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HIPERMETILAÇÃO DO PROMOTOR DE SOMATOSTATINA (SST) EM ASSOCIAÇÃO COM CÂNCER COLORRETAL

SOMATOSTATIN (SST) PROMOTER HYPERMETHYLATION IN ASSOCIATION WITH COLORECTAL CANCER

فرط المثيلة في حفُاز جين السوماتوستاتين بالتزامن مع سرطان القولون والمستقيم

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RESUMO

O câncer colorretal (CCR) é uma das neoplasias malignas mais comuns com diversos fatores de risco, incluindo ambientais e genéticos. Vários genes, chamados genes supressores de tumor, desempenham um papel essencial na inibição desses fatores de risco, impedindo o desenvolvimento de tumores. Um desses genes é a somatostatina (SST). A somatostatina é um peptídeo antiproliferativo com efeitos pró-apoptóticos que aumentam a morte celular para prevenir o crescimento do tumor. Este estudo teve como objetivo investigar a relação entre a metilação do DNA no promotor SST e a progressão do câncer colorretal. Após a conversão do bissulfito de DNA, a metilação do promotor SST foi examinada usando PCR quantitativo específico de metilação (qMSP) em 71 casos (19 metástases CRC, 28 CRC em estágio inicial e 24 controles saudáveis). O PCR específico de metilação quantitativa (qMSP) é um método de PCR em tempo real usado para determinar os resíduos de citosina metilada e não metilada usando um conjunto específico de primers. A porcentagem de hipermetilação no promotor SST foi de 17%, 60% e 79%, em média, para os controles saudáveis, grupos de CRC em estágio inicial e metástase. Os resultados mostraram uma associação significativa entre a hipermetilação do DNA do promotor SST e a progressão do CRC. Os valores de P foram 0,0364 para o grupo de estágio inicial e 0,0138 para o grupo de metástases. Os resultados também confirmaram que a hipermetilação do DNA bloqueia a expressão de SST, que por sua vez induz a carcinogênese. A detecção da hipermetilação do promotor SST no estágio inicial do câncer pode ser usada como um biomarcador para a triagem e prognóstico de CRC.

Palavras-chave: *câncer colorretal, metilação do DNA, gene supressor de tumor.*

ABSTRACT

Colorectal cancer (CRC) is one of the most common diagnosis malignancies with different risk factors, including environmental and genetic. Several genes, called tumor suppressor genes, play an essential role in inhibiting these risk factors by preventing tumor development. One of these genes is somatostatin (SST). Somatostatin is an antiproliferative peptide with pro-apoptotic effects that enhance cell death to prevent tumor growth. This study aimed to investigate the association relationship between DNA methylation in *SST* promotor and colorectal cancer progression. After DNA bisulfite conversion, *SST* promoter methylation was examined using quantitative methylation-specific PCR (qMSP) in 71 cases (19 metastasis CRC, 28 early-stage CRC, and 24 healthy controls). Quantitative methylation-specific PCR (qMSP) is a real-time PCR method used to determine the unmethylated and methylated cytosine residues using a specific set of primers. The percentage of hypermethylation in *SST* promoter was 17%, 60%, and 79% for healthy controls, early-stage, and metastasis CRC groups. The results showed a significant association between DNA hypermethylation of *SST* promoter and CRC progression. P-values were 0.0364 for the early-stage group and 0.0138 for the metastasis group. The results also supported that the DNA hypermethylation block the expression of *SST*, which in turn induce carcinogenesis. The detection of *SST* promoter hypermethylation at early stage of cancer could be used as a biomarker for screening and prognosis of CRC.

Keywords: *colorectal cancer, DNA methylation, tumor suppressor gene.*

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الملخص

يعد سرطان القولون والمستقيم أحد أكثر الأورام الخبيثة شيوعا التي تم تشخصيها والتي لها عوامل خطر مختلفة، بما في ذلك البيئة والوراثة. تلعب العديد من الجينات دورًا مهمًا في تثبيط تأثير عوامل الخطر هذه عن طريق منع تطور السرطان، وهو ما يسمى الجينات الكابتة للورم. أحد هذه الجينات هو السوماتوستاتين السوماتستاتين هو ببتيد يثبط انقسام الخلايا وتكاثرها وله تاثيرات محفزة للموت الخلوي المبرمج ليمنع نمو الورم. تحاول الدراسة الحالية ايجاد علاقة ارتباط بين مثيلة الحمض النووي في حفاز جين السوماتوستاتين وتطور سرطان القولون والمستقيم. تم فحص المثيلة لحفاز جين السوماتوستاين باستخدام تفاعل البلمرة الكمي في 71 عينة (19 عينة تمثل مراحل متقدمة من سرطان القولون والمستقيم و 28 عينه مرحلة مبكرة منه و 24 عينة لحالات سليمة). كانت النسبة المئوية لفرط المثيلة في حفاز جين السوماتوستاتين هي 17% و 60٪ و 79٪ في العينات السليمة وعينات المراحل المبكرة والمتقدمة من سرطان القولون والمستقيم على التوالي. بينت النتائج ان هناك ارتباط كبير بين فرط ميثيل الحمض النووي لحفاز جين السوماتوستاتين وتطور سرطان القولون والمستقيم، اذ كانت القيمة الاحصائية الاحتمالية 0.0364 لمجموعة المرحلة المبكرة و 0.0138 لمراحل المتقدمة من السرطان. وهذا يدعم حقيقة أن فرط ميثيل الحمض النووي يمنع تشفير جين السوماتوستاتين ، والذي بدوره يؤدي الى تطور السرطان. يمكن استخدام الكشف عن فرط ميثيل حفاز جين السوماتوستاتين في المرحلة المبكرة من السرطان كمؤشر حيوي لفحص سرطان القولون والمستقيم والتنبؤ به.

الكلمات المفتاحية: *سرطان القولون والمستقيم، مثيلة الحامض النووي، الجينات الكابحة للورم.*

1. INTRODUCTION:

Colorectal cancer (CRC) is a third cancer type in terms of incidence around the world. In the Global Cancer Observatory database (GLOBOCAN), 1.8 million new cases were registered in 2018, according to the International Agency for Research on Cancer (IARC) (Bray *et al.,* 2018). Globally, the incidence and mortality rates of CRC have been increased 10-fold in 10 years. Specifically, low and middle-income countries show a rapid increase in CRC-related mortality (Arnold *et al.*, 2016). Age is the main risk factor of sporadic CRC; the chance of colorectal cancer developing is increased in patients with age more than 50 years old (Levin *et al.,* 2008). Moreover, patients who have Crohn's disease and/or inflammatory bowel disease have more chance to develop CRC by 2.5% to 3.7% than healthy people. The formation of dysplasia, which considered abnormal growth of mucosal cells could induce the CRC after many years of chronic inflammation (Eaden *et al.,* 2001; Canavan *et al.,* 2006). Other CRC risk related to lifestyle and patient habits a sedentary, obesity (Martinez and Garcia, 2016), unhealthy nutrition (Willett, 2005; Bastide *et al.,* 2011; Santarelli *et al.,* 2008), smoking and alcohol consumption (Pöschl, and Seitz, 2004; Botteri *et al.,* 2008) all of these factors have been elucidated as a risk factor for colorectal cancer.

CRC can be classified depending on genetic alteration as sporadic, inherited, and familial colorectal carcinomas (Mármol *et al.,* 2017; Fearon and Vogelstein, 1990; Smith *et al.,* 2002). Sporadic cancer account for 70% of all CRC cases. Most of this type is caused by point mutations and DNA methylation in specific genes, including oncogene, tumor suppressor genes, and DNA repair genes (Haggar and Boushey, 2009; Kuipers *et al.,* 2015; Lao and Grady, 2011; Sameer and Nissar, 2016). Polyposis and nonpolyposis forms of inherited mutations in specific genes lead to colorectal cancer responsible for only 5% of all CRC cases. The polyposis group recognizes as a formation of potentially malignant polyps in the colon, including familial adenomatous polyposis (FAP) (Lynch and Chapelle, 2003).

In contrast, inherited nonpolyposis CRC is related to mutations in DNA repair genes. This type of malignant primary derived from Lynch syndrome, caused by inherited mutations in DNA repair genes such as *MLH1*, *MLH6*, *PMS*1, and *PMS2* (Umar *et al.,* 2004*).* An inherited mutation in different alleles cannot be included in any inherited cancer variant called familial colorectal cancer, which accounts for 25% of all CRC cases (Stoffel *et al.,* 2014).

During carcinogenesis, mechanisms of genetic alterations and epigenetic aberration modifications affected the normal functions of the oncogene, tumor suppressor genes, cell adhesion molecules, and telomerase activity. All these events induce malignant cell growth (Choi and Lee, 2013). Epigenetics studies have confirmed that DNA methylation in the promoter region of a tumor suppressor gene leads to transcriptional inactivation and is correlated with the carcinogenesis of CRC. DNA methylation is a signature including a panel of methylated CpGs that could show the potential in the screening and prognosis, early diagnosis, or therapy response prediction (Jin *et al.*, 2008; Puccini *et al.*, 2017; Ou *et al.*, 2007). The detection of DNA methylation sheds light on the potential of using methylated CpGs as a biomarker for CRC diagnosis and monitoring (Puccini *et al.*, 2017; Ma *et al.*, 2019). Recently, the U.S. Food and Drug Administration (FDA) approves using methylated septin 9 (*SEPT9*) for a screening of CRC (Ma *et al.*, 2019).

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Somatostatin (SST) is a peptide that suppresses tumor growth through distinct mechanisms that involve inhibition of growth factors and hormones, reduction in vascularization, and regulation of the immune system (Misawa *et al.*, 2015; Reubi and Laissue, 1995). SST is released in different tissues and organs, including the central nervous system, gastrointestinal tract, pituitary, pancreas, and thyroid (Kumar and Grant, 2010; Patel, 1999). It plays an essential role as an endocrine hormone and neurotransmitter (Reichlin, 1995). SST is released by luminal acid stimulation in the gastrointestinal tract and regulates gastric acid (Goo and Kaunitz, 2010). SST inhibits cell proliferation by its antiproliferative and apoptotic effects and induces apoptosis via somatostatin receptor (SSTR) signaling (Grimberg, 2004). Several studies described the CpG hypermethylation of SST promotor in colon cancer (Leiszter *et al.,* 2015), esophageal cancer (Jin *et al.*, 2008), renal cancer (Ricketts *et al.*, 2012), and gastric cancer (Jackson *et al.,* 2010). SST is produced in different tissue and synthesized from a large precursor molecule called (preproSST) (Leiszter *et al.*, 2015).

This study aimed to elucidate SST promoter hypermethylation associated with CRC progression from early-stage to metastasis, which revealed considerable assay to prognosis or early diagnosis.

2. MATERIALS AND METHODS:

2.1. Tissues samples

After informed consent, 71 fresh biopsy tissue samples were collected from the endoscopy unit of Al Hillah Teaching Hospital in Babylon, and Digestive Disease Education Hospital in Bagdad, Iraq. CRC patients were diagnosed clinically, and specialist physicians evaluated the disease. Samples were divided into three groups, including 19 metastasis, 28 early-stage, and 24 healthy controls (Table 1).

2.2. Bisulfite Conversion

According to the manufacturer protocol, the Genomic DNA was extracted from the biopsies by Promega Wizard® Genomic DNA Purification Kit. A total of 30 mg of fresh tissue was added to 700µl of chilled nuclei lysis solution and homogenized for 15 seconds. Following that, 3µl of RNase solution and 200µl of protein precipitation solution was added to the tissue and incubated for 20–30 minutes at 37°C. Then, the contente was submitted to a vortex and chilled on ice 10 minutes before centrifuged at 15.000 rpm for 5min. The supernatant was transferred to a fresh tube containing 800µl of isopropanol. After second centrifugation at 15.000 rpm for 5min, the supernatant was removed, and 600µl of 70% ethanol was added. After the final centrifugation, the DNA rehydration was in 100µl of DNA rehydration solution for 1 hour at 65°C. The next step was to manage all the genomic DNA by DNA methylation modification using spin-column EZ DNA Methylation™ Kit (ZYMO RESEARCH) according to the manufacturer instructions. The protocol included converting the unmethylated cytosine to uracil, while methylated cytosine protected by methyl group from bisulfite conversion. Unmethylated cytidine residues that convert to uridine residues during bisulfite conversion convert to thymidine by PCR (Herman *et al.*, 1996; Lapeyre *et al.*, 1979). The positive methylated controlled DNA has incubated with CpG methyltransferase for 1-2 h at 37°C and terminated following an incubation period at 65°C for 20 min. The modified DNA was then used as a template for downstream analyses, including quantitative methylation-specific PCR.

2.3. Quantitative Methylation Specific PCR (qMSP)

Quantitative methylation-specific PCR (Q-MSP) was used to measured *SST* promoter methylation as described previously (Misawa *et al.*, 2015; SHI *et al.*, 2013) using Techne PrimeQ Real-Time PCR System. The PCR amplification of modified DNA samples consisted of initial denaturation at 94 °C for 6 min, 40 cycles of denaturation at 94 °C for 45 s, primer annealing at 58.8 °C for 45 s, primer extension at 72 °C for 45 s. the PCR product is 102 bp length, Q-MSP primers for methylated SST promoter were Q-MSP-SST- forward primer (5'- GGG GCG TTT TTT AGT TTG ACG T-3') and Q-MSP-SST- reverse (5'-AAC AAC GAT AAC TCC GAA CCT CG-3').

2.4. Statistical Analysis

The association relationships were carried out by Fisher's exact test using GraphPad Software(http://graphpad.com/quickcalcs/contingency1.cfm). *P*-value was calculated with Two-tailed Fisher's exact test as recommended in this software. It was considered significant when the recorded value was less than 0.05.

3. RESULTS AND DISCUSSION:

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The DNA methylation status of SST promoter in the three groups (healthy controls, early-stage, and metastasis) of CRC was detected using qMSP. A single graphic peak elucidated the primer's high quality. When the reaction reached the concentration threshold (CT), the CT value was 15-20 reaction cycles in the amplification curves, demonstrating that the amplification was adequate. The results show that methylated cases were 4 vs. 20, 17 vs. 11, and 19 vs. 4 in healthy controls, early-stage, and metastasis cases, respectively, as shown in Table (2).

The methylation status of SST promoter in the early stage and metastasis CRC group was higher compared with healthy controls. The percentage of methylation for healthy controls, early-stage, and metastasis groups was 17%, 60%, and 79%, respectively (Figure 1).

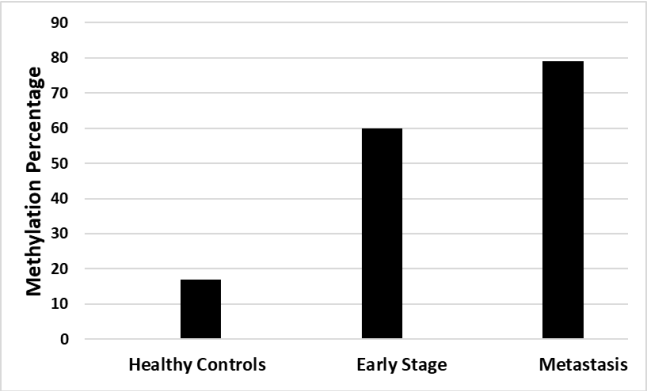

Figure 1. *The percentage of methylation for healthy controls, early-stage, and metastasis*

The two-tailed *P*-values given by the association between methylation status of early-stage and metastasis groups with healthy controls were 0.0364, 0.0138, respectively (Table 2). This association is considered to be statistically significant. Furthermore, a strong association between healthy controls methylation status and all CRC patients (early-stage and metastasis) with a 0.0115 *P*-value.

Somatostatin is a peptide produced in different tissues, including the gastrointestinal tract. This peptide could act as a neurotransmitter or an inhibitory hormone that blocks cell prefiltration (Gorden, 1989). SST directly inhibits cell growth autocrine mechanism via SST receptor type 2. This means that SST has antiproliferative and pro-apoptotic effects (Rauly *et al.,* 1996, Calabró *et al.*, 2002). Several findings involved *in vitro* and *in vivo* studies have suggested that *SST* functions as a tumor suppressor gene in human cancers (Jin *et al.*, 2008).

Epigenetic surveys based on the detection of DNA methylation revealed that the hypermethylation of *SST* in CRC patients was high at an early-stage compared to healthy controls. These findings support that the inactivation of the somatostatin gene by promotor CpG methylation played an essential role in CRC progression (Tariq and Ghias, 2016). Epigenetic silencing of SST promoter could lead to downregulation and silencing of several tumor suppressor genes that enhance more mistakes in cell proliferation (Peng *et al.*, 2008; Clément *et al.*, 2006). Furthermore, the results show an increased percentage of DNA methylation levels with the progression of carcinogenesis. It revealed the maximum DNA methylation in metastasis, which confirmed previous studies (Misawa et al. 2015; SHI *et al.*, 2013; Mori *et al.*, 2006).

The present findings suggested that DNA methylation could be the main mechanism of silencing SST expression in CRC. Several previous findings elucidate that *SST* promoter hypermethylation corresponding with loss of expression of SST in association with cancer progression (Jin *et al.*, 2008). This means that the inactivation of SST may be a critical step in CRC carcinogenesis (Mori *et al.,* 2006).

Modern molecular biology shad the light on the possibility of using epigenetic alteration for new medical strategies in cancer diagnosis and treatment (Hama *et al.,* 2009; Baylin and Ohm, 2006). Several studies attempt to use many methylated genes as biomarkers for CRC diagnosis and monitoring. U.S. Food and Drug Administration (FDA) approves of using methylated septin 9 (*SEPT9*) for a screening of CRC (Ma *et al.*, 2019). This experiment corroborates with the previous results, which show high methylated SST percentage in primary colorectal tumors compared with normal colon mucosa (Patai *et al.*, 2015). CpG methylated penal printed in the early stage of cancer could reveal useful assay to cancer prediction.

4. CONCLUSIONS:

It can be concluded the DNA hypermethylation is the epigenetic mechanism that prevents the expression of *SST* and other types of tumor suppressor genes, which, in turn, induce carcinogenesis. In case other studies confirm these results, the detection of SST promoter hypermethylation at the early-stage of cancer could be used as a biomarker for CRC screening and prognosis.

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Table 1. *Demographic characteristics of studies patients*

Demographic characteristics		Patients	Healthy control
No.		47	24
Gender	Male	29	16
	Female	18	8
Age	Mean age	61	51
	<55	24	19
	>55	23	5
Stage	Early-stage	28	
	metastasis	19	

Table 2. *The association between methylation status of early-stage and metastasis groups with healthy controls*

Biopsies	No. of cases	Methylated cases	*P-value*
Healthy controls	24	4	
Early-stage	28	17	0.0364
Metastasis	19	15	0.0138
Early-stage + Metastasis	47	32	0.0115

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