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Original article

Evaluation of the presence of Epstein-Barr virus (EBV) in Iranian patients with thyroid papillary carcinoma

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ABSTRACT

Background and purpose: Papillary thyroid carcinoma (PTC) is the most common thyroid cancer. EBV is one of the most important viruses related to different types of malignancies. This study investigated the relationship between EBV and papillary thyroid carcinoma.
Material and methods: In this study the presence of Epstein-Barr Nuclear Antigen 1 (EBNA1) gene in papillary thyroid carcinoma tissues were examined by nested-PCR method. Paraffin-embedded tissues (N = 41) blocks of thyroid cancer were used. DNA was extracted from all samples and then samples were evaluated for the presence of EBV gene.
Results: In 41 samples, EBNA1 was detected in 65.8% of patients with papillary thyroid carcinoma which was significantly higher in younger ages.
Conclusion: The significant presence of EBV genome in papillary thyroid carcinoma suggests that this virus may play a role in this cancer especially in younger ages. As a result, monitoring of patients with EBV latent infection for PTC can be very important.

1. Introduction

Studies show that approximately 1–2% of overall cancers occur in thyroid [1]. In Iran, 1.8% of overall cancers and 76.1% of endocrine tumor cancers are types of thyroid cancer [2]. Papillary thyroid carcinoma (PTC) is the most common thyroid cancer and its prevalence in women is twice that of men which occurs in 30–50 years. The disease is attributed to several genetically and environmental factors. Most recently viral agents have also been considered as one of its causes. The main viral agent which infect nasopharyngeal region is Epstein-Barr virus (EBV) which might be associated with development of cancer in this site. EBV which is named HHV4 belongs to Herpesviridae family causes infectious mononucleosis. The virus is associated with Hodgkin's lymphoma, Burkitt's lymphoma, nasopharyngeal carcinoma, lung cancer and gastric cancer [3]. More than 90% of adult people in the world are contaminated with EBV. The virus is transmitted through saliva, during acute infection which infects the stratified squamous epithelium of the oropharynx [4]. It is known that EBV targets different functions of cells and by creating instability in the genome cause cancer [5]. The viral genome consists of a double-stranded DNA with 85 genes including Latent Membrane Protein (LMP1, LMP2A. LMP2B) and Epstein-Barr Nuclear Antigen (EBNA1 and EBNA2B) genes involved in nasopharyngeal carcinoma [6]. LMP1 is one of the most important nasopharyngeal carcinoma oncogenes. Studies show $LMP1^+$ cells have more motility which increase the metastatic potential of tumor, also it can suppress immune responses through effects on IL-6/8, TNFRs(as CD40), NF-kB, JAK/STAT pathway and etc. [7]. EBNA1 attaches the EBV genomes into the cell's genome [8]. EBV increases the oncogenes expression as P53, C-Met and bcl-2 [9].

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Table 1
Primers Used in This Study on the EBV Genome.

Virus gene	Primers Sequences	Product size (bp)
EBV EBNA-1	Outer 5′-GTAGAAGGCCATTTTTCCAC-3′ 5′-CTCCATCGTCAAAGCTGCA-3′	609
	Inner 5′-AGATGACCCAGGAGAAGGCCCAAGC-3′ 5′-CAAAGGGGAGACGACTCAATGGTGT-3′	308

Therefore in this study, the presence of EBNA1 gene in papillary thyroid carcinoma has been studied.

2. Materials and methods

2.1. Patients

In this retrospective study, forty one paraffin-embedded tissue blocks from all patients with papillary thyroid cancer who underwent thyroid surgery at Imam Khomeini Hospital (Tehran, Iran) between 2012 and 2014 were collected and pathology report information were evaluated. Patients were included in the study whose diagnoses were based on the final pathology report after thyroidectomy. Metastatic focus of PTC in other tissues (for example lymphe node) were not used. No other inclusion and exclusion criteria were used.

2.2. Genetic analysis

DNA was extracted from paraffin-embedded tissue blocks. Micro dissected tissue from paraffin embedded block were transferred into sterile tube and xylene added to dissolve the paraffin. After centrifuge and draining the supernatant, samples were rehydrated with ethanol. In order to lyse other proteins, proteinase K and sodium dodecyl sulfate (SDS) were added and samples incubated in 37 °C overnight. After addition of phenol, Isoamyl alcohol and chloroform, samples were centrifuged and supernatant was used for DNA extraction.

2.3. Statistical analysis

All samples were analyzed for the presence of EBNA1 using Polymerase Chain Reaction (PCR),yielding 609 bp. Then nested-PCR was done for EBNA1 (308 bp). Product of the first round of PCR (0.5 μl) was mixed with 12.5 μl of a PCR reaction mixture (Amplinon, UK), containing 10 nM of the primers showed in Table 1 and subjected to 35 cycles of 95 °C for 2 min, 57.5 °C for 1 min, 72 °C for 5 min.

EBV gene frequency was compared between the groups using the Chi-square test. Statistical analyses were performed using SPSS software v.21 for Windows (SPSS Inc., Chicago, IL) and P value <0.05 was considered as statistically significant.

3. Results

3.1. Baseline characteristics

The baseline characteristics of papillary thyroid carcinoma patients are shown in Table 2. The study consists of 41 patients including 9 (22%) males and 32(78%) females. The age was divided into three groups: <40, 40–50 and >50 years old which the most of patients 17(41.4%) were in >50 groups. According to the type of papillary thyroid carcinoma, 35(85.4%) of patients were conventional variant type. In terms of the disease stage, 30(73.2%) were in stage one. Tumor was encapsulated in 19(46.3%) of cases. Perineural invasion and vascular invasion were seen in 2(4.8%) and 15(36.6%) of samples respectively.

Table 2
Demographic characteristics of patients infected with papillary thyroid cancer, N = 41.

Variables		
Age* (years)		**38.5 ± 15.3**
Sex (Male/Female) (%)		**22/78**
Variant of papillary thyroid cancer	Conventional N (%)	**35 (85.4)**
	Follicular N (%)	**3 (7.3)**
	Microcarcinoma N (%)	**3 (7.3)**

* Variable are described based Mean ± standard deviation.

3.2. Molecular tests results

The frequency of EBV genome presence in various groups has been determined. The results showed that EBNA1 was detected in 65.8% of patients with papillary thyroid carcinoma. EBV DNA frequency showed significant association with age. We found that frequency of the virus was higher in younger age. Mean age of patients with EBV DNA positive was significantly different compared to the EBV DNA negative group. The age (mean ± SD) of patients with EBV DNA positive and EBV DNA negative were 35 ± 11 year and 45 ± 15 respectively (P = 0.03).

Table 3 shows total number of EBV positive samples in different variant of PTC. In conventional type of disease, EBV positivity was more frequent in male patients (87.5% of male patients vs 55.5% of female patient) which the difference was not statistically significant. There were no association between EBV positivity and stage, perineural/vascular invasion and encapsulation.

4. Discussion

This study showed that among our patients with papillary thyroid carcinoma large number of samples were positive for presence of EBV EBNA1 gene and also frequency of the virus was higher in younger age which suggests that this virus may play a role in PTC development in younger people.

As mentioned, EBV has been considered as one of the most important factors causing a variety of cancers in lymphoid and epithelioid tissues such as papillary thyroid carcinoma in recent years [10]. Papillary thyroid carcinoma is, a well-differentiated cancer, associated with sever inflammation in 30% of cases and rising in the world in recent decades [10,11]. EBV can cause progression towards cancer in several ways, with creating inflammation in tumors, suppressing the immune responses and leading to alternations in host cells with LMP1, LMP2A. LMP2B, EBNA1 and EBNA2B genes. For example EBNA1 can cause transformation with impact on BRAF (a member of cellular RAF kinase) gene [12,13].

The aim of this study was to determine frequency of EBV presence in cancerous tissues of patients with papillary thyroid carcinoma according to sex and age, and determine whether any correlation exist between them. There are few studies about the association of EBV and PTC which their results are controversial [14]. Some studies demonstrated that the rate of miRNA virus expression in cancerous tissue was higher than normal cells; they attributed the cancer into the virus and its noncoding genes [15]. In 2003, Shimakage et al. showed EBV infection, and its mRNA and protein expression in all carcinoma specimens (including PTC, undifferentiated carcinomas, thyroid squamous cell carcinoma) which were more prominent in the undifferentiated carcinomas [13]. In a report by Stamatiou et al., EBNA2 gene was detected in 90% of thyroid cancer tissue (including PTC) but samples were negative for EBER1 [16]. Above mentioned studies are in line of our results. But there are other studies which do not show any association between EBV and thyroid cancers such as study of Kijima et al., which did not show an association when they studied primary epithelial neoplasms from different organs such as thyroid can-

Table 3
EBV positivity according to tumor variant and sex.

PTC variant	Sex	EBV Positive N (%)	EBV Negative N (%)	P value
Conventional	Female	15 (42.9)	12 (4.2)	0.07
	Male	7 (20.0)	1 (2.9)	
Follicular	Female	3 (100)	–	–
	Male	–	–	
Microcarcinoma	Female	1 (33.3)	1 (33.3)	0.39
	Male	1 (33.3)	–	
Total	Female	19 (46.4)	13 (31.7)	
	Male	8 (19.5)	1 (2.4)	

cer for expression of EBER-1 using in situ hybridization [17]. Same results were obtained in another study by Ludvíková et al., which oncocytic papillary thyroid carcinoma samples were evaluated for presence of EBV using PCR amplification, in situ hybridization, and immunohistochemistry [18]. The difference between studies may be attributed to different evaluated genes, different laboratory methods and different population.

In our study we found that EBNA1 was detected in 65.8% of patients with papillary thyroid carcinoma. The frequency of the positive samples was significantly higher at the younger age. It should be noted that in order to reduce the likelihood of errors and increase the specificity the nested-PCR was performed.

However the results of present study should be interpreted carefully because of small sample size and also lack of further examination for other EBV genes.

Finally high levels of EBV in papillary thyroid carcinoma might suggest that this virus may play a role in this cancer especially at the younger age. Therefore, monitoring of EBV latent infection in these patients can be very important.

Conflict of interest

Authors declare that they have no financial interests related to the material in the manuscript.

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Authors' contributions

MMA and FR proposed the concept of study. FN assisted in sample collection and confirmation of diagnosis. MH did the molecular analysis. MMA and MH analyzed the results. Initial draft of the manuscript was written by MH, which was reviewed and edited by FR, MMA, FN and SAMA. All authors read and approved the final manuscript.

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