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Clinical and immunohistochemical correlates of goitre versus hypoxia-inducible factors: an inferential hospital-based case-control study from Iraq

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Abstract

Goitre, an abnormal enlargement of the thyroid gland, represents one of the most common endocrine disorders encountered in Iraqi patients. Several pathologic conditions, including thyroid malignancies, are correlated with the expression of hypoxia-inducible factors. Those are transcription factors that become activated in response to hypoxia. Our study will attempt to explore those factors in patients with goitre. The study is a hospital-based case-control in design. Individuals were allocated into cases (n=43) of individuals underwent a thyroidectomy procedures for goitre, and controls (n=25) who has conducted a total laryngectomy procedure for conditions unrelated to the thyroid gland. Histological samples were collected and examined via immunohistochemistry for hypoxia-inducible factors specifically HIF-1 and HIF-2. These were quantified and statistically tested with other parameters including age, gender, and the existence of clinical toxicity. Patients with goitre had significantly higher levels of HIF-1 and HIF-2 compared to controls (p-value<0.001). Clinically-toxic patients had substantially higher levels of both HIF-1 and HIF-2 than non-toxic patients (p=0.019, p=0.072). Patients, especially clinically-toxic ones, had notably more elevated levels of HIF-1 and especially when they grew older compared to non-toxic patients. Besides, the levels of HIFs appear to be rising in positive correlation with each other. Although our study has a high level-of-evidence, it may have some limitations. Nevertheless, it represents the first retrospective case-control study from Iraq in connection with the investigation of hypoxia as a cellular-biochemical event in patients from the Iraqi population.

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Introduction

Thyroid goitre is one of the most common endocrine disorders in Iraqi population particularly among adult women (Zhao *et al.* 2014). A multinodular goitre (MNG) is a non-tumorous condition although a thyroid cancer can be identified in more than 13% of patients operated for MNG (Gandolfi *et al.*, 2010). As known, tumour growth and development need the presence of a local vascular network that delivers oxygen and nutrients to tumour cells, and most tumour cells have developed an adaptation to growing in a hypoxic condition. Such hypoxic zones have been proposed to favour tumour progression (Brahimi-Horn *et al.*, 2007).

To date, there are three types of HIF- α subunits, namely HIF-1 α, HIF-2 α, and HIF-3 α, and at present, the HIF-1 α and HIF-2 α gained more attention (Majmundar *et al.* 2010). The hypoxia-inducible factors (HIFs), are well-known heterodimeric transcription factors, that are composed of an oxygen-sensitive alpha subunit (HIF-α) and a constitutive beta subunit (HIF-β), and these are known to be strictly associated with hypoxic microenvironment (Ke & Costa 2007, Vooijs *et al.*, 2007). It is evident that tumour hypoxia and the critical molecular mediators of hypoxia, hypoxia-inducible factors (HIFs), regulate multiple steps of tumorigenesis including tumour formation, progression, and response to therapy (EB Rankin & AJ Giaccia, 2008).

Studies concerning the role of HIFs in thyroid tissue are lacking, and there are even fewer studies which looked at the co-expression of both isoforms in the same histological specimen. Moreover, no one study to date explores the bio-expression of both isoforms in patients with multinodular goitres and in co-existence with clinical toxicity, and we found that there are lack of literitures which investigate the effect of hypoxia and its molecular regulators (HIF-1 and HIF-2) from Iraq and the middle east. Questions need to be answered is that: Are HIF-1α and HIF-2α play a role in the development of thyroid goitres, and are there any relation to clinical toxicity.

Therefore, the aims of our study are

1. To detect the expression of hypoxia-inducible factor (HIF)-1α and HIF-2α in thyroid goitres.
2. To compared expression of these two markers in thyroid goitres with normal thyroid tissues.
3. To compared expression of these two markers between toxic and non-toxic thyroid goitres.

Materials and methods

The study is a hospital-based retrospective case-control in design. All samples were obtained with the consent of the patients. The handling and laboratory manipulation of specimens for histological analysis, including immunohistochemical processing, was approved by the local ethical committee of Al-Mustansiriyah Medical College.

Sample selection

The total sample size is sixty-eight (n=68) that were as forty-three cases within the nodular goitre group and twenty-five controls within another group of patients with healthy thyroid gland (controls). Patients assigned to the cases group underwent surgical resection via a thyroidectomy for goitre, while patients assigned to the control group underwent surgical resection via a total laryngectomy, at the Department of general surgery at Al-Yarmouk teaching Hospital in Baghdad during the period from June 2017 to January 2018. Demographic parameters including age and sex were tabulated for cases and controls, as well as the status of clinical toxicity in some patients (cases).

Proceture

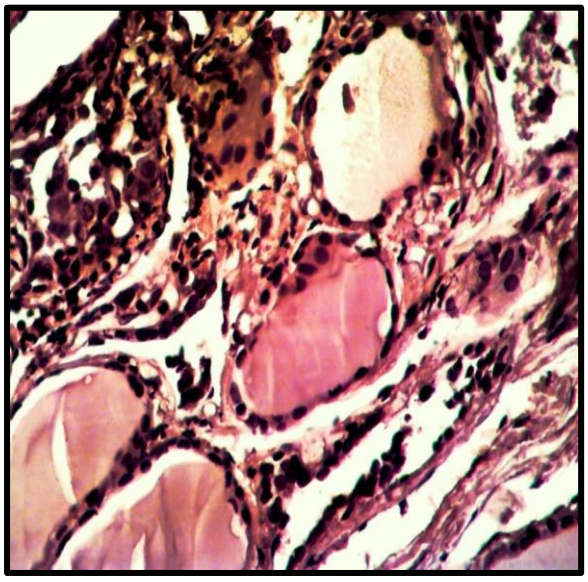
All histological samples (thyroid biopsies) were instantly fixed in 10% neutral buffered formalin for 18-20 hours at room temperature (20-25C˚). Following fixation, tissue samples were processed according to Luna 1968 (Luna 1968, Munro 1971). Then, samples were labelled and embedded in paraffin as blocks.

Paraffin blocks were sectioned into 4-6μm thick slices. From each tissue block, three sections were collected, one for H & E staining for histopathological diagnosis and the other two were for HIF-1 and HIF-2 immunohistochemical staining.

For the immunohistochemical staining, antigen retrieval was performed by boiling the slides with citrate buffer (PH=6) at 95°C, then blocked by a blocking reagent from. Later, all slides were submerged in Peroxidase quenching solution for 15 minutes. The samples were then incubated with an antibody against HIF-1α (1:100; mouse monoclonal, clone; sc-13515; Santa Cruz Biotechnology; USA) and HIF-2α (1:100; mouse monoclonal, clone; sc-13596; Santa Cruz Biotechnology; USA) in a humid chamber and kept at 4°C overnight. Later, slides were incubated for 90 minutes with m-IgGk BP-HRP (1:50; sc-516102; Santa Cruz Biotechnology; USA), and then treated with DAB for 2min. Finally, the sections were counterstained and mounted on slides.

Examination and scoring

Histological slides were examined via a multiheaded microscope by two consultant pathologists who were blinded to the clinical data, patients' demographics, and the outcome of the study. The pathologists performed a semi-quantitative assessment of immunohistochemical (IHC) scoring. The final IHC, on a continuous numeric scale of -3, was calculated based on the multiplication of the intensity of IHC staining and percentage of positive IHC signals within each slide. The intensity score was designated as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining).



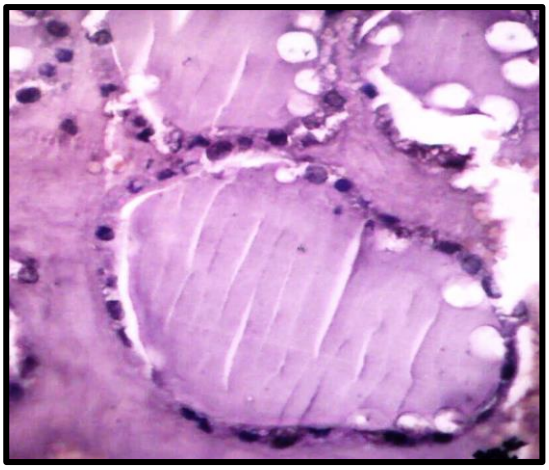


Fig. 1. Immunohistochemical Staining for HIF-1 of a Case Specimen (top) and a Control Specimen (bottom).

Statistical analysis

Statistical analyses were conducted via Microsoft Excel 2016 and SPSS version 20. The inferences were based on the implementation of parametric and non-parametric tests including ANOVA test, Levene's test for equality of variances, Student's t-test, Linear regression models, Chi-Square test and Fisher Exact test, as well as multivariate analysis.

The level-of-evidence for this study rank well within the pyramid of the hierarchy of evidence-based studies. It is estimated to be of level-2 of the categorisation scheme imposed by the *Oxford Center for Evidence-Based Medicine* in 2016 (CEBM 2018).

Evidence base

A systematic review of the literature was carried out on medical and paramedical databases of literature including NCBI-PubMed, the Cochrane Library, Embase, EBSCO, Google Scholar, ResearchGate, and Academia. The grey literature databases were also consulted for relevant information. The reviewed literature of interest was subsequently scrutinised for credibility and reliability via the implementation of the appropriate tools of critical appraisal (Greenhalgh *et al.*, 2014, Zeng *et al.*, 2015).

Results

The total number of participants in this study were sixty-eight (n=68) including 43 cases and 25 controls (Table 1).

The percentile contribution of males and females within cases (patients) was 11.63% and 88.37% respectively, and 44.00% and 56.00% for controls. Toxic goitres were accounted for in 13.95% of cases. Statistical outliers were identified only for age in both cases and controls and were exclusively females (Fig. 2). Cases were approaching normality of distribution with a skewness score of 0.191.

Based on age, cases were distributed into class intervals including those of age 19-27 years (n=4), 27-35 (6), 35-43 (10), 43-51 (15), 51-59 (5), 59-67 (2), and 67-75 (1). Concerning cases, the average values were 43.26 +/- 11.36 (age), 1.41 +/- 0.80 (IHC Score – HIF1), 1.34 +/- 0.62 (IHC Score – HIF2). On the other hand, the mean values for controls were 43.16 +/- 15.66 (age), 0 (IHC Score – HIF1), and 0 (IHC Score – HIF2). IHC scores of HIF-1 averaged 1.39 +/- 0.79 for female cases and 1.57 +/- 0.92 for male cases, while IHC scores of HIF-2 averaged 1.32 +/- 0.62 for female cases and 1.50 +/- 0.66 for male cases.

Cases with non-toxic manifestation had an average of 1.30 +/- 0.77 (IHC Score – HIF1) and 1.26 +/- 0.60 (IHC Score – HIF2), while cases with toxic manifestations averaged 2.06 +/- 0.56 (IHC Score – HIF1) and 1.82 +/- 0.57 (IHC Score – HIF2).

Table 1. Descriptive Statistics of Cases and Controls.

		Gender (cases)	Age (cases)	IHC Score of HIF-1 (cases)	IHC Score of HIF-2 (cases)	Age (controls)	IHC Score of HIF 1 (controls)	IHC Score of HIF 2 (controls)
N	Valid	43	43	43	43	25	25	25
	Missing	0	0	0	0	18	18	18
Mean			43.26	1.4100	1.3419	43.16	.00	.00
Std. Error of Mean			1.732	.12146	.09437	3.133	.000	.000
Median			44.00	1.6700	1.3000	46.00	.00	.00
Mode			49	2.00	2.00	49	0	0
Std. Deviation			11.360	.79648	.61884	15.665	.000	.000
Variance			129.052	.634	.383	245.390	.000	.000
Skewness			.191	-.206	-.036	.520		
Std. Error of Skewness			.361	.361	.361	.464	.464	.464
Kurtosis			.235	-1.169	-.857	.139		
Std. Error of Kurtosis			.709	.709	.709	.902	.902	.902
Range			51	2.80	2.50	60	0	0
Minimum			19	.10	.10	19	0	0
Maximum			70	2.90	2.60	79	0	0
Sum			1860	60.63	57.70	1079	0	0

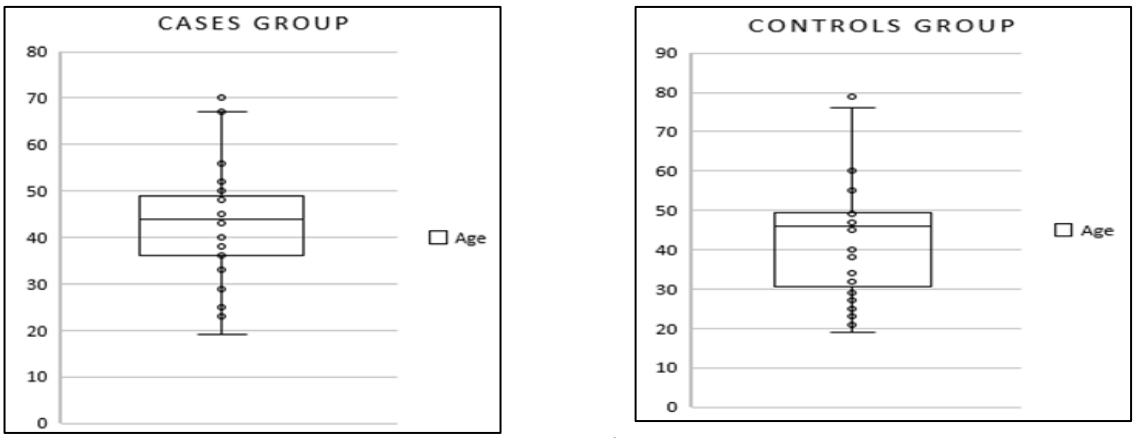


Fig. 2. Boxplot Presentation for Age and Immunohistochemistry Scoring.

The analysis of variance and covariance (ANOVA) confirmed the presence of a statistically significant difference between cases and controls in connection with IHC scoring of HIFs (*p-value*<0.001) (Table 2). This inference was also confirmed via t-test that revealed a significant difference of cases over controls for the IHC scoring of HIF-1 (p<0.001) and HIF-2 (p<0.001). Further, Student's t-test was successful in exploiting an inference about cases stratified based on gender and toxicity of clinical manifestations (Table 3). It has been concluded that there were no significant differences in between males and females in connection with age (42.20 versus 43.39, p=0.718), IHC scoring of HIF-1 (1.57 versus 1.39, p=0.688) and HIF-2 (1.50 versus 1.32, p=0.589). Hypothesis testing also confirmed the absence of significant difference among toxic and non-toxic cases in connection with age (46.00 versus 42.58, p=0.570). However, there is a significant difference existed between clinically-toxic and non-toxic cases for IHC scoring of HIF-1 (2.06 versus 1.30, p=0.019), HIF-2 (1.82 versus 1.28, p=0.072).

Table 2. Analysis of Variance and Covariance of Cases and Controls.

SUMMARY

Groups	Count	Sum	Average	Variance		
Cases, HIF-1	43	60.63	1.41	0.63		
Cases, HIF-2	43	57.70	1.34	0.38		
Controls, HIF-1	25	0	0	0		
Controls, HIF-2	25	0	0	0		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	59.95	**3**	19.99	61.74	<0.001	2.673
Within Groups	42.73	132	0.32			
Total	102.68	135				

Table 3. Independent t-test Statistics of Cases vs Controls, Males vs Females, and Toxic Thyroid vs Non-Toxic Thyroid Status.

	t-test statistics	Age	IHC Score - HIF 1	IHC Score - HIF 2
	Cases vs. Controls (*p-value*)	0.979	<0.001	<0.001
		Age	IHC Score - HIF 1	IHC Score - HIF 2
Mean	Cases	43.26	1.41	1.34
	Controls	43.16	0.00	0.00
St. Dev.	Cases	11.36	0.80	0.62
	Controls	15.66	0.00	0.00
	t-test statistics	Age	IHC Score - HIF 1	IHC Score - HIF 2
	Males vs. Females (*p-value*)	0.718	0.688	0.589
		Age	IHC Score - HIF 1	IHC Score - HIF 2
Mean	Males	42.20	1.57	1.50
	Females	43.39	1.39	1.32
St. Dev.	Males	5.72	0.92	0.66
	Females	11.95	0.79	0.62
	t-test statistics	Age	IHC Score - HIF 1	IHC Score - HIF 2
	Toxic vs. Non-Toxic (*p-value*)	0.570	0.019	0.072
		Age	IHC Score - HIF 1	IHC Score - HIF 2
Mean	T	46.00	2.06	1.82
	Non-T	42.58	1.30	1.28
St. Dev.	T	13.27	0.56	0.57
	Non-T	11.11	0.77	0.60

Based on linear regression models (Table 4 and Fig. 3), it has been inferred that age is positively correlated with IHC scoring of HIF-1 (*df*=1, R^2 *score*=0.158, *p-value*=0.008) though it was not associated with IHC scoring of HIF-2 (*df*=1, R^2<0.001, p=0.984). However, there was a strong correlation in between IHC scoring of HIF-1 and 2 (*df*=1, R^2=0.413, p<0.001). Chi-Square test and Fisher's Exact test were implemented to test the significance of odds ratios in connection with quartile distribution of age versus IHC scoring of HIF-1 (*odds ratio*=3.21, *p-value*=0.067), age versus IHC scoring of HIF-2 (odds=0.83, p=0.768), gender versus

IHC scoring of HIF-1 (odds=1.58, p=0.457), gender versus IHC scoring of HIF-2 (odds=0.12, p=0.040), presence or absence of toxicity versus IHC scoring of HIF-1 (odds=0.16, p=0.078), and the presence or absence of toxicity versus IHC scoring of HIF-2 (odds=0.25, p=0.120).

Table 4. Linear Regression Statistics of Age vs HIF-1.

Regression Statistics								
Multiple R	0.398							
R Square	0.158							
Adjusted R Square	0.137							
Standard Error	0.74							
Observations	43							
ANOVA								
	df	*SS*	*MS*	*F*	*Significance F*			
Regression	1	4.22	4.22	7.708	0.008			
Residual	41	22.43	0.54					
Total	42	26.65						
	Coefficients	Standard Error	t Stat	P-value	Lower 95%	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.204	0.45	0.454	0.653	-0.703	1.110	-0.703	1.110
Age	0.0278	0.01	2.776	0.008	0.007	0.048	0.007	0.048

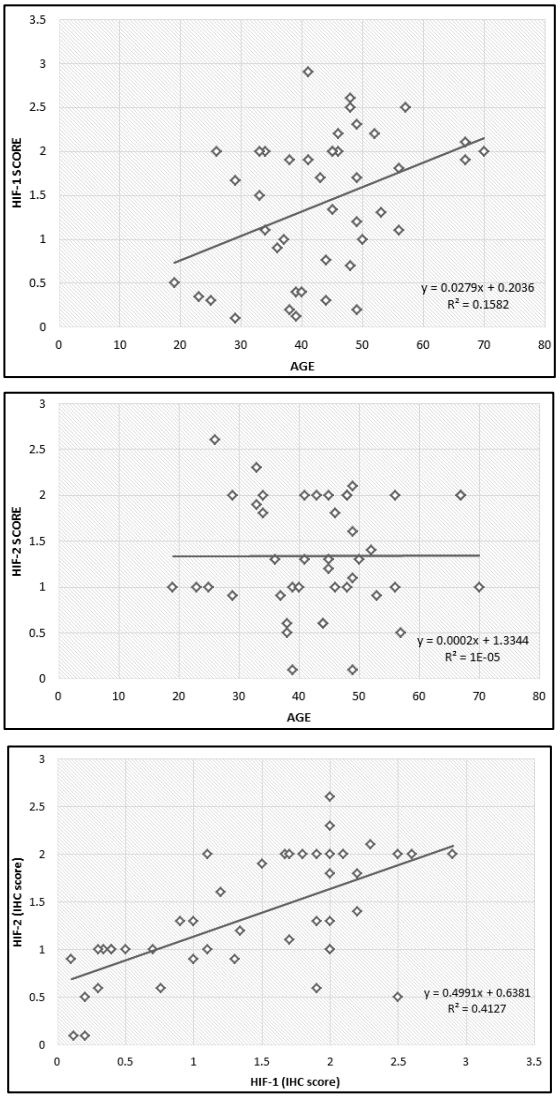


Fig. 3. Scatter Correlation and Linear Regression: Age vs. HIF-1 (top), Age vs. HIF-2 (middle), and HIF-1 vs HIF-2 (bottom).

To summarise the results, cases and controls are of comparable demographics. Most of the cases were females on their 5th decade of life and clinically non-toxic. Patients (cases) had had significantly higher levels of HIF-1 and HIF-2 compared to controls. Male and female patients had similar age and levels of HIFs. Clinically-toxic and non-toxic individuals were of somewhat similar age distribution. Nevertheless, clinically-toxic patients had significantly higher levels of HIFs. Patients, especially clinically-toxic ones, had notably more elevated levels of HIF-1 as the grew older compared to patients with no toxic clinical manifestations. Besides, the levels of HIF-1 and 2 appear to be rising simultaneously in parallel to each other.

Discussion

This study is based on the central hypothesis which state that HIFs facilitate both oxygen delivery and adaptation to oxygen deprivation by regulating the expression of genes that are involved in many cellular processes, including glucose uptake and metabolism, angiogenesis, erythropoiesis, cell proliferation, and apoptosis (Smith *et al.*, 2008).

HIF-1α and HIF-2α have a similar structure and function, but they have different tissue and cell type distributions (Loboda *et al.*, 2012, Bersten *et al.*, 2013, Eales *et al.*, 2016). Recent data indicate that the regulation of HIF-1 target genes depends on tissue

type, lesion type and co-expression with HIF-2 (Koh *et al.*, 2011, Påhlman *et al.*, 2015, Abd-Aziz *et al.*, 2015). Studies concerning the role of HIFs in thyroid tissue are limited in the published body of literature. And there are very few studies which looked at both isoforms expressions in the same specimens. Moreover, there is no one study to date which investigates the expression of both isoforms in multinodular goitres and in connection with clinical toxicity. Therefore, in this study, we tried to examine whether there is any difference in the co-expression of HIF-1α and HIF-2α between toxic and non-toxic multinodular goitres and in contrast with normal thyroid tissues.

We found that most of the cases were females on their 5th decade of life and clinically non-toxic. This finding goes with usual epidemiology of thyroid diseases, which state that thyroid diseases, notably thyroid multinodular goitres are more common in the adult population and more common in females than in males as documented by Castro and Gharib in 2005, Al-Rrawak and co-workers in 2009, Mahdi and colleagues in 2010, and Mandel in 2014 (Mandel *et al.*, 2004, Castro *et al.*, 2005, Al-Rrawak *et al.*, 2009, Mahdi *et al.* 2010). The study found that there is no significant difference between males and females in connection with IHC scoring of HIF-1 (1.57 versus 1.39, p=0.688), and HIF-2 (1.50 versus 1.32, p=0.589).

Interestingly, our study demonstrated for the first time a significantly positive correlation between HIF-1 expression in multinodular thyroid goitre and the age of the patient. We had found that the IHC score of HIF-1 expression in thyroid goitre tissues increases as the patient grew up (*df*=1, *R2 score*=0.158, *p-value*=0.008). To date, there was no study could explain this positive correlation. It is necessary for us in the future to explore mechanisms underlying this correlation. On the other hand, we found that age is not positively correlated with IHC scoring of HIF-2 (*df*=1, *R2 score* <0.001, p=0.984), again there is no study to support or reject this finding. However, there was a strong correlation in between IHC scoring of HIF-1 and IHC scoring of HIF-2 (*df*=1, *R2 score* =0.413, p<0.001). This finding is to be expected as the role of both of these factors (HIF-1 & HIF-2) is the same at the cellular and molecular biological levels, and these findings agree the studies were done by Toschi and co-workers in 2008 and Liu and Xing in 2016 (Toschi *et al.* 2009, Liu & Xing 2016), and this may suggest similar regulation of both genes in the thyroid. In 2010, Burrows and co-authors proposed that the bio-regulation of HIF proteins is influenced by tissue genotype and the local microenvironment (Burrows *et al.*, 2010).

Interestingly, for the first time, this study demonstrated a significantly positive expression of HIF-1 in patients with thyroid goitres as compared with normal thyroid tissue (p<0.001). Another interesting finding is the significantly positive expression of HIF-2 in patients with thyroid goiters as compared with normal thyroid tissue (p<0.001), our results agree with the previously reported data by Wang and colleagues in 1995 (Wang *et al.*, 1995) who showed statistically significant differences in HIF-2α protein expression levels between normal thyroid tissues and nodular hyperplasia tissues [29]. Clinically-toxic patients had higher levels of both HIF-1 (2.06 versus 1.30, p=0.019) and HIF-2 (1.82 versus 1.28, p=0.072), and again this study was the first one to demonstrate such a finding.

This study may have limitations including a relatively small sample size and imbalance in the number of individuals allocated to both arms of the study, i.e. cases versus controls. Besides, the study was based on individuals attending a single hospital rather than attending more than a health care centre which could have led to a more reliable multi-centre study. Nevertheless, our statistically-valid immunohistochemical research is novel and represents the only one conducted in Iraq and the Middle East about hypoxia-inducible factors in patients with goitre. It possesses a high level-of-evidence (level-2) as per the rigour assortment system implemented by the Oxford Center for Evidence-Based Medicine (CEBM 2018). Regarding, the mechanism responsible for the development of

multinodular goitre, we propose that intrinsic factors may stimulate thyroid cells to express HIF-1α and/or HIF-2α, and that, this expression drives follicular remodeling toward follicular hyperplasia. Moreover, we suggest that HIF and/or inhibition HIF be viewed as a novel means of preventing the development of follicular hyperplasia and its progression to thyroid carcinoma. We hope that this study sheds new light on the pathogenesis of multinodular goitre and that it aids those involved in the development of drugs used to treat multinodular goitre.

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Conflicts of interest

The authors have no competing interests to be declared.

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