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Diagnostic Value of *RASSF1A* gene DNA methylation in Differential Diagnosis of Thyroid Benign Tumors and Papillary Thyroid Carcinoma

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A B S T R A C T

Thyroid cancer is the most common endocrine malignancy that its incidence has continuously increased in recent decades all over the world. Thyroid cancer in Iran is the seventh most common cancer in women and the 14th in men and the 11th most common cancer in both genders. The study on the methylation status of RASSF1A gene promoter has shown that this gene is methylated in 35% of benign and malignant thyroid tumors. Hypermethylation of the RASSF1A gene use for the differentiation of benign tumors from malignant of thyroid gland. The aim of this study was to determine the sensitivity and specificity of DNA methylation of RASSF1A gene in the differential diagnosis of benign tumors from papillary thyroid carcinoma.160 samples of patients with malignant thyroid tumors (80 samples) and benign thyroid (80 samples) were entered into this study from all patients referring to Ahwaz medical centers. The Hypermethylation of the gene after DNA extraction was done by COBRA method. Finally, for calculating sensitivity and specificity of the two tests were done by epidemiological calculations.The sensitivity and specificity of Hypermethylation of the RASSF1A gene test were 91.25% and 15% respectively.Hypermethylation of the promoter of the RASSF1A gene as a diagnosis test is more sensitive to differential diagnosis of benign tumors from papillary thyroid carcinoma.

Introduction

Thyroid cancer is the most common endocrine malignancy that its incidence has been increased continuously in recent decades all over the world. (Howlader, Noone, et al.2012) In the United States, the annual incidence of thyroid cancer was 6% from 2000 to 2009, which is the highest among all types of cancers. (Howlader, Noone, et al.2012) Although the mortality rate of this cancer is low, but the rate of relapse or the disease course is high which increases the incapacity of the treatment and mortality (Tuttle, Ball, et al.2010). According to the Iranian Cancer Institute thyroid cancer, 1.8% of the mean age of Iranian patients is 43 years and female to male ratio is 1.8 to 1, Thyroid cancer in Iran is the seventh most common cancer in women and the 14th in men and the 11th most common cancer in both genders (Taghavi, Farzadfar, et al. 2016). 10-year survival of thyroid cancer in middle-aged adults is 80 to 96%. Recurrence is seen in 5% to 20% of cases of papillary thyroid carcinoma. Recurrence may be due to

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inadequate primary treatment or because of a tumor with highly invasive cells (Lee, Lee, et al. 2007). The malignancy risk in cancer suspected FNA samples is reported to be between 10% and 40%, so all patients will need to withdraw suspicious nodules in order to find out more accurately answer through examining pathology biopsy samples and only about 20% of these suspicious samples will have malign neoplasms (Kebebew, Peng, et al. 2005). On the other hand, in 10% of FNA cases, false negative responses are reported, leading to late detection of cancer and so the late treatment and this will negatively affect the disease course (Kebebew, Peng, et al. 2005). RASSF1A gene (Ras association domain domain 1): Another name for this gene is NORE2A and located on chromosome 3 at 3p21.3. This gene encodes a protein similar to the RAS effector proteins. Loss or altered expression of this gene has been associated with the pathogenesis of a variety of cancers, which suggests the tumor suppressor function of this gene. The inactivation of this gene was found to be correlated with the hypermethylation of its CpG-island promoter region. The encoded protein was found to interact with DNA repair protein XPA (Dammann, Yoon, et al.2000). The protein was also shown to inhibit the accumulation of cyclin D1, and thus induce cell cycle arrest (Shivakumar, Minna,et al.2002).The study on the methylation status of RASSF1A gene promoter has shown that this gene is methylated in 35% of benign and malign thyroid tumors (Nakamura, Carney,et al.2005). In a study the promoter hypermethylation of the gene was reported in cancers, including thyroid, and was cited as a molecular biochemical marker that makes possible the detection of cancerous cells (Pfeifer, Dammann, et al.2005). Hypermethylation of RASSF1A has been observed in the blood and body fluids of individuals with certain malignancies, such as thyroid cancers, and therefore can be used as a biological marker for early diagnosis of thyroid cancer (Pfeifer, Dammann, et al.2005). In one study on 89 thyroid tumors and 3 cell lines this has been shown that benign and malign tumors of hypermethylation of RASSF1A were seen in all three cell lines and in 35% of cases. This study for the first time showed that the hypermethylation rate of RASSF1A is related to its expression (Nakamura, Carney,et al.2005). In this research, we decided to investigate the sensitivity and specificity of DNA methylation of RASSF1A gene as molecular diagnostic tests in differential diagnosis of benign tumors of thyroid papillary carcinoma.

Materials and methods

In this study, the sample size was 160 cases; 80 patients with malignant thyroid tumors and 80 patients with benign thyroid tumors were included. A pathologist selected the most suitable paraffined blocks after a re-examination. Ten samples, that in pathologic reports no benign or malignant lesion was not reported for them, were selected as normal tissue.For Microdissection, 6-micron sections of the paraffined blocks were initially given by microtome. These sections were then placed on a microdissection slide in an RNase-free environment. 2slides were prepared from each block and 2 or 3 cuts were placed on each slide, two other for DNA extractions, and one slide was stained with eosin haematoxylin (Sigma) that during microdissections to be used as a guidance to identify the cell boundaries. Specific slides were stored at room temperature for DNA extraction. Then, by comparing between the slides stained with eosin haematoxylin and slides stained with methylene green, and using macrodissection laser (LEICA) device or stereomicroscope and a surgical blade, the required cells were cut from the slides surface and were placed into micro tubes 1.5 µl and stored until the extraction of DNA in a freezer with 80°C temperature. QIAamp DNA Micro Kit made by QIAGEN Co. was used to extract DNA from paraffined samples. Phenol and chloroform method was used to extract DNA from fresh tissue. Combined Bisulfite Restriction Analysis (COBRA) was developed for the first time on 1997 for the quantitative investigation of hypermethylation (Xiong,Laird, et al.1997).In this method, after treating DNA with bisulphite and performing PCR, its products are subjected to enzymatic incision. Due to the rows of primary DNA base, this enzyme is selected to perform incision just on CpG islands with hypermethylation. The amount of hypermethylation in the original DNA is proportional to the quantitative extent of the PCR product incision. If CpG lacks hypermethylation in the original DNA, the during cytosine bisulfite treatment it will converted to Thymine and the enzymatic incision site will be destroyed and so incision will not done. If there is a mix of methylated and non-methylalous Alleles in the original DNA, during the digestion process, the amount of the cuts depends on the amount of methylated CpG. Then, through investigating the concentration of bands in the polyacrylamide gel (using ImageJ software), the amount of hypermethylation is calculated. The percentage of hypermethylation is canulated using formula: X 100 % Methylation= C / (C + B), where B is the band density of the products before incision and C is the band density produced by the enzymatic incision. In the study of hypermethylation, the product of the Millipore Co. called CpGenome ™ Universal Methylated DNA was used as a positive control. In this DNA, all CpG islands have hypermethylation. In addition, the DNA of peripheral blood lymphocytes (PBL) was used as a negative control. The EZ DNA Methylation Kit ™ CA (USA) (ZYMO REASERCH) kit was used to do the bisulfite treatment.The primers required for the investigation of hypermethylation of RASSF1A were designed using the Primer3 site. At first, DNA sequence within the exon 1 domain of

these genes was introduced at the MethPrimer site and the CpG islands were identified in this region. Then, the rows of DNA base were obtained after treatment with bisulphite. Then, an enzymatic incision site having one CpG was selected and the necessary primer was designed around this site. Because DNA samples were obtained from paraffinic tissues, the length of the PCR product was designed to be less than 90 base pair. (Table1)(Figure1)

Table1- Primer Sequence, Length of Product and Enzyme Used to Hypermethylation Test by COBRA Method.

Gene	Primer Sequence	Length	Length After cutting	Enzyme
RASSF1A	**F:GGTTYGYGTTTGTTAGYGTTTAAAGTT R:CTCAAACTCCCCCRACATAA**	**70**	**35-35**	**RsaI**

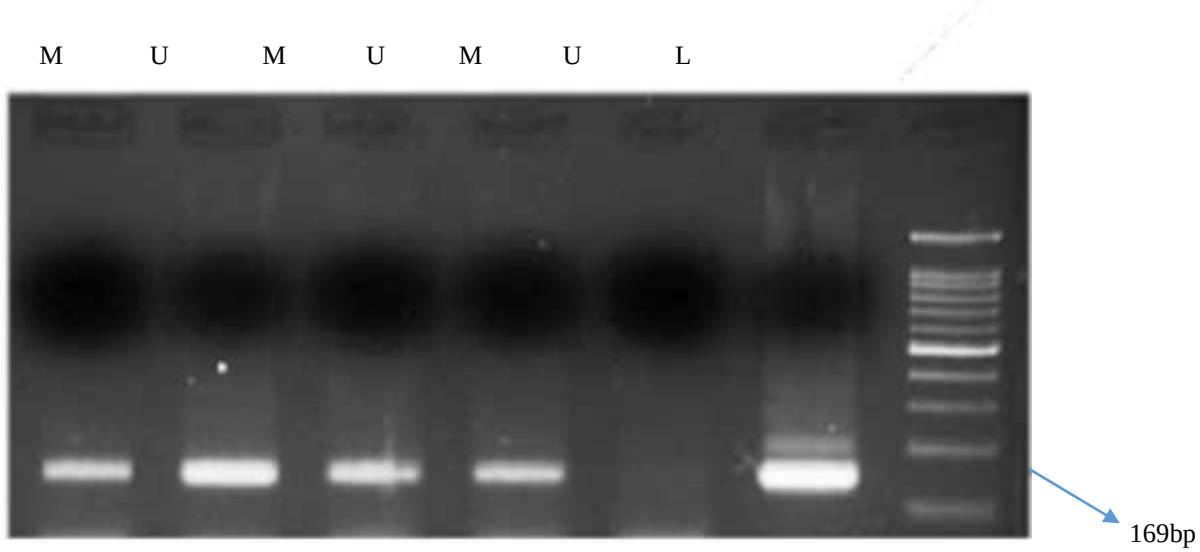

Figure1: Results of *RASSF1A* Gene Methylation MS-PCR Analysis, Lanes M and U Correspond to Methylated and Unmethylated Samples Respectively and Last Lane to a 100bp Ladder as Molecular Weight Marker.

Three enzymes used for the study of hypermethylation had the following incision site. The Taq1 (TCGA) enzyme, the RsaI (GTAC) enzyme, and the BstUI (CGCG) enzyme. Temperature conditions and duration of treatment were performed according to the recommendation of the manufacturer. SPSS software version 16 was used to perform statistical calculations. Chi square test was used to examine the relationship between variables such as age, gender, invasion and metastasis with hypermethylation quality status. In specific cases, Fisher's exact test was used for this purpose. T-test was used to investigate the possible relationship between quantitative hypermethylation with variables such as age, gender, invasion and metastasis. In all of the tests, p value was 0.05.

The following formulas were used to calculate sensitivity, specificity, positive predictive value, negative predictive value and accuracy of test answers. In this calculation, the response of pathologic reports was used as standard test that the true or false results of hypermethylation were assessed compared with pathologic reports.

$$Sensitivy(\%) = \frac{TP}{TP+FN} \times 100$$

$$Specificity(\%) = \frac{TN}{TN+FP} \times 100$$

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$$PPV(\%) = \frac{TP}{TP+FP} \times 100$$

$$NPV(\%) = \frac{TN}{TN+FN} \times 100$$

$$Accuracy(\%) = \frac{TP+TN}{TP+TN+FP+FN} \times 100$$

TP: True-positive
TN: True-negative
FP: False-positive
FN: False-negative
PPV: Positive predictive value
NPV: Negative predictive value

The ROC test was used to calculate the best boundary value in the results of the DNA hypermethylation. This test provides us with the best boundary value for the results of each test, which has the highest sensitivity and specificity, and by calculating the area under curve (AUC), the test efficiency was calculated for the differentiation of benign tumors from malignant ones.

Results

-Investigating the relationship between Hypermethylation of RASSF1A Gene and of tumors type:

Investigating the relationship between Hypermethylation in 160 cases of benign and malignant thyroid tumors showed that the 138 cases of hypermethylation was positive. Among them 67 cases were benign and 71 cases were malignant. The remaining 22 cases did not show hypermethylation, 9 were malignant and 13 were benign. Although the prevalence of hypermethylation in malignant samples (88.75%) was more than benign (83.75%), the difference between the two groups was not statistically significant (p = 0.123).

-Quantitative Assessment of Hypermethylation of the RASSF1A Gene:

A quantitative assessment of hypermethylation in the mean and ups, and it was rate of alleles whereas in the the difference between the two groups was significant (p = 0.001)

-Investigating Hypermethylation of RASSF1A Gene to Differentiate Tumors:

Qualitative hypermethylation assessment showed that if hypermethylation of the RASSF1A gene was used as a diagnostic test for thyroid cancer, 73 cases of malignancy and 12 cases of benign lesions were correctly diagnosed. However, 7 cases were falsely diagnosed as malignant and 68 cases as benign. The sensitivity and specificity of this test were 91.25% and 15% respectively.However, if a quantitative assessment was considered as a diagnostic criterion, the best borderline for the differentiation of benign tumors from malignant was higher than 40% hypermethylation in the RASSF1A gene, and with this boundary point, 22 malignant lesions and 79 benign lesions were correctly recognized. However, 58 cases of were falsely diagnosed as malignancy and 1 case as benign. Therefore, the sensitivity and specificity of this test were 27.5 and 98.75% respectively. (Table2)

Table 2- Results of Diagnosis of benign and Malignant Tumors Based on Hypermethylation Qualitative and Quantitative Assessment of the RASSF1A Gene.

Variable	Qualitative **No/ %**	Quantitative **No/ %**
True Positive	**73**	**22**
True Negative	**12**	**79**
False Positive	**68**	**1**
False Negative	**7**	**58**
Sensitivity	**91%.25**	**27%.5**
Specificity	**15%**	**98%.75**
Negative predictive Positive predictive value value	**51%.77** **63%.15**	**95%.65** **57%.66**
Accuracy	**53%.12**	**6%.12**

Discussion

The development and progression of thyroid tumors are controlled by tumor suppressor genes and oncogenes, of which only a small number of them have been identified (Soares, Maximo,et al.2003. Dammann, Yoon, et al.2000). The RASSF1A gene is a tumor suppressor gene, in 2000 it was identified that its expression is controlled by hypermethylation. This gene is expressed in a variety of body cells (Dammann, Yoon, et al.2000). Its mutated form significantly reduces the activity of cell growth (Dreijerink, Braga, et al.2001). Its re-expression in a variety of cell lines, such as the prostate and the lung, stops cell growth (Dammann, Yoon, et al.2000.). Its function stops cell cycle progression and prevents accumulation of cyclin D1 (Shivakumar, Minna,et al.2002).Its inactivation has been reported in a variety of tumors including the lung, breast, kidney, prostate, ovary, colon and thyroid and other cancers (Schagdarsurengin, Gimm, et al.2002). Hypermethylation of this gene reduces the expression of its protein (Xing, Cohen, et al.2004). This gene is one of the most common tumor suppressor genes that is usually hyper-methylated in neoplasms (Burbee, Forgacs,et al.2001).In the present study, the qualitative study of hypermethylation of the RASSF1A gene showed that hypermethylation is found in both benign and malignant tumors. The frequency of hypermethylation was higher in benign tumors, but in malignant tumors, the hypermethylation level was higher. These findings confirm the previous similar reports, which state that hypermethylation of RASSF1A, is seen in both benign and malignant thyroid tumors. In a study on thyroid tumors, it was found that the hypermethylation is observed in both benign and malignant tumors (Ruebel, Jin, et al. 2005). The results of this study are consistent with the results of Nakamura et al. Their results showed that hypermethylation of the RASSF1A gene was observed in PTC, ATC and FA tumors with relatively similar frequency (Nakamura, Carney,et al.2005). Similar results were found in another study on thyroid tumors. By assessing 22 cases of benign tumors and 22 malignant thyroid tumors in this study, PTC was observed in 62% of cases, UTC in 77% of cases, goiter in 75% of cases and hyperbolic adenomas of RASSF1A gene also was observed in 70% of cases (Tuttle, Ball, et al.2010). The results of this study showed that hypermethylation of the RASSF1A gene is abundant in both benign and malignant tumors, and it can be concluded that the hypermethylation possibly occurs in the early stages of thyroid tumor, which confirms the results of the previous research (Trovisco, Soares, et al.2006). RASSF1A is a tumor suppressor gene that through hypermethylation, its protein expression probably decreases and cell proliferation increases and the cells progress to the next tumorigenic stage. In the study of hypermethylation on thyroid specimens, there was a significant relationship between hypermethylation of RASSF1A gene and age. That is, with the increase in age, the frequency of hypermethylation was also observed more which is consistent with the results of the present study. In the study of hypermethylation of the RASSF1A gene in breast epithelial cells, it has been shown that age can increase the frequency of hypermethylation in epithelial cells (Sakashita, Mimori, et al.2008). Therefore, the results of this study confirm this view that in older people, suppression of some of the tumor suppressor genes such as RASSF1A by hypermethylation may make them more susceptible to thyroid cancer. The results of this study showed that there was no significant correlation between hypermethylation and age in studied gene that confirms the results of previous research and is consistent with them (Hu,Liu, et al.2006). Given that thyroid cancer occurs more often in women, this difference in incidence should be molecular. In this study, it has been shown that in the examined gene there is no difference in the hypermethylation between the two genders. Therefore, it can be concluded from the results of this study that hypermethylation of the RASSF1A gene probably does not cause a difference in incidence of thyroid cancer in both genders. The results of this study showed that there is no significant relationship between invasion and metastasis in malignant thyroid tumors and hypermethylation of RASSF1A gene, which confirms the results of studies of Schagdarsurengin and Hu (Hu,Liu, et al.2006). The lack of association between hypermethylation of these genes with metastases and invasions can be interpreted genes in the process of metastasis and invasion are involved terminal stages of tumorigenicity and forcing the cells to migrate. However, the gene examined showed hypermethylation in benign tumors, so their probable function in tumorigenesis is in the same early stages (Trovisco, Soares, et al.2006). One of the aims of this study was to investigate the status of hypermethylation of the gene, so it can be used to distinguish benign tumors from malignant tumors. The results of this study showed that qualitative studies could not be used for this purpose. Because hypermethylation of this gene is not limited to malignant thyroid tumors and is abundantly seen in benign tumors, this is one of the weaknesses of the hypermethylation study as a biological marker for cancers diagnosis. By assessing the quantitative hypermethylation level, two groups of benign tumors were better distinguished from malignant ones than qualitative assessment. Because in the examined gene, the level of hypermethylation in the malignant group was higher than that of the benign group, and by defining a borderline for the gene we were able to differentiate some of malignant tumors from benign ones. However, this was not practicable in a number of malignant tumors.

Conclusion

Hypermethylation of the promoter of the RASSF1A gene as a diagnosis test is more sensitive to differential diagnosis of benign tumors from papillary thyroid carcinoma.

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حساسیت و ویژگی DNA متیلاسیون ژن *RASSF1A* در تشخیص افتراقی تومورهای خوش خیم از پاپیلاری کارسینومای تیروئید

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چکیده

سرطان تیرویید شایعترین بدخیمی غدد درون ریز است که در دو دهه اخیر در جهان موارد ابتلا به این سرطان افزایش یافته است. بررسی بر روی وضعیت متیلاسیون پروموتر ژن RASSF1A نشان داده است که در ۳۵٪ تومورهای خوش خیم و بدخیم تیرویید این ژن متیله میشود .از هیپرمتیلاسیون پروموتر ژن RASSF1A به عنوان یک بیومارکرمولکولی تشخیص سلولهای سرطانی در سرطانها تیروییدی ذکر شده است. هدف این تحقیق بررسی و محاسبه حساسیت و ویژگی DNA متیلاسیون ژن *RASSF1A* در تشخیص افتراقی تومورهای خوش خیم از پاپیلاری کارسینومای می باشد.در این مطالعه ۱۶۰نمونه از بیماران با تومورهای بدخیم تیرویید(۸۰نمونه) و تومورهای خوش خیم تیرویید (۸۰نمونه) از بین کل بیماران مراجعه کننده به مراکز درمانی اهواز به این تحقیق وارد شدند، برای بررسی هیپرمتیلاسیون ژن پس از استخراج DNA نمونه های مختلف با استفاده از QIAamp DNA Micro Kit به روشCOBRA انجام شد در نهایت میزان حساسیت و ویژگی این تست از محاسبات اپیدمیولوژیکی استفاده شد.در این تحقیق نتایجی حاصل شد: حساسیت تست هیپر متیلاسیون پروموتر ژن*RASSF1A* به شکل کیفی و کمی *به ترتیب۹۱/۲۵ و ۲۷/۵ درصد،همچنین ویژگی* تست هیپر متیلاسیون پروموتر ژن*RASSF1A* به شکل کیفی و کمی *به ترتیب* ۱۵ و ۹۸/۷۵ درصد محاسبه گردید.نتایج حاصل از این مطالعه نشان داد که : تست هیپر متیلاسیون پروموتر ژن*RASSF1A* بصورت کیفی جهت تشخیص افتراقی تومورهای خوش خیم از پاپیلاری کارسینومای تیروئید *حساسیت خوبی دارد* همچنین شکل کمی همین تست جهت تشخیص افتراقی تومورهای خوش خیم از پاپیلاری کارسینومای تیروئید ویژگی عالی دارد.

واژگان کلیدی: هیپرمتیلاسیون، ژن*RASSF1A* ، پاپیلاری کارسینومای تیروئید،حساسیت،ویژگی.

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