Original Article

Immunohistochemical Correlation of Goitre Versus LGR5 Factor: A Case-Control Study From Iraq

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ABSTRACT

Introduction: The thyroid gland is one of the most important endocrine glands in the body. Goitre is an abnormal thyroid enlargement, and it is considered one of the most common endocrinal conditions encountered in the Iraqi population. Numerous pathological conditions, such as thyroid cancers, are associated with LGR5 expression. The LGR5 receptor is similar in its structure to the TSH receptor, this may explain the role of LGR5 in thyroidal pathophysiology.

Objectives: Our study will attempt to explore the expression of LGR5 factor in goitrous patients and try to make a comparison between goitrous and non-goitrous (normal thyroid) ones.

Materials and Methods: Participants were assigned into three groups: toxic goitre cases, non-toxic goitre cases, and controls. Histologic specimens were collected from the patients and studied by immunohistochemical staining for the LGR5 factor. These were quantitated and tested statistically against other indices such as age, gender, and the presence of thyroid toxicity.

Results: Goitrous patients have been shown to express a substantially higher level of LGR5 factors as compared to controls. Patients with toxic goitre had noticeably higher LGR5 levels than patients with non-toxic goitre. Clinically-toxic patients had prominently higher LGR5 levels and in particular when they get older as compared to patients with nontoxic goitre.

Conclusion: Nevertheless, the present study has a high evidence level, but it might possess some limitations. Interestingly, the study denotes the first original retrospective hospital-based case-control research in connection with the examination of LGR5 expression in thyroid tissues of patients from the Iraqi population.

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Key Words: Goitre, iraq, LGR5, thyroid.

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INTRODUCTION

Goitre, an abnormal thyroid enlargement, is considered one of the commonest endocrinal conditions encountered in the Iraqi population predominantly among adult females[1,2]. A multinodular goitre is a benign disorder; however, malignancy can be recognized in about 5-15 %[3].

LGRs "Leucine-rich repeats containing G protein-coupled receptors" are transmembrane group receptors, members of the GPCRs "G protein-coupled receptors" superfamily, that is characterized by having a large extracellular domain which can identify ligands and controls several cellular processes. LGRs are divided into three subclasses depending on their function and structure. Class-A receptors include LGR1-3, Class-B includes LGR4-6, and Class-C includes LGR7 and 8[4,5]. The LGR5 receptor is similar in its structure to the TSH receptor, this may explain the role of LGR5 in thyroidal pathophysiology[6].

In recent years, several kinds of literature have established that LGR5 is overexpressed in many kinds of tumours, including colonic[7], ovary[8], liver[9], skin[10], oesophageal[11], and thyroid cancers[12]. Works of literature investigating the LGR5 role in the thyroid gland are few, furthermore, there is a lack of literature which investigate the effect of LGR5 in the thyroid gland in Iraq. Questions required to be investigated are: Is the LGR5 factor play a role in thyroid goitre development, and is there any correlation with thyroid toxicity? Consequently, this study has the following objectives:

1. Detection of LGR5 co-expression in thyroid tissues.
2. Comparison of this expression between goitrous and non-goitrous (normal) thyroids.
3. Comparison of this expression between toxic and non-toxic goitre.

MATERIALS AND METHODS

Sampling

The total sample size is sixty. It was divided into three groups according to their diagnosis (Table 1). Participants allocated to the cases groups (toxic goitre and non-toxic goitre) had undergone a thyroid resection for goitre, whereas

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participants allocated to the controls group had experienced a total laryngectomy for nonthyroidal conditions, at the general surgery department of at Al-Yarmok Hospital (July 2018-February 2019). Demographical indices such as age and gender were tabularized for all groups together with the clinical toxicity state. The patient's ages ranged from 19 to 69 years. They were divided according to their age into three groups (Table 2).

Table 1: Patients' categories (sample size in the three different group)

Group	Type	N	Gender	
			Male	Female
I	Normal thyroid (NT)	19	8	11
II	Non-Toxic goitre (NTG)	24	14	10
III	Toxic goitre (TG)	17	4	13
Total		60	26	34

Table 2: Age distribution of various groups

	Normal thyroid (NT)	Non-toxic goitre (NTG)	Toxic goitre (TG)	Total
<20 years	0	4	6	10
20-40 years	3	15	3	21
>40 years	16	5	8	29
Total	19	24	17	60

Inclusion criteria

Any adult patient (age 18 years and older) with goitre (toxic or non-toxic) admitted to the general surgical department for thyroidectomy was included in the cases group, and any patient admitted to the ENT department for laryngectomy, who have apparently normal thyroid was included in the control group.

Exclusion criteria

Regarding cases: any patient who shows a feature of malignancy, either clinically or during the histological examination was excluded from the study.

Regarding controls: any patient who shows a feature of thyroid diseases, either clinically or during the histological examination was excluded from the study.

Immunohistochemical procedure

The biopsy specimens were immediately fixated in a 10% solution of neutral buffer formalin at ambient temperature (20-25C˚) for about 24 hours. Subsequently, tissue specimens underwent processing according to Luna method[13,14] and were embedded in paraffin oil blocks. The blocks then were sectioned into 4µm thick sections, via Histoline microtome. Three sections from each block had been collected. One section was mounted on a charged slide to be used for immunohistochemical (IHC) staining, and the other two sections were mounted on an ordinary slide to be used for Haematoxylin and Eosin (H. &E.) staining which was used for histopathological examination[15].

The procedure of the IHC staining adopted by this study was according to the immunohistochemistry detection kit, HRP (DAB, Broad Spectrum) from Us Biological.

Antigenic retrieval was achieved by slide boiling in a buffered citrate at 90°, followed by antibody blocking via a blocking agent. Thereafter, slides were immersed in a solution of a peroxidase-quenching agent for 10 minutes. specimens then were incubated with a GPCR49 primary antibody (Us Biological Catalog # G8600-53) in a moist chamber and reserved at 4° for 30 minutes. Then, slides were incubated for 10 mins. in HRP polymer conjugate to each section (Us Biological Catalog # I7506-07), and after that they were treated with DAB for 5 minutes. Lastly, the specimens are counterstained and get mounted.

Semiquantitative methods for IHC scoring

Histologic slides were inspected through a multi-head microscope by two specialists in histopathology who were blinded to the study data. Examiners executed a semiquantitative calculation of IHC scoring. The ultimate IHC was considered based on the multiplication of the staining intensity and ratio of the positive signal inside each slide. Scoring was nominated as score-0 (negative stain), score-1 (weakly positive stain), score-2 (moderately positive stain), and score-3 (strongly positive stain)[16].

Data analysis

Analysis of the data were conducted by SPSS version 26 (IBM-Chicago-USA). The interpretations had been based on the application of nonparametric and parametric tests such as the Chi-Square test, ANOVA, Student's t-test, Linear regression test as well as multivariate analysis. A *P- value* <0.05 was regarded as statistically significant[17].

A systemic review of the literature had been conducted on databases of literature (including medical and paramedical ones) like "Cochrane, the ResearchGate, Embase, PubMed, Google-Scholar, and Academia". Old literature was similarly checked for related evidence. The revised works of literature of interest were then scrutinized for reliability and credibility through the application of suitable critical appraisal tools[18].

Ethical approval

This study was conducted under the ethical principles that have their origin in the WMA Declaration of Helsinki. It was carried out with patients' verbal and analytical approval before the sample was taken. Sampling, manipulation and handling of specimens for histologic examination, including the immunohistochemistry process, were ethically approved by the local ethical committee of Al-Mustansiriya Medical College.

RESULTS

The participants number in this study was sixty (n=60), divided as 41cases (68.3%) and 19 controls (31.7%). Their age ranges from 19-69 years with a mean of 42.27 +/- 16.28 years. The gender distributions within the case group were

43.9% (males) and 56.1 % (females), while for the controls were 42.1 % and 57.9 % respectively.

Regarding cases, the average values were 36.66 +/- 15.46 (age), 1.2 +/- 0.80 (LGR5 score), while for the control group, the mean values were 54.37 +/- 10.57 (age), 0.53 +/- 0.61 (LGR5 score). LGR5 scores were 1.39 +/- 0.79 for female and 1.57 +/- 0.92 for male cases. Non-toxic goitre cases had an average value of 35.50 +/- 14.11 (age), 0.38 +/- 0.70 (LGR5 score), while toxic goitre cases averaged age of 38.29 +/- 17.50 and LGR5 score of 1.71 +/- 0.92 (Figure 1, Table 3).

Cases (thyroid goitres) vs Controls (normal thyroid)

The ANOVA "analysis of variance and covariance" test confirmed the existence of a statistically significant difference amongst cases and controls in correlation to age (*p-value*=0.000) and LGR5 score (*p-value*=0.005). These findings were also established through a t-test which exposed a statistically significant difference between cases and controls regarding age (36.66 versus 54.37, *p-value*=0.000) and for the LGR5 scoring (1.20 versus 0.35, *p-value*=0.005) (Tables 4,5, Figure 2).

Moreover, the independent t-test exploits an implication about cases and control groups according to age, gender, and LGR5 scoring. It was determined absence of differences between different genders of the cases group in connection with age (36.17 vs 37.04, *p-value*=0.860) and of the controls group in connection with age (54.50 vs 54.27, *p-value*=0.965), also there was no significant difference between different genders of cases in connection with the LGR5 scoring (1.11 vs 1.26, *p-value*=0.604), and of controls in connection with LGR5 scoring (0.38 vs 0.64, *p-value*=0.373) (Table 6).

Toxic vs Non-Toxic Cases

The hypothesis test established that there were no significant differences between the toxic and the non-toxic case groups in connection to age (38.29 vs 35.50, *p-value*=0.575). But there was a statistically significant difference between the two groups regarding to the LGR5 score (1.71 vs 0.83, *p-value*=0.001) (Table 7). These findings were further established by Pearson Chi-square test (*p-value*=0.019) (Table 8, Figure 3).

Linear regression tests concluded that there was a significant correlation between age and the LGR5 scoring in clinically toxic cases (R2=0.218, *p*=0.029), but no such correlation presents in the non-toxic cases (R2=0.003, p=0.807) (Table 9).

In summary, cases and controls possess somewhat equal demographical parameters. Most cases were women in their 4th decade of life and are clinically toxic. In comparison to controls, cases had notably significantly high LGR5 score levels. Gender in both cases and control groups had no significant effect on age and LGR5 levels. Individuals with toxic and non-toxic goitre were of fairly similar age distribution. Interestingly, patients with toxic goitre had significantly higher LGR5 levels and they had particularly more elevated LGR5 levels as they get older.

Table 3: Descriptive Statistic

Cases vs Controls								
	Cases				Controls			
	Gender		Age	LGR5 Score	Gender		Age	LGR5 Score
	M	F			M	F		
N	18	23			8	11		
Mean			36.66	1.20			54.37	0.53
SD			15.46	0.90			10.57	0.61
Variance			238.88	0.811			111.80	0.37
Skewness			0.45	0.46			-0.42	0.70
Kurtosis			-0.66	-0.39			-0.96	-0.31
Non-Toxic vs Toxic Cases								
	Non-Toxic Cases				Toxic Cases			
	Gender		Age	LGR5 Score	Gender		Age	LGR5 Score
	M	F			M	F		
N	14	10			4	13		
Mean			35.50	0.83			38.29	1.71
SD			14.11	0.70			17.50	0.92
Variance			198.96	0.49			306.346	0.85
Skewness			0.73	0.24			0.16	0.13
Kurtosis			0.12	-0.812			-1.16	-0.92

Table 4: ANOVA test (Cases vs Controls)

Descriptives						
		N	Mean	Std. Deviation	95% Confidence Interval of means	
					Lower 95%	Upper 95%
Age	Case	41	36.66	15.456	31.78	41.54
	Control	19	54.37	10.574	49.27	59.46
LGR5	Case	41	1.20	0.901	0.91	1.48
	Control	19	0.53	0.612	0.23	0.82
ANOVA						
		SS	df	MS	F	Sig. F
Age	Between Groups	4072.093	1	4072.093	20.417	0.000
	Within Groups	11567.64	58	199.442		
	Total	15639.73	59			
LGR5	Between Groups	5.807	1	5.807	8.598	0.005
	Within Groups	39.176	58	0.675		
	Total	44.983	59			

Table 5: Independent t-test Statistics

Cases vs Controls		No.	Mean	Std. D	t	df	*p-value*
Age	Cases	41	36.66	15.46	4.519	58	0.000
	Controls	19	54.37	10.57			
LGR5	Cases	41	1.20	0.90	-2.932	58	0.005
	Controls	19	0.53	0.61			

Table 6: Independent t-test Statistics

Male vs Female (Cases)							
		No.	Mean	Std. D	t	df	*p-value*
Age	Males	18	36.17	15.007	-0.178	39	0.860
	Females	23	37.04	16.123			
LGR5	Males	18	1.11	1.079	-0.524	39	0.604
	Females	23	1.26	0.752			
Male vs Female (Controls)							
		No.	Mean	Std. D	t	df	p-value
Age	Males	8	54.50	13.480	0.045	17	0.965
	Females	11	54.27	8.603			
LGR5	Males	8	0.38	0.518	-0.915	17	0.373
	Females	11	0.64	0.674			

Table 7: Independent t-test Statistics (Toxic vs Non-Toxic Cases)

		No.	Mean	Std. D	t	df	*p-value*
Age	Toxic	17	38.29	17.503	0.565	39	0.575
	Non-Toxic	24	35.50	14.105			
LGR5	Toxic	17	1.71	0.920	3.447	39	0.001
	Non-Toxic	24	0.83	0.702			

Table 8: Chi-square tests Statistics (Thyroid toxicity vs LGR5 scoring)

		Value	df	*p-value*
P - Chi-Square		9.967	3	0.019
L-Ratio		11.985	3	0.007
Linear/Linear Association		9.342	1	0.002
No. of Valid Cases		41		
		Value	SD	t	*p-value*
Interval/Interval	Pearson's R	.483	.113	3.447	0.001
Ordinal/Ordinal	Sp. Correlation	.464	.123	3.273	0.002

Table 9: Linear Regression Statistics (Age vs LGR5 Scoring)

Toxic Cases					
Regression Statistics					
R	0.530				
R^2	0.218				
Adjusted R^2	0.233				
ANOVA					
	SS	df	MS	F	Sig. f
Regression	3.80	1	3.800	5.86	0.029
Residual	9.73	15	0.649		
Total	13.53	16			
Non-Toxic Cases					
Regression Statistics					
R	0.053				
R^2	0.003				
Adjusted R^2	-0.043				
ANOVA					
	SS	df	MS	F	Sig. f
Regression	0.031	1	0.031	0.061	0.807
Residual	11.302	22	0.514		
Total	11.333	23			

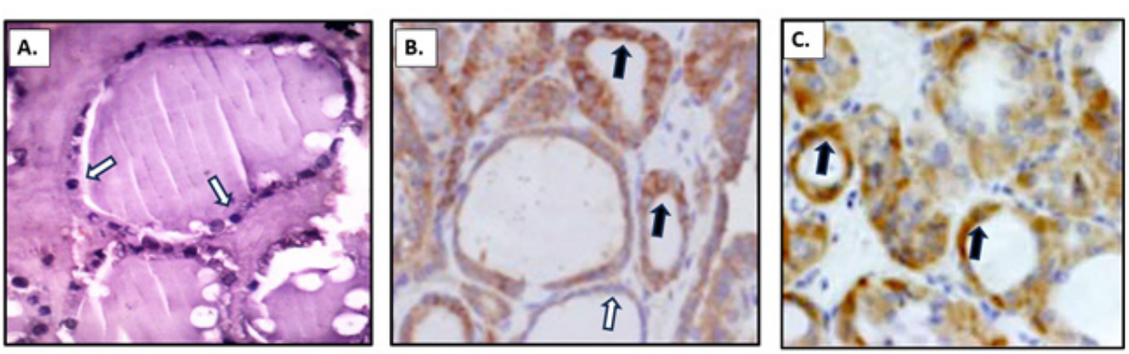

Fig. 1: LGR5 immunohistochemical staining in human thyroid tissues (white arrows = Lgr5 negative follicular cells. Black arrows= LGR5 positive follicular cells) X 100
A. Sample from control group shows negative staining (score 0)
B. Sample from non-toxic goitre group shows a moderately positive staining (score 2)
C. Sample from toxic goitre group shows a strong positive staining (score 3).

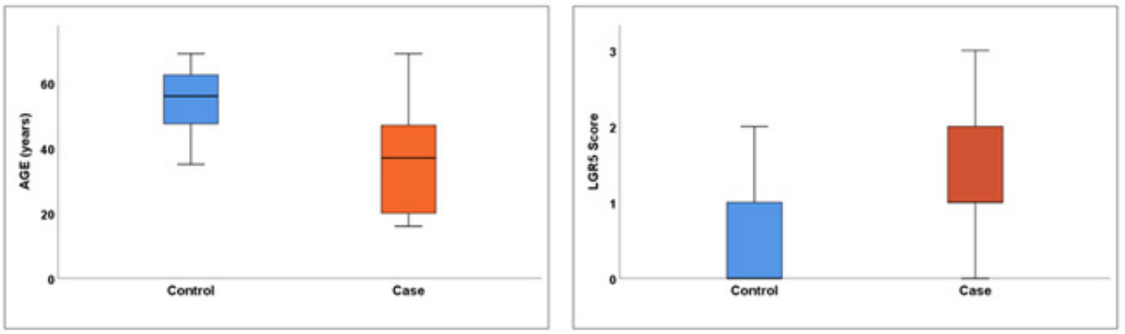

Fig. 2: Boxplot Presentation for Age (left) and LGR5 Score (right) between Cases and Controls

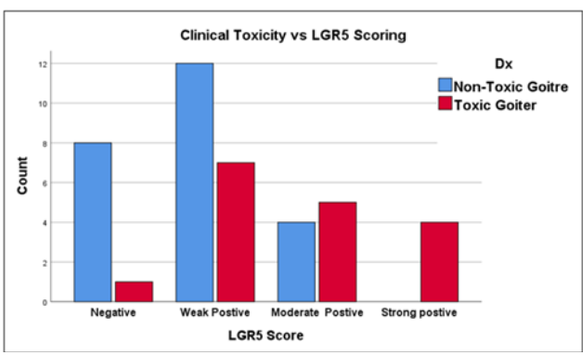

Fig. 3: Bar Chart Presentation for LGR5 Soring in Toxic vs Non-Toxic Goiter

DISCUSSION

This study is constructed according to a central proposition which states that the LGR5 receptor is similar structurally to the TSH receptor, consistent with a role for LGR5 in thyroid gland physiology and pathology[4]. Researches regarding the LGR5 role in thyroid pathophysiology are limited. Furthermore, there is a lack of studies which examines the LGR5 expression in thyroid goitre and in correlation to thyroid toxicity. Hence, the study tries to scrutinize whether or not there are any significant differences in the LGR5 expression between toxic and non-toxic goitre and in comparison, to normal thyroidal tissues.

The study revealed that most goitre cases were women in their forties and were clinically toxic. This result is in accord with the traditional epidemiology of thyroid disorders, which states that thyroid disorders, particularly thyroid goitre have a higher prevalence in adults and are commoner among women than men as recognized by Assi and colleagues in 2018[18], Mandel in 2014[19], Mahdi and coworkers in 2010[20], Al-Rrawak and coworkers in 2009[21], and Castro in 2005[22].

The study revealed that the gender of the cases and controls has no effect on the LGR5 scoring.

Remarkably, the study confirmed for the first time a significant positive linear correlation between the LGR5 score and the patient's age in the thyroid goitre. It had been confirmed that the LGR5 co-expression in thyroid goitre tissues of toxic cases rises as the patient's age increases. In contrast, it had been found that there is no correlation between age and the LGR5 score in thyroid goitre tissues of clinically non-toxic cases. Up to the present time, no research might clarify these findings. It is essential for us in the future to discover the mechanism responsible for such correlation.

Interestingly, this study confirmed for the first time, a significant positive LGR5 expression in thyroid goitres as compared with normal thyroid, once more there are no researches to support or reject this result.

The study had got limitations including a fairly small size sample and inequity in the number of participants assigned to both arms of the study (namely case group vs control group). In addition, the trial was based on participants from one health centre rather than multiple ones which might have led to a more dependable multi-centre trial. However, this statistical-valid immunohistochemical study is novel and denotes the first original study performed in Iraqi and Middle-Eastern populations about the LGR5 factor in thyroid goitre. The study holds a high level of evidence as per the rigour system of assortment applied by CEBM "Oxford Centre for Evidence-Based Medicine"

CONCLUSION

Concerning, the mechanisms responsible for thyroid goitre development, the study suggests that internal factors may be responsible for the stimulation of the thyroidal cells to express LGR5, and that, this co-expression may initiate the remodelling of thyroid follicles towards follicular cellular hyperplasia. Furthermore, this study proposes that LGR5 may be regarded as an innovative means of prevention the thyroid hyperplasia development and its evolution to malignancy, hoping that our research may throw new light on the thyroid goitre pathophysiology and that it may help persons concerned in the development of medicines that used in the treatment of goitres.

CONFILECT OF INTERESTS

There are no conflict of interest.

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الملخص العربى

دراسة أرتباط العامل (LGR5) بمرض تضخم الغدة الدرقية في الانسان : دراسة كيمانسيجية مناعية

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الجامعة المستنصرية، كلية الطب، التشريح البشري، العراق

المقدمة: تعتبر الغدة الدرقية واحدة من أهم الغدد الصماء في الجسم وان تضخم الغدة الدرقية (الدراق) هو من أكثر أمراض الغدد الصماء شيوعاً بين سكان العراق. يرتبط تعبير المستضد LGR٥ بعدد من الحالات المرضية، مثل سرطانات الغدة الدرقية، وبما ان البنية التركيبة لهذا المستضد تشابه البنية التركيبية للمستقبل TSH، هذا قد يفسر دور LGR٥ في الفيزيولوجيا المرضية للغدة الدرقية.

الهدف من العمل: الكشف عن التعبير المشترك للمستضد LGR٥ في انسجة الغدة الدرقية. الطبيعية وقارنتها مع التعبير لنفس المستضد في انسجة الغدة الدرقية المصابة بالدراق السام والغير سام.

المواد وطرق العمل: شملت الدراسة ٦٠ عينة من انسجة الغدة الدرقة. تم تقسيم العينات إلى ثلاث مجموعات: حالات تضخم الغدة الدرقية السامة، وحالات تضخم الغدة الدرقية غير السامة، وحالات الغدة الدرقية الطبيعية. تم جمع العينات النسيجية من المرضى ودراستها عن طريق الصبغ المناعي الكيميائي لعامل LGR٥ وتم قياسها واختبارها إحصائيًا مقابل مؤشرات أخرى مثل العمر والجنس ووجود سمية الغدة الدرقية.

النتائج: لقد ثبت الدراسة أن التعبير النسيجي للعامل LGR٥ عند مرضى الغدة الدرقية أعلى بكثير من تعبيره مقارنة بالعينات المأخوذة من الغدد الطبيعية. كما تبين ان التعبير هذا العامل كان أعلى بشكل ملحوظ في المرضى الذين يعانون من تضخم الغدة الدرقية السام. وخاصة عندما يتقدمون في السن مقارنة بالمرضى الذين يعانون من تضخم الغدة الدرقية غير السام.