-adenovirus. Consistent with the *in vivo* lung tumor data, proliferation of $BRAF^{V600E}/KRAS^{G12D}$ double-mutant MEFs was lower than in MEFs expressing either oncogene alone; and they expressed higher levels of pERKs, showed strong SA-β-galactosidase staining, and expressed higher levels of cell cycle inhibitors $p16^{Ink4a}$, $p15^{Ink4b}$, and $p19^{Arf}$. The $p21^{Cip1}$ tumor suppressor was not involved in the senescence response, as its mRNA and protein levels were unaltered. Moreover, expression of the MAPK pathway negative regulators of the Dusp, Sprouty, or Spread families was unaltered. The lack of activation of $p21^{Cip1}$ was surprising given that $p19^{Arf}$ is a positive regulator of p53, the main transcriptional activator of $p21^{Cip1}$. One possibility is that the levels of $p19^{Arf}$ were not sufficiently high to stabilize the p53 protein to a point where it obtains robust transcriptional activity. To further elucidate the requirement for both $KRAS^{G12D}$ and $BRAF^{V600E}$ expression for the induction of senescence, we knocked down the expression of the $KRAS^{G12D}$ oncogene in the double-mutant MEFs using retrovirally–delivered shRNAs. Knock down of $KRAS^{G12D}$ expression increased proliferation of $BRAF^{V600E}/KRAS^{G12D}$ MEFs, suggesting that oncogenic KRAS is functionally involved in senescent response induced by both oncogenes. Similarly, knockdown of either $p16^{Ink4a}$ or $p15^{Ink4b}$ significantly increased $BRAF^{V600E}/KRAS^{G12D}$ MEFs proliferation.

Cell death may underlie the mutual exclusivity of other oncogenes

Interestingly, tumor burden in the double-mutant mice was similar to the tumor burden observed in $KRAS^{G12D}$ mice. This finding raised the possibility that $KRAS^{G12D}$ expression inhibits efficient $BRAF^{V600E}$-induced tumorigenesis. Mutant KRAS was previously shown to induce apoptosis, primarily by stimulating the RASSF1/Nore/Mst1 tumor suppressor pathway,[28] or by being phosphorylated by protein kinase C (PKC), and translocated to mitochondria.[29] We stained lung sections for cleaved caspase-3 and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) – well-established markers of apoptosis. We reasoned that any proapoptotic effect of $KRAS^{G12D}$ expression should be rapid and therefore evaluated apoptosis in lungs of mice, 4 and 10 d after *Cre*-adenovirus inhalation. However, the number of apoptotic cells in $BRAF^{V600E}$, $KRAS^{G12D}$, and double-mutant lungs did not differ suggesting that apoptosis does not contribute to the mutual exclusive nature of the oncogenes. We cannot, however, exclude the possibility that double-mutant cells underwent another form of cell death. Along those lines, a recent study by Varmus and co-workers[30] showed that lung cancer cells coexpressing mutant KRAS and mutant epidermal growth factor receptor (EGFR) – which are mutually exclusive oncogenes – undergo methuosis, a cell death pathway characterized by vacuolization and ruffled cell membrane.[31-33] Consistent with our findings, lung cancer cells expressing both KRAS and EGFR displayed higher pERK levels.[30]

Ras mutations may coexist with mutations in some of its downstream pathways

Mutations in KRAS are not mutually exclusive with mutations in all of its downstream protein mediators. Activating mutations in PI3KCA frequently coexist with mutant KRAS in lung cancer.[34,35] This may be related to the fact that KRAS is a relatively poor activator of the PI3K-AKT pathway,[36] so it may benefit from activation of this pathway without the risk of inducing senescence. Accordingly, it has been proposed that activation of PI3K-AKT pathway in mutant KRAS tumor cells may stimulate tumor growth by reducing KRAS-induced senescence.[37] In keeping with this notion, KRAS mutations frequently co-occur with mutations in HRAS, a much stronger PI3K activator,[36] in soft tissue sarcomas;[13] but HRAS mutations are mutually exclusive with loss of the PTEN tumor suppressor in skin cancer.[38] On the contrary, HRAS is recruited to the plasma membrane and activates CRAF less efficiently than KRAS.[36] It is possible that RAF-MEK-ERK activation by HRAS is suboptimal and may benefit from additional activation by *e.g.,* mutant BRAF.

Consistent with the mutual complementation of the MAPK-ERK and PI3K-AKT pathways, mutations in BRAF have been found together with mutations in PI3K or with PTEN deletions in human tumors, and were shown experimentally to increase tumor aggressiveness in mice.[39-42] Mechanistically, activation of both pathways seems to enable escape from senescence.[43] However, another study revealed that expression of $KRAS^{G12V}$, or expression of either of 3 KRAS mutants that specifically activate one of the 3 major KRAS downstream pathways MAPK-ERK, PI3K-AKT, and RalGDS, markedly reduces endogenous $BRAF^{V600E}$-induced lung cancer, suggesting that activation of canonical KRAS pathways is incompatible with $BRAF^{V600E}$ mutation.[25] This interesting result, should, however, be interpreted with caution as high level of transgene expression of the 3 KRAS mutants was used. Regardless, it seems clear that the mechanism that underlies the mutual exclusive nature of oncogenic RAS and RAF is potent, and effectively eliminates double-mutant cells in tumor development and progression. Intriguingly, mutations in KRAS and BRAF may sporadically co-occur in advanced, metastatic disease[44] likely

due to inactivation of several tumor suppressive mechanisms.

A key pathway downstream of both ERK and AKT is the mTOR pathway which is responsible for new biomass synthesis required for cell growth and proliferation. It has been proposed that excessively strong mTOR signaling may stimulate OIS, especially when the cell cycle is inhibited by CDKs.[45] Our data support this scenario. Lung tumors that developed in $BRAF^{V600E}$/$KRAS^{G12D}$ mice showed higher levels of phosphorylated AKT^{Ser437}, a known downstream "feed-back-target" of mTORC2 and $pS6^{Ser235/236}$ protein which is downstream of mTORC1. Why does expression of both oncogenes synergistically increase mTOR activation is unclear, but may involve cumulative activation of Akt pathway directly by $KRAS^{G12D}$, and indirectly by $BRAF^{V600E}$ through activation of p90 ribosomal S6 kinase (RSK),[46] inhibition of PTEN expression via AP-1 transcription factor,[47] negative regulation of LKB1/AMPK signaling,[48,49] or through a yet another mechanism. However, those possibilities require an experimental verification.

Summary

Our paper aimed at elucidating the mechanisms behind the mutual exclusive nature of 2 major human oncogenes, $KRAS^{G12D}$ and $BRAF^{V600E}$ using a mouse model of lung cancer.[22] In this paper we propose that cell cycle arrest and senescence are causally involved in this response. Our results imply that if a $BRAF^{V600E}$-mutant cell acquires an additional $KRAS^{G12D}$ mutation, or vice versa, it will hyperactivate MAPK-ERK pathway and senesce, and be outcompeted by single mutant cells. This concept fits well in the so-called "Goldilocks Principle," the idea that certain biological processes require precise levels in order to promote fitness, where either too little or too much is detrimental.[50] The evidence that ERK signaling obeys this principle was nicely illustrated in a mouse model of breast cancer in which mutant KRAS expression levels were doxycycline-regulated. Low levels of KRAS activity promoted tumor formation, whereas high levels induced growth arrest and senescence.[51]

Presently, it is unclear whether the mutual exclusive nature of oncogenes may be exploited therapeutically. Some light on this matter was shed by an intermittent use of kinase inhibitors like $BRAF^{V600E}$ and MEK inhibitors. When both inhibitors were withdrawn from addicted cells, the pERK levels in those cells rebound, and their growth was inhibited.[52] Moreover, pharmacologic inhibition of CDK4/6, which *de facto* mimics the function of the endogenous CDK inhibitors, $p16^{INK4a}$ and $p15^{INK4b}$, is undergoing clinical trials in a number of cancer types.[53]

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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