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Long noncoding RNA HOTAIR and Midkine as biomarkers in thyroid cancer

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Abstract

Thyroid cancer is the most common endocrine malignancy, and its incidence is increasing. Differentiated thyroid cancer is the most common type and papillary thyroid carcinoma is the most common type of differentiated thyroid cancer. This work aimed to study long noncoding (Lnc) RNA homeobox transcript antisense RNA (HOTAIR) expression in plasma and serum midkine, a heparin binding growth factor, as biomarkers of thyroid cancer. This study included 27 thyroid cancer patients, 29 patients with benign thyroid disease and 26 individuals as normal controls. HOTAIR expression was assessed by real time polymerase chain reaction and midkine by ELISA. These biomarkers were elevated in thyroid cancer patients than patients with benign thyroid diseases and controls. Patients with thyroid cancer stage III had higher midkine levels in comparison to those with stage-I and stage-II (p<0.001). Patients with grade II had higher midkine in comparison to those with grade I (p<0.001). Statistically significant elevation of HOTAIR expression was found in stage III and stage II (p=0.001), compared to stage I. However, no difference was observed between stage II and stage III (p=0.533). There was no difference in both biomarkers in different histopathological types of thyroid cancer. ROC analysis was used for detection of thyroid cancer, midkine had AUC of 0.95 at a cutoff 897.5 pg/ml with a sensitivity of 98.0%, and specificity of 81.5% (p<0.001). HOTAIR had AUC of 1 at a cutoff 11.8-fold change with a sensitivity and specificity of 100 %, (p<0.001). We concluded that HOTAIR has high sensitivity and specificity in detection of thyroid cancer. It was correlated with tumor stage but not with histopathological types.

Keywords: Thyroid cancer, HOTAIR, midkine.

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Introduction

Thyroid cancer is the most common malignant disease in endocrine system and is rapidly increasing in incidence.[1] and it is the fifth most common cancer in women.[2] In Egypt, thyroid cancer represents about 1.5% of all cancers and constitutes about 30% of endocrine malignancies, the rate among Egyptian females is 0.0027%. These epidemiological data suggest a role of estrogen in the pathogenesis of thyroid diseases.[3]

Midkine is a heparin-binding growth factor, was originally reported as the product of a retinoic acid-responsive gene during embryogenesis.[4] Increased midkine expression

has been reported in many types of malignancies. Midkine is thought to contribute to tumor development and progression by enhancing the growth, survival, migration, epithelial-mesenchymal transition (EMT), and angiogenic activity of tumors.[5] Because of such a wide range of cancer-related biologic activities and the antitumor effect after midkine inhibition, midkine has been suggested to be a good molecular target for cancer monitoring.[6]

Long noncoding RNAs (LncRNAs) represent a class of noncoding RNAs that are longer than 200 nucleotides without protein-coding potential and function as novel master regulators in various human diseases, including cancer.[7] An increasing number of studies have documented that lncRNAs play diverse roles in regulating gene transcription, post-transcription, translation, and epigenetic modification.[8,9] Furthermore, a link between aberrant expression of lncRNAs and human diseases was reported.[10] Homeobox (HOX) transcript antisense RNA (HOTAIR) is an example of lncRNA that has *trans*-regulatory function. [(11)] HOTAIR is closely associated with the onset of cancers such as breast cancer, liver cancer, colorectal cancer, laryngeal cancer, and nasopharyngeal carcinoma.[12]

HOTAIR is expressed by the homeobox C gene (HOXC) locus, located in the HOXC cluster between the HOXC11 and HOXC12 genes on human chromosome 12q13.13.[13] Increasing evidence suggests that HOTAIR is strongly associated with different types of cancer.[14] It has been reported to promote papillary thyroid carcinoma (PTC) proliferation, invasion, and migration. HOTAIR plays a critical role in various areas of cancer, such as proliferation, survival, migration, drug resistance, and genomic stability.[15] HOTAIR expression inhibits thyroid cancer cell growth *in vivo* and *in vitro.*[16] Functional investigations showed that miR-1 can decrease cell proliferation and migration in papillary, follicular and anaplastic thyroid cancer tissues via targeting cyclin D2 (CCND2) which belongs to the conserved cyclin family, and controls the cell cycle. HOTAIR enhances thyroid cancer (TC) cell proliferation, invasion, and migration through the miR-1/CCND2 axis.[17]

This study aimed to evaluate the utility of HOTAIR and midkine in diagnosis of thyroid cancer and their relation to tumor stage and histopathological types.

Subjects and Methods

The study protocol was reviewed and approved by the Institutional Review Board of the Faculty of Medicine, Assiut University (dated April 2018). A written informed consent was obtained from each participant before enrollment. This study included 56 patients who were presented to the endocrine outpatient clinic with thyroid enlargement (malignant or benign) during the period from April 2021 to April 2022, and 26 control subjects. All patients' data were recruited from the General Surgery Department, Assiut University Hospital and Surgical Oncology Department, South Egypt Cancer Institute, Assiut University.

Diagnosis of thyroid cancer depended on fine-needle aspiration cytology (FNAC), tumor staging was based on TNM system (tumor size, regional lymph nodes and distant metastasis) according to The American Joint Committee of Cancer staging system.[18] Body mass index was calculated according to this formula: weight (kg)/(height (m)^2. The laboratory work and interpretations were carried out at Clinical Pathology Department, Assiut University Hospital.

Subjects

Subjects were classified into 3 groups: Group I, included 27 patients (7 males and 20 females) with thyroid cancer. They were subclassified according to TNM (tumor, node, metastasis) staging system into stage I (14 patients), stage II (4 patients) and stage III (9 patients). Thyroid cancer patients were classified according to the histopathological type of thyroid cancer into papillary thyroid carcinoma (20 patients) and follicular thyroid carcinoma (7 patients). Group II included 29 patients (one male and 28 females) with benign thyroid lesions. Group III included 26 apparently healthy individuals (19 females and 7 males) who were selected as a control group for comparison.

Patients with undifferentiated thyroid cancer and those already received any type of treatment such as chemotherapy, radiotherapy, or surgical treatment or those with history of benign or malignant tumors in other organs, were excluded from the study.

Blood samples

A blood sample (2 ml) was collected for separation of serum, used in assessment of serum midkine. Another blood sample (2 ml) was collected into ethylene-diamine tetra acetic acid (EDTA) coated tube, centrifuged at approximately 2200 xg for 10 min, and then plasma was carefully transferred into an RNase–free tube for extraction of RNA. Plasma was stored at -80 °C until used.

Methods

A commercial ELISA kit (Human Midkine (MK) ELISA Kit, catalog No: SG-10623 provided by Sinogene, China) was used for determination of serum midkine, according to the manufacturer's instructions. Results were determined using a microtiter plat reader (EVOLIS, BIORAD, France).

Relative expression of plasma HOTAIR was carried out by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) using a 7500 fast real time PCR machine (Applied Biosystems, USA). The first step was extraction of RNA from stored plasma samples, using the commercial miRNeasy Mini Kit (Cat. No. 217004; Qiagen, Germany), according to the manufacturer's instructions. Then RNA reverse transcription into complementary DNA (cDNA) was done using the commercial RTII lncRNA kit, (Cat. No. 330404; Qiagen - USA), according to the manufacturer's instructions. Then cDNA amplification and detection were done using the RTII lncRNA qPCR assay kit (Catalog Number: 330710, QIAGEN, USA) and the RTII SYPER Green ROXTM qPCR Mastermix 2 kit (Catalog Number: 330520, QIAGEN, USA), according to the manufacturer's instructions. The qRT-PCR used the following primer set: F: GGCGGATGCAAGTTAATAAAAC; and R: TACGCCTGAGTGTTCACGAG. The glyceraldehyde 3phosphate dehydrogenase (GAPDH) was used as an internal control to normalize RT-qPCR readout.

The 7500 fast real time PCR machine was programmed according to the following conditions: incubation period at 95°C for 15 min as a preliminary activation step for DNA polymerase, followed by 40 amplification cycles, each cycle included three consecutive steps of DNA denaturation at 94°C for 15 sec., annealing at 55°C for 30 sec., and extension at 70°C for 30 sec. Fluorescence measurement was made at every cycle.

The expression levels of the investigated long noncoding RNA were evaluated using ΔCt method (Livak method for relative gene expression analysis). The results were expressed as fold changes compared to the control sample, considered the normal value, and assumed to equal 1.

Delta-Delta method for comparing Relative Quantitation results in Real-time PCR

$\Delta Ct\ Sample = Ct_{\ hotair} - Ct_{\ GPDH}$
$\Delta Ct\ Control = Ct_{\ hotair} - Ct_{\ GPDH}$
$\Delta\Delta Ct\ Sample = \Delta Ct\ Sample - \Delta Ct\ Control$
Relative quantitation (Fold Change, FC) of sample = $2^{-\Delta\Delta Ct}$
Relative quantitation (Fold Change, FC) of control $_{Mean} = 1$.

Statistical analysis

All statistical calculations were done using SPSS (statistical package for the social science; SPSS Inc., Chicago, IL, USA) version 22. Data were statistically described in terms of mean ± standard deviation (±SD), or median and range when not normally distributed, frequencies (number of cases) and relative frequencies (percentages) when appropriate. One Way ANOVA test with post-hoc analysis was used for comparing more than two groups. Correlation between various variables was done using Pearson correlation test. Receiver Operating Characteristic Curve (ROC) analysis was used to evaluate the diagnostic performance of midkine and HOTAIR for diagnosis of thyroid cancer. A p value ≤ 0.05 was considered significant.

Results

The demographic data of the studied participants are reported in Table 1. Patients

with thyroid cancer had significantly higher serum midkine levels and higher plasma HOTAIR expression in comparison to those with benign thyroid diseases (p<0.001) and the control group (p<0.001). Also, patients with benign thyroid diseases had significantly higher midkine levels in comparison to the control group (p<0.001). But no difference was found in plasma HOTAIR expression in comparison of patients with benign thyroid lesions and the control group (p=0.974; Table 2).

Table 1. Demographic data of the studied groups.

Variable name	Malignant Group I (n=27)	Benign Group II (n=29)	Controls Group III (n=26)	*p*-value*
Age (years)				
Mean ± SD	42.30±7.79	39.83±12.51	37.77±6.99	NS
Range	25 – 66	19 – 63	25 – 55	
Sex				
Male	7 (25.9%)	1 (3.4%)	7 (26.9%)	0.020
Female	20 (74.1%)	28 (96.6%)	19 (73.1%)	
BMI (kg/cm^2)				
Mean ± SD	32.88±3.14	25.88±3.69	22.85±2.65	0.000
Range	29 – 40	19 – 33	19 – 28	
p value**				
I v II		0.000		
I v III		0.000		
II v III		0.003		

* ANOVA test was used to compare mean difference between groups. ** Post-hoc with bonferroni corrections Quantitative data are presented as mean ± SD, qualitative data are presented as number (percentage), p > 0.05 is not significant (NS).

Table 2. Serum midkine and plasma HATAIR among the studied groups.

	Malignant Group I	BenignGroup II	Control Group III	*p* value
Midkine (pg/ml)				
Mean ±SD	1039.82±180.67	805.88±57.32	556.73±119.67	0.000
Range	757.0 – 1378.0	726.0 – 928.5	329.5 – 726.0	
p value**				
I v II		0.000		
I v III		0.000		
II v III		0.000		
HOTAIR (fold change)				
Mean ± SD	49.40±27.82	1.99±1.80	1.01±0.34	
Median	39	1.75	0.9	0.000
Range	15.0 – 120.0	0.19 – 8.6	0.45 – 1.70	
p value**				
I v II		0.000		
I v III		0.000		
II v III		NS		

* ANOVA test was used to compare mean difference between groups. ** Post-hoc with bonferroni corrections. Quantitative data are presented as mean ± SD. * P > 0.05 is not significant (NS).

Serum midkine levels and plasma HOTAIR expression were statistically significantly higher in patients with stage III thyroid cancer compared to those with stage I and stage II (p<0.001). Also, patients with stage II thyroid cancer showed significantly higher levels of serum midkine than those with stage I (p<0.001). However, no difference was found in plasma HOTAIR expression between stage II and stage III (p=0.533), as shown in Table 3. There was no difference in the serum level of midkine and plasma HOTAIR expression in different histopathological types of thyroid cancer cases, papillary and follicular thyroid carcinoma (p=0.699 and p=0.952, respectively), as shown in Table 4.

Table 3. Serum midkine and plasma HOTAIR in thyroid cancer stages.

	Stage I N = 14	Stage II N = 4	Stage III N = 9	*p* value	III vs I	II vs I	III vsII
S. midkine (pg/ml)							
Mean ± SD	889.68±61.79	1063.00±45.06	1263.06±57.7	0.000*	0.000**	0.000**	0.000**
Range	757 – 954	1022 – 1126	1170 –1378				
Plasma HOTAIR (fc)							
Mean ± SD	29.30±8.33	68.19±15.43	78.87±25.19	0.000*	0.000**	0.000**	NS**
Range	15 – 42.97	54.5 – 89.5	51.5 – 120				

* ANOVA test was used to compare mean difference between groups. ** Post-hoc with bonferroni corrections. Quantitative data are presented as mean ± SD. * P > 0.05 is not significant (NS).

Table 4. Serum midkine and plasma HOTAIR in histopathological types of thyroid cancer.

	Histopatholoy		
	Papillary thyroid carcinoma (n= 20)	follicular thyroid carcinoma (n= 7)	*p* value*
Midkine			
Mean ± SD	1040.55 ± 189.27	1037.71 ± 167.33	NS
Range	757 – 1378	836 – 1272	
HOTAIR			
Mean ± SD	49.22 ± 27.83	49.87± 30.00	NS
Range	36.6 (16.5 – 120.0)	40 (15 – 89.5)	

Quantitative data are presented as mean ± SD and range. * P > 0.05 is not significant (NS).

The ROC curve analysis was performed to assess the diagnostic performance of serum midkine and plasma HOTAIR for detection of thyroid cancer. For midkine, at a cutoff 897.5 pg/ml and area under the curve (AUC) of 0. 95, the sensitivity was 81.5%, and specificity 98.0%, (p<0.001). Regarding HOTAIR, at a 11.8 FC; AUC was 1 with a sensitivity of 100.0%, and specificity of 100.0%, (p<0.001), as shown in Figure 1.

A strong significant positive correlation was found between both studied biomarkers (HOTAIR and midkine) (r=0.824, p<0.0001), as shown in Figure 2. Also, a strong significant positive correlation was found between HOTAIR and body mass index (BMI) (r=0.648, p<0.0001), as shown in Figure 3.

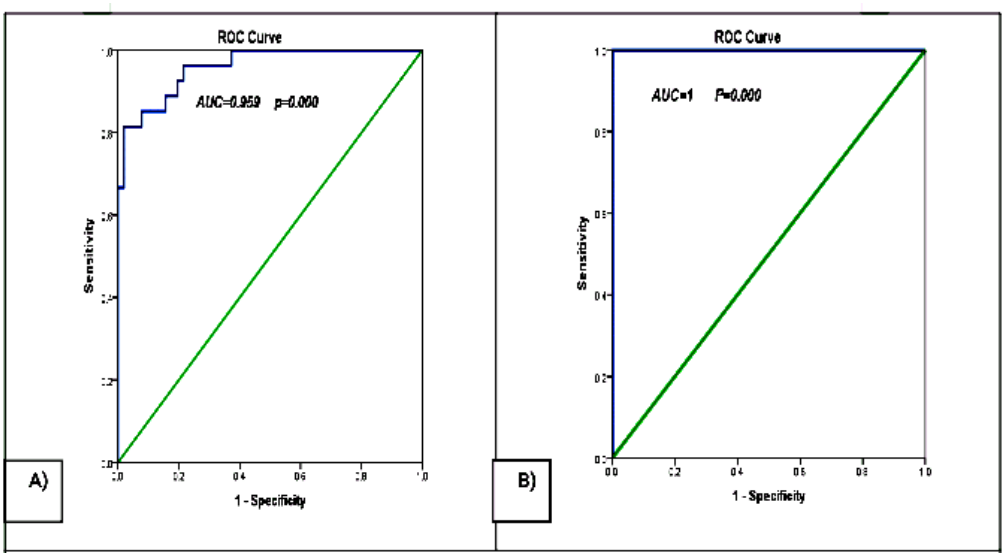

Figure 1. ROC curves for detection of thyroid cancer in studied participants. A) Midkine (blue) and Reference line (green, AUC 0.959, p< 0.0001.) B) HOTAIR (blue) and Reference line (green), AUC = 1, *p*<0.0001.

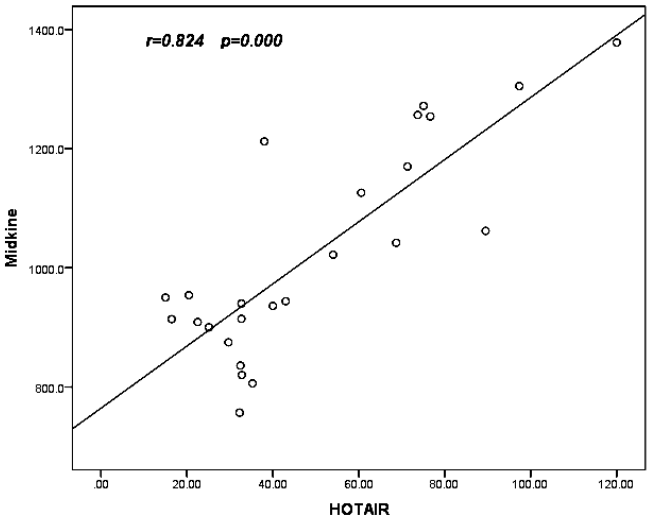

Figure 2. Scatter dot diagram showing the correlation between HOTAIR and midkine.

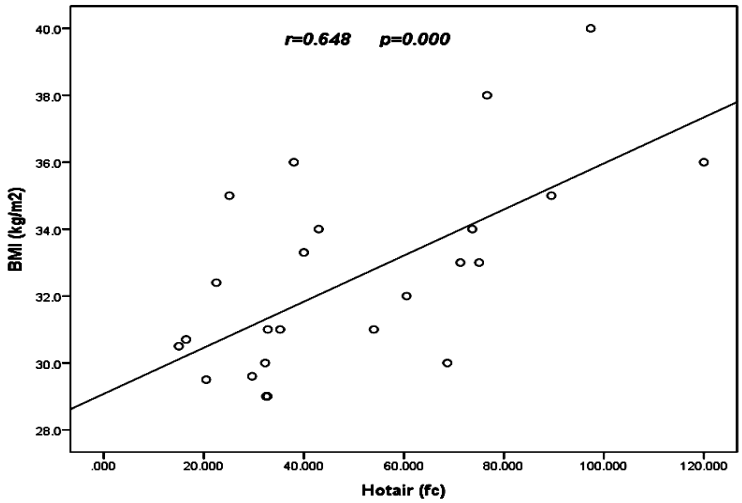

Figure 3. Scatter dot diagram showing the correlation between HOTAIR and BMI

Discussion

TC is the most common endocrine malignancy, accounting for 3.4% of all cancers diagnosed annually.[2] In the past ten years, the incidence of thyroid cancer has been increasing yearly, and it has become the fourth highest in women. Part of the reason might be due to the rapid development of imaging detection technologies and continuously increasing awareness of people's health.[20]

The vast majority (85–95%) of thyroid nodules are benign. For this reason, the ability to distinguish between benign and malignant nodules is important to separate preoperative patients from those with no need or unnecessary surgery.[21] Moreover, evidence suggested that for patients with indeterminate thyroid biopsy results, a combined assessment including the initial ultrasound risk stratification or molecular markers, can further clarify the risk of thyroid cancer and the management strategies.[22]

The present work aimed to evaluate the role of LncRNA HOTAIR and midkine (MK) as noninvasive biomarkers in differentiating malignant from benign thyroid nodule. Also, this work aimed to study their relation to staging and histopathological types of thyroid cancer. The current study included 27 thyroid cancer patients, 29 patients with benign thyroid lesions and 26 apparently healthy individuals as a control group. Patient groups (malignant and benign) had female predominance. This may be attributable to increased incidence of thyroid disease among females, which occurs 2–4 times in females than in males.[23] In a previous study by Shobab et al., 2022,[24] it was reported that TC was the only nonreproductive cancer with striking female predominance with three- to four-fold higher incidence among females.

Sex hormones mediate effects by specific nuclear receptors for gene expression and regulation of tumor cell biology. Therefore, polymorphism of the estrogen receptor may be a risk factor for differentiated thyroid cancer (DTC).[19] While α-estrogen receptor density in PTC tumor cells is low, physiologic estrogen stimulation accounts for significant upregulation and increase of cell proliferation.[19] Estrogen is associated with cell adhesion, invasion, and migration in thyroid carcinoma cell lines, effects that are reversible with use of estrogen antagonists.[25]

PCT is the predominant form of thyroid cancer accounting for 80-85% of all thyroid cancer cases.[26] PTC is a major differentiated adenocarcinoma which consists of 90% of thyroid cancers.[27] Papillary adenocarcinoma is the most common histopathological type and accounts for 50-60% of-all thyroid cancers. In women, this tumor occurs three times more often than in men.[28]

In the present study, we found that BMI was significantly higher in patient with TC than those with benign thyroid diseases and the control group. In 2016, the International Agency for Research on Cancer (IARC) reported an increased risk of TC with every 5 kg/m2 increase in BMI.[29] Previous studies have confirmed that obesity and BMI are positively correlated with the increased risk of thyroid cancer.[30, 31]

In obesity, the release of proinflammatory factors from adipose tissue increases, while the release of adipokines decreases. Proinflammatory factors act as signal mediators in tumor growth and progression. As the adipose tissue increases, leptin synthesis increases, and the chronic inflammation in enlarged adipose tissue augments the secretion of the cytokines IL-6 and TNF, which contribute to cancer development, growth, progression, and metastasis.[32]

Aberrant expression of leptin and/or its receptor has been found in a variety of malignancies including thyroid carcinoma. In vitro studies have shown that leptin modulates the growth, proliferation, and invasion of thyroid carcinoma cell lines via activation of various signaling pathways such as Janus kinase/signal transducer and activator of transcription, phosphoinositide 3-kinase/protein kinase B/Akt, and/or mitogen-activated protein kinase (MAPK).[33] We also found a positive correlation between BMI and MK which is in line with those reported by Gebur and Ali (2021).[34]

In the present study, serum MK was found to have statistically significant higher level in patients with malignant thyroid nodules than in

patients with benign thyroid nodules and the studied controls Also, patients with benign thyroid disease had significantly higher MK in comparison to the control group. These results are consistent with previous studies, which reported that serum MK was significantly higher in patients with TC compared to patients with benign lesions and normal controls.[6, 34, 35] Kuzu et al., 2016[5] found that both serum MK and nodular MK levels were higher in malignant nodules compared with benign nodules. Also, they found that serum MK and nodular MK levels were higher among patients with suspicious ultrasound features for malignancy. MK promotes tumor cell proliferation, transformation, and epithelial-to-mesenchymal transition (EMT). It has angiogenic, mitogenic, antiapoptotic, and antitumor immunity roles, and it has also been involved in chemoresistance of tumors.[36]

In our study there was no significant difference in the mean value of midkine levels regarding the type of pathology of thyroid cancer among studied cases. Based on stages of thyroid cancers, serum midkine was found to have statistically significant higher levels in patients with stage III thyroid cancer compared to those with stages I and II. Also, patients with stage II thyroid cancer had statistically significant higher midkine in comparison to those stage I. Previous studies found that MK expression significantly differed according to TNM staging and a high level of midkine expression has been associated with advanced tumor stage and a poor prognosis.[6] Shao et al., 2014,[37] reported that strong midkine positivity and high expression scores were associated with clinicopathological features of PTC, e.g., extrathyroidal invasion, lymph node metastasis and tumor stages III/IV.

MK binds to heparin sulfate and chondroitin sulfate and activates several signaling pathways contributing to cell growth and proliferation. In general, MK in a receptor mediated manner promotes cancer cell growth, migration, metastasis, and angiogenesis via the activation of downstream signaling cascades,[4] and this can demonstrate the role of MK in thyroid cancer.

In a similar manner, Ibrahim and Hamam 2019,[6] reported that ROC curve analysis of serum MK in solitary thyroid showed sensitivity of 76% and specificity of 86% in detecting malignant/suspicious nodules. As well, Meng et al., 2015,[38] found a diagnostic capability of midkine to discriminate differentiated thyroid cancer (DTC) from benign thyroid nodules before surgery with a diagnostic accuracy of 75.31%.

In our study, the plasma expression of HOTAIR biomarker was found to be significantly higher in the patients with malignant thyroid nodules than in the patients with benign thyroid lesions and the studied controls. Our result agreed with those of Li et al., 2021,[39] who reported higher serum expression of LncRNA HOTAIR in PTC patients than in patients with benign thyroid tumor.

HOTAIR can increase cancer cell proliferation, invasion, and migration, and coupling with the regulation of apoptosis. HOTAIR inhibits endonuclear miR-34a, promoting the development of EMT. Besides, HOTAIR affected the expression level of the E-cadherin protein, a hallmark of EMT. It negatively regulates miR-1 by direct competitive binding to the miR-1 locus and participates in the regulation of thyroid cancer cell carcinogenesis.[40] So, there are much evidence that HOTAIR may be closely related to the pathogenesis of thyroid cancer. Another study by Chen et al., 2021a,[41] reported that the expression level of HOTAIR was notably higher in the thyroid cancer tissues than in the normal tissues and nodular goiter tissues. Also, HOTAIR could be detected in TC patient plasma, whereas there was almost no HOTAIR expression in the plasma of the healthy volunteers.[42]

In this study there was significant elevation of plasma HOTAIR in patients with stage III thyroid cancer compared to stage I and II thyroid cancer patients. Also, patients with stage II thyroid cancer were found to have significantly higher levels of plasma HOTAIR than those with stage I. These results are in consistence with those reported by Guo et al., 2021,[43] who found that HOTAIR was correlated with TNM stages. Also, in previous studies, the relation of HOTAIR expression level with the clinicopathological features, CT characteristics

and prognosis of PTC patients were analyzed. They reported that the high expression of HOTAIR was significantly correlated with tumor size, depth of invasion, lymph node metastasis, TNM stage and distant metastasis.[40, 41, 44, 45] HOTAIR promotes proliferation, invasion, and migration, and inhibits apoptosis in cancer cells. HOTAIR also participates in the pathogenesis and progression of cancer by regulating inflammation and immune signaling.[46]

There was no significant difference in the plasma expression of HOTAIR according to the type of pathology of thyroid cancer cases. In contrast to these results, Zhang et al., 2018,[47] reported that HOTAIR expressions were significantly higher in the PTCs compared to the other groups (follicular thyroid cancers, and atypical thyroid cancer), when HOTAIR expression levels were studied in thyroid cancer tissues. This difference in results may be attributed to different types of samples in our study and their study. To evaluate the role of HOTAIR in thyroid cancer diagnosis, ROC analysis was performed and showed high sensitivity and specificity of 100.0%.

In conclusion, our study demonstrated a strong significant positive correlation between both studied biomarkers (HOTAIR and midkine). Therefore, they may be considered useful biomarkers for differentiating malignant from benign thyroid nodule and to determine TC stages.

Author Contributions

AAM, methodology, analysis and interpretation of data, review and editing. HOM, project administration, conception and design of the study and supervision. AMAA, methodology, analysis and interpretation of data. HSA, writing the original draft, review and editing and practical work. MAJ, review and editing, clinical evaluation of patients and resources.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study protocol was reviewed and approved by the Institutional Review Board of the Faculty of Medicine, Assiut University (dated April 2018).

Informed consent

A signed informed consent form was obtained from each study participant before included in the study.

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