Immunohistochemical Detection of Latent membrane Protein 1 and Human Twist Protein in Patients with Thyroid Cancer

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ABSTRACT
Epstein Barr virus is tumour virus that expresses cancer genes and immortalizes infected lymphocytes. This study is designed to determine the presence of the LMP-1 and human Twist protein and study the association between them in a patient with thyroid cancer and benign lesion. The retrospective study consists of 40 formalin-fixed, paraffin-embedded thyroid tissue blocks were obtained, from 20 patients with thyroid papillary carcinoma and 20 patients with thyroid follicular carcinoma, and compared with 10 tissues from follicular hyperplasia without atypia were collected from Teaching Laboratories in Medical city, Baghdad, during the period from July 2019 till February 2020. Tissue blocks were used for the detection of LMP1 and human twist protein by immunohistochemistry technique. The results of LMP-1 was (45%, 40% and 30%) respectively and high proportion was noticed in females than males, (77.78%), (78%), (100%) respectively, and the results reveal high percentage 66.67% in age (20-40 years). Human twist protein shows that the most favourable cases 9 (45%), 8 (40%), in patients with PTC and FTC while no instances in the control group. In conclusion, LMP1 and human Twist protein may have an essential role in the progression of thyroid cancer.

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INTRODUCTION
Thyroid cancer is common in endocrine malignancy whose incidence has increased in recent years; several internal and external risk factors are involved in the development of this cancer, such as infectious agents. Evidence supporting the role of viral infection as an aetiology for the invasiveness of thyroid cancer is increasing (Moghoofei et al., 2019).

Epstein Barr virus (EBV) is a ubiquitous virus which belongs to the gamma-herpes virus are known as tumour viruses that express virus cancer genes and immortalize infected lymphocytes (Kimura et al., 2013). Among all the EBV genes, most of the recent investigations focused on studying the latent membrane protein 1 (LMP-1) oncogene because of its high degree of polymorphism and association with tumorigenic activity. There are two main EBV genotypes, 1 and 2, distinguished by the differences in the Epstein Barr virus nuclear antigen-2 gene (EBNA-2). Further subgenotype can be characterized by analyzing the Latent membrane protein 1 (LMP-1) gene variation (Smatti et al., 2018).

Transmission occurs mainly through bodily fluids, especially saliva. These viruses can also spread through blood and semen during sexual contact, blood transfusion, and organ transplantation (Balfour et al., 2015).
Primary infection usually asymptomatic, although a subset of the person in whom primary infection is delayed until adolescence or young adulthood develop infectious mononucleosis (Young and Richardson, 2004). Past EBV infection is associated with lymphomas and may also result in certain allergic and autoimmune diseases (Dittfeld et al., 2016). Epstein-Barr virus has been studied as a potential cause of thyroid infection due to the herpesviral group known for its association with certain types of tumour and underlying infection. Human thyroid inflammation is a common disease and is classified into different types depending on the causative agent and a clinical and immunological condition (Al-Tamimi and Rushdi, 2014; Almeida et al., 2017).

Twist a basic helix loop transcription factor has indicated to play a critical role in the progression of numerous malignant disorder (Zhuo et al., 2015). Its role in tumour progression is associated with the metastatic process also it’s over expression increases the invasive and metastatic ability of cancer cells by downregulation of E-cadherin expression and induction of EMT (Yang et al., 2004). Furthermore, growing evidence indicates that twist1 also plays a crucial role in supporting tumour initiation by evading p53 induced cell senescence and apoptosis, the well-known program to counter cell transformation (Ansieau et al., 2008). So this study to detect the presence of LMP-1 and to evaluate the amount of anti-Twist in tissues of patients with thyroid cancer and benign lesion, and to study the correlation between EBV infection and different parameters such as age, gender and type of cancer.

MATERIALS AND METHODS

The retrospective study consists of 40 formalin-fixed, paraffin-embedded thyroid tissue blocks were obtained, from 20 patients with thyroid papillary carcinoma and 20 patients with thyroid follicular carcinoma, 32 (female) and 8 (males). Their age ranged from 20-80 years, there were 10(female), and their age ranged from 22 to 68 as the control group.

Formalin-fixed, paraffin-embedded thyroid tissue block collected from Teaching laboratories in Bagdad during the period from July 2019 till February 2020, while the pathological samples belong to the period from 2015 to 2019. The diagnosis of these tissue blocks was primarily based on the obtained histopathological records of thyroid tissue samples that had accompanied in the hospital laboratory such as the age of patients, gender and tumour type.

All slides were reviewed by Dr Abdul-Razak Naif at Histology Department of Kirkuk General Hospital and given the WHO classification of thyroid tumour, so each case was reassigned accordingly (Hedinger, 1988).

All formalin-fixed, paraffin-embedded tissue blocks were sectioned (4μm thickness, two-section were mounted on charged slides to be used for (LMP-1 and human twist), and one section was stained with Haematoxylin and Eosin.

The slides were dehydrated by graded alcohol concentration (100%, 95%, 70%) and distal water and phosphate-buffered saline then treated with endogenous peroxidase and dehydrated. Protein block added and dehydrated after that primary antibody was applied to cover slides, the primary antibody was prepared at dilution 1-50 by mixing 1μl from the concentrated protein with 49 μl of PBS for EBV LMP-1, and dilution 1-100 by mixing 1 μl from the concentrated protein with 99 μl of PBS for a human twist, and incubated for 1 hour in a humidity chamber at 37° C, then drops of secondary antibody reagent were added and incubated for 10 minutes, streptavidin-HRP antibodies were applied on tissue section and incubated for 10 minutes at room temperature, a drop of substrate chromogen was applied on tissue and incubated for 1-10 minutes. Slides were then counter stained, and sections were mounted with a permanent-mounting medium (DPX). Finally, the examination under a light microscope at power 40 by a pathologist. Immunohistochemistry of the twist expression was scored according to (Wang et al., 2013). [The intensity score was noted with 0 (no staining), 1 (low intensity), 2 (moderate intensity) and 3 (high intensity), the Score 1(5 %), Score 2 (6-25%), Score 3(26-57%) and score 4 (≥75%).]

Statistical Analysis

The analysis was conducted to describe different variables and parameters in this research and to describe relationships with each other as well. Chi-square test was used to find out the effect of different patients’s criteria on the reading of each marker of immunohistochemistry.

RESULTS

Immunohistochemical results demonstrate that 45% (9 cases out of 20) of papillary thyroid carcinoma, 40% (8 cases out of 20) of thyroid follicular carcinoma and 30% (3 cases out of 10) of thyroid follicular hyperplasia without atypia are positive for LMP-1 but statistically non-significant, as shown in Table 1 and Figure 1.
Table 1: Immunohistochemical detection of LMP-1 in the study groups

| IHC results of LMP-1 | Thyroid papillary carcinoma | Thyroid follicular carcinoma | Thyroid follicular hyperplasia without atypia | P-value |
|----------------------|----------------------------|-----------------------------|---------------------------------------------|---------|
| Positive results     | 9 (45%)                   | 8 (40%)                     | 3 (30%)                                     | 0.73 Not significant at p<.05 |
| Negative results     | 11 (55%)                  | 12 (60%)                    | 7 (70%)                                     |         |
| Total                | 20 (100%)                 | 20 (100%)                   | 10 (100%)                                   |         |

Table 2: Distribution of patients with LMP-1 according to gender and age in the studies group

| Variable factors | Thyroid papillary carcinoma No.% | Thyroid follicular carcinoma No.% | Thyroid follicular hyperplasia No.% | P-value |
|------------------|----------------------------------|----------------------------------|------------------------------------|---------|
| Gender           |                                  |                                  |                                    |         |
| Female           | 7 (77.78%)                       | 6 (75%)                          | 3 (100%)                           | 0.92 Not significant at <.05 |
| Male             | 2 (22.22%)                       | 2 (25%)                          | 0                                  |         |
| Total            | 9 (100%)                         | 8 (100%)                         | 3 (100%)                           |         |
| Age group        |                                  |                                  |                                    |         |
| 20-40y           | 6 (66.67%)                       | 2 (25%)                          | 0                                  | 0.49 Not significant P <.05 |
| 41-60y           | 3 (33.33%)                       | 4 (50%)                          | 3 (100%)                           |         |
| 61-80y           | 0                                | 2 (25%)                          | 0                                  |         |
| Total            | 9 (100%)                         | 8 (100%)                         | 3 (100%)                           |         |

Table 3: Immunohistochemistry detection of human twist protein in study groups

| IHC results of human twist | Thyroid papillary carcinoma No.% | Thyroid follicular carcinoma No.% | Thyroid follicular hyperplasia No.% | P-value |
|---------------------------|----------------------------------|----------------------------------|------------------------------------|---------|
| Positive results          |                                  |                                  |                                    |         |
| High                      | 9 (45%)                          | 8 (40%)                          | 0                                  | 0.65 Not significant at p <.05 |
| Intermediate              | 2 (10%)                          | 1 (10%)                          | 2 (20%)                            |         |
| Low                       | 3 (15%)                          | 0                                | 7 (70%)                            |         |
| Negative results          |                                  |                                  |                                    |         |
| Low                       | 5 (25%)                          | 10 (50%)                         | 7 (70%)                            |         |
| Total                     | 20 (100%)                        | 20 (100%)                        | 10 (100%)                          |         |

Figure 1: IHC staining of LMP-1 in thyroid follicular carcinoma section stained by DAB chromogen and counter stained with haematoxylin (Magnification power, 400), A-LMP-1 positive expression, B- LMP-1 negative expression
Table 4: Number and proportion of cases with positive twist antibody according to gender and age in studies group

| IHC results of anti-twist antibody | Thyroid papillary carcinoma No. | Thyroid follicular carcinoma No. | Thyroid follicular hyperplasia without atypia No. | P-value |
|-----------------------------------|-------------------------------|-------------------------------|-------------------------------------------------|---------|
| Gender                            | Females                       | 13 (86.67%)                  | 8 (80%)                                         | 3 (100%)| 0.41*   |
|                                   | Males                         | 2 (13.33%)                   | 2 (20%)                                         | 0       |         |
| Total                             |                               | 15 (100%)                    | 10 (100%)                                       | 3 (100%)|         |
| Positive results                   |                               | 20-40y                       | 8 (53.33%)                                      | 2 (20%) | 1 (33.33%)|
|                                   | 41-60y                        | 6 (40%)                      | 6 (60%)                                         | 0       |         |
|                                   | 61-80y                        | 1 (6.67%)                    | 2 (20%)                                         | 2 (66.67%)|   |
| Total                             |                               | 15 (100%)                    | 10 (100%)                                       | 3 (100%)|         |
| Negative                          |                               | 5                             | 10                                              | 7       |         |
| Total                             |                               | 20 (100%)                    | 20 (100%)                                       | 10 (100%)|   |

* for age group

Table 5: Number and proportion of positive LMP-1 human twist protein according to age groups

| Positive human twist protein       | Thyroid papillary carcinoma Positive LMP-1 No. | Thyroid follicular carcinoma Positive LMP-1 No. | Thyroid follicular hyperplasia Positive LMP-1 No. | P-value |
|-----------------------------------|-----------------------------------------------|-----------------------------------------------|-------------------------------------------------|---------|
| Gender                            | Female                                        | 6 (85.71%)                                    | 7 (87.5%)                                       | 1 (100%)| 0.91    |
|                                   | Male                                           | 1 (14.29%)                                    | 1 (12.5%)                                       | 0       | 0.44*   |
| Age groups                        |                                               |                                              |                                                 | Not significant at p<.05 |
| 20-40y                            | 4 (57.14%)                                    | 2 (20%)                                       | 0                                               |         |
| 41-60y                            | 2928.58%(                                    | 4 (50%)                                       | 1 (100%)                                       |         |
| 61-80y                            | 1 (14.28%)                                    | 2 (20%)                                       | 0                                               |         |
| Total                             | 7                                             | 8                                             | 1                                               |         |

* for gender

Figure 2: Immunostaining for human twist protein stained by DAB chromogen and counter stained with hematoxylin (Magnification power, 400). A- Human twist positive expression, B- Human twist negative expression
Distribution of positive LMP-1 according to gender and age groups are summarized in Table 2, show that females high proportion than males in thyroid papillary carcinoma group (77.78%), thyroid follicular carcinoma group (75%) and thyroid follicular hyperplasia without atypia group (100%). High frequency (66.67%) were noticed in age group (20-40) years but statistically non-significant

Most positive cases for human twist protein showed in high score 9 (45%) in patients with thyroid papillary carcinoma, 8 (40%) in patients with thyroid follicular carcinoma, while no high score among the control group, (Table 3 and Figure 2).

The distribution of gender and age had revealed that higher percentage of human twist positive cases is detected in females 13 (86.67%), 8 (80%), 3 (100%) in patients with thyroid papillary carcinoma, thyroid follicular carcinoma, and thyroid follicular hyperplasia without atypia respectively. While most positive cases of thyroid papillary carcinoma patients in age group (20-40). The results of the frequency distribution of positive human twist showed no significant differences between each group and age of patients (P>0.050) as shown in Table 4.

Based on results the Table 5, the association between LMP-1 and overexpression human twist protein in patients with thyroid nodules found that 7 cases positive for both among thyroid carcinoma, 8 cases among thyroid follicular carcinoma, while one example among the control group. Most cases were in female and age (20-60) years. However, the difference failed to reach the level of statistical significances (P=0.05) between LMP-1 and human twist protein overexpression in studies groups.

DISCUSSION

The present study focused on the association between LMP-1 and thyroid cancer using IHC, the results which demonstrated that LMP-1 was observed in 45% of patients with thyroid papillary carcinoma, 40% thyroid follicular carcinoma and 30% thyroid follicular hyperplasia without atypia. This result was consistent with the finding of Shimakage (2003) explored the potential involvement of EBV expression in the progression of thyroid cancer by examining different thyroid neoplasm specimens ranging from PTC to anaplastic thyroid carcinoma. Also, agreement with Homayouni et al. (2017) reported the presence of Epstein Barr virus nuclear antigen 1 gene in papillary thyroid carcinoma tissue by the nested-PCR method in Iran. The result of the present study is relatively higher than a study done by Stamatiou et al. (2016) identified Epstein-Barr virus DNA in 15 out of 100 thyroid cancers, and 7 of them turned out to be EBER-positive. Epstein Barr virus is detected in thyroid lymphoma, and 30 cases of Hashimoto’s thyroiditis by in situ hybridization EBV related mRNA, as well as the associated proteins, were investigated in 32 cases using ISH and IHC on routinely processed tissue sections (Takahashi et al., 1995). The result of the present study is lower than others, such as Stamatiou et al. (2015), who detected LMP1 in 50% (15/30) of the nodules and 46% (14/30) of the adjacent healthy tissue, other study done by Homayouni et al. (2017) reported the presence of Epstein Barr virus nuclear antigen 1 was 65.8% in papillary thyroid carcinoma tissue by nested polymerase chain reaction method in Iranian patients.

In contrast to the studies mentioned above, negative results regarding the association between thyroid tumours and EBV have been reported by several studies such as Kijima et al. (2001). None of the 45 PTCs resected from patients in the southern part of Kyushu, Japan and subjected to EBER1 in situ hybridization tested positive for EBV. One study reported PCR revealed no significant positive signal for EBV, ISH for EBER and IHC, performed in cases (11 females/ 1 male) of oncocytic PTC with lymphoid stroma (Warthin-like tumour) (Ludvikova et al., 2001).

This study detects LMP-1 (30%) among the control group. This agrees with Stamatiou et al. (2015) high EBV detection percentage in thyroid nodules consisting of PTCs and multinodular hyperplasia specimens, as well as adjacent healthy thyroid tissue. Another study investigated the frequency of the 30-bp deletion in EBV healthy carriers from Argentina and found that it was present in 28% of these healthy people (Correa et al., 2004).

In the present study, most, signals noticed in nuclear. At the same time, other found in cytoplasmic, so this consist of study is done by Tomita et al. (1995) who reported LMP1 is also expressed in the cytoplasm of lymphoma cells in patient’s chronic lymphocytic thyroiditis.

The differences and similarities between the results of the previously mentioned studies and with the results of the present study could be related to many factors, like the type of the tumour, whether papillary or follicular, the methodology, the affinity of the antibody, the sensitivity of the test, number of the study population as well as the lifestyle.

Latent membrane protein -1 was detected by IHC technique in the present study and the result revealed that the prevalence of LMP-1 was found to be higher in females, this result agreed with Tomita et al. (1995) who found (10 males and 48 females)
among 30 patients with thyroid lymphoma and 28 with chronic lymphocytic thyroiditis. Regarding the comparison of LMP-1 expression results obtained according to age group with thyroid lesions in the present study showed a high percentage of 66.67% in thyroid papillary carcinoma was noticed in the age group (20-40) years. In comparison, thyroid follicular carcinoma 50% and thyroid follicular hyperplasia 100% was more common in age group (41-60) years but statistically non-significant, this result consistent with Kilfoy et al. (2009) found patient under 45 years of age. Those aged 45 or over can have a different prognosis for tumours of similar characteristics.

Little is known concerning the role of twist 1 in thyroid cancer. In the present study, the expression of human twist is measured in thyroid papillary carcinoma, thyroid follicular carcinoma, and thyroid follicular hyperplasia without atypia. The results showed high expression of human twist among study population but statistically non-significant, this result consists with a study done by Wang et al. (2013); He et al. (2008), while the outcome of present study disagrees with Buehler et al. (2013) investigated that the expression of Twist 1 in 28 papillary thyroid carcinomas and 27 follicular carcinomas including tall cell and follicular variants found no reactions in all cases, the immunostaining being present only in anaplastic forms of tumours. Twist, is detected by immunohistochemistry only in human anaplastic thyroid carcinomas (49%) but not in well-differentiated or poorly differentiated thyroid cancers (Salerno et al., 2011).

Many reports found Twist overexpression is associated with high-grade cancer aggressiveness and reduced patient survival rate (Mani et al., 2008; Qin et al., 2012; Khan et al., 2013). Overexpression of twist might be associated with lymph node metastasis of thyroid cancer and development (Stamatiou et al., 2016).

The present study found twist protein in the cytoplasm, and this agreed with research done by Mozafari et al. (2018) it is found Twist staining found in both nucleus and cytoplasm. Still, during evaluated the epithelial cells, it is found 76.6% (23 out of 30) of the oral squamous cell carcinoma were positive, and the cytoplasmic twist expression was positive in all samples.

In this study, a higher percentage of the twist positive occurred in females than males, and there is no significant association between twist and gender. Previous research has found the same result, such as Wang et al. (2013); Calangiu et al. (2014). Concerning the age distribution of the present study, it is observed that most of the patients with thyroid carcinoma have the age range of (20-60) years, these results are in accordance with the studies of other researchers Calangiu et al. (2014). Who found the most positive case is in age group more than 45 years, through analysis of clinicopathological parameters of the 43 thyroid differentiated carcinomas and Ghamari (2019) show an increasing trend in the number of patients recently with a minimum age of three-years-old, in 84% of the patients, the age ranged from 20 to 60 years old in Iran.

The current study focusing on the association between LMP-1 and overexpression human twist in patients with thyroid nodules were investigated. Which revealed that 7 cases among thyroid papillary carcinoma were positive for both, and 8 cases among thyroid follicular carcinoma, while among the control group was found in one case but statistically non-significances. A study done by Horikawa et al. (2007) reported in human nasopharyngeal tissue, expression of twist and LMP-1 is directly correlated, and interpretation of twist is associated with metastasis clinically. So, induction of twist by a human viral oncoprotein LMP-1 directly contributes to the metastatic nature of NPC.

CONCLUSION
Prevalence of thyroid cancer in female more than male, and age group (20-40). A strong association between LMP-1 and human twist in pathogenicity of patients with thyroid carcinoma.

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Conflict of Interest
The authors declare that there is no conflict of interest for this study.

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