

## **THE EXPRESSION OF INTERFERON- ALPHA IN THYROID TUMORS CORRELATED TO HUMAN PARVOVIRUS B19 EXISTENCE**

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### **ABSTRACT**

The present study was carried out to detect the expression of INF- $\alpha$  by immunohistochemical technique in Iraqi patients of malignant and benign thyroid tumors under the effect of parvovirus B19. A total number of (94) formalin-fixed paraffine-embedded thyroid tumors tissue collected from archivesdepartmentof histopathology laboratories during the period from July 2013 till November 2013. Tissue blocks were divided into three groups. First one included (53) tissue blocks of malignancy thyroid whereas the second group included (41) tissue blocks of benign thyroid tumors and the third group included (21) tissue blocks were selected from the same benign cases which have normal tissue. By using immunohistochemistry, B19 virus was detected in 66% of malignant and in 48.8%of benign tumors. INF- $\alpha$

expressed positively in 83% of malignant tumors compared to 63% of benign tumors, while it was detected only in 54 % of normal tissue. There was a significant correlation between human parvovirus B19 score category and INF- $\alpha$  score ( $r=0.296$ ,  $p=0.031$ ). It can be concluded that the expression of INF- $\alpha$  in thyroid tumors is significantly affected by the existence of human parvovirus B19.

**KEYWORDS:** thyroid tumors, human parvovirus B19, Interferone –alpha.

## INTRODUCTION

The thyroid is a major human endocrine gland that controls heat rate, blood pressure, temperature and metabolism. Most thyroid tumors are benign.<sup>[1]</sup> Thyroid cancer is the most common neoplasm of the endocrine system, which originates from follicular thyrocytes or parafollicular C-cells of the thyroid gland, represent a model of malignant transformation from benign adenomas and well differentiated carcinomas to poorly differentiated carcinoma.<sup>[2,3]</sup> Although relatively rare, thyroid cancer is the seventh most frequent human malignancy, and is increasing in incidence more rapidly than any other cancers. From 1995 to 2008, thyroid cancer age-adjusted incidence rates more than doubled in the U.S., whereas the incidence of most major cancers (lung, prostate, breast and colorectal) decreased during the same period<sup>[4]</sup>, Thyroid cancer occurs at ages from childhood to old age. Women are three times more likely than men to be afflicted.<sup>[5]</sup>

In Iraq thyroid cancer ranks the eighth among commonest ten cancers in female.<sup>[6]</sup> Therefore, thyroid cancer is a growing health problem. Depending on the cell origin and histological characteristics, thyroid carcinomas are generally classified to papillary thyroid carcinoma, follicular thyroid carcinoma, anaplastic thyroid carcinoma (poorly differentiated), and medullary thyroid cancer. Most of these thyroid tumors arise from the follicular thyrocytes whereas medullary thyroid cancer is the only C-cell-originated tumor.<sup>[7]</sup>

Exposure to ionizing radiation, iodine prophylaxis, external beam irradiation, and <sup>131</sup>I have also been put forth as possible reasons, but whether there are other causes, such as viruses, has not been determined.<sup>[8]</sup>

Human parvovirus B19 has been shown to be a cause of several, wide-ranging human illnesses<sup>[9]</sup>, including aplastic crisis, erythema infectiosum (fifth disease), arthritis, thrombocytopenia, hydropsfetalis, and myocarditis. B19 has also been highly associated with neurological disorders<sup>[10, 11]</sup> and autoimmune disorders with symptoms similar to rheumatoid arthritis and lupus erythematosus.<sup>[12]</sup> Recently, B19 infection has been strongly associated with hashimotos 's thyroiditis and thyroid cancer<sup>[13, 14, 15, 16]</sup>. Interferon-alpha (IFN- $\alpha$ ) It is predominantly characterized as an antiviral and antiproliferative agent which stimulates both macrophages and natural killer cells to elicit an antiviral response, and it is also active against tumors. Besides, a very important property of IFN- $\alpha$  is its ability to arrest the cell in the G1-G0 phase, which is what determines static effect of IFN- $\alpha$  on growth of many tumors.<sup>[17]</sup>

## MATERIALS AND METHODS

### Tissue samples

The retrospective study included ninety four formalin-fixed, paraffin –embedded tissue blocks, which have been diagnosed as thyroid tumors. Fifty three of these were malignant thyroid tumors, forty one were benign thyroid tumors and twenty one case selected from benign tumors which have normal tissue as control from period 2010 till 2013. The study samples were collected from the pathology archives of the Teaching laboratories Department, Medical/ city, Al-shaheed Ghazi hospital lab, Al-Kindy hospital lab, Al-karama hospital lab and central public health lab. All Hematoxylin and Eosin stained tissue sections were reviewed, the best sections and Those representing the original tumor site from each specimen were selected. In period from August 2013 to April 2014 all the preparations for immunohistochemistry were performed in histopathology unit in central public health lab. From each block, three sections of 4µm thickness were taken, one section was stained with Hematoxylin and Eosin (H&E) and the other two sections were immunohistochemically stained for human parvovirus B19, INF- $\alpha$ .

### Immunohistochemistry and scoring

Paraffin sections of 4µm thickness were deparaffinised and treated with hydrogen peroxide to block endogenous peroxidase activity. Heat-induced antigen retrieval was performed in sodium citrate (pH 6.0) and protein block at room temperature to block the non specific antibody-antibody-binding site. Then the sections were incubated overnight at 4° C with mouse monoclonal antibody against the B19 proteins VP1/VP2 (1:30) dilution; and INF- $\alpha$  (1:50) dilution (abcam, UK).

IHC was performed to detect and determine of B19 capsid protein in each tissue section. Each case was assigned a positive score based on the value by multiplying the average intensity of each positive area by the total positively of the whole tissue section. Cases with score less than five were considered negative (-), with those from 5.1-20 ranking (+), and greater than 20.1 ranking (++). <sup>[16]</sup>

Two experienced pathologists examined the integrated slides without information regarding patients. The percentage of positive cells and the intensity of positive staining were scored according to the Axiotis score standard based on evaluation of five randomly chosen fields at 400-fold magnification. If the histology scores of the two pathologists were different for the same sample, we chose the mean as the final result. Percentage of tumor cells staining: 0,

0%-10%; 1 +, 11% - 25 %; 2 +, 26% - 50 %; 3 +, 51 % - 75 %; 4+, 76 % - 100 %; and intensity of immuno- staining: 1 +, weak staining; 2 +, moderate staining; 3+, strong staining.<sup>[18]</sup>

**Statistical Analysis:** Data were translated into a computerized database structure. An expert statistical advice was sought for. Statistical analyses were done using SPSS version 21 computer software (Statistical Package for Social Sciences).

Some of the quantitative outcome variables were measured on an ordinal scale (like intensity). Compliance of quantitative random variables with Gaussian curve (normal distribution) was analyzed using the Kolmogorov-Smirnov test. The percentage and score measures of selected markers were shown to be non-normally distributed quantitative variables. These variables in addition to those measured on an ordinal scale (like intensity) can be described by median and inter-quartile range. The difference in median between 2 groups was assessed by non-parametric test (Mann-Whitney), while between more than 2 groups Kruskal-Wallis test was used. Associations between 2 categorical variables were explored by cross-tabulation. The statistical significance of such associations was assessed by Chi-square ( $\chi^2$ ) test. The statistical significance, direction and strength of linear correlation between 2 quantitative normally variables, one of which being non-normally distributed was measured by Spearman's rank linear correlation coefficient. P value less than 0.05 level of significant were considered statistically significant.

## RESULTS

**Immunohistochemical expression of human parvovirus B19 –capsid protein in patients with thyroid tumors:** The results of B19 protein detection in patients with thyroid tumors, using immunohistochemistry (IHC) technique revealed that 66% (35 out of 53) of malignant thyroid tumors, 48.8% (20 out of 41) of benign thyroid tumors and 4.8% (1 out of 21) of normal thyroid tissue blocks have positive reaction. Statistically, the positive rate of B19 VP1/VP2 antigen in thyroid malignancy cases was higher than benign but not significantly while cases of thyroid malignancy and benign cases were both significantly higher than normal thyroid tissue  $p < 0.001$  (Table 1).

Classification of the cases of carcinoma, benign and control groups into different grades of intensity (negative, weak, moderate, and strong) showed significant difference among these groups.

**Table 1: Human Parvovirus B19 Expression In Study Groups.**

	Healthy control (normal thyroid tissue)	Diseased controls (Benign thyroid tumor)	Cases (Thyroid malignancy)	P value
Human Parvovirus B19	N (%)	N (%)	N (%)	<0.001
Negative	20 (95.2)	21 (51.2)	18(34)	
Positive	1 (4.8)	20(48.8)	35(66)	
Total	21 (100)	41(100)	53(100)	
<b>P (Chi-square) for 2 groups comparison:</b>				
Cases (Thyroid malignancy) x Positive controls (Benign thyroid tumor) = 0.09[NS]				
Cases (Thyroid malignancy) x Healthy control (normal thyroid tissue) <0.001				
Positive controls (Benign thyroid tumor) x Healthy control (normal thyroid tissue) = 0.001				

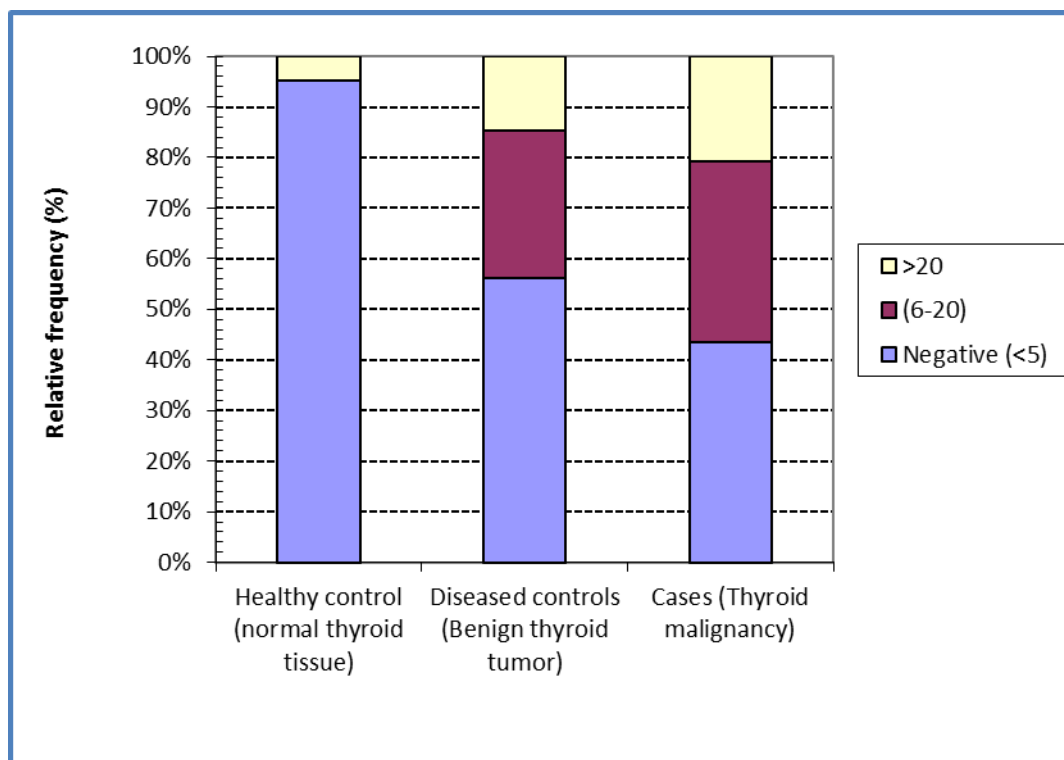
**Table 2: Immunohistochemical scoring of human parvovirus B19 intensity.**

	Healthy control (normal thyroid tissue)	Diseased controls (Benign thyroid tumor)	Cases (Thyroid malignancy)	P value
Human Parvovirus B19-intensity	N (%)	N (%)	N (%)	0.001
Negative	20 (95.2)	21 (51.2)	18 (34)	
Weak intensity	0 (0)	0 (0)	2(3.8)	
Moderate intensity	1 (4.8)	16 (39)	24(45.3)	
Strong intensity	0 (0)	4 (9.8)	9(17)	
Total	21 (100)	41 (100)	53(100)	
Median	negative	negative	moderate	
Mean rank	32.5	58.0	68.1	
<b>P (Mann-Whitney) for difference in median between 2 groups</b>				
Cases (Thyroid malignancy) x Positive controls (Benign thyroid tumor) = 0.11[NS]				
Cases (Thyroid malignancy) x Healthy control (normal thyroid tissue) <0.001				
Positive controls (Benign thyroid tumor) x Healthy control (normal thyroid tissue) = 0.001				

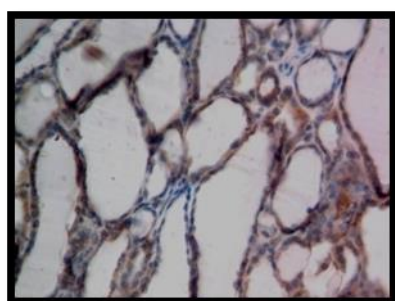
Strong human parvovirus B19 capsid protein staining was mainly seen in (9 out of 53) 17% of malignant cases in comparison with benign cases (4 out of 41) 9.8% and healthy tissue (0 out of 21) 0%. While the frequency of moderate staining was (24 out of 53) 45%, (16 out of 41) 39% and (1 out of 21) 4.8% in malignant, benign and healthy tissue, respectively (Table 2).

The scoring system and cut-off value were used depending on (Adamson, 2013). Figure (1) shows the difference in median of human parvovirus B19 score categories, the score category

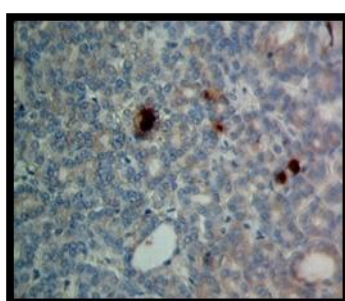
that got the higher percentage (6-20) of expression of B19 was in malignant cases (19 cases, 35.8%) and in benign cases it was (12 cases, 29.3%). While lowest score categories were (11 cases, 20.8%), (6 cases, 14.6%) in malignant and benign cases respectively, which was among the score ( $>20$ ) compared with healthy tissue which have (1 case only 4.8%) in score ( $>20$ ).



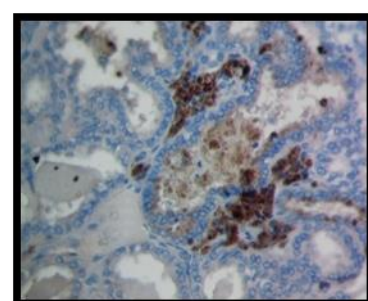
**Figure 1:** Component bar chart showing the difference in score categories of human Parvovirus B19 marker between the 3 study groups.



**A**



**B**



**C**

**Figure. 2.** Immunohistochemical expression of B19 capsid protein in thyroid tumors, (A) Normal thyroid follicle with cytoplasm expression, (B) Adenoma which express nuclear staining (C) papillary carcinoma which express nuclear staining, thick arrow and cytoplasm expression, thin arrow. X40.



### Immunohistochemical expression of INF- $\alpha$

The results revealed positive expression of INF-  $\alpha$  in cytoplasm of malignant cells as a diffuse brown pigment. In benign cases 63% were positive for INF- $\alpha$ , and 37% were negative. From the total 53 malignant thyroid cases, 44 (83%) were positive and 9 (17%) negative. Statistically there is a significant difference between thyroid malignant and benign tumors  $p=0.046$  also between thyroid malignancy and healthy tissue  $p=0.007$  (Table 3).

The majority of those 44 cases, 37.7% were weak intensity, 34% were moderate and 11.3% were strong intensity for INF-  $\alpha$ . According to benign tumors the majority of these 41 cases, 33.3% were moderate, 25.9% were weak and 3.7% were strong while the majority of healthy tissue 42.9% were weak intensity positive expression (Table 4).

Figure (3) shows the results in median score of INF-  $\alpha$  were 4 in malignant and benign thyroid tumor and 1 in normal tissue with significant correlation.

In the present study, there was a statistically significant positive linear correlation between INF- $\alpha$  score and human parvovirus b19 score category ( $r=0.296$ ,  $p=0.031$ ) as presented in (table 6).

**Table.3 Immunohistochemical expression of INF- $\alpha$  in study groups**

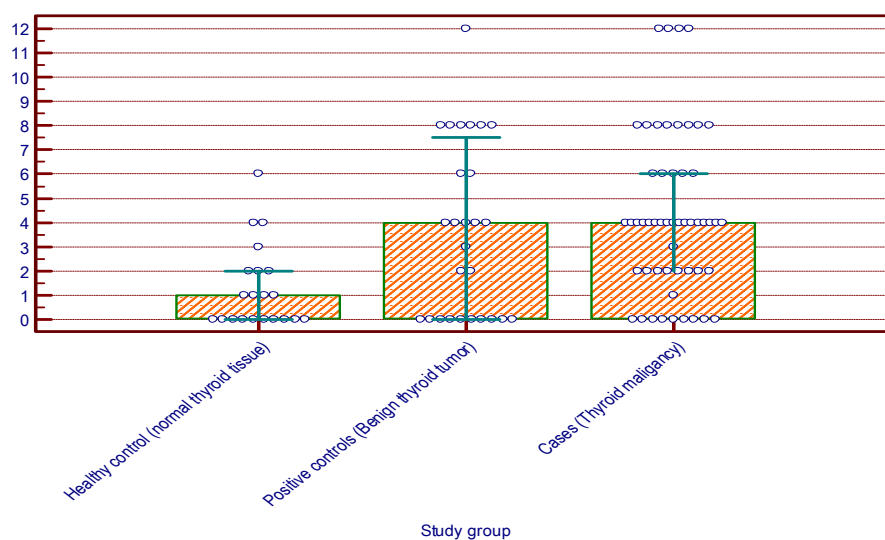
	Healthy control (normal thyroid tissue)	Diseased controls (Benign thyroid tumor)	Cases (Thyroid malignancy)	P .value
INF- $\alpha$	N (%)	N (%)	N (%)	0.017
Negative	10 (47.6)	10(37)	9(17)	
Positive	11 (52.4)	17(63)	44(83)	
Total	21 (100)	41(100)	53(100)	
P (Chi-square) for 2 groups comparison:				
Cases (Thyroid malignancy) x Positive controls (Benign thyroid tumor) = 0.046				
Cases (Thyroid malignancy) x Healthy control (normal thyroid tissue) =0.007				
Positive controls (Benign thyroid tumor) x Healthy control (normal thyroid tissue) =0.46[NS]				

**Table4:Immunohistochemical score of INF-intensity.**

	Healthy control (normal thyroid tissue)	Diseased controls (Benign thyroid tumor)	Cases (Thyroid malignancy)	P .value
INF- $\alpha$	N (%)	N (%)	N (%)	0.004
Negative	10(47.6)	10 (37)	9 (17)	
Weak intensity	9 (42.9)	7 (25.9)	20 (37.7)	
Moderate intensity	2 (9.5)	9 (33.3)	18 (34)	
Strong intensity	0 (0)	1 (3.7)	6 (11.3)	
Total	21 (100)	41 (100)	53(100)	
Median	weak	weak	weak	
Mean rank	35.1	48.2	58.7	
<b>P (Mann-Whitney) for difference in median between 2 groups</b>				
Cases (Thyroid malignancy) x Positive controls (Benign thyroid tumor) P= 0.12[NS]				
Cases (Thyroid malignancy) x Healthy control (normal thyroid tissue) P =0.001				
Positive controls (Benign thyroid tumor) x Healthy control (normal thyroid tissue) P =0.13[NS]				

**Table 5: Difference in expression of human parvovirus B19 and INF- $\alpha$  among histopathological types of malignant cases.**

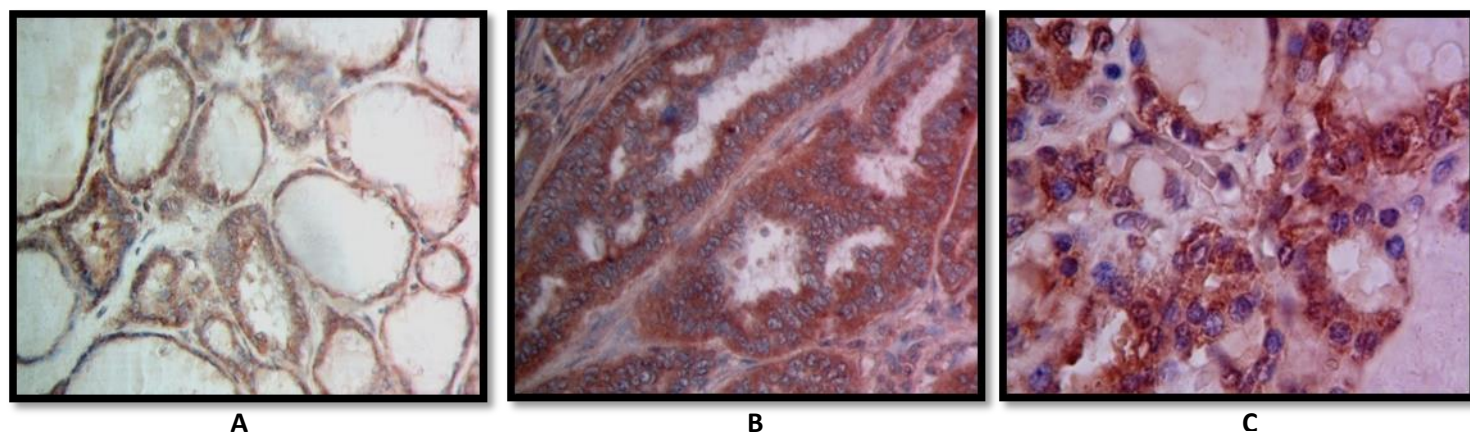
	Follicular Thyroid Carcinoma (FTC)		Papillary Thyroid Carcinoma (PTC)		P
	N	%	N	%	
<b>Human Parvovirus B19</b>					0.41[NS]
Negative	1	12.5	10	30.3	
Positive	7	87.5	23	69.7	
Total	8	100.0	33	100.0	
<b>Interferone alpha</b>					0.12[NS]
Negative	3	37.5	4	12.1	
Positive	5	62.5	29	87.9	
Total	8	100.0	33	100.0	

**Figure.3: Bar chart showing the difference in median score of interferone alpha marker between the 3 study groups.**



**Table 6: linear correlation for cases goup.**

Parameters correlated	Linear correlation (r)	P-value
INF- $\alpha$ score & B19 virus score category	0.296	0.031



**Figure.4 Immunohistochemical expression of Interferon- $\alpha$  (A) Normal thyroid follicles with cytoplasm staining,(B)Adenoma (C) papillary carcinoma with strong cytoplasm staining X40**

## DISCUSSION

In the present study, B19 capsid proteins were frequently detected in malignant thyroid tumors tissue 66% compared to benign 48.8% and normal tissue 4.8%. These results support that recorded by Wang *et al.* <sup>[13]</sup> who found B19 infection in papillary thyroid cancer tissue samples using nested PCR even in 95%-97%, insitu hybridization (ISH) in 83.3% and immunohistochemistry (IHC) in 63% of their cases .The results also agreed with Adamson *et al.* <sup>[15]</sup>who extend the data available on B19 detection in thyroid to show 21 of 24 (88%) PTC tumors and 3 of the 3 ATC undifferentiated tumors tissue samples were positive for B19 capsid protein by IHC. Adamson <sup>[16]</sup>results also showed that B19 infection in the thyroid tissue increased capsid protein detection in adenoma and tumor. These studies were the only three attempts which have been done in China and Florida trying to correlate B19 and thyroid tumors while the present study, according to our best knowledge was the first one in Middle East.

There was no significantly difference between papillary and follicular thyroid carcinoma in expression of B19 capsid proteins which were 69.7% and 87.5%, respectively as shown in

table(5). These results were higher than that in benign and normal tissue, among the other finding in this respect, (1 out of 3) cases with anaplastic carcinoma revealed a positive expression of B19 while no one of the three cases with medullary carcinoma was with positive expression. These findings came in agreement with that of Wang *et al.*,<sup>[13]</sup> in respect to papillary and medullary carcinoma but not in follicular cases, who interpreted such findings depending on the well known observation that B19 can only infect the cells that have the proper receptors to which the virus can bind.<sup>[19]</sup> Human thyroid follicle epithelia have been shown to have globoside.<sup>[20]</sup> Therefore; the expression of the specific cellular receptor of B19 on these cells might interpret their susceptibility to B19 infection. While the absence of such receptors on medullary thyroid carcinoma cells may justify the insusceptibility to infection with this virus.<sup>[13]</sup>

However, In the last few years Norja *et al.*<sup>[21]</sup> have found that the persistence of B19 genome in human tissues is ubiquitous and lifelong. Another publication has demonstrated short regions of sequence identity between B19 and human genes, and these sequence identity may be biologically relevant to the persistence of the viruses in human tissues.<sup>[22]</sup> B19 DNA has been detected in sera and may persist in various tissues liver, thyroid, testis, brain, tonsils, skin, myocardial endothelium and synovial.<sup>[23, 21, 24, 15, 25]</sup>

The increased presence of B19 capsid could reflect the efficacy of B19 infection in these tumors or of B19 replication, the persistence of B19 genome mainly denotes latent infection. While, usually persistence of viral genome with expressed viral proteins in the tumor cells indicates a productive infection of virus in pathways leading to the development/or progression of cancer.<sup>[26]</sup> Our findings that B19 capsid protein expression in papillary and follicular thyroid carcinoma can be interpreted depending on the above mentioned which may indicate that the virus was active in papillary thyroid carcinoma and follicular thyroid carcinoma lesions, and the productive infection of the virus might play some role, directly or indirectly, as a cofactor in the development of the tumor. Keeping in mind that benign thyroid proliferative disease may become neoplastic<sup>[27, 28]</sup> and some benign Thyroid diseases without histopathological evidence of papillary thyroid carcinoma harbor similar molecular genetic changes with papillary thyroid carcinoma.<sup>[29]</sup>

INF- $\alpha$  has a variety of biologic properties, including antiviral, immunomodulatory, anti-proliferation, anti-angiogenic effects.<sup>[30]</sup> It is type one interferon that has widely used as a therapeutic agent mostly, but not exclusively, for infectious and malignant disease.<sup>[31]</sup>

The present study is the first study that assessed INF- $\alpha$  in thyroid tumors by immunohistochemistry technique. The scoring system used according to Axiotis.<sup>[18]</sup> Two parameters were used intensity of the stain and proportion of the stained cells.

This study showed a statistical difference between benign and malignant expression and between malignant thyroid tumors and adjacent healthy tissue. study of Kobawala *et al.*, reported ( By using immunoassay EIA) that serum INF- $\alpha$  was significantly higher in all the patients with thyroid disorders (goitre, autoimmune thyroid disease, and thyroid carcinoma) as compared to healthy individuals they also found significantly increased levels of INF- $\alpha$  in patients with papillary and follicular carcinoma. So, the authors suggest attributed such elevation to the excessive production of this cytokine in tumor cells and subsequent release into the circulation.<sup>[32]</sup> INF- $\alpha$  stimulates both macrophages and natural killer cells to elicit an anti viral response, and it is also activate against tumors, Besides, a very important property is it's ability to arrest to cells in the G1-G2 phase, which is what determines static effect of INF- $\alpha$  on many tumors.<sup>[17]</sup> Few studies to date examined the direct effects of INF- $\alpha$  on thyroid cells. It was shown to inhibit TSH-induced gene expression of thyroglobulin (TG), TPO, and sodiumiodidesymporter (NIS) in cultured human thyrocytes.<sup>[33]</sup>

Up to our best knowledge there is no study describing the correlation between thyroid tumor patients with human parvovirus b19 and INF- $\alpha$  co- expression but still these are some comparable attempts have been done by Wu *et al.*<sup>[34]</sup> who studied the correlation between intrahepatic INF- $\alpha$  expression levels and disease severity using liver biopsy specimens from HBV-infected patients with different outcomes and reported that there was no significant correlation between intrahepatic INF- $\alpha$  expression and HBV DNA levels in liver biopsy samples in chronic hepatitis B, sever hepatitis B and liver cirrhosis, while the IHC scores of intrahepatic INF- $\alpha$  expression in the SHB group were significantly higher than those observed in the LC and NC groups. This differences between the result of the current study and this one can be attributed to difference in tissue, disease, the different technique for HBV was ISH to asses DNA with IHC to assay INF- $\alpha$  while for b19 was IHC to asses capsid protein with IHC INF- $\alpha$  also to asses protein. Previous studies have shown that INF- $\alpha$  can active the expression of hundereds of genes, and generate some INF-inducible proteins to establish the antiviral state<sup>[35]</sup>. INF-  $\alpha$  also directly enhance NK cell mediatating cytotoxicity through activating NK cell recptors, and also regulates cytotoxic T.cells differentiation.<sup>[36]</sup>

## CONCLUSION

Detection of parvovirus B19 capsid protein in malignant and benign thyroid tumors suggesting the noxious role of such virus in thyroid tumors. In addition to INF- $\alpha$  expression is increased with progression of benign and malignant thyroid tumors indicating that INF-  $\alpha$  may play a role in pathogenesis of such tumors and there is a significant correlation between INF-  $\alpha$  score category and B19 score.

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