Determining How MSI Impacts Progression And Invasiveness Of BRAF-Driven Serrated Colon Cancer

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Abstract

Colorectal cancer is the third most common cancer in the United States and is the second leading cause of cancer-related deaths. While the most common type of colon cancer is the WNT-driven adenoma, ~20% of colon cancers follow the more lethal "serrated" pathway -which is commonly driven by the oncogene BRAF (Patai, Molnár, Tulassay, & Sipos, 2013). Furthermore, serrated colon cancers can also be classified by Microsatellite Instability (MSI) status, which is characterized by the impaired function of DNA Mismatch Repair (MMR). It is suggested that BRAF-driven serrated cancers are driven by MSI status and are required for the hyperplasia- to-dysplasia transition (Rad et al. 2013). The Verzi lab previously reported that the oncogenic allele BRAF$^{V600E/+}$ alone is inefficient at tumorigenesis (Tong et al. 2017). However, the loss of tumor suppressor SMAD4 greatly accelerates BRAF-driven tumorigenesis and significantly upregulates WNT signaling. Interestingly, tumors that arose from these mice were primarily Microsatellite Stable (MSS). Thus, we wanted to determine whether MSS or MSI has a substantial difference in dictating how BRAF-driven serrated tumors form. We generated a mouse model in which the oncogenic BRAF mutation was combined with a knockout of both tumor suppressor SMAD4 and DNA mismatch repair protein MSH2 in a Villin-Cre system. Strikingly, SMAD4^{KO} BRAF$^{V600E/+}$ MSH2^{KO} mice develop multiple macroscopic tumors and serrated invasive tumors within three months. In contrast, SMAD4^{KO} BRAF$^{V600E/+}$ mice developed fewer serrated tumors within the same time frame, suggesting that MSH2^{KO} accelerates serrated tumorigenesis and progression. Furthermore, the SMAD4^{KO} BRAF$^{V600E/+}$ MSH2^{KO} tumors show elevated levels of WNT signaling, supporting the notion that elevated WNT signaling is required for serrated tumorigenesis. Whole Exome Sequencing (WES) analysis reveals that WNT-effector β-catenin (Ctnnb1) is commonly mutated in tumor organoids derived from both SMAD4^{KO} BRAF$^{V600E/+}$ MSH2^{KO} and SMAD4^{KO} BRAF$^{V600E/+}$ mouse models. To determine whether elevated WNT is critical for serrated tumorigenesis, the Verzi Lab generated a mouse model in which β-catenin was added into a SMAD4^{KO} BRAF$^{V600E/+}$ mouse model. By doing this, WNT had become significantly upregulated and caused tumors to produce as soon as 7 days while in the SMAD4^{KO} BRAF$^{V600E/+}$ it took at least 1 month for tumors to be seen. This suggested that a high WNT environment is needed to produce tumors in a shorter amount of time. This supports the initial claim that WNT is needed to progress tumorigenesis and inducing MSI can accelerate it.

Background

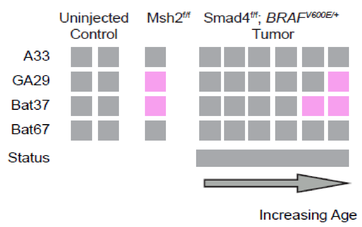

Figure 1: Smad4^{KO} Braf$^{V600E/+}$ Tumor organoids are mainly microsatellite stable (MSS)
MSI analysis of Smad4^{KO} Braf$^{f/+}$ tumor organoids derived from mouse model. Organoids derived from MMR repair deficient mice (Msh2f/f) reveal mutational shifts at specified microsatellite loci (pink), indicative of MSI. Smad4^{KO} Braf$^{V600E/+}$ tumor organoids are predominately MSI negative suggesting loss of SMAD4 bypasses the MSI pathway in serrated cancers.

Microsatellite Instability (MSI) is caused by having certain genes that are involved in DNA mismatch repair (MMR) downregulated, which allows cells to escape the DNA damage checkpoint being more prone to obtaining mutations. This predisposition to mutations allows cells to pick up oncogenic mutations to accelerate proliferation and cancer progression (Li, G. 2008). Studies show that MSI causes patients to be at a higher risk of having a poorer prognosis since their tumors are more susceptible to progressing quicker as opposed to MSS patients and can also lead to higher drug resistance (Goldstein, J. et al. 2014). The Verzi lab has previously generated a Smad4^{KO} Braf $^{V600/+}$ mouse model which accelerates serrated tumorigenesis (Tong et al 2017). Interestingly, though tumor formation was accelerated, most tumors were found to be microsatellite stable (MSS). This then prompted to determining if there were any distinctive characteristics between MSS and MSI BRAF-driven serrated colon cancer. Thus, it is possible that the addition of MSI to the Smad4^{KO} Braf $^{V600/+}$ mouse model may also further accelerate the progression of serrated tumors.

Results

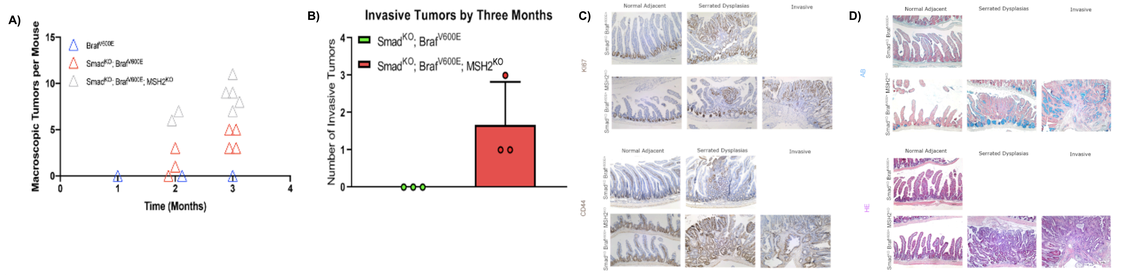

Figure 2: High levels of MSI in a Smad4^{KO} Braf$^{V600E/+}$ mice produce serrated and invasive tumors in 3 months
Smad4^{KO} Braf$^{V600E/+}$ and Smad4^{KO} Braf$^{V600E/+}$ MSH2 ^{KO} Villin-Cre mice treated with Tamoxifen 4 consecutive days to induce recombination in the intestinal epithelium. (A) Counts of macroscopic tumors in Villin-cre mice were based off 3 biological replicates of Smad4^{KO} Braf$^{V600E/+}$ MSH2 ^{KO}, Smad4^{KO} Braf$^{V600E/+}$ and uninjected control within 3 months post tamoxifen treatment. The number of tumors found within the Smad4^{KO} Braf$^{V600E/+}$ MSH2 ^{KO} mice are higher than in the Smad4^{KO} Braf$^{V600E/+}$ mice. (p-value=0.0050, Unpaired T-Test). (B) Counts of invasive tumors in Villin-cre mice were based of 3 biological replicates of Smad4^{KO} Braf$^{V600E/+}$ MSH2 ^{KO} and Smad4^{KO} Braf$^{V600E/+}$ within 3 months post tamoxifen treatment. The number of tumors found within the Smad4^{KO} Braf$^{V600E/+}$ MSH2 ^{KO} mice are higher than in the Smad4^{KO} Braf $^{f/+}$. (p-value=0.0668, Unpaired T-Test) (C) Histology of invasive tumors found in Smad4^{KO} Braf$^{V600E/+}$ and Smad4^{KO} Braf$^{V600E/+}$ MSH2 ^{KO} Villin-Cre mice. Mice were collected 3 months post tamoxifen treatment based on the weight loss of Smad4^{KO} Braf $^{f/+}$ MSH2 ^{KO}. KI67 and CD44 of the duodenum and tumors were identified. Images are representative of 3 biological replicates. (D) Histology of Smad4^{KO} Braf$^{V600E/+}$ and Smad4^{KO} Braf$^{V600E/+}$ MSH2 ^{KO} Villin-Cre mice. H/E and Alcian Blue of the duodenum and tumors were identified. Images are representative of 3 biological replicates. Histology reveals that both Smad4^{KO} Braf$^{V600E/+}$ and Smad4^{KO} Braf$^{V600E/+}$ MSH2 ^{KO} had extended protrusions from the epithelial-lumen interface with deep pockets of mucin that are concentrated at the base of the epithelium. But in the Smad4^{KO} Braf$^{V600E/+}$ MSH2 ^{KO} there are deeper pockets of mucin and the protrusions extend from the muscle layer. This suggest that MSH2 has the ability to form tumors quicker to the point of becoming invasive.

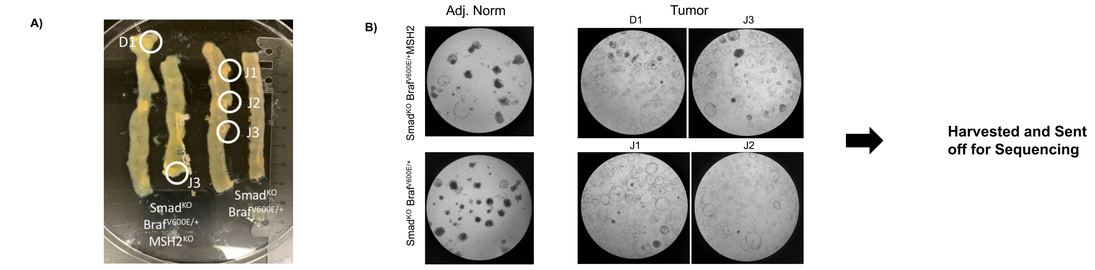

Figure 3: Tumor Organoids Submitted for Whole Exome Sequencing
(A) Whole mount of Smad4^{KO} Braf$^{V600E/+}$ MSH2 ^{KO} and Smad4^{KO} Braf$^{V600E/+}$ showing visible macroscopic tumors in Duodenum and Jejunum. (B) Tumor Organoids were isolated from macroscopic tumors and cultured in 3D-Matrigel. Tumor organoids show very distinct morphology when compared to non-tumor (Adj Norm) organoids. Passaged tumor organoids were seeded and cultured for 3 days prior to genomic DNA isolation and submission for sequencing.

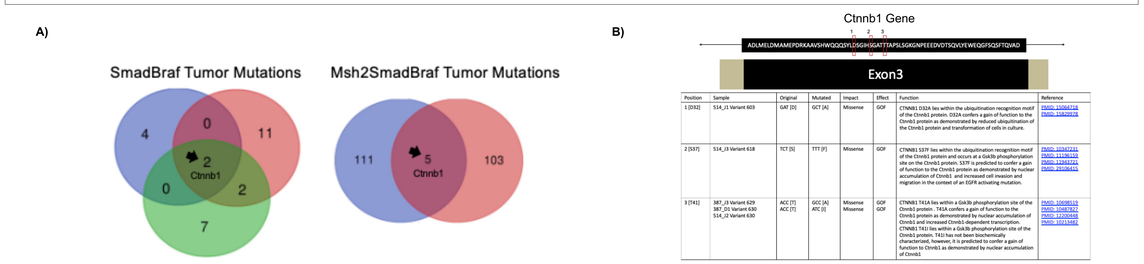

Figure 4: Smad4^{KO} Braf$^{V600E/+}$ MSH2 ^{KO} Tumors Acquire Mutations Quicker Than Smad4^{KO} Braf$^{V600E/+}$ Tumors, but commonly share gain-of-function mutations in Ctnnb1
(A) Venn Diagrams of annotated gene variants from Smad4^{KO} Braf $^{f/+}$ (n=3) and Smad4^{KO} Braf$^{V600E/+}$ MSH2 ^{KO} (n=2) tumor organoids. Both Smad4^{KO} Braf$^{V600E/+}$ MSH2 ^{KO} and Smad4^{KO} Braf$^{V600E/+}$ tumor samples had Ctnnb1 in common. (B) Mutational variants of Ctnnb1 gene occur in exon3. Three of the tumors were mutated at the same position (T41) while the other two were at mutated at different positions (D32 and S37) – documented gain-of-function mutation hot-spots.

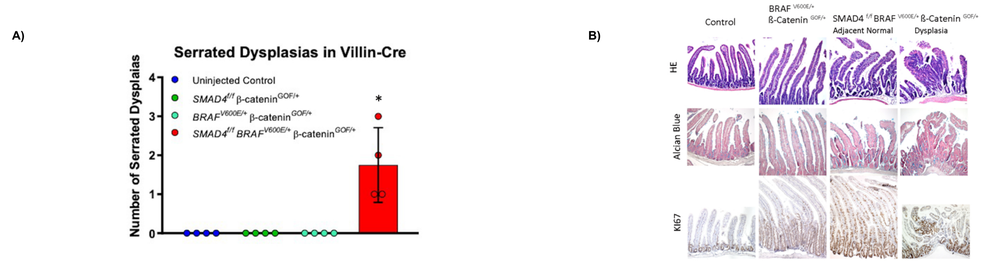

Figure 5: Activation of WNT in Smad Braf mouse model accelerates serrated tumorigenesis
(A) Histology of Uninjected control, Braf$^{V600E/+}$ β-catenin$^{Exon3/+}$, Smad^{KO} Braf$^{V600E/+}$ and Smad^{KO} Braf$^{V600E/+}$ β-catenin$^{Exon3/+}$ Villin-Cre mice treated with Tamoxifen to induce recombination in the intestinal epithelium. Mice were collected 7 days after the initial tamoxifen treatment, due to the declining health of the triple mutant mice. H/E, Alcian Blue, and Ki67 of the duodenum revealed hyperplasia in both mutant mice epithelium, and dysplasias were identified in the triple mutant model. Images are representative of 4 biological replicates. (B) Counts of serrated dysplasias in Villin-cre mice were based on 4 biological replicates of Braf$^{V600E/+}$ β-catenin$^{Exon3/+}$, Smad^{KO} Braf$^{V600E/+}$ β-catenin$^{Exon3/+}$ and uninjected control within 3 days post tamoxifen treatment. The number of dysplasias identified within the Smad^{KO} Braf$^{V600E/+}$ β-catenin$^{Exon3/+}$ mice is significantly higher than the number of dysplasias found within the Braf$^{V600E/+}$ β-catenin$^{Exon3/+}$ mice and the Smad^{KO} β-catenin$^{Exon3/+}$ mice (p-value =0.0004 by ANOVA).

Methods

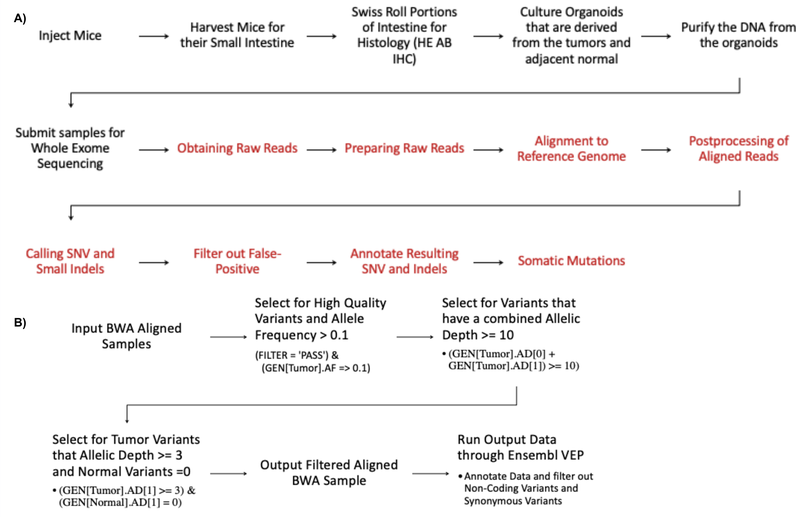

Figure 6: Diagram of Workflow for Whole Exome Sequencing Pipeline
(A) The steps written in black explain how we got our samples ready to be sent off to be sequences . The steps in red is the bioinformatics steps in which the raw sequencing data was processed. (B) The diagram describes the code necessary to filter out false-positives and annotate the resulting SNVs.

Conclusion

Serrated cancers with MSI cause patients to be at a higher risk of having a poorer prognosis since their tumor are more susceptible to progressing quicker as opposed to MSS patients and can lead to higher drug resistance (Goldstein, J. et al.2014). The Verzi lab has previously developed a mouse model that aggressively develops serrated tumors. However, we show that the majority of tumors that arose from this model were microsatellite stable (MSS). Thus, it was necessary to see if there was a difference between MSS or MSI BRAF-driven serrated tumors. Loss of MSH2, which is a key regulator in MMR, and can induce MSI in the SMAD4^{KO} BRAF$^{V600E/+}$ mouse model, resulted in accelerated development of macroscopic serrated tumors when compared to the original SMAD4^{KO} BRAF$^{V600E/+}$ mouse model. This finding suggests that MSI can accelerate the development of serrated tumorigenesis. Unexpectedly, not only did incorporating MSI result in faster tumorigenesis, the Smad4^{KO} BRAF$^{V600E/+}$ MSH2^{KO} mouse model had also developed invasive tumors faster than the Smad4^{KO} BRAF$^{V600E/+}$ mouse model. Furthermore, IHC reveals elevated levels of WNT signaling in the SMAD4^{KO} BRAF$^{V600E/+}$ MSH2^{KO} mouse model then in the Smad4^{KO} Braf$^{V600E/+}$ mouse model which supports previously published findings that elevated WNT is necessary for serrated tumorigenesis and promotes invasion (Rad et al. 2013, Tong et al. 2017, Silva, A. L. et al. 2014). This finding suggests that loss of MSH2 could be hypermutating WNT genes to upregulate expression which could be pushing tumors to become invasive.

We next sought to determine the mutational profile of the serrated tumors to determine what mutations were critical for serrated tumor development. WES of serrated tumor organoids derived from SMAD4^{KO} BRAF$^{V600E/+}$ and SMAD4^{KO} BRAF$^{V600E/+}$ MSH2^{KO} mice revealed that independent tumor isolates shared very few common mutations, indicating highly divergent mutational profiles. Stunningly, all tumors were found to have mutated the gene Ctnnb1, which transcribes the WNT-effector protein β-catenin. Furthermore, all annotated mutations were found to affect phosphorylation sites in Exon3 of the Ctnnb1, which results in a gain-of-function mutation of β-catenin (Gao, C. et al. 2017). The Verzi lab has a genetic mouse model in which β-catenin is hyperactivated by altering the 3rd exon of the gene (Perekatt, Ansu. et al 2018). When the β-catenin hyperactivation was induced in our SMAD4^{KO} BRAF$^{V600E/+}$ model, tumors developed as soon as 7 days. This supports our claim that elevated levels of WNT along with MSI will cause tumors in a BRAF-driven serrated cancer model to develop and progress much quicker.

One possible future direction for this project would be to assess how the SMAD4^{KO} BRAF$^{V600E/+}$ MSH2^{KO} tumor organoids respond to certain therapeutics when compared to SMAD4^{KO} BRAF$^{V600E/+}$ tumors. Patients that have MSI-positive tumors are not able to go through the same treatments as patients with non-MSI tumors. For example, previous studies have shown that MSI tumors are resistant to fluorouracil drug due to not being able to inhibit the repair of DNA damage and also promote apoptosis (Li, K. et al. 2020). Using our tumor organoids, we could compare the effectiveness of different drugs on organoids that are derived either from SMAD4^{KO} BRAF$^{V600E/+}$ and SMAD4^{KO} BRAF$^{V600E/+}$ MSH2^{KO} tumors. One example would be to treat both organoid genotypes with Nivolumab, which has been proven to be an effective drug in combating MSI/DNA mismatch repair-deficient colorectal cancers (Overman, M.J. et al.2017). Organoids will be seeded in 6 wells with about 50 organoids per well. Organoids will then be treated with either vehicle or various working concentrations of Nivolumab. We will assess the viability in the organoids by seeing which one has more of their structural borders intact and quantify organoids by counting the number of viable organoids present on day zero and then counting after treatment to see if there is a decrease or increase of organoids present. If Nivolumab is specifically effective at treating MSI tumors, we expect to see a decrease in viability in SMAD4^{KO} BRAF$^{V600E/+}$ MSH2^{KO} when compared to either vehicle or SMAD4^{KO} BRAF$^{V600E/+}$ organoids. These findings would help in figuring out the mechanism behind BRAF-driven serrated colon cancer and finding new therapeutic treatments that are specific to MSI-positive tumors.

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