EXPERIMENTAL ONCOLOGY GROUP

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OVERVIEW

We recently demonstrated that ablation of RAF1 induces significant levels of tumour regression in mice bearing lung adenocarcinomas induced by KRas/Tp53 mutations. We observed similar results in mice bearing pancreatic tumours providing that RAF1 ablation is combined with elimination of the EGF Receptor. We are now attempting to translate these observations to a pharmacological scenario. Unfortunately, none of the available putative RAF1 inhibitors has shown antitumour activity in the clinic. Therefore, we decided to interrogate by genetic means the best strategy to block RAF1 activity. Unexpectedly, expression of 2 kinase dead isoforms of RAF1 failed to exhibit therapeutic activity, indicating that RAF1 does not contribute to tumour development via its kinase activity. Hence, pharmacological targeting of RAF1 will require the use of other strategies such as the use of degrons, small chemotypes capable of inducing the degradation of their target proteins.

"The protein kinase activity of RAF1 is dispensable for KRAS induced tumour development."

"The oncogenic form of the KRAS4A protein isoform is sufficient to induce lung adenocarcinomas that undergo proximal metastasis."

The *KRAS* locus encodes 2 protein isoforms, KRAS4A and KRAS4B, which differ in intracellular trafficking and location in the plasma membrane. *KRAS* mutations in human cancer affect both protein isoforms. Efforts to selectively target the KRAS4B isoform are under development. We have observed that expression of the endogenous KRAS4A mutant oncoprotein is sufficient to induce lung adenocarcinomas. Hence, effective therapeutic strategies against KRAS mutant tumours must take into account inhibition of both protein isoforms.

RESEARCH HIGHLIGHTS

RAF1 kinase activity is not required for KRAS/p53-driven tumour progression

We generated mouse strains that express conditional knocked-in alleles that encode 2 independent RAF1 kinase dead isoforms, RAF1^{D468A} and RAF1^{K375M} (FIGURE 1A). Surprisingly, systemic expression of these kinase dead isoforms under the control of the endogenous *Raf1* locus in mice bearing advanced *Kras/Trp53* tumours failed to induce tumour regression (FIGURE 1B). Previous studies indicated that, in addition to its role in MAPK signalling, RAF1 has anti-apoptotic activity. This effect is mediated, at least in part, by its ability to inhibit the pro-apoptotic kinases ASK1 and MST2. Moreover, *in vitro* studies suggested that this anti-apoptotic activity is likely to be kinase-independent. To interrogate whether the anti-proliferative effect of ablating RAF1 is mediated by these kinases, we blocked proliferation of human A549 lung adenocarcinoma cells as well as of cells obtained from a patient-derived xenograft (PDX) model, PDX-dc1, with lentiviral vectors expressing 2 independent shRNAs against *RAF1* (FIGURE 1C). Co-infection of these *RAF1* shRNAs with two independent shRNAs against *ASK1* or *MST2* restored the proliferative properties of these human lung tumour cells (FIGURE 1D). Re-expression of a cDNA encoding the murine RAF1 protein, whose sequences could not be recognised by the human *RAF1* shRNAs, restored proliferation of the A549 and PDX-dc1 cells to levels similar to those observed upon co-infection with *ASK1* or *MST2* shRNAs. These results demonstrate that the anti-proliferative effect of silencing RAF1 expression in human lung adenocarcinoma cells is mediated by the pro-apoptotic properties of ASK1 and MST2. These results, taken together, have important implications for the design of effective therapeutic strategies to block progression of *KRAS* mutant human cancers. They also help to explain, at least in part, the poor results obtained so far in the clinic with RAF inhibitors that either block their kinase activity or other features that regulate their involvement in MAPK signalling. In summary, these studies strongly suggest that current therapeutic strategies based on inhibition of RAF1 kinase activity are unlikely to produce anti-tumour results in the clinic. Instead, pharmacological targeting of RAF1 will require novel strategies that prevent the anti-apoptotic activity of RAF1, either by blocking its interaction with the ASK1 or MST2 kinases or, more directly, by inhibiting RAF1 expression with selective RAF1 degraders.

KRAS4A induces metastatic lung adenocarcinomas *in vivo* in the absence of the KRAS4B isoform

In mammals, the *KRAS* locus encodes 2 protein isoforms, KRAS4A and KRAS4B, which differ only in their extreme C-terminus via alternative splicing of distinct fourth exons. Previous studies have shown that whereas KRAS expression is essential for mouse development, the KRAS4A isoform is expendable. To unveil the unique properties of the KRAS4A isoform, we generated a mouse strain that carries a point mutation in exon 4B that causes the selective degradation of KRAS4B while leaving KRAS4A expression unaffected. Mice selectively lacking KRAS4B developed to term but died perinatally due to hypertrabeculation of the ventricular wall, a defect reminiscent of that observed in midgestation embryos lacking the *Kras* locus. Introduction of an oncogenic mutation (G12V) into the *Kras*^{FSFG12V} allele allowed expression of an endogenous KRAS4A^{G12V} oncogenic isoform in the absence of KRAS4B. Exposure of *Kras*$^{+/FSF4AG12V4B-}$ mice to Adeno-FLPo induced lung tumour formation with complete penetrance, albeit with increased latencies than control *Kras*$^{+/FSFG12V}$ animals that expressed both oncogenic isoforms. Interestingly, a significant percentage of these mice developed proximal metastasis, a feature seldom observed in mice expressing both mutant isoforms, probably due to their shorter tumour latencies. These results illustrate that expression of the KRAS4A^{G12V} mutant isoform is sufficient to induce lung tumours, thus indicating that effective anti-tumour strategies against KRAS mutant tumours must take into account inhibition of both protein isoforms. ■

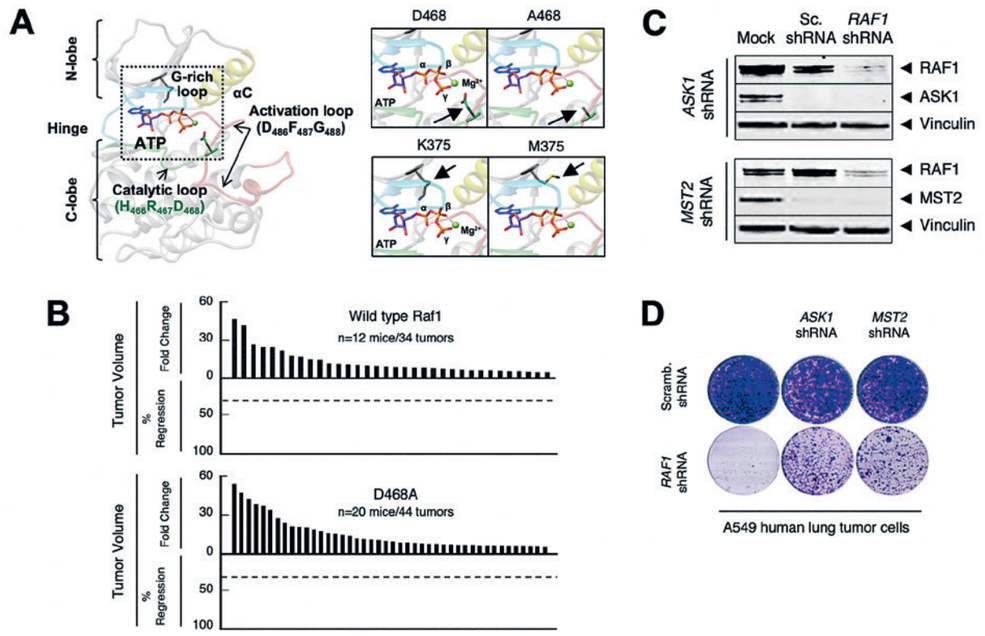

FIGURE 1 RAF1 kinase activity is dispensable for KRAS/p53-driven tumour progression. (**A**) Human RAF1 kinase domain structure highlighting the position of the D468 residue along with its respective mutations A468. (**B**) Waterfall plots representing the change in tumour volume of individual tumours expressing the wild type RAF1 or the mutant RAF1D468A proteins in mice exposed to a tamoxifen (TMX) containing diet for 2 months. (**C**) Western blot analysis of RAF1, ASK1 and MST2 expression in lysates obtained from A549 human lung tumour cells either not infected or infected with representative shRNAs against *ASK1* (top panels) or *MST2* (bottom panels) in combination with either a scrambled shRNA or a representative shRNA against *RAF1*. Vinculin was used as loading control. (**D**) Colony formation in A549 human lung tumour cells infected with a scrambled shRNA and representative shRNAs against *RAF1*, *ASK1* and *MST2* sequences.

PUBLICATIONS

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PATENT

- Barbacid M, Guerra C, Blasco MT, Navas C (2018). Combined therapy against cancer. *WO 2020/020942.A1*. National phase entry (2021).

AWARDS AND RECOGNITION

- *Premio Fundación CRIS contra el Cáncer, CRIS* Foundation, Spain.
- Inaugural Conference, *SEMERGEN* National Congress, Zaragoza, Spain.
- Keynote Lecture, IV International Workshop on Genomic Testing in Cancer, Pamplona, Spain.