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Targeting SRC as a novel strategy to overcome resistance to BRAF-targeted therapy in BRAFV600E-mutated colorectal cancer.

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Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related death worldwide. 10% of CRC present BRAF mutations (most commonly V600E). In contrast with other BRAFV600E-mutated (BRAFm) tumors, BRAF-targeted therapy has proven largely ineffective (response rates < 5%). These subgroup of patients remains particularly challenging in the clinic, with limited treatment options and a dismal prognosis. Based on previous results, our hypothesis is that this resistance may partially pass through SRC as a common signal transduction node. We have previously described that activation of SRC is associated with a more aggressive phenotype, increased resistance to chemotherapy and a worse prognosis. Accordingly, our **main goal** is to demonstrate that SRC activation is involved in resistance to conventional and targeted therapies in BRAFm CRC patients, and that SRC-targeted therapies overcome drug resistance in this context. The **specific objectives** are:

-To define the potential functions of SRC in BRAFm CRC *in vitro.*

-To explore the role of SRC in resistance to BRAF inhibition *in vitro.*

-To assess the antitumor efficacy of the combination of SRC and BRAF inhibition in BRAFm CRC *in vitro* and *in vivo*.

Methods: Five BRAFm CRC cell lines were used (HT29, WiDr, SW1417, LS411N, RKO). Overexpressing (OE) and knock-out (KO) cell lines for Src were generated by lentiviral infection and CRISPR/Cas9 system, respectively. Cell lines were treated with different drugs (BRAF inhibitors: vemurafenib and encorafenib; and SRC inhibitors: dasatinib). Protein extract was evaluated by Western Blot or by Proteome Profiler Human Phospho-Kinase Array. Cell viability was analysed by crystal violet and MTS assay. For cell migration, transwell assays were used. For synergy assays, the combination Index (CI) were obtained with CompuSyn: CI< 1, synergism. For apoptosis and cell cycle analysis, cells were analysed by FACS after being stained with Annexin V FITC and 7AAD, and Propidium iodide respectively. Cell line-derived xenograft (CDX) and patient-derived xenograft (PDX) models were performed. All *in* vivo treatment were intraperitoneally administered for a total of 20 doses (except Cetuximab, 8 doses).

Results: The five BRAFm CRC cell lines, both those with microsatellite stability, MSS (HT29, WiDr and SW1417) and instability, MSI (RKO and LS411N), displayed variable levels of SRC expression. Src-OE cells showed significantly higher clonogenicity, migration and proliferation capacities. Conversely, Src KO cells lead to a significant reduction in colony formation, migration and proliferation. Interestingly, treatment of the different cell lines with BRAF inhibitors (BRAFi), vemurafenib (V) and encorafenib (E), lead to p-SRC upregulation, indicating that SRC activation occurred as an adaptive response to BRAF inhibition. Moreover, SRC overexpressing cells

were significantly more resistant to BRAFi, while SRC depletion resulted in an increased sensitivity to these agents. As expected, CRC BRAF WT cells (DIFI) did not show differences of response to BRAFi when the expression of SRC was modified. Overall, these data support the implication of SRC in the sensitivity to BRAFi in BRAFm CRC. Furthermore, a strong synergistic effect was found after dual SRCi and BRAFi treatment in all BRAFV600E cell lines, independently of MSI status. Moreover, *in vitro* studies showed that the novel combinatorial treatment significantly decreased cell viability, increased apoptosis and induced G0/G1phase cell cycle arrest in the whole set of cell lines.

Consistently, combination therapy also showed synergistic anti-tumor effects *in vivo,* with a significant reduction in tumor size in HT29 CDX and in two different patient-derived xenograft (PDX) mouse models treated with both BRAFi (vemurafenib and encorafenib). As anticipated, BRAFi or SRCi alone were largely ineffective in controlling tumor growth, while tumors treated with the combination therapy were significantly smaller. Finally, we observed that our combinatorial therapy showed a similar effective tumor growth inhibition as the recently EMA-approved regimen based on BRAFi+EGFRi, highlighting the relevance of our novel drug combination.

Conclusions

-SRC is a master regulator of oncogenic functions such as proliferation, migration and clonogenicity in BRAFm CRC.

-SRC plays a pivotal role driving resistance to BRAF inhibitors in BRAFm CRC cells. SRC inhibition overcomes resistance to BRAF inhibition.

-Dual BRAF and SRC inhibition shows synergistic anti-tumor effects in BRAFm CRC *in vitro*, significantly decreasing cell viability, clonogenicity and migration, increasing apoptosis and inducing cell cycle arrest.

-Dual treatment (BRAFi+SRCi) potentiates tumor growth inhibition in both MSS and MSI BRAFm CRC *in vivo* models

-These promising data provide a solid rationale to assess this novel therapeutic strategy (BRAFi+SRCi) in clinical practice, to potentially improve the dismal prognosis of BRAFm CRC patients.