DNA Methylation Status of RETN and ADIPOQ Genes in Sporadic Colon Cancer

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Research Article

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Abstract

Background: Colon cancer develops through a complex process that involves epigenetic alterations. Compelling evidence has been achieved that adipocytokines link obesity with colon cancer progression. Therefore, understanding the epigenetic modifications in adipokine genes might help in clarifying their role in colon cancer pathogenesis. The aim of the present project was to study the DNA methylation status of \textit{RETN} and \textit{ADIPOQ} genes in sporadic colon cancer patients.

Methods: 70 cancerous colon tissue and adjacent paired non-cancerous tissue was used to determine the DNA methylation status using methylation-specific polymerase chain reaction (MS-PCR) assay. Quantitative real-time PCR (qRT-PCR) was used to determine the expression level of \textit{RETN} and \textit{ADIPOQ} genes.

Results: In colon cancer tissues, the CpG sites in the three selected promoter regions of \textit{ADIPOQ} and \textit{RETN} were hypermethylated in all samples. DNA methylation level at the CpG sites in exon one of the \textit{RETN} gene exhibited a lower level in the non-cancerous tissue compared to the cancerous tissue and paired blood samples. The \textit{RETN} mRNA was upregulated.

Conclusion: We postulate that DNA methylation status at the CpG sites in exon one of the \textit{RETN} gene might help uncover cancer signatures in sporadic colon cancer and may be used as a biomarker. The upregulation of the \textit{RETN} mRNA level might play a role in sporadic colon cancer tumorigenesis.

Introduction

Colon cancer is a hyperplasia of the large intestine and one of the most common causes of cancer-related death in the world\textsuperscript{1}. Colon carcinogenesis is a slow and stepwise process that can take years to develop\textsuperscript{2,3}. The initiation of colon cancer exhibits additional gene mutations, oncogene activation, loss and gain of chromosomes, microsatellite instability, and CpG island methylator phenotype (CIMP)\textsuperscript{3}. As reported previously in the literature, 20% of patients diagnosed with colon cancer have familial or congenital mutations in genes that accelerate carcinogenesis to an early age onset. The remaining 80% tend to develop colon cancer later in life and do not exhibit any obvious genetic causes\textsuperscript{1}. Therefore, lifestyle factors that might modify epigenetic patterns, including obesity, have been linked to colon cancer development\textsuperscript{3}. A large number of research studies have examined the association between obesity and colon cancer\textsuperscript{4–6}. The literature suggested that increased body mass index correlates with colon cancer development\textsuperscript{7}. Therefore, adipokines such as leptin, adiponectin, resistin, and visfatin were measured to investigate their association with colon cancer\textsuperscript{8–10}. Resistin, also known as an adipose tissue-specific secretory factor, is a cysteine-rich peptide secreted by adipocytes, immune cells, and epithelial cells that in humans is encoded by the \textit{RETN} gene\textsuperscript{11,12}. The \textit{RETN} gene is located on chromosome 19p13.2 with four exons and three introns, considering exon one as the untranslated region\textsuperscript{13}. Resistin protein is comprised of 90 amino acids and 18 amino acids as a signal sequence. Therefore, resistin pre-peptide has 108 amino acids with 12.5 kDa. Resistin is found in two different biological active forms: an oligomer and a trimer\textsuperscript{14}. Although resistin is a small adipokine, it is involved in the activation of cell proliferation, survival, and anti-apoptosis\textsuperscript{15}. Furthermore, resistin is also involved in cell inflammation that causes cell adhesion and migration that promotes tumorigenesis\textsuperscript{14}. \textit{ADIPOQ} is the gene of adiponectin located on chromosome 3q27 and comprises three exons and two introns, where exons 2 and 3 are coding regions. Adiponectin protein is 30 kDa with 244 amino acids that is expressed exclusively in adipocytes\textsuperscript{16}. It has been acknowledged that a low adiponectin level may influence colon cancer carcinogenesis\textsuperscript{17–19}. It has been suggested that adiponectin may have a role in the development and progression of cancer via its pro-apoptotic
and/or anti-proliferative effects. Normally, adiponectin attenuating cancer progression through adenosine monophosphate-activated protein kinase (AMPK).

Epigenetic research has shown that human cancer cells harbor epigenetic alterations\(^\text{20}\). Advancements in the rapidly evolving cancer epigenetic regulations have shown that changes in the epigenetic landscape are a hallmark of cancer. Therefore, disruption of epigenetic mechanisms can lead to altered gene activity and malignant cellular transformation. Significant progress has been made in the basic understanding of how various epigenetic changes such as DNA methylation, histone modification, miRNA expression, and higher-order chromatin structure affect gene activity. DNA methylation is the most broadly investigated epigenetic alteration of genomic DNA and regulating genomic function\(^\text{21}\). Therefore, changes in the methylation state at discrete loci are potentially associated with the control of specific genes related to cancer pathogenesis\(^\text{22}\). DNA hypermethylation has many roles in tissue-specific transcription control. Although tissue-specific DNA hypermethylation occurs more frequently in actively transcribed gene bodies and in intragenic or intergenic enhancers, promoter hypermethylation influences gene expression most notably at promoter regions that are rich in CpGs\(^\text{23}\). Abnormal increases in methylation at specific DNA sequences can serve as biomarkers for a variety of diseases\(^\text{24}\).

Previous studies have examined \textit{RETN} DNA methylation\(^\text{25,26}\). But to date, none has focused on the effect of \textit{RETN} DNA methylation on sporadic colon cancer. Understanding the epigenetic role in \textit{RETN} may serve as a prognostic, diagnostic marker, and the reversibility of the changes makes it possible to treat sporadic colon cancer disease. Therefore, the present study aimed to study the DNA methylation status of \textit{RETN} and \textit{ADIPOQ} genes in sporadic colon cancer patients.

**Materials And Methods**

**Bioinformatics analysis**

The presence of promoter CpG islands in the \textit{RETN} sequence region ranging from 500 bp upstream to 500 bp downstream of the transcription start site was evaluated using the online CpG Island Searcher software (http://dbcat.cgm.ntu.edu.tw/) with default settings. For MS-PCR primers design, bioinformatics link was used as DNA bisulfite conversion tool (https://www.zymoresearch.com/pages/bisulfite-primer-seeker). Then, the design of MS-PCR primers was followed as described by Herman and his team\(^\text{27}\). For the \textit{ADIPOQ} gene, two sets of primers were chosen from the literature\(^\text{27–29}\).

**Subjects**

Sporadic colon cancer Saudi females (n = 39, age = 59.46 ± 2.05 years) and males (n = 31, age = 60.10 ± 2.35 years) patients were included in the study. All participants provided written informed consent after receiving information about the purpose of the study. In some cases, first-degree relatives signed the consent form on behalf of the patients. Tissue samples were obtained from patients assigned for colectomy or endoscopy procedures at Prince Sultan Military Medical City (PSMMC; Riyadh) and King Khalid University Hospital (KKUH; Riyadh). Two samples were collected from each patient, one from cancerous tissue and one from adjacent (>10cm) non-cancerous tissue. After surgical removal, the tissue samples from the colectomy procedure were either snap freeze by liquid nitrogen for two minutes or saved in RNA later solution (Invitrogen by Thermo Fisher Scientific, USA) and stored at -80°C.

The volunteer's exclusion criteria required individuals to have hereditary colon cancer, treated with either chemo- or radiotherapy.
The Institutional Review Board approved the study at Prince Sultan Military Medical City (PSMMC), Oncology Department (project no. 995 ref no. HAP-01-R-015; approval date: 10 October 2017; Riyadh; Saudi Arabia and King Khalid University Hospital (KKUH), College of Medicine (project no. E-17-2732 ref no. 18/0068/IRB; approval date: 28 December 2017). All experiments were performed in accordance with relevant guidelines and regulations.

DNA extraction and bisulfite treatment

Genomic DNA was extracted from peripheral blood samples (2.5 ml) using the Puregene Blood Core Kit C (Qiagen, Germany) and from tissue using DNeasy Blood and Tissue Kit (Qiagen, Germany). DNA integrity and concentration were assessed by NanoDrop 2000c spectrophotometer (Thermo-Scientific). DNA (up to 1µg DNA) was then treated with sodium bisulfite using the EpiTect Fast DNA Bisulfite Kit (Qiagen, Germany) according to manufacturer protocol. CpG Methylated Human Genomic DNA Control (Thermo scientific, USA) was used to ensure conversion efficiency.

Methylation Specific-PCR (MS-PCR)

To determine the methylation status of the two selected genes (RETN and ADIPOQ), MS-PCR was used for tissues and blood samples. To validate primer design, fully methylated and fully unmethylated DNA controls (EpiTect Control DNA and Control DNA Set (Qiagen) were used. Also, MS-PCR primers were tested against un-treated DNA with bisulfite to test primer's specificity. It was considered only the specific primers. The experiment was duplicated for methylated results to confirm methylation status. Bisulfite-treated DNA templates were PCR amplified using HotStarTaq (5units/µl) from Qiagen (Germany), dNTPs (20mM) from Thermo (USA), 10X buffer from Qiagen (Germany), 10µM each primer, and nuclease-free water up to 25µl. Amplification conditions for region and exon one at the RETN gene were: 95°C for 15 minutes; 95°C for 30 seconds, 59°C for 30 seconds, 72°C for 1 minute, for 40 cycles, and 72°C for 10 minutes. Amplification conditions for promoter region two and three at the ADIPOQ gene were: 95°C for 15 minutes; 95°C for 10 seconds, (variable for each primer, Table 1) for 10 seconds, 72°C for 30 seconds, for 40 cycles, and 72°C for 10 minutes. The primers for the MS-PCR are shown in Table 1. All MS-PCR amplicons (10µl) were separated on 2% gel electrophoresis containing GelStarTM Gel Stain (Lonza, USA) and visualized under UV transillumination.
Table 1
List of primers for MS-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer 5'-3' seq</th>
<th>Length (bp)</th>
<th>Annealing temperature (ºC)</th>
<th>Product size (bp)</th>
<th>Number of CpG</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RETN*</td>
<td>MF1-GTGTAGGAATTCGTGTGTCG</td>
<td>20</td>
<td>59</td>
<td>166</td>
<td>2</td>
<td>Designed manually</td>
</tr>
<tr>
<td></td>
<td>UF1-GTGTAGGAATTTGCTGTGTTG</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UM-R1-AATCTACCCCTAAACCTAAACC</td>
<td>22</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RETN**</td>
<td>MFEX1-GTTGTAGGTTTCGTCGGTATCG</td>
<td>22</td>
<td></td>
<td>223</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UFEX1-GTTGTAGGTTTTGTTTATTTGGTT</td>
<td>26</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UM-REX1-CTCCAAATTATTTCACAATCC</td>
<td>22</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADIPOQ*</td>
<td>MF2-TTAGGTTGGAGTGTAATGGTGCC</td>
<td>22</td>
<td>60</td>
<td>171</td>
<td>1</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>MR2-TAACGAAAATAATAAAAACCGTCCT</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UF2-TTAGGTTGGAGTGTAATGGTGTC</td>
<td>23</td>
<td>58</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UR2-CTAAACAAAAATAATAAAACCGTCCT</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADIPOQ**</td>
<td>MF3-TAATTGGTAGTATTTGGGAGATCGA</td>
<td>25</td>
<td>54</td>
<td>140</td>
<td>1</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>MR3-AATTACAAACACCTACCACGTCCCAG</td>
<td>23</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>UF3-GTTGTAGGTTTTGTTTATTTGGTT</td>
<td>26</td>
<td></td>
<td>142</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>UR3-AAATTACAAACACCTACCACGTCCCAG</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

M = methylated, U = unmethylated sequences. RETN*=region one, RETN**=exon one, ADIPOQ*=region one, and ADIPOQ**=region two.

RNA extraction and real-time qPCR

Total RNA was isolated from blood using PAXGene Blood RNA Kit (Qiagen, Germany) and from tissues using RNeasy Plus Mini Kit (Qiagen, Germany) as the manufacturer described. Total RNA concentration and purity were determined using NanoDrop 2000c spectrophotometer (Thermo Scientific).
**Results**

**Frequency of methylation**

All the tissues and the paired blood samples at the CpG sites in the two promoter regions of the *ADIPOQ* gene showed a high frequency (92–100%) of DNA methylation in all representative samples. There was also a high level of DNA methylation at the CpG sites in the promoter region of the *RETN* gene. Hypermethylation was found in 85.7% cancerous tissue, 91.1% non-cancerous tissue, and 83.3% paired blood samples. The region that is downstream of the transcriptional start site (TSS) in exon one of the *RETN* gene showed 67.1%, 51.1%, and 62.5% methylation in colon cancerous tissue, non-cancerous tissue, and paired blood samples, respectively (Fig. 1). The Fisher test did not show any significant differences in the frequency of methylation between cancerous tissue paired with non-cancerous tissue at the CpG sites in the region of *RETN* promoter (*P* = 0.14) and in exon one (*P* = 0.83).

**Correlation of hypermethylation between cancerous tissue and paired blood samples**

A positive correlation was found between cancerous tissue paired with blood for both targeted genes (Table 2). The result suggests that blood is a useful non-invasive sample to study both genes for the level of DNA methylation.
Table 2
Correlation of hypermethylation between cancerous tissue and paired blood samples

<table>
<thead>
<tr>
<th>Gene</th>
<th>r</th>
<th>Pvalue</th>
</tr>
</thead>
<tbody>
<tr>
<td>RETN*</td>
<td>0.9848</td>
<td>0.1112</td>
</tr>
<tr>
<td>RETN**</td>
<td>0.8030</td>
<td>0.4065</td>
</tr>
<tr>
<td>ADIPOQ*</td>
<td>0.9351</td>
<td>0.2307</td>
</tr>
<tr>
<td>ADIPOQ **</td>
<td>0.7190</td>
<td>0.4892</td>
</tr>
</tbody>
</table>

RETN*=region one, RETN**=exon one, ADIPOQ*=region one and ADIPOQ**=region two.

**Gene expression for ADIPOQ and RETN genes**

Gene expression was examined quantitatively for ADIPOQ and RETN using TaqMan gene expression assay. Sixty-eight tissue samples (cancerous paired non-cancerous tissue) and 21 paired blood samples were assayed. The TaqMan assay could not detect the ADIPOQ gene transcript, either in tissue or blood. The single most striking observation to emerge from the data comparison using paired t-test between cancerous and adjacent non-cancerous tissue revealed that RETN expression is downregulated (P = 0.154). This result may implicate DNA methylation in exon one may have an effect on gene activity. Interestingly, there was a significant positive correlation between cancerous tissue and paired blood (P = 0.0006). This correlation suggests that blood is a good representative sample to study the RETN gene expression in colon cancer.

**Discussion**

Genetic cancer cannot explain sporadic cancer or cancer development in individuals with no family history of cancer. Nonetheless, epigenetic mechanisms can describe the increased risk for the development of sporadic cancer. Thus, epigenetic markers that are consistently dysregulated among cancers present an opportunity to use them as cancer biomarkers for diagnosis, risk assessment, and prediction of therapeutic response. Our study shed light on the DNA methylation of RETN and ADIPOQ genes and their involvement in the progression of sporadic colon cancer to serve as a base for future studies. It is well known that obesity causes dysregulation of adipokines, and that is associated not only with obesity but also with malignancy. Studying aberrant DNA methylation on promoter regions on adipokine genes might help decipher the molecular mechanisms underlying the effect of obesity in sporadic colon cancer development. To the best of our knowledge, this is the first study to investigate DNA methylation status at CpG sites of RETN and ADIPOQ genes in sporadic colon cancer tissue.

The number of genes known to undergo promoter hypermethylation in cancer has grown substantially. Our exploring study using cancerous tissue, matched normal tissue, and paired blood samples from colon cancer patients demonstrated hypermethylation at the CpG sites in the two promoter regions of the ADIPOQ gene. Additionally, the expression level of adiponectin was undetectable. Our result agrees with previous studies. Haghiac and his team showed that obesity in pregnancy is associated with an increase in ADIPOQ DNA methylation and lower mRNA concentration. A year later, another group have observed hypermethylation in the same promoter region that we explored (region one) and confirmed down-regulation for adiponectin level in prostate cancer tissue via promoter hypermethylation. Given that hypermethylation is an important factor in regulating gene expression, we hypothesize that the undetected ADIPOQ gene transcript in our study is due to the frequency or location of the DNA
methylation on the *ADIPOQ* promoter. Another possible explanation is the developmental stages of cancer. It is well
known that the role of adiponectin in colorectal neoplasm is a direct anti-tumorigenic effect. So, deficiency of
adiponectin seems to be associated with the development of an early neoplasm rather than advanced colon cancer.
Therefore, we suggest that the promoter hypermethylation that we observed in the present study is not sporadic
colon cancer-associated methylation but might be associated with the repression of the *ADIPOQ* mRNA transcription.

We also observed hypermethylation at the CpG sites in the promoter region of *RETN* gene in cancerous tissue,
adjacent normal tissue, and paired blood samples. Whereas DNA methylation level at the CpG sites in exon one of
the *RETN* gene exhibited a lower level in the non-cancerous tissue compared to the cancerous tissue, and the blood
samples. The region that is downstream of the transcriptional start site (TSS) in exon one of the *RETN* gene showed
a cancer-specific methylation pattern. This region was more informative because less methylation in the adjacent
non-cancerous tissue than the cancerous tissue was detected. This finding raises the hypothesis that methylation at
the CpG sites in exon one might contribute to the disease and can be suggested as a biomarker to sporadic colon
cancer. Also, the outcome also suggests that the methylation pattern in exon one of the *RETN* may be important for
understanding the molecular mechanisms linking to the development of the disease. Here we believe we are
identifying novel colon cancer DNA methylation changes associated with sporadic colon cancer. We postulate that
DNA methylation at the CpG sites in exon one of the *RETN* gene might help uncover cancer signatures in colon
cancer and may be used as a prognostic biomarker.

Moreover, our data showed that the *RETN* mRNA was upregulated in the tissues and blood samples. Previous
studies confirmed a higher level of resistin in patients with colon cancer than healthy controls, indicating that resistin
levels may be positively correlated with the risk of colon cancer. In 2002, Sadashiv and his group measured
resistin mRNA in subcutaneous adipose tissue (SAT) in postmenopausal obese and non-obese women and showed
that the expression of resistin in serum and the level of *RETN* mRNA were upregulated in postmenopausal obese
women. In contrast, when resistin was measured in visceral adipose tissue (VAT), it was found that the expression
of resistin was downregulated at the transcriptional level and upregulated in the serum of the postmenopausal
obese group. They postulated that the mRNA resistin level variation in both tissues could pinpoint a
posttranslational mechanism occurrence. Therefore, they suggested that additional research is needed to explore the
regulation and biological function of resistin in humans.

Although the role of resistin in colon cancer is far from being elucidated, several mechanisms may be involved in
explaining these outcomes. In fact, a few years later it was reported that the plasma resistin was inversely
associated with the extent of methylation at SNP – 420 in the promoter of the *RETN* gene. They suggested that the
association could have genetic and epigenetic effects on the expression of the *RETN* gene and the level of plasma
resistin. The result was supported by another group who reported the effect of hypomethylation on the *RETN* gene
on PCOS causing upregulation of resistin mRNA. Given that hypermethylation is an important factor in regulating
gene expression, we suggest that the hypermethylation at the CpG sites in the promoter region of the *RETN* gene is
not colon cancer-associated methylation. It might have an effect on *RETN* gene expression, and hence, resistin
action.

While DNA methylation as cancer biomarkers is still a relatively new area, the advantages of using DNA methylation
as a cancer marker are evident. Furthermore, the upregulation of *RETN* mRNA in the tissues and blood samples may
regard as a fundamental role of DNA methylation in the regulation of gene activity. It is known that the development
of CpG island hypermethylation profiles for every form of human tumors will yield valuable pilot clinical data in
monitoring and treating cancer patients. Therefore, more analysis is needed to determine if DNA methylation in exon
one region is truly relevant for sporadic colon cancer tumorigenesis and the increased expression of the \textit{RETN} gene. The expectations are high.

Although the sample size in this study was small, the design of this study was relatively strong because the normal tissue samples near the cancerous tissue were taken from the same colon cancer patients. Therefore, upcoming research should address the prognostic potential of methylation profiles in sporadic colon cancer tissue, especially primary tumors of early-stage colon cancer.

In the future, the investigation of DNA methylation can be helpful in early cancer detection. Understanding the differentiation-related roles for tissue-specific DNA hypermethylation is important for evaluating the biological functionality of disease-related DNA hypermethylation. Therefore, challenging future clinical applications to colon cancer is the inclusion of epigenetic markers in diagnostic, treatment-decision assays, and the development of treatments aiming at improving patients' outcomes and enhance the quality of life.

\section*{Conclusion}

DNA methylation is a useful biomarker for predicting tumor recurrence patterns and colon cancer patient survival. Our findings provide further characterization of the methylation profile in the sporadic colon cancer disease that might help in the identification of novel targets to be used for improving patients' outcomes. Our study indicates that hypermethylation at the CpG sites in the two regions of the \textit{ADIPOQ} promoter has an effect on gene expression and may play a role in colon cancer tumorigenesis. We postulate that DNA methylation at the CpG sites in exon one of the \textit{RETN} gene might help uncover cancer signatures in colon cancer and may be used as a prognostic biomarker.

\section*{Declarations}

\subsection*{Compliance with ethical standards}

\textbf{Funding}: There has been no financial support for this work.

\textbf{Disclosure statement}: The authors declare that they have no competing interests.

\textbf{Data availability statement}: The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

\textbf{Ethical approval}: This study was approved by the Research Ethics Committee of Prince Sultan Military Medical City, Riyadh, Saudi Arabia and King Khalid University Hospital (KKUH), College of Medicine, Riyadh, Saudi Arabia.

\textbf{Contributorship}: Rowyda N. Al-Harithy and Eman A. Al-Abdulsalam are equally contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

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\section*{References}


**Figures**
Figure 1

MS-PCR results of RETN and ADIPOQ genes. Bands represent the MS-PCR results using a set of primers that upper raw shows the results obtained from the (M) methylated primers, whereas lower raw presents (U) unmethylated results for the same samples for each gene targeted region. C=colon cancerous tissue, B= blood pair samples, N=non-cancerous colon tissue, (+)= positive control, and (−)=negative control. RETN*=region one, product size= 166 bp. RETN**=exon one, product size= 223 bp. ADIPOQ*=region one, product size 171 bp. ADIPOQ**=region two, product size for M=140, U=142 bp. Marker 50 bp was loaded for all the MS-PCR amplicons.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalinformation.pdf
- Additionalinformation2.pdf