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RESEARCH ARTICLE

Investigation of *Mir-34b/c* Gene Methylation in Patients with Papillary Thyroid Disease Compared to Healthy Individuals

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ABSTRACT

Background and Objective: Papillary thyroid carcinoma (PTC) is the most frequent type of differentiated thyroid cancer. Recent advances have shown that cancer cells can have some epigenetic changes involved in all stages of cancer. It has also been shown that *Mir-34b/c* acts as a gene expression regulator in many biological processes, including angiogenesis. In the current study, to identify the potential role of *Mir-34b/c* in PTC progression, the methylation status of the *Mir-34b/c* promoter region has been evaluated. **Methods:** In this case-control study, blood samples were taken from 60 patients with papillary thyroid and 60 healthy people. The method of polymerase chain reaction method was used to investigate gene promoter methylation. The results obtained from the study were studied using Statistical Package for the Social Sciences and Chi-square tests respectively. **Results:** Findings confirm that there was a significant association between *Mir-34b/c* and gene promoter methylation. **Conclusion:** The results of the study showed that the reduction of gene promoter methylation in papillary thyroid cancer can be used as a biomarker in the diagnosis of the disease.

Keywords: DNA methylation, mir34b/c, papillary thyroid carcinoma, tumorigenesis

INTRODUCTION

Thyroid cancer, which is the most common type of thyroid cancer, accounts for about 80% of all thyroid cancer cases.[1,2] This disease is one of the fastest-growing types of cancer with more than 10,000 new cases per year. It is the eighth most common cancer among women and the most common cancer in women under the age of 25. Although a person may develop papillary thyroid cancer at any age, most patients are diagnosed before the age of 40. Causes of papillary thyroid cancer include radiation exposure and a family history of thyroid cancer. It is important to note that most patients have no risk factors at all.[3,4] Papillary carcinomas are sometimes referred to as differentiated thyroid cancers, which are easier to treat than other types. Thyroid cancer occurs when a change occurs in the DNA within thyroid cells that causes them to grow uncontrollably and produce masses.[5] It is not usually known what causes this change, but some things can be risk factors for the disease, such as thyroiditis or goiter, a family history of thyroid cancer, and radiation exposure such as radiotherapy, Acromegaly, an intestinal disease called familial adenomatous polyposis. Thyroid cancer treatment depends on the type of thyroid cancer and its extent, which includes surgery, radioactive iodine treatment, external radiotherapy, chemotherapy, and targeted treatments.[6,7] microRNAs are short RNA molecules of 22 nucleotides that play a role in regulating gene expression and present in all human cells. There are different types of miRNAs that are effective in regulatory and physiological processes of the body such as cell growth and proliferation, homeostasis, metabolism, and cell differentiation. Studies have shown that they play an effective role in biological processes,

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for example, microRNAs can silence or degrade mRNA by binding to a complementary sequence in the untranslated region. Among the different types of miRNAs, we can mention the family of three miRNAs that result from the processing of two primary miRNAs. MiR-34bmi and miR-34ca are processed from a common primary transcript. These miRNAs have tumor suppressor activity due to the inhibition of target genes.[8,9] They are involved in the regulation of various processes related to cancer such as proliferation, apoptosis, migration, invasion, and metastasis.[10] The coding gene has 90 polymorphic sites, one of the most important of which has been studied in some cancers. The miR-34 family includes three types of *miR-34a*, *miR-34b*, and *miR-34a*, among which *miR-34b* and miR-34c are located in the same chromosomal location.[11,12] Methylation of these genes leads to silencing. It seems to be stronger than *miR-34a*, which can be related to the metastasis of cancers in such a way that it can be a factor inhibiting the growth of cancer cells, invasion, and metastasis. Therefore, their methylation can be used as a prognostic indicator.[13,14] Epigenetic mechanisms are involved in the silencing of miRNAs in cancer. Studies have shown that *miR-34a* acts as a tumor suppressor and can directly target the proto-oncogene BCL6, and the result of this mechanism is that the upregulation of *miR-34a* with methylation DNA. *miR-34a* is a tumor suppressor that under CpG island hypermethylation causes the transcriptional inactivation of various cells in human tumors. Among the molecular processes of cancer is CpG dinucleotide methylation, which is common in all types of cancer.[15] Today, the promoter hypermethylation process has opened new horizons in the direction of obtaining cancer molecular markers. Under normal conditions, CpG islands that include the promoter and the transcription start site are unmethylated, and if this region is hypermethylated, gene expression stops.[16] Of course, this is even though the pattern of genomic methylation in somatic cells is stable and heritable. A change in the methylation pattern occurs in many types of cancer, which inhibits the expression of genes and destabilizes the entire genome. The stability of the normal methylation position in the target genes makes it easier to detect its presence than multiple mutations.[17,18] The location of such mutations in the gene may be different even for a specific type of tumor from one patient to another, but for each gene, promoter methylation abnormalities can be checked with a diagnostic test. One of the uses of methylation as a tumor marker is that the CpG islands of the promoter region of genes are not methylated in normal cells, so observing the hyper-methylated region is a positive signal indicating the presence of a normal tumor.[19,20] Another advantage of using hypermethylation of CpG islands as a tumor marker is the existence of very sensitive methods such as methylation specific polymerase chain reaction (PCR) to identify methylated sequences, which can reveal small amounts of methylated DNA. The purpose of this study is to investigate gene methylation as one of the most important molecular processes common in all types of cancers, including papillary thyroid.

METHODS

In this experiment, the study group included 60 people with papillary thyroid cancer whose disease was confirmed by a pathologist. The average age of this group was 55.3 ± 14.5. The control group consisted of 60 people with an average age of 62.3 ± 12.8 who did not have any cancer or autoimmune disease. All ethical conditions were followed for the experiment and written consent was obtained from the patients. The sample was prepared from among the patients referred to Ali Ibn Abi Talib Hospital in Zahedan City. The study was approved as a research project in the Faculty of Medicine of the Azad University of Zahedan and has a code of ethics (IR.IAU.ZAH.REC.1400.035). 5 cc of venous blood was taken from the subjects and transferred to tubes containing ethylenediaminetetraacetic acid. The genomic DNA of salting out was extracted. In the following, the information obtained from the patients and samples was carefully examined, and some samples were excluded from further study in this project. These were samples that did not meet the following requirements: If the primary site of cancer was unclear, if the patient had another type

of cancer besides thyroid, if a diagnosis other than papillary thyroid was confirmed histologically for the tumor; if detailed information about the history or pathology reports of the patients was not available and if the patients had a family relationship. The characteristics of patients with papillary thyroid cancer are listed in Table 1.

Methylation in Gene Promoter Mir 34 b/c

To check the methylation or non-methylation of the gene, the specific methylation method of PCR was used. To perform the test, the genome of the studied subjects was first tested using the bisulfite kit manufactured by Qiagen, Germany. The recipe for the kit was prepared. As a result of these actions, cytosine bases that are methylated remain intact, and if they are not methylated, they are converted into uracil bases. Specific primers were used to check the methylation of the promoter regions of the gene. Two specific primers were needed for each gene. One to check methylation and the other to check non-methylation. To perform the reaction, two separate tubes were needed for each sample, one for methylation and the other for non-methylation, whose specific primers were different. The procedure was as follows: MSP analysis was performed on a total volume of 10 μL reaction mixture, which consisted of 1 X PCR Buffer, 1.5 mM MgCl2, 0.2 mM dNTP, 0.5 u/μL AmpliTaq DNA polymerase, 0.2 μM forward and reverses primers, approximately 50 ng of genomic bisulfite-treated DNA template, and distilled deionized water (ddH2O). MSP amplification of all genes was done and optimized using the PCR protocol and the following cycling conditions: 10 min of initial denaturation (95°C), 14 cycles of 20 s of denaturation (94°C), 1-min annealing (variable temperature), and 1-min elongation (72°C). Amplification was run for further 32 cycles of 20 s of denaturation (94°C), 1-min annealing (57°C), 1-min elongation (72°C), and final elongation at 72°C for 5 min and storage at 10°C. 4 μL of PCR products were loaded into 3% agarose gel, electrophoresed and visualized under UV light, and its picture was captured by a UV Transilluminator. The sequence of the primers used to check the methylation of the mentioned gene is shown in Table 2.

Statistical Analysis

A statistical study was done using Statistical Package for the Social Sciences version 18 and the Chi-square test and Fisher Kappa. In this study, $P < 0/05$ was considered significant.

RESULTS

The results showed that there is a significant relationship between the methylation of the mir-34 b/c gene promoter and the development of the disease, so that the lack of methylation of the *mir-34b/c* gene promoter increased more in healthy people than in patients. The results are shown in. The results showed that there is a significant relationship between the methylation of the mir-34 b/c gene promoter and the development of the disease so that the lack of methylation of the

Table 1: Patient characteristics of PTC and clinic pathologic features

Patient characteristic	Number of patients (%)	*P*-value
Gender		
Male	33 (61.4)	0.023
Female	27 (38.6)	
Age (years)		
<45	30 (52.7)	0.4
>45	30 (47.3)	
Grade		
G1	15 (24.6)	0.071
G2	15 (24.6)	
G3	30 (50.8)	
Tumor size		
<5.8 cm	34 (52.2)	0.344
>5.8 cm	26 (47.8)	

PTC: Papillary thyroid carcinoma

Table 2: Specific primers for MSPPCR reaction of mir34b/c gene

GGTGGCATACTCCGTCTG	MF
AGACAATGGTGGCATACTCC	MR
TCTTCTCTCGCCACTGGA	UMF
CCGCTAGGAAAGACAATGGT	UMR

MF: Methylation forward, MR: Methylation reversed, UMF: Unmethylation forward, UMR: Unmethylation reversed

mir-34b/c gene promoter increased more in healthy people than in patients. The results are shown in Table 3.

DISCUSSION

Papillary thyroid cancer is one of the most common cancers that can affect many people without symptoms.[21,22] Studies have shown that the occurrence of inappropriate methylation in the areas of islets CpG which are unmethylated leads to cell death and transformation, which is done through inactivating the transcription of tumor suppressor genes.[23-25] Mapping of methylation patterns in islands CpG can be used as an important tool in understanding gene expression events in cells in both normal and pathological conditions such as cancer.[26] MiRNAs are regulators that regulate gene expression after transcription. These regulators act by binding to mRNA. The result of this interaction is the destruction of mRNA suppression of protein synthesis and reduction of protein expression, which can affect the expression of tumor suppressor genes.[27] One of the effective types of MIRNs is MiRNA34b/c, which has an effective function in tumor suppressor transcription, signaling, and gene expression, which can suppress tumor growth by inhibiting cell proliferation, apoptosis, and metastasis.[28] In many studies, methylation of the *MiR-34b/c* gene promoter has a high prognostic value in many types of cancer. For example, in lung adenocarcinoma, its expression leads to a less aggressive phenotype.[29] In one study, Ng *et al*. showed that abnormal methylation of *miR-34b/c* genes can be considered as a prognostic tumor marker for non-invasive screening.[30] In another study, Roy *et al*. showed that methylation of the miR-34c promoter led to a decrease in protein expression in colon cancer.[31] The results of Szyf and Bick research showed that the methylation of the *miR-34b/c* gene in small cell lung cancer (SCLC) is significantly higher than in non-SCLC, and therefore, it can be concluded that aberrant methylation of the *miR-34b/c* gene in the pathogenesis of SCLC is important and a useful therapeutic target for SCLC.[32] Tanaka *et al*.'s study showed that *miR-34b/c* gene promoter methylation affected the risk factor of chronic lymphocytic leukemia by suppressing cell proliferation and increasing cell death.[33] Also, the Varriale study showed that abnormal methylation of the *miR-34b/c* gene promoter is related to high recurrence of the disease. Therefore, methylation of the *miR-34b/c* promoter can be considered as a prognostic and independent factor.[34] The research results have shown that there is a significant difference in the amount of methylation in the promoter of the gene in healthy people compared to people with papillary thyroid and considering that this gene is an effective factor in suppressing tumor cells, it seems that methylation or the lack of methylation of this gene is an effective factor in the risk factor of contracting this disease. The Vogt *et al*. study showed that the increase in gene methylation is related to the decrease in gene expression in people with papillary thyroid.[35] The Wang *et al*. study showed an increase in gene methylation.[36] It has been directly related to the risk factor of papillary thyroid disease that this gene can be investigated as a tumor marker. Liao *et al*. also showed in their studies that the increase in gene methylation is related to papillary thyroid disease.[37] Our study showed that the methylation level of the Mir 34b/c gene promoter in the group of patients with papillary thyroid was significantly higher than the normal control group. The results showed that *miR-34b/c* gene promoter methylation is an important mechanism in the pathogenesis of papillary thyroid carcinoma (PTC). In fact, in this study, we looked at the relationship between gene promoter methylation and clinical features of the disease. This is the first study to show that *miR-34b/c* DNA hypermethylation correlates with the clinical stages of patients with PTC. In line with our study, the research results of Huang *et al*. and his colleagues

Table 3: Comparison of mir34b/c gen promoter methylation in patients and control group

	Study subjects		
Mir34b/c	**Case (%)**	**Control (%)**	**Pv**
M	27 (47.5)	18 (45)	0/4
UM	21 (31.5)	34 (51)	0/03
M/UM	12 (21)	8 (4)	0/1
Total	60	60	

M: Methylation, UM: UN methylation

showed that DNA methylation of the gene promoter is more in women with papillary thyroid than in men.[38] The study of Li *et al*. showed that there is a significant relationship between methylation of the mir gene promoter and non-malignant progression and decreased survival of patients with papillary thyroid.[39] The results of the study by Yang *et al*. also showed that the methylation level of *miR-34b/c* was significantly higher in elderly patients.[40] Our study showed that mir gene promoter methylation is an important prognostic parameter in the papillary thyroid. However, it should be noted that in our study, the number of samples is small, and in future studies, it is better to consider a larger volume for a more accurate evaluation Mapping.

CONCLUSION

According to the study and considering the inhibitory role of the *mir-34b/c* gene, it seems that the methylation or non-methylation of the promoter of the gene has an effect on turning the gene off or on, which leads to the susceptibility or resistance of people. It is called papillary thyroid cancer. The results of our study showed that methylation of *miR-34b/c* CpG islands gene promoter is a common event in PTC and is significantly related to clinical symptoms in patients, so it can be an effective prognosticator for oncogenesis and pathogenesis of diseases such as papillary thyroid.

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CONFLICTS OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

AUTHORS' CONTRIBUTIONS

Study concept and design: H.H. Analysis and interpretation of data: A.S. Drafting of the manuscript: H.H. Statistical analysis: SH.SH.

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