Decitabine treatment induces demethylation of partially hypermethylated SOX21 DMR in HCT-116 colorectal cancer cell line

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Abstract
Colon cancer is one of the most prominent cancer types in the world, being in second place as the leading cause of cancer death. Even though the molecular mechanisms underlying this disease are not yet understood, epigenetic modifications have been attributed to possible involvement in tumorigenesis. In particular, DNA methylation is frequently seen across multiple cancer patients. There are high-confidence differentially methylated regions (DMRs) that could provide insight into this modification and its role in cell survival and proliferation. In this study, the researchers use the HCT-116 colorectal cancer cell line as a model to study the effects of treatment with the demethylating anti-cancer drug, decitabine, on the methylation sites of the SOX21 DMR. For this, decitabine effects on cell growth and demethylation were analyzed by global demethylation assay using methylation-sensitive restriction enzyme HpaII. Followed by bisulfite PCR Sanger sequencing analysis to quantify CpG methylation for HCT-116 cells and after treatment. It was found that decitabine is a good agent for demethylating DNA and inhibiting cell growth. As of SOX21 DMR, it appeared that it was originally unmethylated, and thus, decitabine partially demethylated specific CpG sites at the promoter region. The data was uploaded to the UCSC genome browser and compared with previous ENCODE Project data, which indicated new CpG sites near known methylated sites for SOX21 DMR. For future research, the researchers aim to investigate chromatin accessibility along with methylation sites to investigate gene expression and possible mechanisms involved in colon cancer tumorigenesis, expanding site-specific understanding of this DMR.

Acknowledgements
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Introduction

Colon cancer is one of the most prominent cancer types in the world affecting mostly older adults. According to the Colon Cancer Coalition, colorectal cancer – indicating colon and rectal cancer - is the third most diagnosed and the second, in terms of, leading cause of death (Colon Cancer Facts). Not only is it diagnosed in 1 out of 24 people, but also, it has expanded to almost all ages, usually common at the age of 66 or younger (Colon Cancer Facts). Nonetheless, the molecular oncogenic pathway of colon cancer remains a mystery with different mechanisms as potential models. Epigenetics has opened the research field for its further understanding and treatment.

DNA methylation is one of the most important epigenetic modifications in mammalian genomes that controls gene expression. It is characterized by cytosine methylation. This happens at the C5 position of cytosine, where a methyl group is added onto it, converting the normal cytosine into a 5-methylcytosine (Kasai and Kawai 2009). This conversion can occur due to DNA methyltransferases (DNMTs), such as DNMT1, that help carry out the process of methylation of nucleotides (Robertson 2002). The patterns of DNA methylation are not randomly distributed, they follow different regions in the DNA. Those regions with parasitic and repetitive DNA are hypermethylated, while those with high CpGs are hypomethylated (Robertson 2002). As mentioned before, cytosine methylation is identified as a determinant of gene expression, and therefore, studied for colon cancer-associated genes, for its possible role as the underlying cause.

For example, Rhee et al. (2002) (Rhee et al. 2002) were interested in investigating the unknown mechanism of global methylation by knocking out the activity of the DNA methyltransferase (DNMT1 and DNMT3b) in the HCT-116 colorectal cancer cell line. They found that when lacking activity of both, the promoter region of the tumor suppressor gene p16INK4a - which is silencing-sensitive to methylation - was demethylated greater than 95%, as well as, having inhibited cell growth. All of this suggested that DNA methylation seems to play a role in silencing genes and cell survival. Furthermore, it also demonstrated that the colorectal cancer cell line HCT-116 is sensitive to DNA methylation, making it a good cell line for further studying of DNA methylation and its effects on colon cancer associated-genes.

Other studies have also suggested the negative effect of DNA methylation on colon cancer contributing to a poor prognosis. One study conducted by Merry et al. showed that by studying one specific colorectal cancer cell line, HCT-116, DNMT1 could be identified and found their role in the process of tumorigenicity (Merry et al. 2015). They showed that DACOR1m, a DNMT1-associated long non-coding RNA is downregulated in cancer cells. However, if the right conditions were administered, they found that the colon crypts present in colon cancer were maintained by DACOR1.This demonstrated the idea that disruption of normal DNA methylation patterns is one of the most common features of transformed cells and these changes are early events in the formation of cancerous cells.

At the same time, different genome-wide analyses of DNA methylation sites for colorectal cancer have been investigated to compare the differential patterns of methylation for tumor and normal tissue. As Simmer et al. (2012), by using the MethylCap-seq approach they were able to identify 2,687 frequently hypermethylated regions, referred to as DMRs (differentially methylated regions). Once again, they suggested that hypermethylation happened most frequently in promoter regions, such as the region of interest for Rhee et al. (2002) and in CpG islands. This study only provided evidence for a list of high-confidence hypermethylated DMRs that could potentially be related to colorectal cancer, as well as possible genes that are co-localized with the DMRs identified.

The researchers sought to investigate one of the genes that are co-localized with these DMRs that are not known to have a role in colorectal cancer. The region of interest in this study is SOX21, which is a transcription factor with high-confidence of hypermethylation at the promoter region (Simmer et al. 2012). The role of the SOX21 gene on colon cancer is still an under-researched area that the researchers intended to explore in this study. As a member of the SOX protein family and as a transcription factor, their role is tied to the regulation of different development and differentiation of cells, including proliferation, and turning off and on genes. Even though no specific connections have yet been made about SOX21 and colorectal cancer, it is proposed as a biomarker for colorectal cancer diagnosis since methylation of this gene was present in cancer patients. Moreover, hypermethylation of this causes deregulations on their function granting neoplastic proliferation leasing to tumorigenesis (Simmer et al. 2012, Moradi et al. 2020, Yang et al. 2016).

For this research, the colorectal cell line HCT-116 will be the cell model used. With Rhee et al. (2002) effective use of this cell line and their findings, it gives evidence of its prominent role in identifying DNA methylation and its influence on genes. The HCT-116 cell line was first isolated from an adult male, making it both a human and a colorectal carcinoma cell line (Imanis Life Sciences). As seen, methylation of HCT-116 cell lines decreases growth and cell survival (Merry et al. 2015) and they are both useful for either in vitro or in vivo experiments (Imanis Life Sciences). This cell line will be used for this study to analyze the SOX21 DMR when treated with decitabine (5-aza-2-deoxycytidine) - an anti-cancer drug identified as a demethylation agent (Chemocare).

In this report, the researchers sought to study if decitabine demethylates the global DNA methylation present in the SOX21 DMR in HCT-116 cells. From the consensus on the role of decitabine and methylation on colorectal cancer, it was predicted that SOX21 DMR, after treatment with decitabine, will be demethylated and HCT-116 cell survival will be inhibited. To accomplish this goal, the reesearchers started by analyzing the gene of interest to determine if it was globally methylated looking at the UCSC Genome Browser. This was followed by extracting genomic DNA from the HCT-116 cell line, treating it with decitabine or DMSO (control group - unaltering methylation regions), and counting cells to identify the effect of each treatment on cell growth. Then, a glob-

al demethylation analysis was carried out with restriction enzyme HpaII, bisulfite conversion of extracted DNA, bisulfite PCR reactions, and lastly, sequencing analysis to study CpG sites demethylation of SOOX21 DMR.

In this study, the researchers found that decitabine is an effective agent to demethylate DNA and inhibit cell growth for the HCT-116 colorectal cancer cell line. However, it partially demethylated the FBSOX21 DMR, which seemed to be originally demethylated. Our findings indicated new CpG sites previously not identified in the genome as hypermethylated in the promoter region of SOX21 DMR.

Results

Decitabine treatment negatively affects HCT-116 cell growth

To determine if the anti-cancer drug decitabine decreases HCT-116 colon cancer cell survival, genomic DNA was isolated and extracted from the HCT-116 cell line using the enzymes Proteinase K and RNase A, following the Monarch®Genomic DNA Purification Kit Protocol (for detailed protocol refer to Methods section). The cells were separated and treated twice with DMSO or decitabine for two days, and then counted using a hemocytometer. The researchers expected to see fewer cells in the decitabine sample as compared to the DMSO, which is a control group and should affect the cells' survival. It was observed that for the cells treated with 0.25μM decitabine, the cell count was much lower than those cells treated with 0.1% DMSO (Figure 1). The researchers concluded that decitabine inhibits cell growth for the HCT-116 cell line. It doesn't inhibit it completely, even though there are much fewer cells in the decitabine treatment sample than in the DMSO, there will still be some present, suggesting that decitabine treatment slowed cell growth.

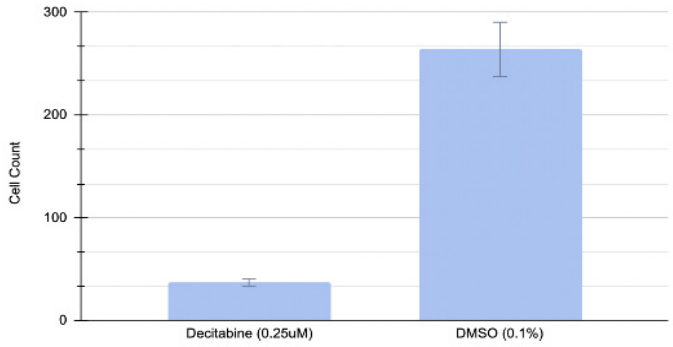

Figure 1. 0.25μM Decitabine slowed HCT-116 cell growth. The number of counted HCT-116 cells, using a hemocytometer, after decitabine or DMSO treatment is done twice for 2 days. Decitabine-treated sample, with an average of 37 cells in the hemocytometer chamber, showed a much lower cell count than DMSO, with an average of 264 cells.

Decitabine demethylates HCT-116 genomic DNA

The researchers previously showed that Decitabine can inhibit HCT-116 cell growth. This anti-cancer drug is also classified as a demethylating drug, which means it can demethylate DNA. To test whether it demethylates DNA, to the extracted and treated gDNA the restriction enzymes *HpaII* and *MspI* were added, and gel electrophoresis was done to visualize demethylation. Before any analysis could be done, the extracted gDNA was tested for purity and quantity using the BioTek Synergy spectrophotometer and the spectrometer absorbance data. It was observed that the gDNA was present in good concentration and with an accepted value of A260/280 (above 1.8), which indicates purity (Table 1).

Moreover, the restriction digest enzyme reaction uses the HpaII restriction enzyme, which is sensitive to methylation. This is to say it won't cleave the DNA if methylation is present. As seen previously, since decitabine demethylates DNA, the researchers expected that decitabine-treated sample would show higher sensitivity to HpaII, and thus, it will be cleaved by HpaII more than the DMSO sample. As for MspI, this restriction enzyme is used as a control to see if the DNA can be cleaved or not (not methylation-sensitive). From the results with the different digest conditions - no enzymes, or either one of them - it was observed that decitabine DNA sample with HpaII was cleaved as compared with DMSO DNA sample, which was not, or either sample for no digest enzyme condition (Figure 2). All in all, these findings show that decitabine can global demethylate DNA from HCT-116 cells.

	Concentration (ng/μl)	A26 0	A28 0	A32 0	A260/A28 0
DMSO (0.1%)	155.3	0.06 2	0.06 0	0.05 4	2.001
Decitabine (0.25μM)	398.6	0.05 6	0.05 4	0.05 1	2.049

Table 1. Extracted genomic DNA is pure and of good concentration. The concentration of DNA samples for each group by UV-vis spectrophotometer and Nucleic Acid Quantification program was revealed to be of good purity.

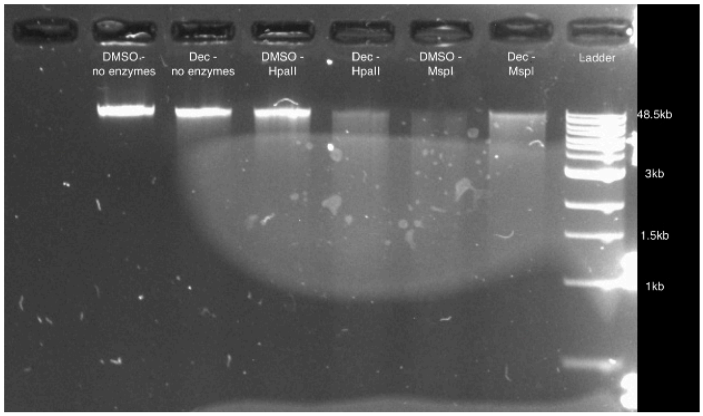

Figure 2. 0.25μM Decitabine demethylated genomic DNA from HCT-116 cell line. The gel from gel electrophoresis, after PCR reaction, separating DNA bands for each six-digest condition - no enzyme for both DMSO or decitabine sample, only the methylation-sensitive *HpaII* for each treatment sample, or with *MspI* restriction enzyme for each treatment sample. The expected result for no enzymes was 50bp, seen for samples with no enzymes and DMSO with *HpaII*, which cannot cleave DNA when there is methylation. Dimmer bands were seen for all three remaining samples, decitabine-treated with *HpaII*, and each treatment with *MspI*. Using Quick-Load DNA Ladder 50kb with band marks indicated in the figure.

Unsuccessful amplification of extracted DNA by known primer pair

After finding that decitabine decreases cell growth and demethylates DNA, the effect of decitabine in demethylating the DMR of interest is still unknown. For this, first, the extracted DNA was tested using a known set of primers, the FIGN primers from the BIOL322X course from Spring 2020 adapted for bisulfite-converted DNA, to determine if the DNA can be amplified. The extracted DNA is, first, bisulfite-converted and then a bisulfite PCR reaction and gel electrophoresis were followed. Due to time constraints, the PCR reaction was cut short from 39 cycles to 32 total cycles. The researchers observed that no sample presented a DNA band, and odd contamination was present at around 1kb (Figure 3). These findings suggest that the extracted gDNA was unsuccessful in amplification.

To further test the extracted DNA in the positive control PCR reaction, the lab instructor WHC carried out another PCR reaction with the extracted DNA from this study from two lab members - 2019 gDNA - and DNA extracted in 2020 using the known primers. This reaction completed the 39 cycles. The results showed that all samples were successfully amplified by the FIGN primers, except for the negative control labeled as water (no DNA was added, and no expression should have been seen) (Figure 4). The odd contamination was still present for the second PCR reaction. All of these findings suggest that the extracted gDNA from both 2019 - this study - and 2020 can be amplified by the known primers FIGN pair. Yet, the reason for unsuccessful amplification could be the unfinished PCR reaction and not an issue with the DNA. The gDNA from 2020 is labeled as the "4-" for DMSO- treated and "4+" for Decitabine treated, these were used in the previous section of BMB322X and analyzed for the initial components - demethylation and growth survival - and the results agreed with

the ones found in this study. Decitabine inhibits cell growth and demethylation - information provided by WHC. Moreover, the findings also suggest that the odd contamination most arguably comes from the reagents used, which were the only components re-used for both PCR reactions.

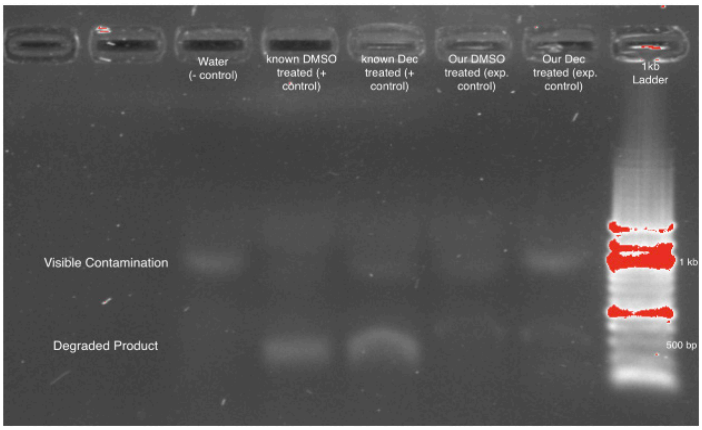

Figure 3: The experimental bisulfite-converted gDNA samples were oddly, almost not, amplified by the known primer pair. The negative control didn't show any bands at the expected amplification length; however, it showed contamination at approximately 1kb. Moreover, the known DMSO and Dec treated showed a light, degraded band at the expected amplicon length (approx. 500 bp), which was brighter than the DNA bands obtained for our samples (LAF) of DMSO, and Dec treated gDNA. These were extremely light and presented an odd length, bigger than expected. Using O'generuler 1kb DNA ladder with band marks indicated in the figure.

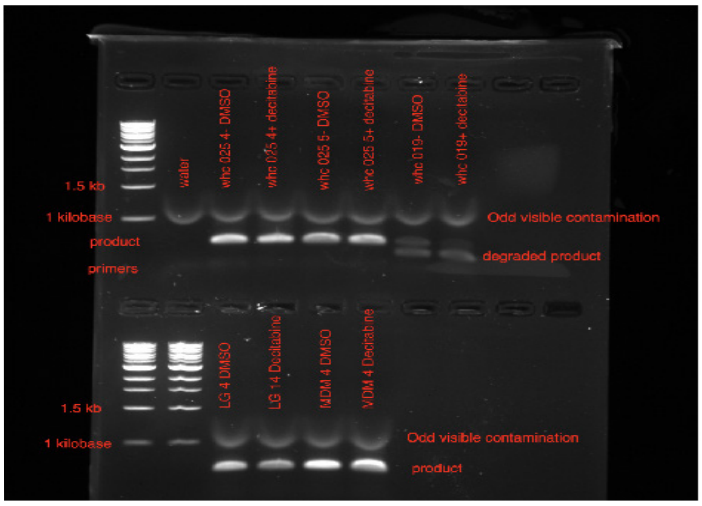

Figure 4: All the bisulfite-converted gDNA samples, by the lab instructor and two lab members, were amplified by the known primer pair. The negative control still showed contamination at approx. 1kb, but no DNA bands. The samples on lane 2 (whc025 4- DMSO), lane 3 (whc025 4+Decitabine), lane 4 (whc025 5-DMSO), 5 (whc025 5+ Decitabine), 8 (LG4 DMSO), 9 (LG14 Decitabine), 10 (MDM4 DMSO), and 11 (MDM4 Decitabine) showed good, bright DNA bands at the expected 500 bp length. These DNA samples are from 2020, not used previously by the LAF group. Although, in lanes 6 (whc019- DMSO) and 7 (whc019+ Decitabine) the products seem to be degraded.

Methylated sites in SOX21 at the promoter region have fewer transcription factors activity

After analyzing the effects of decitabine on the HCT-116 cells and finding that the 2020 gDNA can be amplified using known primers, the researchers sought to determine if the DMR of interest, SOX21, can be unmethylated by decitabine. To do this, first, a region of interest for the SOX21 gene with the coordinates chr13:95,364,162-95,364,771 and the transcription factors' activity at this region using the UCSC genome browser were analyzed using the data from the ENCODE project. Methylation is thought to silence gene expression, and thus, the researchers expected to see less binding of transcription factors at the promoter region of the SOX21 gene. Therefore, this region was analyzed specifically. From the view of the UCSC genome browser, it was observed that for the selected DMR there are fewer binding of transcription factors, and for the most common one POLR2A is not even bound to it. Contrastingly, a transcription factor that can be found to be bound most prominently in comparison to SUZ12 or RBBP5 is EZH2, which is a gene function suppressor. This data suggests that this DMR seems to be a silenced region, or highly prone to silencing. Even more with methylation being present, and thus, disrupting the expression of the gene. The other transcription factor that is present, SUZ12, is also attributed to this gene silencing.

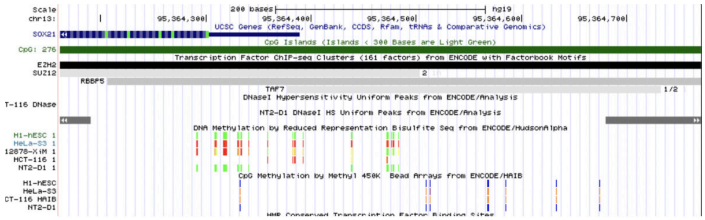

Figure 5. UCSC Genome browser view of the region of interest of SOX21 DMR with transcription factors that bind to it. Screenshot of the view from UCSC genome browser for the SOX21 DMR. The top section indicates the scale followed by the position on chromosome 13. The gene SOX21 is shown in blue under the title UCSC Genes, followed by the presence of CpG islands in green and the transcription factors bound to this region in black/gray - the darker the color the more present it is from the ENCODE project data. Lastly, CpG methylation sites are presented in the following tracks from the RRBS data (100% methylation marked with red, and 0%methylation marked with green) and the HM450 data for different cell lines.

Designed primer pair for SOX21 DMR has an optimum annealing temperature of 56°C

The researchers assessed the amplification of the gDNA using the known FIGN primers that resulted in successful amplification and analyzed the region of interest for the SOX21 DMR. To continue studying the effects of demethylation of decitabine for the DMR of interest, the researchers sought to design a primer pair (both forward and reverse) that would amplify this region. For this, the position on the chromosome from the UCSC genome browser was selected and the MethPrimer website was used to obtain a primer pair labeled FBSOX21 (Figure 6). Once this was received, the bisulfite-converted DNA was amplified using the FBSOX21 primer pair for the SOX21 region with different annealing temperatures to determine which one was the optimum one for the primers. The results showed that there was partial success in the amplification of the SOX21 gene region. For the experimental conditions, five gave a DNA band at the expected 100bp mark (Figure 7).

From the results, the only samples with the specific temperatures that gave a DNA band were FB 4- DMSO (52°C), FB 4- DMSO (56°C), FB 4+ Dec (56°C), FB 4- DMSO (60°C), and FB 4+ Dec (60°C) - with FB 4- DMSO (56°C), FB 4+ Dec (56°C) having the brightest band, best amplification of DNA. Additionally, in lane 3, for FB 4+ Dec (52°C), there were no bands and no contamination, but this sample was lost when preparing the reaction, and thus, no conclusion can be made for the 52°C for the amplification of the Decitabine- treated DNA. For the remaining temperature of 64°C, no amplification resulted for either group. The reagents used were kept, and thus, the odd contamination from them is still present at around 1kb. These results suggest that the optimum temperature for the FBSOX21 primer pair is 56°C and at very high temperatures, such as 60°C, the primers were not successful in aligning and binding to the DNA, resulting in no amplification.

BSP Pair 1

	Primer name	Sequence (5'-3')	Len	Start	End	Degenerate	CpG's	C's	Self_any	Self_end	Tm	GC%	Stability	Score
Pair	F1	GAGGAAATTAATGTTGGTTGT	21	200	220	No	0	3	600	0	53.4	33.3	6.4	3.6
	R1	CTAAACTAATCCCCCAACCC	20	306	287	No	0	6	200	0	57.4	50.0	9.4	3.4
	Product size	Pair start	Pair end	CpG's	Comp_any	Comp_end	Tm	Tm_diff			Score			
	107	200	306	14	500	300	69.2	4.0			8.1			

Figure 6. FBSOX21 primer pairs with both reverse and forward primers. The amplicon size is indicated with a product size of 107 nucleotides for the FB SOX21 primer pair. The sequence of each primer is noted with

the forward primer being F1 and the reverse primer being R1. Information for the primer pair for SOX21 gene is provided by MethPrimer Website.

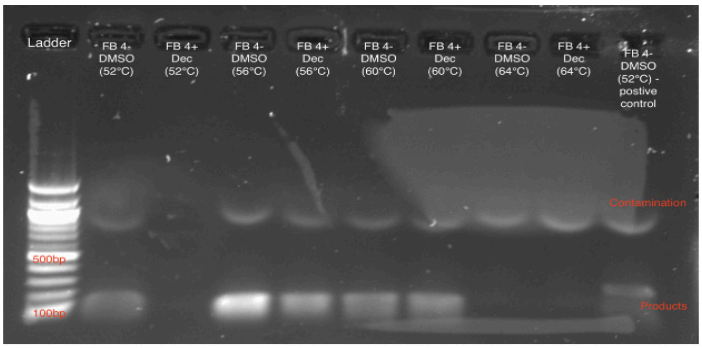

Figure 7: Half the samples had a successful amplification of the SOX21 region with the designed primers, these were for FB 4- DMSO (56°C), FB 4+ Dec (56°C), FB 4- DMSO (60°C), and FB 4+ Dec (60°C). Even though these showed degraded products, the expected band size can be seen, especially from FB 4- DMSO (56°C), which showed the brightest band out of all of them. Moreover, FB 4- DMSO (52°C) also showed a degraded product band much lighter than any other, but with the indicated size. FB 4+ Dec (52°C), FB 4- DMSO (64°C), and FB 4+ Dec (64°C) showed no bands. There is again some odd contamination present, and an odd product for the positive control with a degraded band at approximately 300bp, which is 200bp shorter than expected.

Amplification of bisulfite-converted gDNA from HCT116 cells using SOX21 gene- designed primers in PCR

The optimum annealing temperature was determined to be 56°C for the FBSOX21 primer pair, and thus, the researchers sought to amplify the bisulfite-converted genomic DNA samples (both DMSO and Decitabine treated), that were extracted from the HCT116 colorectal cancer cells, in 2020, with the FBSOX21 primer pair. To be able to study the demethylation of the CpG sites in the DMR, the amplification of this region is prominent for later sending it for sequencing and sequencing analysis that will allow for quantification of both the methylation levels in the HCT-116 cells, as well as the methylation levels after Decitabine treatment. This all leads to the major goal of this lab project to study if the Decitabine treatment decreases methylation for the SOX21 DMR. A bisulfite PCR reaction was done again using the FBSOX21 primers and an annealing temperature of 56°C with the 2020 gDNA, as well as the "4- and 4+" samples provided by WHC. The gel resulted in DNA bands for all samples including the negative control at the expected 100bp for the expected amplicon size of 107 nucleotides (Figure 8). Importantly, the negative control showed contamination most arguably of DNA samples. This could have been a product of adding DNA to the sample mistakenly. Since the negative control showed contamination, no conclusions were made from the gel and the PCR was re-done using the same conditions.

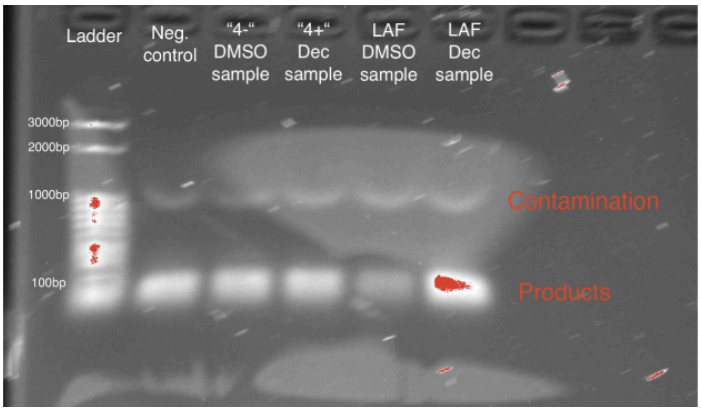

Figure 8: Successful amplification of DNA samples with FB-SOX21 primers and contamination of negative control. Lane 1, negative control, showed a DNA band at approximately 100bp, this is a contaminated sample with genomic DNA. Lanes 2-5, for all the DNA samples, were successfully amplified as seen from the DNA band also at approximately 100bp. Odd contamination from reagents was also present at approximately 1kb. The ladder DirectLoad™ 50 bp DNA Step Ladder, SIGMA, was used for band marks on the figure.

After re-doing the bisulfite PCR reaction, the researcherscobserved the same results as before, where the negative control seems to be contaminated with DNA sample from an unknown source of which condition or sample either the ones provided by WHC or the LAF samples - the samples from this study (Figure 9). Despite not obtaining non-contaminated samples, there seems to be a successful amplification of the other DNA samples, and thus, these were sent for Sanger sequencing for further analysis of CpG methylation and decitabine treatment effects.

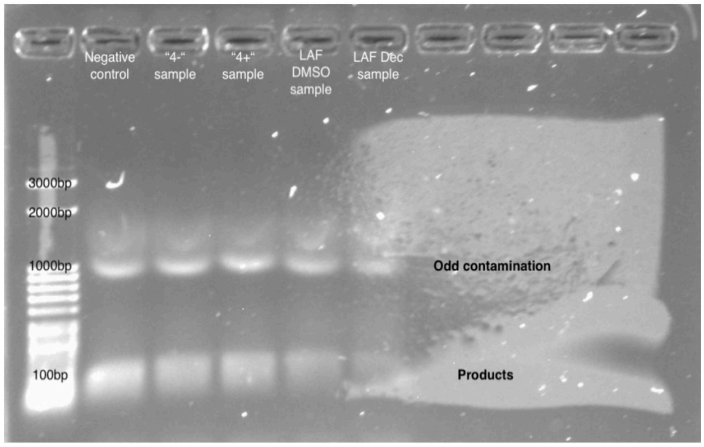

Figure 9: DNA contaminated negative control and successful amplification of all DNA samples. All samples were amplified with the FBSOX21 primers, lane 1, negative control, showed a DNA band at approximately 100bp, this is a contaminated sample with genomic DNA. Lanes 2-5, for all the DNA samples, were successfully amplified as seen from the DNA band also at approximately 100bp. Odd contamination from reagents was also present at approximately 1kb.

SOX21 DMR is partially demethylated by decitabine treatment

The DMR of interest was able to be amplified with designed pairs specific for it (Figure 8 and 9) and was sent for sequencing. Previously, it was seen the characteristics of decitabine as a demethylating agent (Figure 2), and thus, the researchers sought to investigate the demethylation, if any, of the amplified sequence of the HCT-116 cells' DNA after decitabine treatment. The samples were sent for Sanger Sequencing at the University of Chicago, the sequence methylation was observed by quantifying methylation in HCT-116 cells and methylation after the decitabine treatment by looking at the peak height of the C in the sequencing relative to the C and T present, which all would indicate the percent methylation on the sample, referred as %mC. A characteristic of bisulfite conversion is that all the unmethylated cytosines were changed to uracil, and thus, after sequencing these will either appear as non-recognizable or T's. The sequencing data showed that decitabine was successful in demethylating some regions of the amplified product (Figure 10). One crucial note is that the sanger sequencing for the decitabine sample wasn't as successful as the researchers would have hoped. The initial sequence showed to be very messy with low confidence in the readings of signals from the nucleotides.

The quantity of methylation for each treatment was done as mentioned with the peak height of C relative to the signal strength of C's and T's (Figure 11), as well as the standard deviation for each %mC. The data wasn't as expected. There wasn't a decrease in %mC for decitabine treatment as compared to DMSO. On the contrary, the decitabine- treated sample showed a higher %mC than DMSO. This was followed by looking at the data obtained from sequencing on the UCSC genome browser to compare it to other known methylated sites. The view on the browser suggests new CpG sites that were previously unknown (Figure 12). Ultimately, all these results could have been a result of partially unsuccessful sequencing affecting the signal readings. However, this data also suggests that the region of interest in this study, to begin with, was already partially unmethylated. There are new CpG sites identified for the DMR that are near known methylated sites. These CpG sites seem to be unmethylated.

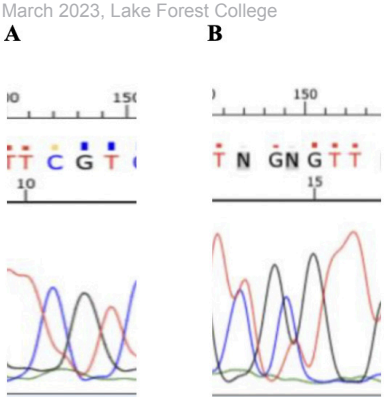

Figure 10. Decitabine demethylated the SOX21 DMR. Information from this website was used to compare the obtained sequence and the expected sequence from the initial amplicon by using the EMBOSS Needle website as well as analyze the %mC by looking at the peak height of C per signal strength of both C and T. (a) Screenshot of a chromatogram from ThermoFisher website with sequenced data for the bisulfite-converted and amplified DMSO- treated sample. Signal strength of C is indicated in red. (b) Screenshot of a chromatogram from the ThermoFisher website with sequenced data for the bisulfite-converted and amplified decitabine-treated sample. Signal strength of C is indicated in red and of T in blue.

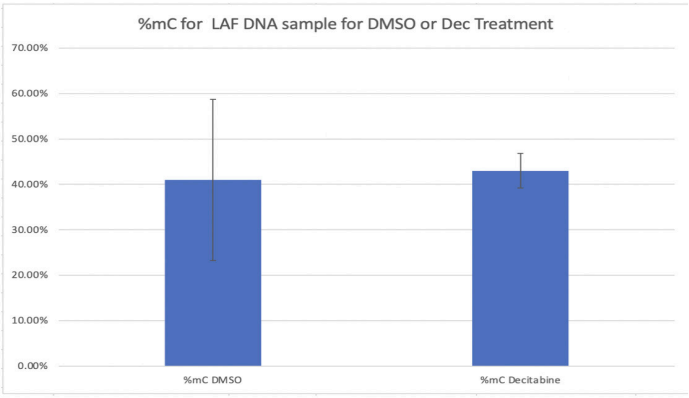

Figure 11. Decitabine- treated showed higher %mC than the DMSO-treated sample. The calculated %mC, using the signal strength of C's and T's obtained from the ThermoFisher VA analysis app. for DMSO and decitabine- treated sample for the bisulfite-converted and sequenced SOX21 DMR. The results contradicted the expected results of lower levels of %mC for decitabine because of demethylating the DNA. The Decitabine sample showed 43%mC and the DMSO sample showed 41%mC, each standard deviation is indicated with error bars in the figure.

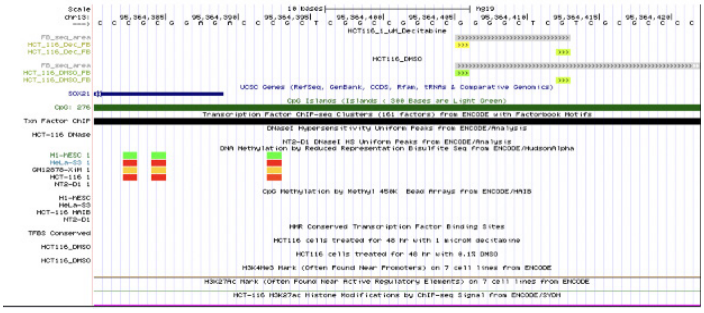

Figure 12. New CpG sites were discovered to be near known methylated regions for the SOX21 DMR. Screenshot of UCSC Genome browser view for chromosome 13 with uploaded data of bisulfite-converted, amplified, and sequenced SOX21 DMR depicted in track with FB-seq-area for both Decitabine and DMSO-treated samples.

Discussion

The results showed that decitabine has an important role in inhibiting HCT-116 cell growth (Figure 1) and the previously associated demethylating abilities (Figure 2). Moreover, through looking at high-confidence DMRs in colon cancer (Simmer et al. 2012), The researchers were able to study SOX21 specifically at the promoter region. This gave insight into hypermethylated CpG sites that seem to be globally methylated in the HCT-116 colon cancer cell line. Although these sites were identified and new were discovered (Figure 12), it seemed that this region was originally partially demethylated from the beginning, regardless of decitabine treatment, and could serve as an answer to the higher %mC for decitabine- treated sample as compared to DMSO sample.

These findings are important since it suggests that the techniques used are not biased to just methylated DNA and exclude this as a possible limitation of the study. In addition, these findings delve deeper into the possible roles of SOX21. As seen, these genes seem to be prone to be silenced, either by DNA methylation at the promoter region or by the same transcription factors such as EZH2 (Figure 5). This leads us to speculate that the region of interest studied in this report is highly relevant in the DKO1 phenotype. The lack of transcription factors and silencing characteristic provides possible evidence to indicate that this region of the DNA is more highly compacted, decreasing accessibility of transcription factors, and thus inhibiting expression of the gene. The DKO1 phenotype presents a lack of DNA methylation due to the engineered negative interference with the DNA methyltransferases (Pandiyan et al. 2013). It was hypothesized that the DKO1 phenotype and the HCT-116 cells will have rather similar demethylated sites since the SOX21 DMR was shown to be partially demethylated from the beginning of the study (Figure 11 and 12). It is noteworthy to see this relationship since it could present a possible explanation for SOX21 DMR as a mechanism for colon cancer tumorigenesis. In cancer, a mutation causing methylation to this region could mean that it is even harder to make transcription factors bind and inhibit gene expression. The alteration of the protein-coding gene which has been related to regulating cell development can cause neoplastic proliferation.

Further work is needed to assess this possible mechanism, however, another important finding related to this is that decitabine did show demethylation properties. Not as much as expected, but some at last. Following up with the speculated molecular mechanism, 5-aza-2-deoxycytidine could potentially be another element that would demethylate the sites that were known to be methylated helping in expressing the gene, which is prone to be silenced (Figure 5). Because DNA methylation doesn't seem to be as present as in other sites for other genes, the effects of 5-aza-2-deoxycytidine would substantially be more relevant for damaged DNA.

In the future, there are many areas that can be further explored such as expanding the quantification of DNA methylation for the totality of the SOX21 DMR. In this study, the researchers only focused on the promoter region which was speculated to be relevant for the expression of the gene. Nonetheless, it would be interesting to study whether the pattern of demethylated DNA is present throughout the entire DMR or only in the promoter region and see what effects this could have on gene expression. The steps would be similar to this, amplifying the amplicon size with different primer pairs. Furthermore, as mentioned before, it would be insightful to study DNA methylation along with the compressibility of the genes in chromatin, whether it is present in a more loose or packed manner, affecting DNA accessibility for transcription factors. These could be done by using the accessibility of chromatin analysis such as ATAC-Seq (Buenrostro et al. 2015) and then followed by a chromatin immunoprecipitation assay (Pandiyan et al. 2013). An additional future experiment could also be comparing the DKO1 phenotype to the HCT-116 cell line and studying their relative similarities if any.

Methods

Materials:

1. Mid-log culture of HCT-116 cells
2. Molecular biology lab bench equipment including P2.5, P20, P200, and P1000 pipettes and tips, serological pipet and pipet-aid, biohazard tip waste, liquid waste beaker, bleach, 70% ethanol, 15 ml, and 50 ml conical centrifuge tubes, 1.5 ml microcentrifuge tubes, microfuge, and centrifuge tube racks, kim wipes and paper towels, lab markers
3. T25 flasks
4. McCoy's 5A media (complete w/ antibiotic)
5. PBS
6. Trypsin
7. Hemocytometers
8. Cell counters

9. Compound microscopes
10. UV-vis spectrometer
11. RNase A
12. Proteinase K
13. Elution buffer
14. Cell Lysis Buff
15. Vortex
16. Microcentrifuge
17. Wash buffer
18. Agarose
19. 1x TBE buffer
20. sybr safe
21. O'gene ruler 1kb DNA ladder
22. 5x DNA loading dye
23. Parafilm
24. TE buffer
25. M-elution buffer
26. ddH2O
27. Mold rig with lid
28. Narrow comb (1.0mm)
29. Power supply
30. EZ DNA Methylation kit (zymo research D5001; CT conversion reagent, M-dilution buffer, M-binding buffer, and M-Desulphonation buffer all carry health hazards -> sodium metabisulfite, sodium hydroxide, guanidinium HCl, and alcohol with sodium hydroxide respectively. Wear safety glasses, gloves, and a lab coat.

Overview of protocols given for this study, protocols of instructions, and procedures provided in methods (William 2021, Conrad 2021 1, Conrad 2021 2, Conrad 2021 3).

Cell culture for HCT-116 cell line

To grow the HCT-116 cells in a physiological condition they had to be cultured in 50ml of McCoy's 5A media according to WHC instruction in Lab 1 in LabArchives (William 2021). The process was carried out by WHC and lab TA, however, it was explained to the class by WHC. On day 1, into 3 mid-log T25 flasks, 800,000 cells / well were transferred to 20 T25 flasks making a total of 16 million cells from the HCT-116 cell line. After a one-day incubation, 0.1% DMSO was added to half of the flasks and 0.25 uM decitabine to the other flasks - this concentration was created from the 10 mM decitabine stock by WHC. After another day of incubation, the media was replaced by adding the same concentration of DMSO or decitabine to the corresponding flask. On Day 3, the media was replaced once again, but no more drugs were added, only McCoy's 5A media. The cells were left incubating for 72 more hours.

Genomic DNA isolation and bisulfite conversion of extracted DNA

To isolate and extract the genomic DNA from the cultured HCT-116 cells, the genomic DNA extraction was done according to the protocol in the PureLink® Genomic DNA Kit and Monarch Genomic DNA purification kit (NEB T3010S) following the instructions by the manufacturers. By trypsinizing the flasks with Trypsin, the cells for DMSO and decitabine samples were collected and counted by using a hemocytometer (15 µl were used in each chamber). These were calculated to obtain a final volume of 4 million cells which were collected per condition and followed by the PureLink® Genomic DNA Kit until part 3, after this the purification kit using RNase A and Proteinase K, counting with two parts (Genomic DNA Binding and Elution) was used. The only variation between protocols is a change in enzyme volumes. This was later quantified and tested for purity by using nanodrop UV-vis spectroscopy, the DNA was located on a BioTEK plate reader. The concentration and A260/A280 ratio were obtained per treatment (Conrad 2021 1).

To study demethylation sites on a specific region of interest, the DNA must be bisulfite-converted changing the unmethylated cytosines to uracil's by following the protocol of the EZ DNA methylation kit. Briefly, after obtaining the quantity of DNA using the nanodrop, the necessary volume of these cells is calculated to obtain a final DNA concentration of 400ng. This was treated with 5 µl of M-dilution buffer, ddH2O, and incubated for 15 minutes at 37 ºC. Then, 100 µl freshly prepared CT Conversion Reagent was added and followed by two incubations in the thermal cycler, one at 50ºC for 12-16 hours and the second for 10-20 minutes at 0-4ºC. The sample was then transferred to a Zymo-Spin™ IC Column with 400 µl of M-Binding Buffer, centrifuged, and followed by repetitive sequences of adding M-Wash and M-Elution Buffer.

Global demethylation assay

To measure the demethylation caused by decitabine treatment on the HCT-116 cells, a restriction digest procedure using *HpaII* (NEB R0171S) and *MspI* (NEB R0106S) was used according to the lab manual by WHC labeled Lab 3 SOP and *intro: gDNA quality control and HpaII digest* (Conrad 2021 1). Both enzymes cleave at the cut site C/CGG, but HpaII is the only one that is methylation-sensitive (). After extracting and calculating the volume for 400ng of gDNA and different samples, 0.5 ul of the indicated restriction enzyme were added and proceeded by a bisulfite PCR reaction. The results were analyzed by performing gel electrophoresis on the amplified products.

Bisulfite PCR reaction

To test the amplification of the DNA samples for each corresponding section, a bisulfite PCR reaction was performed according to EpiMark® Hot Start Taq DNA Polymerase Guidelines for PCR (M0490) and adaptations presented in the Lab Manual by WHC (Conrad 2021, 2). Primarily, the extracted gDNA samples, for both DMSO and Decitabine treatment, were added to a Master Mix containing the fundamental PCR components: DNA polymerase (qpiTaq 5 U/ ul), 10 mM dNTPs, diH2O, bisulfite-specific primers (reverse and forward of 10uM each) and 5x epiTaq buffer. The reaction was set at 56°C for a total of 39 cycles and ran for about 1 hour. There were multiple PCR reactions carried out in this study. A ll the concentrations for the reagents were adapted from their initial volume needed for one sample (1x) depending on the corresponding samples - either 4 or 5 regardless of which there was always a little more of each reagent added getting it to 4.4x or 5.5x times (Table 2). Variations of the reaction included the DNA sample, which is indicated in each figure, the annealing temperature (Table 3), and the primer pair used. There were two pairs used in this study, the FIGN primers and the FBSOX21 primers obtained from the MethPrimer website - both designed for bisulfite-converted DNA - the use of either of these were indicated in the figures too.

Reagent	1x	4.4	5.5x
5x epiTaq buffer	4	17.6	22
diH2O	13.5	59.4	74.25
10 mM dNTPs	0.4	1.76	2.2
genomic DNA (8 ng/ul)	1	4.4	5.5
Primer F (10 uM)	0.5	2.2	2.75
Primer R (10 uM)	0.5	2.2	2.75
epiTaq 5 U/ ul	0.111	0.4884	0.61

Table 2. Reagents table with indicated volume for one sample 1x and the necessary volume for more samples either 4.4x or 5.5x.

PCR reaction	Annealing Temperature
Restriction enzymes PCR	56°C
Positive control PCR with FIGN primers	56°C
WHC positive control PCR with FIGN primers	56°C
Optimum temperature PCR with FBSOX21 primers	52°C, 56°C, 60°C, and 64°C
PCR reactions with FBSOX21 primer pairs	56°C

Table 3. PCR condition for each reaction with corresponding annealing temperature.

Gel Electrophoresis

To test the quality of the DNA samples' amplification after bisulfite PCR reaction, gel electrophoresis protocol was adapted from the DOE JGI "Genomic DNA QC Using Standard Gel Electrophoresis (for collaborators)" (Lin 2012). To set up the technique, the agarose gel was prepared by mixing, in a 250ml Erlenmeyer flask, 0.5g of agarose, and 50 ml of 1x TBE buffer. To dissolve this mixture, the defrost setting in the microwave was used and set for 30sec-1min. After heating and dissolving it, the solution had to be cooled down with water, and added to it 2.5ul of sybr safe – swirled to mix it well. This solution was poured into the mold rig with the narrow comb (1.0mm side) and left to solidify. Once this is done, the PCR samples obtained are prepared by adding 5ul of 6x DNA loading buffer and then loaded into the gel with the mold filled with 1x TBE buffer. The order of the samples in the gel varied according to each gel, they are indicated in each figure. The gel was left to run for 40-45 min, and then gel imaging was used to visualize the gel.

Quantifying DNA methylation

To analyze the CpG sites methylated at the SOX21 DMR and quantify the %mC for the CpG sites, analysis of the sequence after Sanger sequencing was performed according to lab manual entry Lab11: Sequence analysis (Conrad 2021 3). After amplifying the gDNA with the FBSOX21 primer pair with the optimum annealing temperature, the samples were prepared with 1µl ExoI and 2µl of rSAP and submitted to the University of Chicago for sequencing. From the data, the peak height was obtained first using the Thermo Fisher Cloud Variant Analysis app. Then, EMBOSS Needle was used to align the sequence data with the known position of the nucleotides on the amplicon, and with this determine the location of the CpG sites. The percent methylation (%mC) was calculated by looking at the signal strength (peak heigh), of Cytosines related to the signal strength of C's and T's, using the formula %mC = C/(C+T) x 100. Using Microsoft Excel, the %mC for DMSO and decitabine-treated samples were compared, and a t-test was used to calculate for statistical significance. Lastly, the data obtained from the VA app of the Thermo Fisher Cloud was also uploaded to the UCSC Genome Browser to identify and align the observed CpG sites with the DMR of interest. This allowed for comparison with already known methylated regions of the DMR.

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