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Epigenetic modulation in BRAF mutated metastatic colorectal cancer

Van Morris

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EPIGENETIC MODULATION IN BRAF-MUTATED METASTATIC COLORECTAL CANCER

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EPIGENETIC MODULATION IN BRAF-MUTATED METASTATIC COLORECTAL CANCER

**A
THESIS**

**Presented to the Faculty of
The University of Texas Health Science Center at Houston, and
The University of Texas MD Anderson Cancer Center
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**In Partial Fulfillment
of the Requirements
for the Degree of
MASTER OF SCIENCE**

**By
Van Morris, M.D.**

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EPIGENETIC MODULATION IN BRAF-MUTATED METASTATIC COLORECTAL CANCER

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Introduction: BRAF V600E mutations are associated with poor clinical outcomes for patients with metastatic colorectal cancer (mCRC). Unlike other tumors with the same mutation, BRAF inhibitors are ineffective as monotherapy. CRC tumors with BRAF V600E mutations are associated with global hypermethylation, which may turn off tumor suppressor gene expression. We studied demethylation in BRAF V600E mCRC to restore sensitivity to BRAF inhibitors.

Methods: Tumor databanks were investigated for genes differentially expressed according to BRAF mutation status to identify genes which may be particularly susceptible to epigenetic influence. Mouse xenograft models of BRAFV600E mCRC were treated with vemurafenib or azacitidine, alone or in combination, to assess for changes in tumor size. Tumors and cell lines exposed to azacitidine were analyzed for methylation status and for gene expression differences, with particular emphasis on genes identified from the bioinformatics analysis.

Results: The addition of azacitidine did not restore sensitivity to vemurafenib in two xenograft models of BRAF V600E mCRC. Genes critical to negative regulation of Wnt/ β -catenin signaling like RNF43 and Axin2 were significantly underexpressed in BRAF V600E mutant tumors when compared to their wild-type counterparts. These genes were hypermethylated in the xenograft models, which could be reversed with a demethylating agent.

Conclusions: The combination of a BRAF inhibitor and a demethylating agent does not appear to have promising anti-tumor activity in preclinical models of BRAF V600E mCRC. Negative regulators of Wnt/ β -catenin signaling are influenced by hypermethylation. Future clinical trials incorporating these genes as integral biomarkers should consider gene expression given the relevant non-genomic alterations.

Dedication

This thesis is dedicated to all my family, who has supported me wholeheartedly through all my academic endeavors; to my mentors, Dr. Scott Kopetz, Dr. Waun Ki Hong, Dr. Robert Wolff, Dr. Cathy Eng, and Dr. Diane Bodurka, all of whom have empowered me with the knowledge and belief in my quest to become the best oncologist that I can be; and mostly to patients afflicted with colorectal cancer, who inspire me to strive for new discovery every day.

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Chapter I

INTRODUCTION

Colorectal Cancer in the United States: Colorectal cancer remains the second leading cause of cancer mortality in the United States, with over 50,000 deaths expected in the United States in 2016¹. Over this past several decades, survival for patients with metastatic disease has increased due to the incorporation of increased surgical resection for patients with oligometastatic disease in which few sites of distant metastases are present²⁻⁴. During this time, however, there have not been many significant advances with regards to the use of systemic agents in the management of metastatic colorectal cancer. Cytotoxic chemotherapies including 5-fluorouracil/oxaliplatin (FOLFOX) and 5-fluorouracil/irinotecan (FOLFIRI) remain the backbone of treatment for metastatic disease⁵⁻⁸. The introduction of biological agents with monoclonal antibodies which target VEGF (bevacizumab) and EGFR (e.g., cetuximab and panitumumab) do improve survival outcomes further when used in combination with cytotoxic chemotherapy⁹⁻¹⁴. Nonetheless, utilization of such biologic agents must incorporate clinical comorbidities and a detailed knowledge of the specific genomic profiling of a given patient. For example, KRAS and NRAS mutations, which occur in 45–55% of all patients with metastatic colorectal cancer¹⁵⁻¹⁷, are contraindications to the administration of anti-EGFR therapies, as these activating mutations occur downstream of EGFR and are associated with inferior outcomes when used in patients with RAS-mutated metastatic colorectal cancer^{9,18-21}.

BRAF Mutations in Colorectal Cancer: Valine to glutamic acid substitutions (V600E) in the BRAF oncogene occur independently of mutations in KRAS and NRAS^{22,23}. Here, mutations in the BRAF oncogene, found in approximately 5-10% of colorectal cancers²², cause constitutive activation of the MAPK pathway²⁴⁻²⁶, which promotes tumor cell proliferation and anti-apoptotic activity²⁷. Along the MAPK signaling pathway, EGFR stimulation triggers activation not only of

the separate PI3K/Akt pathway but also of downstream RAS signaling proteins, including KRAS and NRAS. Activation of these kinases results in subsequent phosphorylation and increased activity of BRAF, which in turn activates MEK1 and MEK2. These proteins subsequently are able to phosphorylate ERK1 and ERK2, which can then be translocated into the nucleus in order to propagate the downstream affect her functions of the MAPK signaling pathway²⁸.

The BRAF V600E mutation is understood to be an adverse prognostic marker in patients with metastatic colorectal cancer²⁹. Patients with metastatic colorectal cancer who harbor this mutation have inferior responses to standard cytotoxic chemotherapy agents relative to their BRAF wild type counterparts³⁰⁻³³. In addition, BRAF V600E mutations are associated with unique patterns of distant metastatic spread in patients with metastatic colorectal cancer, with higher rates of involvement of peritoneum, brain, and bone³⁰. For patients with BRAF V600E mutated metastatic colorectal cancer, survival in the metastatic setting has been estimated at only 10 months, less than the 35 months expected in patients with metastatic BRAF wild-type colorectal cancer (BRAF^{WT}-colorectal cancer)³⁰.

BRAF Inhibitors in the treatment of BRAF-mutated colorectal cancer: Unlike metastatic melanoma, in which use of targeted inhibitors against the BRAF V600E kinase-like vemurafenib and dabrafenib are associated with response rates as high as 50–60 percent^{34,35}, success with such agents in the treatment of metastatic colorectal cancer is far inferior. A phase I study of vemurafenib noted only one partial response among 21 patients with metastatic BRAF-mutated colorectal cancer, for a response rate of 5%³⁶. A separate phase I/II study of a BRAF inhibitor/MEK inhibitor reported similar modest efficacy, with a 12% partial response rate with this combination³⁷. These findings suggest that inhibition of the MAPK pathway alone does not

adequately inhibit BRAF-mutated colorectal cancer tumor progression. Indeed, subsequent preclinical work has shown that inhibition of BRAF by vemurafenib triggers compensatory activity of EGFR^{38,39}, which can bypass the inhibited BRAF to trigger downstream PI3K/Akt and MAPK signaling. Concomitant inhibition of BRAF and EGFR leads to tumor regression in preclinical xenograft models of BRAF mutated metastatic colorectal cancer not seen with agents which block BRAF and/or EGFR alone. Translating these findings into the clinical setting, early results from phase I/II clinical trials provide promising data that this approach may have some success in the treatment of BRAF mutated metastatic colorectal cancer. Nonetheless, resistance to target therapies invariably develops, and additional understanding is required in order to deep in the antitumor response⁴⁰. Understanding better the complex biology underlying these tumors is essential in order to design improved treatment options for this subset of patients.

Hypermethylation in BRAF-mutated colorectal cancer: Analysis of 239 tumors from The Cancer Genome Atlas (TCGA) revealed that BRAF mutations occur predominantly within a subpopulation of colorectal cancer that is characterized by a high genomic mutational burden, a phenotype which is also observed with the presence of microsatellite instability⁴¹. In colorectal cancer, microsatellite instability leads to deficient mismatch repair of DNA and can be caused either by germline mutations in critical DNA mismatch repair proteins or by tumor hypermethylation^{42,43}. Here, promoter hypermethylation of the MLH1 gene leads to downstream silencing of this gene and lack of activity of this critical DNA mismatch repair protein.

Hypermethylation in colorectal cancer is associated with the CpG Island methylation phenotype (CIMP). The vast majority (>90%) of BRAF-mutated colorectal cancer is CIMP-positive and features promoter regions of DNA enriched with cytosine-guanine (CpG) dinucleotides^{44,45}. Hypermethylation of tumor suppressor genes leads to gene silencing and

loss of cell cycle regulation, among other functions^{46,47}. Without concurrent hypermethylated genomes, BRAF-mutated colonocytes remain senescent⁴⁸⁻⁵⁰. Together, BRAF mutation and hypermethylation comprise key molecular features of the “serrated adenoma” pathway to colorectal cancer tumorigenesis^{51,52}, which is named for the unique precursor lesion, highlighting the key role of epigenetics in the pathogenesis of BRAF-mutated colorectal cancer.

Wnt/ β -catenin signaling in metastatic colorectal cancer: Alterations of the Wnt/-catenin pathway are present in >90% of colorectal cancers⁴¹, and inhibition of this pathway remains the "holy grail" for targeted therapies in this disease. Inactivating mutations in APC and activating mutations in CTTNB1 account for the majority of these alterations⁵³, and are difficult to target pharmacologically⁵⁴. The remaining fraction of colorectal cancer tumors are wild-type (WT) for APC and CTTNB1 and often feature aberrancies in tumor cell surface proteins like RNF43 and R-spondin (RSPO)⁵⁵, which also lead to deregulated Wnt/-catenin signaling^{56,57}. These tumors derive predominantly from the sessile serrated adenoma pathway and have a high proportion of BRAF mutations.

Wnt/ β -catenin signaling is important in the pathogenesis of colorectal cancer. Most colorectal tumors are driven by aberrant Wnt signaling predominantly via APC and CTTNB1 mutations^{58,59}. RNF43 mutations and RSPO fusions have been found in approximately 20% of colorectal cancer tumors and are exclusive to APC and CTTNB1 mutations. The common endpoint of these irregularities is nuclear translocation of β -catenin and upregulation of the T-cell factor transcriptional complex, promoting cell proliferation, anti-apoptotic behavior, and the epithelial-to-mesenchymal phenotype^{60,61}.

Preclinical work has suggested that targeted agents can dampen Wnt/-catenin signaling in

colorectal cancer models harboring RNF43 mutations or RSPO fusions⁶². However, additional alterations, including epigenetic modulation, also activate Wnt signaling in a ligand dependent manner in a subset of patients. Predictive biomarkers with available matched targeted therapies against Wnt/-catenin signaling are needed in order to tailor novel agents for patients with metastatic colorectal cancer.

CHAPTER II

METHODS

Xenograft Studies

Tumors were collected under an IRB-approved protocol from 2 patients with BRAF–mutated metastatic colorectal cancer who were treated at M.D. Anderson for establishment of patient-derived xenografts. The specimens were called “C0999” and “B1003” in order to provide names for these models that included no personally identifiable patient information. These xenograft tumors had been previously established by our group and were removed from storage at -80° Celsius. The samples were thawed, and for each of the 2 models individually, the thawed tumor was divided by scalpel at room temperature into 10 equal parts for an approximate volume of 4 x 4 x 4 mm. These smaller tumors were implanted subcutaneously into the right lateral flank of 10 individual female, NOD–SCID–gamma mice (purchased from Experimental Radiation Oncology at M.D. Anderson).

Once it had been determined that the tumors were growing in the mice in which they had been implanted, for each of the 2 different models, 6 mice were selected for further use once their mean tumor volume was approximately 200 mm³. These 6 mice were randomized into 3 equal arms (N=2 per arm) for initial treatment randomization. Here, an F1 generation was treated intraperitoneally with placebo (phospho-buffered saline, Sigma Aldrich) or with 5–azacitidine (Sigma Aldrich) at 2 different doses (0.25 mg/kg or 0.50 mg/kg). Intraperitoneal injections were administered twice weekly. The F1 generation was utilized to allow for prolonged exposure a demethylating agent prior to the experimental studies regarding a BRAF inhibitor in the F2 generation. Once these 6 tumors had reached a mean volume had reached a mean volume of 1500 mm³, the mice were sacrificed, and the tumors were harvested. Here, the tumors were immediately divided using a scalpel into equal parts with approximate volumes of 4 x 4 x 4 mm.

Tumors from the placebo/control mice in the previous F1 generation were implanted into 30 female NSG mice, and tumors from the 5-azacitidine treated mice were implanted into 30 different female NSG mice. Given that toxicities appeared to be similar/insignificant between the mice in the F1 generation treated with azacitidine at doses of 0.25 mg/kg and 0.50 mg/kg, we chose to continue with the tumors that had been exposed to azacitidine at a dose of 0.50 mg/kg for the experimental studies as part of the F2 generation. We surmised that doing so would optimize the exposure to a demethylating agent.

For the 2 sets of mice in the F2 generation which were treated with saline/placebo or

azacitidine, they continue to

receive twice-weekly

intraperitoneal injections until

the mean tumor size was

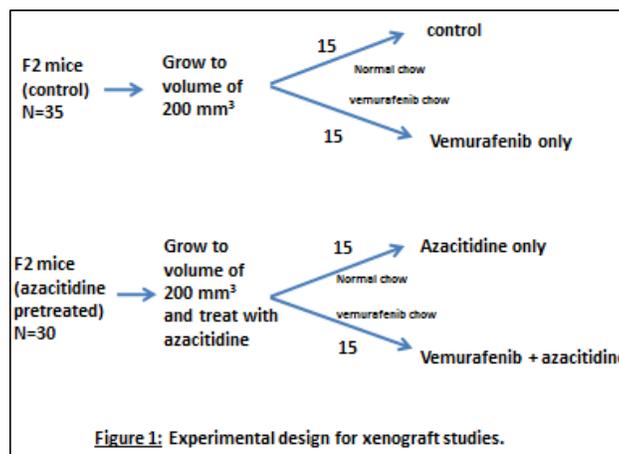
approximately 200 mm³. At that

time, mice were randomized in

a 1:1 fashion to receive or not to

receive concomitant

vemurafenib (see Figure 1 for



experimental flow). Vemurafenib was administered continuously as a chow at a dose of 417 mg/kg (Plexikon/Scientific Diets), and control chow was provided by scientific diets as well.

This resulted in 4 different groups for analysis: untreated control, azacitidine only, vemurafenib only, and azacitidine plus vemurafenib. Tumor volumes were measured twice-weekly and calculated as the product of length² x width/2, whereby length represents the longest axis of the cross-sectional measurements of the particular tumor.

The primary endpoint here was tumor size after a five-week treatment period, and the primary objective was to assess if the combination treatment resulted in smaller tumor size compared to

BRAF inhibitor treatment alone. For the primary comparison of tumor size at 5 weeks, we fit a one-way ANOVA model to assess the difference among all four treatment groups in order to derive the pairwise comparison results.

Bioinformatic Analysis

The CIMP-High status and the BRAF mutation data of the TCGA CRC samples were extracted from the Memorial Sloan Kettering Cancer Center cBio portal (www.cbioportal.org). The 36 corresponding CIMP-High samples were only retained in the RNAseq and Methylation datasets, and BRAF mutation status was identified from the identified samples. Probes/genes were queried from the 450,000 probes provided by the Illumina 27K methylation assay (Illumina, San Diego, CA) to compare the BRAF wild-type and mutated populations. From these cases, univariate analysis was performed to identify differentially expressed transcripts and differentially methylated transcripts using t-test from the various probes of interest. The p-values obtained by multiple t-tests were corrected with a false discovery rate using the beta-uniform modeling (BUM) method. The same methodology strategy was performed using the available data internally from the MD Anderson Integromics dataset. Again, RNAseq datasets were analyzed according to BRAF mutant and BRAF wild-type specimens from patients with colorectal cancer. Given the higher number of gene expression differences in various gene probes for this dataset, we constructed a heat map to illustrate the differences in expression patterns between these two populations of interest. Lists of genes with differential expression were compared for the two independent analyses to provide a better validated identification of genes which may be preferentially overexpressed (or underexpressed) in the BRAF-mutant CRC population. The list of genes identified here was used as input into the IPA Ingenuity BioProfiler list (www.ingenuity.com) to identify signaling pathways which may be most influenced by the variations in gene expression for the selected genes of interest from the gene expression analysis.

Methylation Profiling of Xenograft Tumors

We were interested as well in comparing the methylation profiling of the xenograft tumors exposed or not exposed to azacitidine. Here, 15 xenograft tumors were analyzed – 7 control tumors (4 from B1003 and 3 from C0999) and 8 experimental (3 from C0999 and 5 from B1003). Frozen tumors stored at -80°C were thawed, and DNA was extracted and isolated using a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA). To check the overall methylation pattern 10,000 highly variable probes were chosen from the Illumina 450k dataset. A next generation heat map was generated using the highly variable probes for visualization.

To test whether or not azacitidine was successful in demethylating biologically relevant markers for the presence of CIMP, three genes commonly hypermethylated in CRC tumors and universally accepted as markers for the presence of CIMP-high status – CACNA1G, RUNX3, and TIMP3 – were selected for further study. Here, mean methylation values (with associated standard deviation) from the 5'-methylation probe for each of the individual genes were calculated for the C0999 and B1003 tumors in the control and experimental/azacitidine arms. Results were compared between control and experimental arms each of the two xenograft models separately using a t-test in order to examine whether or not a difference in methylation was noted for the tumors exposed to a demethylating agent.

Given that the Wnt/ β -catenin signaling pathway had been demonstrated to be preferentially affected for BRAF mutant versus wild-type tumors, various genes in this pathway were also selected to assess for differences in methylation between the control and experimental/azacitidine groups for the C0999 and B1003 models separately. Here, the following genes were examined: APC2, AXIN1, AXIN2, CSNK1A1, CSNK1D, CSNK1E, CSNK1G1, CSNK1G2, CSNK1G3, DVL1, DVL2, DVL3, FZD1, FZD2, FZD3, FZD5, FZD6, FZD8, FZD9, FZD10,

GSK3A, GSK3B, LRP1, LRP4, LRP5, LRP6, RNF43, and RSPO1. Results were compared separately using a t-test for each of the given genes.

Gene Expression Profiling of BRAF-mutated CRC Models

To test further whether or not differences in gene expression were noted by the use of azacitidine, we first extracted RNA (Qiagen RNeasy kit) from the same xenograft tumors which had been analyzed for methylation for the C0999 and B1003 models. We were interested in looking at the genes in which methylation differences had been noted between the control and experimental (azacitidine-treated) arms in the xenograft studies to see if a corresponding differential gene expression possibly affected by methylation was observed. Once isolated, RNA was assessed by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) with probes specific for selected genes (APC2, AXIN1, AXIN2, CSNK1A1, CSNK1D, FZD9, LRP1, LRP4, LRP5, RNF43). Mean expression levels for the selected genes were compared between control tumors and experimental tumors for each of the two xenograft models. To look at another data set, we had gene expression data generated from prior work with the BRAFV600E CRC cell line HT29 treated with and without a demethylating agent. Cells were treated for 14 days with or without azacitidine, and harvested for RNA to compare differences in gene expression of selected genes. qRT-PCR was performed using probes from the same genes in the Wnt/ β -catenin signaling pathway as listed above. Differences between control and experimental/treated cells were compared using a Student's t-test.

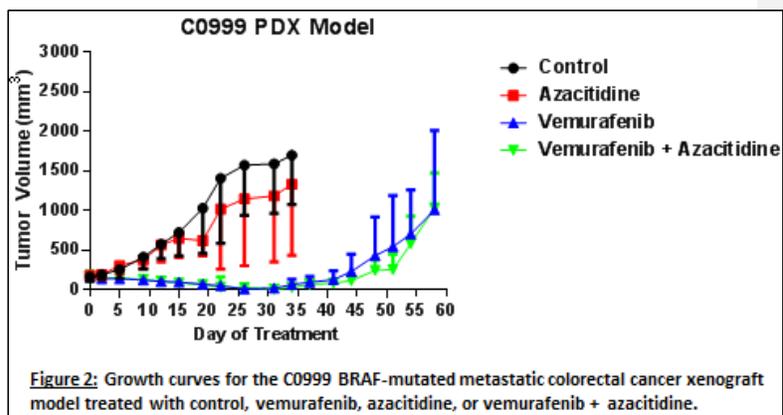
CHAPTER III

RESULTS

Xenograft Studies

Two different xenograft models of BRAF–mutated metastatic colorectal cancer – C0999 and B1003 –were utilized for these experiments to assess whether or not the addition of azacitidine to vemurafenib generates additional tumor regression. The results are seen in Figures 2 and 3. In both experiments, 40 mice with established C0999 or B1003 BRAF–mutated metastatic colorectal cancer xenografts were divided into 4 separate arms of 10 mice each. Mice were treated as either untreated controls, or with vemurafenib, azacitidine, or the combination of vemurafenib plus azacitidine. As seen in Figure 2, which represents the C0999 xenograft model, the addition of vemurafenib, with or without azacitidine, generated a significant tumor

regression, with no palpable tumor in either arm, after 21 days of treatment. However, these tumors quickly grew back with no persistent response to BRAF inhibition.

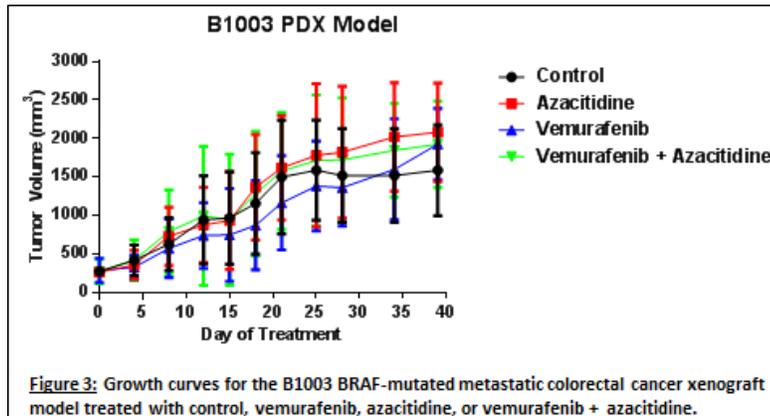


ANOVA analysis was used to demonstrate that a significant difference ($P < .0001$) in tumor volume at day 28 was detected among before groups here. Specifically, although there were no differences in tumor volume between control/azacitidine groups and vemurafenib/vemurafenib plus azacitidine groups, there were differences between the 2 groups of mice which received vemurafenib in the 2 groups of mice which did not receive vemurafenib.

In Figure 3, the growth curves for the B1003 BRAF mutated metastatic colorectal cancer xenograft model are shown, with individual arms for the untreated control, vemurafenib, azacitidine, and

vemurafenib plus azacitidine combination groups. Unlike the prior

xenograft model, ANOVA analysis



demonstrated no difference in tumor sizes between the four groups here ($P=.66$). For all cohorts, the tumors demonstrated no regression noted for any of the mice treated with experimental agents.

Collectively, for both xenograft models used here, C0999 and B1003, azacitidine did not cause regression in mean tumor volume, independent of inhibition of BRAF with vemurafenib.

Bioinformatic Analysis of Gene Expression/Methylation Patterns

In order to perform differential expression analysis between the BRAF mutant and wild-type sample groups in the RNAseq and methylation data of TCGA CRC dataset, we examined 226 samples with RNAseq and Methylation datasets both available. Of these 226 samples in this dataset, 36 samples were in CIMP-High group. Sixteen were BRAF mutated, and twenty were BRAF wild-type. With a False-Discovery Rate (FDR) threshold of 0.3, there were 55 significantly different transcripts in the RNAseq data (see Figure 4A) within the CIMP-high group distinguished according to BRAF mutation status. Even with an FDR threshold of 0.3 there were no significant probes in the methylation data (Figure 4B).

We next turned to the Integromics cohort of patients at MD Anderson, a separate group of patients independent from the samples analyzed in the TCGA, for whom genomic (mutation) profiling, RNAseq gene expression data, and methylation data had already been characterized. Here we performed hierarchical clustering using a heat map

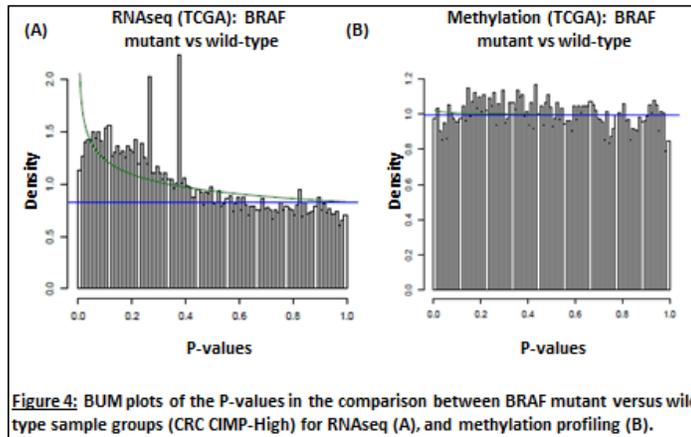


Figure 4: BUM plots of the P-values in the comparison between BRAF mutant versus wild type sample groups (CRC CIMP-High) for RNAseq (A), and methylation profiling (B).

generated by the gene expression data, and analyzed according to BRAF mutation status. As seen in Figure 5, a clear difference in selected genes was noted for patients in this cohort according to BRAF mutation status. There were 5954 genes which demonstrated differential gene expression according to BRAF mutation status in the analysis of the RNAseq gene expression data for the Integromics cohort. CIMP high vs CIMP low status was not available to select only for the CIMP-high patients, as had been done in the analysis of the TCGA data. Genes of interest which were preferentially underexpressed for the BRAF-mutated population relative to the BRAF wild-type population in the

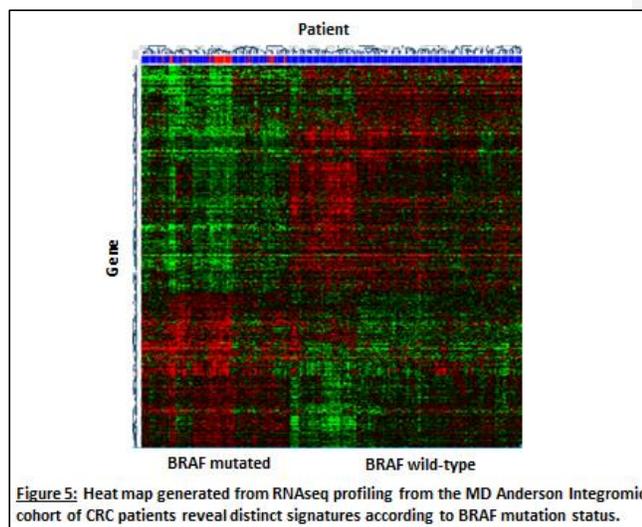


Figure 5: Heat map generated from RNAseq profiling from the MD Anderson Integromics cohort of CRC patients reveal distinct signatures according to BRAF mutation status.

Integratics cohort (with adjusted p-value to quantify the significance of difference) include AXIN2 (2.8×10^{-13}), FOXD1 (5.2×10^{-13}), RNF43 (1.3×10^{-12}), MLH1 (2.8×10^{-11}), PTPRD (3.7×10^{-12}), JUN (5.9×10^{-9}), RBP2 (1.4×10^{-8}), APC2 (4.9×10^{-8}), and RNF44 (1.1×10^{-6}).

Next, we were interested in identifying the union genes which were differentially expressed according to BRAF mutation status for both the Integratics and TCGA separate cohorts. We compared the 250 genes of highest significance of difference for each of the two datasets and identified genes common to both lists. These genes are listed in Table 1 below, along with the direction of change of gene expression (overexpressed or underexpressed) of the BRAF-mutated patients relative to the BRAF wild-type population:

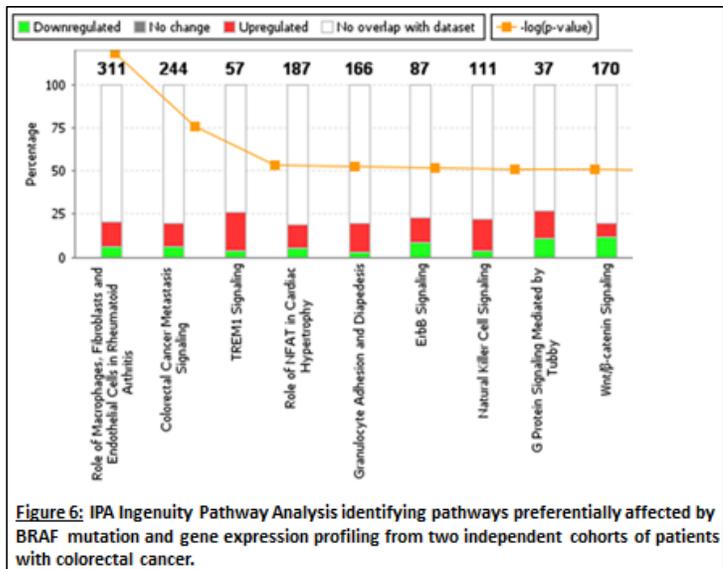
Gene	Relative expression in BRAF-mutated cohorts	Function of gene
AXIN2	Underexpressed	Phosphorylates CTTNB1 in preparation for degradation
EPDR1	Underexpressed	Cell-ECM adhesion
KHDRB53	Underexpressed	Inhibits cell proliferation
RNF43	Underexpressed	Negative regulator of Wnt/beta-catenin signaling
MLH1	Underexpressed	Mismatch repair of DNA
RAB32	Underexpressed	Binds to regulatory unit of PKA
TDGF1	Underexpressed	Cripto1:EGF signaling
KDSR	Overexpressed	Sphingosine/ceramide synthesis

Gene	Relative expression in BRAF-mutated cohorts	Function of gene
JUN	Underexpressed	
GTF21RD1	Underexpressed	Transcriptional regulator under control of Rb
PTPRO	Underexpressed	Induced by Wnt signaling; negative regulator of Wnt/beta-catenin signaling
PLAGL2	Underexpressed	Inhibits differentiation to generate stem cell phenotype; upregulates Wnt signaling
RBP2	Underexpressed	Retinal-binding protein
TDGF3	Underexpressed	
ID1	Underexpressed	Inhibitor of DNA binding
DDX27	Underexpressed	RNA helicase involved in cellular growth/differentiation
HPSE	Overexpressed	Facilitates cell migration via ECM degradation; cleaves HSPGs
IHH	Underexpressed	Indian hedgehog
LM04	Overexpressed	Transcription factor for oncogene activity
ZIC5	Overexpressed	DNA binding, activation of transcription factors
CYP2B6	Underexpressed	Retinoic acid production

Gene	Relative expression in BRAF-mutated cohorts	Function of gene
FERMT1	Underexpressed	Links extracellular matrix to cytoskeleton
NR112	Underexpressed	Binds CYP3A4 and RXRA
GPR56	Underexpressed	Cell adhesion; in melanoma, overexpression inhibits metastases
MOSC2	Overexpressed	
GRAMD1A	Underexpressed	
KDEL3	Overexpressed	Golgi: ER processing
TINAG	Underexpressed	
SPATA2	Underexpressed	
SEC22B	Overexpressed	

Table 1: Genes preferentially expressed (direction of change listed in middle column) for BRAF-mutated CRC tumors in the Integromics cohort and the TCGA cohorts, with description of the functional relevance of selected genes in the third column.

With many genes here demonstrating differential gene expression among the BRAF mutated and BRAF wild-type cohorts, we were next interested in understanding what signaling pathways may be preferentially affected by the presence or absence of a mutated BRAF. We performed an Ingenuity IPA pathway analysis incorporating the identified genes, common to both the TCGA and Integromics data sets, with the results for the most significant signaling pathways listed in Figure 6:

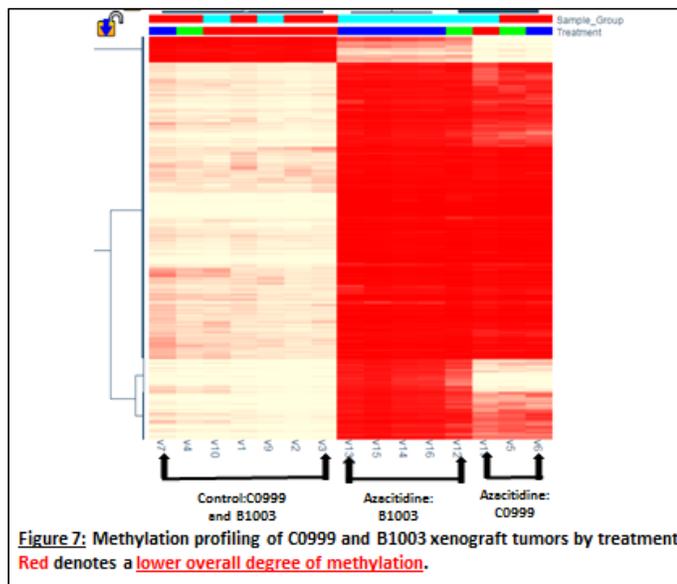


One of the strongest links was in genes affecting “colorectal cancer metastasis signaling,” which serves as a confirmatory positive control for the biological relevance of this analysis here. Other pathways of interest included pathways involved in inflammation/immune activity (“role of macrophages, fibroblasts, and endothelial cells in rheumatoid arthritis,” “granulocyte adhesion and degradation,” “natural killer cell signaling,” and “B cell receptor signaling”). Of great interest, the Wnt/β-catenin signaling pathway was also associated with preferential activity in the BRAF-mutated colorectal cancer cohorts per the results of this pathway analysis.

Methylation Profiling of Xenograft Tumors

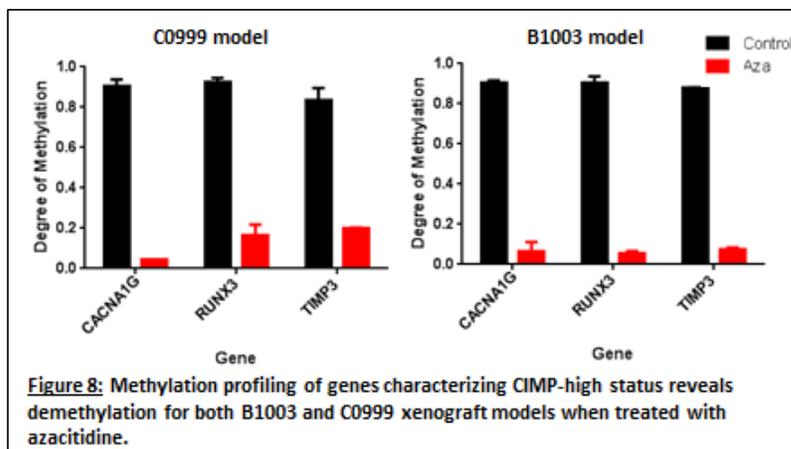
Given that no significant differences in tumor volumes were noted in the xenograft studies with the use of a demethylating agent, we next performed methylation profiling for the various tumors treated with or without azacitidine to assess whether or not changes in methylation were occurring. To check the overall methylation pattern 10,000 highly variable probes were chosen

from the Illumina 450k dataset. The next generation heat map from this analysis is demonstrated in Figure 7.



Clearly, for the C0999 and B1003 models alike, profiles for methylation are very similar. In this figure, red represents an overall lower degree of methylation, whereas the whiter hue represents a higher level of methylation. In the untreated controlled groups, for both xenograft models, for the selected probes there is a methylation profiling which demonstrates clear higher levels of methylation overall relative to the 2 groups (C0999 and B1003) which were exposed to azacitidine. In addition, the individual xenograft tumors treated with azacitidine clustered together between the individual models. In other words, all of the C0999 xenograft tumors that were treated with azacitidine had a methylation profiling that was separate and distinct from the B1003 xenograft tumors that were treated a demethylating agent. These results lend support to the notion that azacitidine was indeed capable of performing his task in the xenograft tumors – i.e., reversing methylation of specific genes. Therefore, it cannot be concluded that no

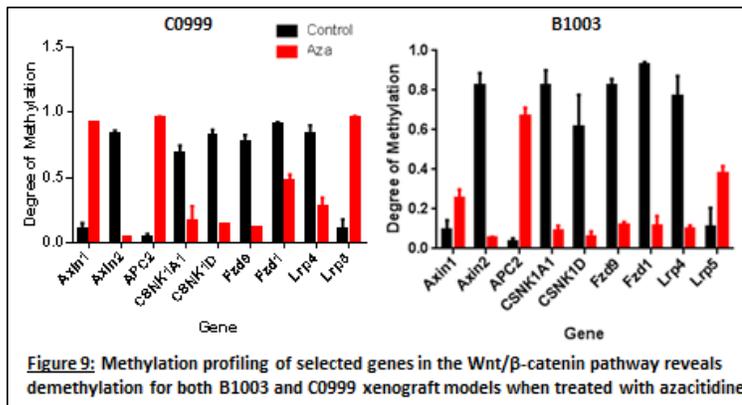
differences in tumor volumes were observed due to the lack of demethylation by azacitidine. Instead, this drug was able to alter the overall methylation patterns within the given tumor. We also looked at genes which are known to be under epigenetic regulation and, when methylated, characterize the presence of CIMP–high status for a given tumor. Specifically, we looked at the 3 genes CACNA1G, RUNX3, and TIMP3. As seen in Figure 8, for both xenograft models, the addition of azacitidine resulted in a significantly lower degree of methylation in the experimentally treated group when compared to the untreated controls. This provided additional evidence to us that the administration of a demethylating agent resulted in changes in methylation profiling and serves as a positive pharmacodynamics biomarker that azacitidine was indeed performing its specified task in these studies ($P < .005$ for all six comparisons).



Methylation/gene expression analysis of Wnt/ β -catenin targets

The Wnt/ β -catenin signaling pathway was a pathway previously identified to show differential gene expression for colorectal tumors according to the presence or absence of a BRAF mutation. We next proposed to examine specific genes of interest in this pathway in order to assess whether their expression may be affected by demethylation. Specifically, we compared methylation levels between untreated control groups and their corresponding azacitidine groups

for each of the two xenograft models for the following genes: Axin1, Axin2, APC2, CSNK1A1, CSNK1G2, CSNK1D, FZD9, FZD1, LRP4, and LRP5. Statistically significant changes in methylation ($P < .05$ for all) were noted with the addition of a demethylating agent, as seen in Figure 9:



Here, methylation levels of genes like Axin2, CSNK1A1, Fzd9, Fzd1, and Lrp4 were reduced in both models of BRAF-mutated metastatic colorectal cancer with the addition of azacitidine, whereas higher methylation of Axin1 and APC2 were noted with the same intervention.

Comparison of Gene Expression Profiling of selected genes in the Wnt/β-catenin signaling pathway for the Xenograft models of BRAF-mutated colorectal cancer

Given that we found significant differences between the various previously mentioned genes in this signaling pathway, we next analyzed gene expression profiling on the xenograft tumors individually and compared the mean values between the control groups and the azacitidine groups to assess whether or not these changes in methylation correlated to matched changes in gene expression profiling. Table 2 lists the magnitude of change (ratio) of the mean level of gene expression by RNAseq for the azacitidine group relative to that of the untreated control group. In all instances, a t-test was performed to compare the mean values the P value to

compare for a difference between the untreated control and the experimental groups exceeded 0.05, and therefore none of the differences noted were statistically significant for these experiments.

Here, the direction of change for CSNK1A1, CSNK1D, Fzd9, and Fzd1 were all increased, which corresponded to the direction of change for the matched change in methylation noted by the addition of azacitidine in the respective experiments, which was decreased. Therefore, the methylation changes were biologically consistent with the changes in the gene expression.

Gene	C0999	B1003
Axin1	1.21	.93
Axin2	1.16	.76
APC2	4.02	1.22
CSNK1A1	1.07	1.34
CSNK1D	1.00	1.04
Fzd9	25.7	1.37
Fzd1	1.09	1.79
Lrp4	1.00	.73
Lrp5	1.12	.97

Table 2: Magnitude of change in mean gene expression of various genes in Wnt/ β -catenin signaling upon addition of azacitidine.

qRT-PCR Analysis of BRAF-mutated xenograft models

To investigate further whether or not there were differences in gene expression upon the addition of azacitidine for the C0999 and B1003 models, we performed quantitative reverse-transcriptase PCR (qRT-PCR) on frozen specimens of xenograft tumors in the untreated controls and in the cohort exposed to azacitidine. Mean values (with standard error) are demonstrated for each of these genes in Figure 10: Here, no significant changes in gene expression according to qRT-PCR were noted in any of the genes assessed in this pathway with the exception of RNF43 in the B1003 model, whose expression did increase upon exposure to azacitidine. Interestingly, RNF43 was also the gene with the strongest association with preferentially decreased expression in the gene expression profiling for the BRAF-mutated colorectal tumors relative to the BRAF wild-type tumors.

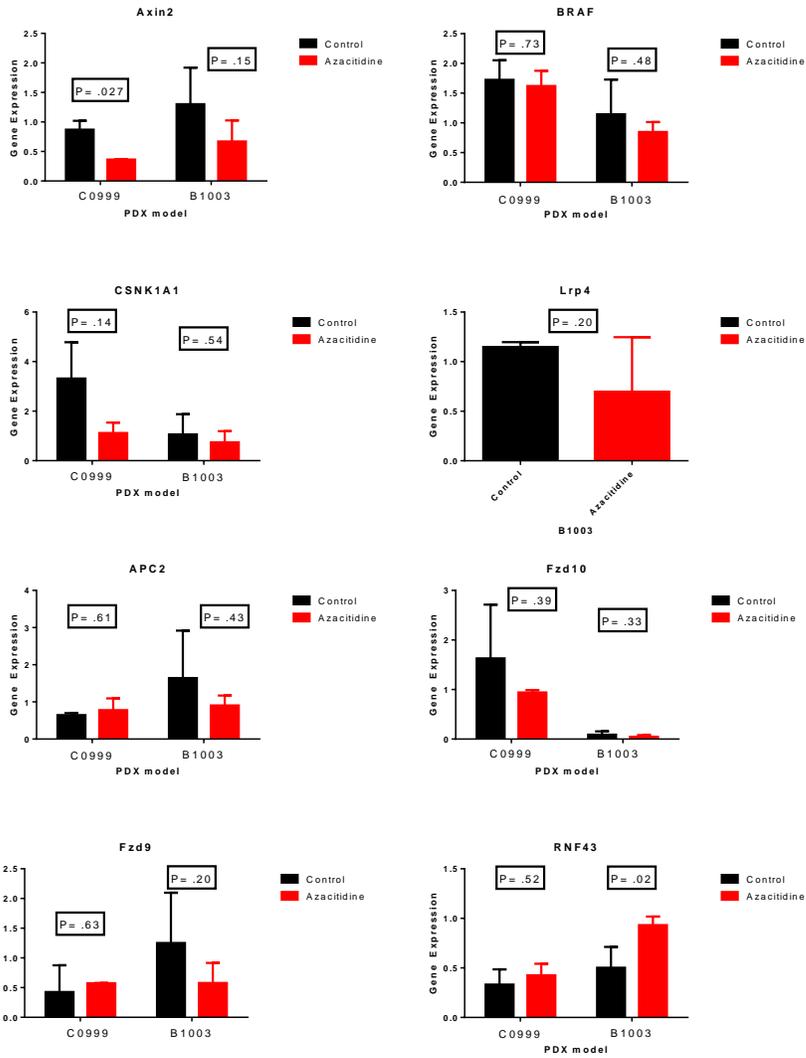


Figure 10: Changes in gene expression for selected genes in the Wnt/ β -catenin pathway with or without azacitidine using qRT-PCR.

qRT-PCR Analysis of BRAF-mutated cell line models We also looked at the HT29 BRAF-mutated colorectal cancer cell line for changes in methylation and in gene expression upon the introduction of azacitidine. Here, we compared mean values for differences in methylation and in gene expression for untreated controls versus cells treated with a demethylating agent using qRT-PCR provided from an outside, publicly available data set. Table 3 demonstrates the magnitude of change in the ratio is for both methylation and corresponding gene expression in mean is azacitidine: untreated control for each of the listed genes important in the signaling pathway. Values with significant differences are listed, but otherwise only trends, but no statistically significant differences, were observed upon the addition of azacitidine. Notably, decreases in mean levels of methylation were observed for Fzd9 and Lrp4 with an associated increase in gene expression upon addition of azacitidine. Increases in methylation were likewise observed with Axin1 and Lrp5 with associated decrease in gene expression of these genes. In addition, expression of the surface proteins RSPO2 and RSPO4 were observed upon exposure to azacitidine, and neither of these genes were expressed at detectable levels by qRT-PCR in the untreated control HT29 cells.

Gene	Methylation change	RNAseq change (for azacitidine)
Axin1	↑	↓ (P=.04)
Axin2	↓	↑
APC2	↑	↑
CSNK1A1	↓	↓
CSNK1D	↓	↓
Fzd1	↓	↑ (P=.03)

Gene	Methylation change	RNAseq change (for azacitidine)
Fzd9	↓	↑
Lrp4	↓	↑ (P=.06)
Lrp5	↑	↓ (P=.04)
RNF43	↓	↓
RSPO2		↑ (0 -> detected)
RSPO 4		↑ (0 -> detected)

Table 3: Direction of change in mean methylation and gene expression of various genes in Wnt/ β -catenin signaling upon addition of azacitidine in HT29 BRAF-mutated colorectal cells.

CHAPTER IV

DISCUSSION

BRAF–mutated metastatic colorectal cancer represents a rare subset of the colorectal cancer population with characteristic clinical and pathological features. Notably, these tumors are associated with concomitant microsatellite instability and global hypermethylation. As patients whose colorectal tumors harbor these BRAF V600E mutations demonstrate poor responses to standard cytotoxic chemotherapy regimens and rapid clinical deterioration that lead to terrible survival outcomes, new therapies are desperately needed in order to provide this group of patients otherwise effective treatment. This proposal sought to address the role of hypermethylation in these BRAF mutated colorectal tumors by a set seeing the role of a demethylating agent in combination with a BRAF inhibitor and by identifying signaling pathways/potentially targets which have the potential to be manipulated by demethylating agents.

Azacitidine is a nucleoside analogue which forms covalent bonds with DNA methyltransferases and inhibits further methylation of downstream nucleotides, thereby promoting an overall demethylated state⁶³. Demethylating agents have FDA–approval for clinical use in hematologic disorders such as myelodysplastic syndrome. However, the clinical utility of azacitidine in solid tumors is less understood. Preclinical studies have shown that brief exposure of epithelial tumor cell lines to low doses of demethylating agents generates a sustained anti-tumor effect, even against subpopulations of solid tumors not sensitive to standard chemotherapy⁶⁴. These findings provided us with the rationale to study lower doses of azacitidine over prolonged treatment time with the goal of understanding whether or not demethylation can restore sensitivity to BRAF inhibitors in preclinical models of colorectal cancer.

We examined two xenograft models of BRAF mutated metastatic colorectal cancer to investigate the interplay between BRAF inhibitors and demethylating agents. To our surprise, the C0999 model showed sensitivity to single agent therapy with the BRAF inhibitor vemurafenib. Here, after approximately 3 weeks, all tumors developed complete regression with this therapy. Eventually, all tumors treated with the BRAF inhibitor develop resistance to this therapy, and detailed analysis of these findings was performed and has been described elsewhere (manuscript under review). Our conclusions, based on mutational profiling at the time of acquired resistance, were that these tumors were dependent on continued MAPK signaling as a means to propagate tumor growth. Regardless, in both the C0999 model and the B1003 model, the addition of azacitidine had no effect on treatment response/tumor volume either by itself or in combination with vemurafenib.

Given these findings, we were interested in understanding whether or not these azacitidine was even affecting methylation patterns at the low dose at which was being administered. To assess this further, we performed genomic methylation profiling on the tumors in the control arms and in the tumors treated with a demethylating agent. Clearly, differential methylation profiling was seen in the tumors that were treated with azacitidine. Some of the methylation probes were noted to be hypermethylated in the control tumors and then convert to a less methylated state upon exposure to azacitidine, whereas other probes were noted to be more demethylated in the control and become more methylated with the addition of azacitidine. Notably, many of the latter cases happen to be probes that were assessing methylation within the bodies of genes of interest (downstream from the 5'-promoter sequence). On the other hand, many of the probes which were relatively more methylated in the control group were found on the 5'-promoter foci.

Previous work on the X-chromosome studying the relationship between epigenetic modulation and gene expression has shown that, in genes which are actively express, an inverse relationship exists in methylation patterns between the promoter sequence and gene body

sequence⁶⁵. In other words, decreased methylation at the promoter site with corresponding relative hypermethylation at the gene body can be associated with higher levels of gene expression. Alternatively, higher methylation levels in the promoter region with corresponding lower methylation patterns in the gene body sequence can be associated with relatively lower levels of gene expression. These patterns could explain why there were regions of both hypermethylation and relative hypomethylation in the xenograft tumors randomized to the control arm, which were reversed with use of a demethylating agent. As evident by our data, methylation patterns were reversed by azacitidine. Despite the fact then that there was no change in tumor volume with the addition of azacitidine, this drug was nonetheless pharmacodynamically effective in reversing methylation patterns with the use of a lower dosed, prolonged exposure to a demethylating agent treatment strategy.

Next, we were interested in identifying genes which may be preferentially affected by methylation changes in the BRAF mutated colorectal cancer population relative to the BRAF wild type counterparts. Based on gene expression characterization, genes like MLH1 were preferentially under expressed in the BRAF mutated population. Decreased expression of this gene is associated with deficient mismatch repair in colorectal cancer^{43,66}, and, given that BRAF mutations are likewise associated with microsatellite instability, this findings served as a positive control that provided validation to our data within the correct clinical context for the biologic understanding of colorectal cancer. Interestingly, two of the most significantly underexpressed genes in the BRAF–mutated population were AXIN2 and RNF43. Both of these genes serve as negative regulators of Wnt/ β -catenin signaling, and, when not fully present, can be associated with increased activity of this pathway which is seminal important in the pathogenesis of colorectal cancer. RNF43 mutations often occur mutually exclusively to APC mutations⁵⁵, and appear to co-occur more commonly with BRAF mutations (unpublished data estimated RNF43 mutations present in 64% of BRAF mutated colorectal tumors). This protein is found at the

surface of the tumor cell and functions to downregulate the presence of the Wnt ligand receptor Frizzled (Fzd). Similarly, AXIN2 is also important in modulating activity of the Wnt/ β -catenin pathway. Lower expression levels of this gene prevent APC binding to and eventual phosphorylation/degradation of β -catenin, thereby permitting continued effector activity upon nuclear translocation of β -catenin. Given that these were two of the strongest associations for preferential gene under expression and given that we know that Wnt/ β -catenin signaling is deregulated in greater than 90% of colorectal cancers⁴¹, even in the absence of an APC mutation, we were next interested in investigating whether or not non-APC genes may be susceptible to the effects of epigenetic modulation in Wnt/ β -catenin signaling via changes in methylation.

For both xenograft models, AXIN2 was relatively over methylated in the untreated controls, and methylation levels at the 5'-promoter and were significantly reversed from a hyper-methylated state. This was also seen in other tumor suppressor genes imported in Wnt/ β -catenin signaling like APC2, FZD9, and LRP4. Corresponding gene expression profiling differences were noted in FZD9 (25.7x) and APC 2 (4.02x) were noted with corresponding reversal in methylation by azacitidine in both xenograft models. However, no statistically significant differences were noted despite the high differential magnitude of these trends, which may have been statistically limited by the low number of tumor specimens that were able to be assessed by this project. To investigate further, we performed RT-PCR on RNA extracted from both control and experimentally treated xenograft tumors to assess for any differences in gene expression using a different methodology. Interestingly, RNF43 was preferentially increased in terms of gene expression in the B1003 model that was exposed to azacitidine. Using a different model, an HT29 BRAF-mutated colorectal cancers line, use of a deep blebbing agent was associated with a decrease in methylation profile with corresponding trend towards increase in gene expression for genes like AXIN2, FZD1, LRP4, and LRP5. Collectively, these findings may suggest that

negative regulators important in Wnt/ β -catenin signaling may be influenced by epigenetic modifications in methylation, and have the potential to be targeted in the future.

The implications of these findings are important when translating towards clinical applications. A recent phase II clinical trial assessing the addition of a porcupine inhibitor to agents against BRAF and EGFR completed for patients with BRAF mutated metastatic colorectal cancer harboring concomitant RNF 43 mutations. While current clinical trials often consider only genomic aberrations (e.g., mutations) in determining inclusion criteria for assessing tumors with a targetable drug against a known/defined aberrant signaling pathway, our data suggest that non-genomic/epigenetic aberrations, and their corresponding influence on the pathogenesis of a given tumor, should be considered as well when seeking to identify patients who may benefit from a novel therapeutic agent under study. From our results, given that methylation can be associated with potential gene expression, we support the idea that gene expression profiling may better include patients with aberrant Wnt/ β -catenin signaling of interest in future clinical trials.

Chapter V

LIMITATIONS

We did not see any antitumor response with the addition of a demethylating agent to treatment with a BRAF inhibitor. However, since the time that this experimental design was planned, preclinical work has shown that inhibition of BRAF in the BRAF–mutated colorectal cancer is associated with an upregulation in EGFR, and that antitumor responses can be restored with the addition of an anti–EGFR therapy. Clinical trials that are currently ongoing will provide additional insight into the efficacy of combined targeted therapies against both BRAF and EGFR. Given this updated knowledge, the more clinically applicable experimental design would have been to test the addition of azacitidine to therapies targeting both BRAF (vemurafenib) and EGFR (cetuximab).

Although trends were seen in differential gene expression by both RT–PCR of RNA extracted from xenograft models treated with azacitidine and from cell line models of BRAF mutated colorectal cancer treated with azacitidine, no statistically significant differences were detected. Given the finding that was available for this project from the ASCO Young Investigator Award, we were able to perform analyses only on a few selected tumors. The trends that we observed may have been limited by high standard deviations reflective of a low sample size. Increasing the sample size and including additional tumor samples may have detected statistically a difference back only be inferred at present with the current trends.

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Chapter VI

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FUTURE DIRECTIONS

We are collaborating with a company in the Texas Medical Center who has created a novel inhibitor of Wnt/ β -catenin signaling whose activity is very downstream in the nucleus. Given that we have this increased understanding of the role of this pathway that it is important and BRAF mutated colorectal cancer pathogenesis based on these data, we would like to test this in combination with targeted therapies against both BRAF and EGFR in preclinical xenograft models. If promising activity is observed, then we propose translating these findings into a clinical trial.

We are also set to open a clinical trial at M.D. Anderson in the coming months which incorporates an antibody against RSPO, another regulator important in signaling of the Wnt/ β -catenin pathway, in combination with the cytotoxic chemotherapy regimen FOLFIRI. Inclusion criteria for the study will build upon our conclusion here that patients must have high levels of expression of RSPO in order to be candidates for the dose expansion portion of the study. Therefore, gene expression profiling will be incorporated into the screening process.

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VITA

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