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ORIGINAL ARTICLE

Comparison of the Validity of Quantitative and Qualitative Methods of *RASSF1A* Gene Hypermethylation Molecular Test in the Diagnosis of Thyroid Malignancies

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SUMMARY

Background: **Within the past two decades, the incidence of thyroid cancer has increased across the globe. In the differential diagnosis of benign tumors from malignant tumors in the thyroid gland, hypermethylation of the RASSF1A gene is used. The aim of this study is comparing the validity of quantitative and qualitative methods of the RASSF1A gene hypermethylation molecular test in the diagnosis of thyroid malignancies.**
Methods: **One hundred sixty samples from patients with malignant thyroid tumors (80 samples) and benign thyroid tumors (80 samples) were entered into this study from all patients referred to Ahvaz Medical Centers. First DNA was extracted from samples embedded in paraffin. After DNA extraction, hypermethylation of the gene was done by COBRA method. Finally, to calculate the effects of sensitivity, specificity, predictive value and accuracy of the test, epidemiological calculations were done.**
Results: **The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of the qualitative method of the RASSF1A gene hypermethylation molecular test in the diagnosis of thyroid malignancies are: 91.25%, 15%, 51.77%, 63.15%, and 53.12%, respectively and for the qualitative method are: 27.5%, 98.75%, 95.65%, 57.66%, and 6.12%, respectively.**
Conclusions: **The quantitative level of hypermethylation differentiated groups of benign tumors from malignant groups better than the qualitative evaluation but the qualitative studies are not applicable for this purpose.**
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INTRODUCTION

Thyroid cancer is the most common endocrine malignancy, which has increased in the world in the last two decades [1]. In the United States, the annual increase in the incidence of thyroid cancer between 2000 and 2009 is 6 percent, the highest among all cancers [1]. Although the mortality rate of this cancer is low, the rate of recurrence or treatment cycle is pretty high, leading to an increase in disability in treatment and mortality [2]. According to statistics given by the Cancer Institute of Iran, thyroid cancer is the seventh most common can-

cer in women and 14th in men and 11th in both sexes [3]. The 10-year survival of thyroid cancer in middle-aged adults is 80 - 96%. Recurrence occurs in 5 to 20% of thyroid papillary carcinomas. Recurrence may be due to incomplete initial treatment or due to a tumor with highly invasive cells [4]. The risk of malignancy in FNA (Fine Needle Aspiration) suspected of cancer specimens is reported to be between 10% and 40%. Therefore, all patients will require suspected nodule resection for more accurate diagnosis by examining pathologic biopsy specimens, and only about 20% of these suspected specimens will have malignant neoplasm [5]. On the other hand, in 10% of FNA cases, false-negative results are reported, leading to late diagnosis of cancer and consequently late treatment, which negatively affects the course of the disease [5].

RASSF1A Gene (Ras association domain family 1), also called NORE2A, is on chromosome 3 at 3p21.3. This gene encodes a protein that resembles the RAS effector proteins [6]. The change of gene expression is associated with the pathogenesis of various types of cancers that suggest that this gene is a tumor suppressor. One type of inactivation of this gene is the hypermethylation of CpG islands in its promoter. The produced protein reacts with the DNA repair system proteins such as XPA [6]. It also prevents the accumulation of cyclin D1, thereby stopping the cell cycle [7]. Examination of the promoter methylation status of the RASSF1A gene has shown that it is methylated in 35% of benign and malignant thyroid tumors [8]. In a study, promoter hypermethylation of this gene was reported in cancers, including thyroid cancer, and was cited as a biomolecular marker that enables the detection of cancer cells [9]. RASSF1A hypermethylation has also been observed in the blood and body fluids of people with certain malignancies, such as thyroid cancer and can, therefore, be used as a biomarker in the early detection of thyroid cancer [9].

This study compares the validity of quantitative and qualitative methods of the RASSF1A gene hypermethylation molecular test in the diagnosis of thyroid malignancies.

MATERIALS AND METHODS

The sample size in this study was 160 cases, 80 patients with malignant thyroid tumors and 80 patients with benign thyroid tumors were included in this study. Paraffin blocks were re-examined by a pathologist and the most suitable ones were selected. Three specimens with no benign or malignant lesions reported in the pathology report were selected as normal tissue. For the microdissection, 6-micron cuts were first made from paraffin blocks by a microtome. Two slides from each block were used for DNA extraction and one slide was stained with hematoxylin-eosin (Sigma) to be used as a guide to identify the range of cells at the time of microdissection. Specific slides for DNA extraction were stored at room temperature. Then, by comparison between slides stained with hematoxylin-eosin and slides stained with methylene green and with the help of laser microdissection device (LEICA) or with stereomicroscope and razor blade, the required cells were cut from the surface of the slides and placed in 1.5 µL microtubes and stored at -80°C until DNA extraction. QIAGEN company's QIAamp DNA Micro Kit was used to extract DNA from paraffin samples. The phenol and chloroform method was used to extract DNA from fresh tissue. In 1997, for the first time, the Combined Bisulfit Restriction Analysis (COBRA) method was developed for the quantitative evaluation of hypermethylation. In this method, after DNA treatment with bisulfite and performing PCR, its products are subjected to enzymatic cleavage. According to the initial basic row of DNA, this enzyme is selected to perform cleavage only in the CpG islands with hypermethylation. The extent of hypermethylation in the primary DNA is proportional to the number of PCR products [10]. If CpG in the primary DNA lacks hypermethylation, it becomes thymine due to the cytosine bisulfite treatment and the cleavage site of the enzyme is destroyed and the cleavage will not be performed. If there is a mixture of methylated and nonmethylated alleles in the primary DNA at the time of enzymatic digestion, the amount of cleavage depends on the amount of methylated CpG. Then, hypermethylation quantity was calculated by measuring the concentration of bands in gel (using ImageJ software). The percentage of hypermethylation was calculated based on the formula X 100% Methylation = C / (C + B) where B is the density of the product bands before cleavage and C is the density of the enzymatic cleavage (Figure 1).

In the study of hypermethylation of DNA, Millipore Company's CpGenome™ Universal Methylated DNA was used as a positive control. In this DNA all CpG islands have hypermethylation. Peripheral blood lymphocyte DNA (PBL) was also used as a negative control. EZ DNA Methylation Kit™ kit (Zymo Reaserch, CA, USA) was used for the treatment of sulfite.

The primers required for RASSF1A hypermethylation were designed using the Primer 3 website (Table 1). First, DNA sequences in exon 1 of these genes were inserted into the Meth Primer website and CpG islands were identified in this region. Then a basic row of DNA was obtained after treatment with bisulfite (according to the methylation kit guide). An enzyme cleavage site containing a CpG was then selected and the necessary primers were designed around the site. Because DNA samples were obtained from paraffin-embedded tissues, the goal was to obtain a PCR product with size less than 90 bp.

The three enzymes used for the investigation of hypermethylation had a cleavage site as follows. Taq1 enzyme (TCGA), RsaI enzyme (GTAC), BstUI enzyme (CGCG). Temperature conditions and duration of treatment were based on the recommendation of the manufacturer. SPSS software version 16 was used for statistical analysis. Chi-square test was used to investigate the relationship between variables such as age group, gen-

der, aggression, and metastasis with hypermethylation qualitative status. In certain cases, Fisher's exact test was used for this purpose. *t*-test was used to investigate the possible relationship between the quantity of hypermethylation and mRNA expression with variables such as age, gender, invasion, and metastasis. In all tests, the p-value was 0.05. The following formulas were used to calculate Sensitivity, Specificity, Positive Predictive Value, Negative Predictive Value, and Accuracy. In these calculations, the pathology reports were considered as the standard test, in which the truth or falseness of hypermethylation results was compared with pathological reports. (True Positive (TP): The patient is diagnosed correctly. False Positive (FP): Healthy person, mistakenly diagnosed as a patient. True Negative (TN): Healthy person, properly diagnosed healthy. False Negative (FN): The patient is mistakenly diagnosed as healthy).

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

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

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

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

$$\boldsymbol{Accuracy}(\%) = \frac{\boldsymbol{TP + TN}}{\boldsymbol{TP + TN + FP + FN}} \times \mathbf{10}$$

RESULTS

Results of descriptive information of patients

In the current study, 160 paraffin blocks of archival specimens from 2006 to 2016 were collected from pathological laboratories of Ahvaz Medical Centers after being reviewed by a pathologist. The specimens consisted of 80 malignant tumors and 80 benign tumors including 40 goiter, 13 Hashimoto's thyroiditis, and 12 follicular adenomas, which were investigated in the present study. Also, 23 specimens of fresh thyroid tissue including 4 cases of CV-PTC tumors and 19 cases of benign tumors were collected directly from the operating rooms during the project. Table 2 provides information on the range of gender, age, and histologic type of tumors (Table 2).

Evaluation of the association of RASSF1A gene hypermethylation with tumor type

Evaluation of hypermethylation in 160 cases of benign and malignant thyroid tumors revealed 138 cases of positive hypermethylation, 67 of these were benign and 71 were malignant. Nine cases of the remaining 22 samples that did not show hypermethylation but were malignant and 13 cases were benign (Table 2). Although the frequency of hypermethylation in malignant specimens (88.75%) was more than benign ones (83.75%), the difference between the two groups was not statistically significant ($p = 0.123$).

Quantitative evaluation of RASSF1A gene hypermethylation

Quantitative evaluation of hypermethylation in the RASSF1A gene was performed as average and standard deviation of different groups. Hypermethylation alleles were found to be 28.28% in malignant tumors whereas in benign tumors it was 11.40%. The difference between the two groups was significant ($p = 0.01$).

Evaluation of RASSF1A gene hypermethylation in the differentiation of tumors

A qualitative study of hypermethylation showed that if RASSF1A gene hypermethylation was used as a diagnostic test for thyroid cancer showed 73 malignant and 12 benign cases were diagnosed correctly. However, 7 malignant and 68 benign were diagnosed falsely. The sensitivity and specificity of this test were 91.25 and 15%, respectively (Table 3).

However, if the quantitative evaluation was considered as a diagnostic criterion, the best cutoff point for distinguishing benign from malignant tumors was over 40% hypermethylation in the RASSF1A gene, and with this boundary point, a total of 22 malignant and 79 benign cases were diagnosed correctly. However, 58 malignant and one benign case were diagnosed falsely. Therefore, the sensitivity and specificity of this experiment were 27.5% and 98.75%, respectively (Table 3).

DISCUSSION

The evolution and development of thyroid tumors are controlled by oncogenes and tumor suppressor genes, of which only a few have been identified so far [11,12]. The RASSF1A gene is a tumor suppressor gene that was identified in 2000, its expression is controlled by hypermethylation. This gene is expressed in different types of body cells [13]. Its mutated form significantly decreases cell growth arrest activity [14]. Its re-expression in a variety of cell lines, such as the prostate and lung, inhibits cell growth [13,14]. Its function is to inhibit cell cycle progression and prevent cyclin D1 accumulation [7]. Its inactivation has been reported in a variety of tumors including lung, breast, kidney, prostate, ovary, colon, thyroid gland, and other cancers [13,15]. Hypermethylation of this gene decreases its protein expression [13,16,17]. This gene is one of the most common tumor suppressor genes that is typically encountered in the hypermethylation in neoplasms [13,18].

In the present study, qualitative evaluation of RASSF1A gene hypermethylation showed that it was found in both benign and malignant hypermethylation tumors. Hypermethylation was more frequent in benign tumors, but in malignant tumors, the quantitative level of hypermethylation was slightly higher. These results are con-

Table 1. Base row, product length, and enzyme used to evaluate hypermethylation using the COBRA method.

Gene	Basic row of primers	Product length	Length after cleavage	Enzyme
RASSF	F: GGTTYGYGTTTGTTAGYGTTTAAAGTT R: CTCAAACTCCCCCRACATAA	70	35 - 35	RsaI

Table 2. Average and standard deviation of the age of diagnosis (year) and gender frequency in patients with benign and malignant thyroid tumors by histopathological classification (n = 183).

Tumor type		Histopathology type	Gender (number)		Age distribution	Mean ± SD (min - max)
Paraffinic	malignant (n = 80)	CV-PTC	32	18	41.20 ± 21.17	15 - 82
		TC-PTC	6	6	48.01 ± 39.18	21 - 78
		FV-PTC	1	5	13.00 ± 0.00	13 - 13
		UTC	6	6	71.51 ± 18.19	58 - 85
	benign/natural (n = 80)	HT	12	1	44.33 ± 11.22	29 - 56
		FA	10	2	56.41 ± 15.78	38 - 83
		GT	35	5	46.74 ± 14.87	22 - 68
		N	9	6	49.33 ± 26.99	22 - 72
Fresh tissue	malignant (n = 4)	CV-PTC	4	0	47.00 ± 12.16	28 - 57
	benign/natural (n = 19)	HT	1	0	42.00 ± 0.00	42 - 42
		FA	5	0	38.27 ± 13.42	26 - 55
		GT	10	1	38.82 ± 13.68	25 - 65
		N	1	1	38.52 ± 22.93	23 - 56

CV-PTC - Classic Variant Papillary Thyroid Carcinoma, TC-PTC - Tall cell variant papillary thyroid carcinoma, FV-PTC - follicular variant papillary thyroid carcinoma, UTC - Undifferentiated thyroid carcinoma, HT - Hashimato's thyroiditis, FA - Follicular adenoma, GT - goiter, N - Normal.

Table 3. Accuracy of diagnosis of benign and malignant tumors based on qualitative and quantitative evaluation of RASSF1A gene hypermethylation.

Variable	Qualitative results of hypermethylation	Quantitative results of hypermethylation
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%
Positive predictive value	51.77%	95.65%
Negative predictive value	63.15%	57.66%
The accuracy of the test	53.12%	6.21%

sistent with previous reports that have suggested that RASSF1A hypermethylation is observed in both benign and malignant thyroid tumors. In a study by Xing et al. [16] on thyroid tumors, it was found that hypermethylation was observed in both benign and malignant tumors. The results of this study are in agreement with the re-

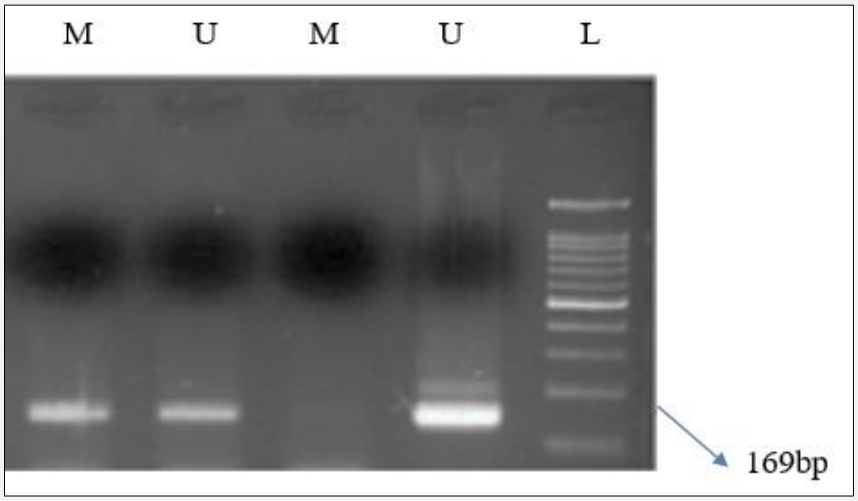

Figure 1. Results of the RASSF1A hypermethylation analysis.

The results of MS-PCR analysis for the RASSF1A gene are methylated and non-methylated in the M and N bands. As intended, the end band (L) was a marker of 100 bp molecular mass.

sults of Nakamura et al. study [8]. In which, hypermethylation of the RASSF1A gene was observed in PTC, ATC, and FA tumors with relatively similar frequency. Also, similar results were obtained in another study of thyroid tumors. In that research, studying 22 benign and 22 malignant thyroid tumors, it was found that hypermethylation of the RASSF1A gene was observed in 62% of PTC, 77% of UTC, 75% of goiter, and 70% in follicular adenoma patients [2]. The results of this study showed that in both groups of benign and malignant tumors, RASSF1A gene hypermethylation is abundant. Therefore, it can be concluded that hypermethylation most likely occurs in the early stages of thyroid tumorigenesis, which confirms the results obtained in previous studies [8,19]. RASSF1A is a tumor suppressor gene which, when hypermethylated, possibly reduces protein expression and increases cell proliferation; therefore, cells progress towards the next stages of tumorigenesis. In the study of hypermethylation by Schagdarsurengin and his colleagues on thyroid samples, there was a significant relationship between hypermethylation of the RASSF1A gene and age. That is, with an increase in age the frequency of hypermethylation was seen more [2], which is in agreement with the results of the present study. Studies on hypermethylation of the RASSF1A gene in breast epithelial cells have shown that age can increase the frequency of hypermethylation in epithelial cells [20]. Therefore, the results of this study support the theory that in elderly people, probably by suppressing some tumor suppressor genes such as RASSF1A by hypermethylation, they are more susceptible to thyroid cancer.

The results of this study showed that in the studied gene, there was no significant relationship between hypermethylation and gender of the patients, which confirms the results of previous studies and is consistent with them [21]. Since thyroid cancer occurs more frequently in women, this difference should have a molecular basis. This study showed that there was no difference in hypermethylation between the two sexes in the studied gene, so it can be concluded from the results of this study that hypermethylation of the RASSF1A gene is not a difference factor in thyroid cancer outbreak in both sexes.

CONCLUSION

The results showed that there was no significant relationship between invasion and metastasis in malignant thyroid tumors with RASSF1A gene hypermethylation, which confirms the results of Schagdarsurengin and Hu studies [2,21]. The lack of association between hypermethylation of these genes with metastasis and invasion can be interpreted as the genes involved in the process of metastasis and invasion are involved in the late stages of tumorigenesis, forcing the cells to migrate. But the genes examined in the present study also showed hypermethylation in benign tumors, so their likely function in tumorigenesis is in the early stages [8,19].

One of the aims of this study was to differentiate benign and malignant tumors by investigating the status of gene hypermethylation.

The results of this study showed that qualitative studies are not applicable for this purpose. Because hypermethylation of this gene is not only specific for malignant thyroid tumors and is abundantly seen in benign tumors, this is one of the weaknesses of investigating hypermethylation as a biomarker in the diagnosis of cancers. By examining the quantitative level of hypermethylation, groups of benign tumors were differentiated from malignant groups better than qualitative evaluation. Because in the studied gene, the quantitative level of hypermethylation was slightly higher in the malignant group than in the benign group and by defining a cutoff point for the gene, we were able to differentiate some malignant tumors from benign. However, in some malignant tumors, this was not practical.

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Declaration of Interest:

The authors declare that they have no conflicts of interest.

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