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Detection of Epstein Barr Virus DNA in Thymic Epithelial Tumor Using Nested PCR

Forozan Mohammadi [1,2], Seyed Ali Reza Nadji [1], Shirin Karimi [1,2], Seyed Davood Mansoori [3,4], Sara Sharifkashani [1], Moslem Bahadori [1]

[1] Department of Clinical Anatomical Pathology, [2] Mycobacteriology Research Center, [3] Department of Infectious Disease, [4] Lung Transplantation Research Center, NRITLD, Shaheed Beheshti University of Medical Sciences and Health Services, TEHRAN-IRAN.

ABSTRACT

Background: *Thymus is a lymphoepithelial organ composed of epithelial cells and lymphocytes. Primary tumors of the thymus are uncommon and a definite risk factor has not been found. There are some reports regarding the association of the (EBV) Epstein Barr Virus with thymic epithelial tumors. This study was conducted to evaluate the presence of EBV genome in thymic epithelial tumor.*

Materials and Methods: *EBV genome, EBNA2 was examined from DNA extracts of 41 paraffin embedded specimens including 16 thymic epithelial tumors as subject cases and 25 mediastinal lymph nodes as controls.*

Results: *Nested PCR assay revealed that 31.25% of cases were positive for EBV genome.*

Conclusion: *The presence of EBV genome EBNA2 in thymic epithelial tumor suggesting that this association may be due to the endemic nature of EBV infection.* ***(Tanaffos 2006 5(4): 9-13)***

Key words: *Epstein Barr virus, Thymic epithelial tumor, Nested PCR*

INTRODUCTION

The Epstein-Barr virus (Human herpes 4 genus lymphoma cryptovirus, subfamily of Gamma herpes virus, family of *Herpes viridae*) is a ubiquitous B cell lymphocytotropic virus which is known to cause infectious mononucleosis. It has been found in tumor cells of malignancy of lymphoid origin including Burkitt's lymphoma, Hodgkin's disease and non-Hodgkin's lymphoma (1-3). It is also implicated with malignant cells of epithelial neoplasms. The association of the virus with neoplasm of epithelial cells is puzzling, but studies have shown that the virus can penetrate and replicate in some neoplastic epithelial cells including nasopharyngeal carcinoma and gastric adenocarcinoma (1, 2). The thymus is a lymphoepithelial organ composed of interconnecting meshwork of epithelial cells in the lymphocytic background. Primary tumors of thymus are uncommon. Among them the thymic epithelial tumor presents at the top of the list followed by thymoma,

Correspondence to: Mohammadi F

Address: NRITLD, Shaheed Bahonar Ave, Darabad, TEHRAN 19569, P.O:19575/154, IRAN

Email address: frmohamadi@nritld.ac.ir

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invasive thymoma and thymic carcinoma (4). Although, for development of thymic epithelial tumor no well documented etiologic factor has been reported, in the first series of investigations Leyvraz demonstrated EBV genome in thymic carcinomas (5), but consequent studies showed conflicting results (4, 5, 6, 7, 8). In the present study, we assessed the possible association of EBV genome with thymic epithelial tumor in Iranian patients and, the presence of EBNA2 gene in tissue specimens was analyzed using nested PCR.

MATERIALS AND METHODS

1. Specimen selection

A total of 41 blocks of paraffin-embedded tissue including 16 samples diagnosed as thymic epithelial tumor and 25 non- thymic tumor samples as controls were retrieved from the archive of Masih Daneshvari Hospital.

The specimens were either surgical biopsy or complete excisions. All hematoxylin-eosin stained and immunohistochemistry slides were reviewed for confirmation of diagnosis. Paraffin blocks of lymph nodes resected for staging of lung cancer devoid of any epithelial or lymphoid malignancy were used as controls.

2. Preparation of samples for PCR assay

Genomic DNA from tissue sections were prepared according to the methods described by Impraim et al. (9). Two 5 µm-thick sections were cut from each block using a microtome with disposable blades , deparaffinized by xylene, and rehydrated in alcohol. Lysis of the sample was carried out by resuspending the dried tissue in 150 µl of TES buffer (10 mM/L Tris PH=8.00, 1.5 mM/L Nacl,10 mM/L 1% SDS, 200 mg/ml proteinase k [Bioline, USA]) and incubated at 45°C over night .The extraction was then followed by phenol/ chloroform- isoamyl alcohol/ chloroform as a standard protocol. Finally the DNA dissolved in 40 µl of D.D.W or TE buffer. The extracted DNA was frozen at -20°C until use. The adequacy of DNA in each specimen for PCR amplification was determined by detection of a 110- or 268-base pair fragment of the β-globin gene using the PC03/PC04 and GH20/PC04 primer set, respectively (10).

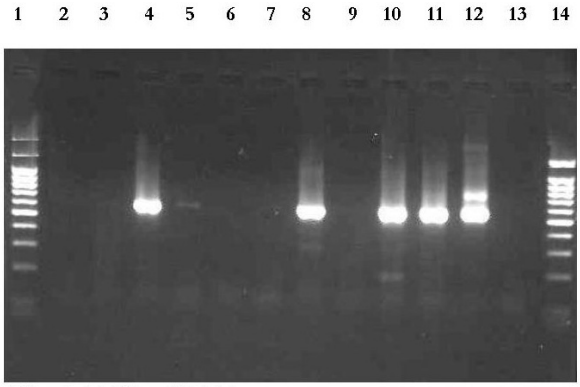
3. Nested-PCR assay

The 1st PCR round was performed with 10 µl of DNA sample in 50 µl of reaction mixture containing 2 U of Taq DNA polymerase (Super Taq, U.K),1.5 mM/L MgCl2 , 5 µl of 10 x PCR buffer (Super Taq), 0.2 m M/L dNTPs (Pharmacia) and 20 pmoles of each primer common for both EBV genotypes (E2P1: 5'-AGG GAT GCC TGG ACA CAA GAG-3', E2P2: 5'-TGG TGC TGC TGG TGG YGG CAA T-3') for amplification of a 596 bp fragment in EBNA-2 gene (3). After denaturation of the template DNA at 94°C for 3 min, the PCR was performed for 35 cycles using a Perkin-Elmer thermocycler (Rocsh diagnostic label). Each cycle consisted of denaturation at 94°C for 35 sec., primer annealing at 60 °C for 35 sec. and primer extension at 72 °C for 45 sec.

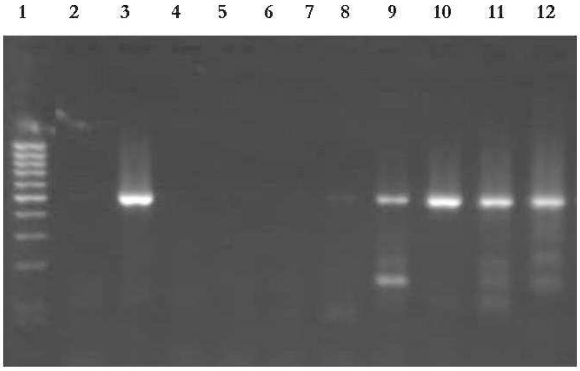
The 2nd PCR round was performed with 5µl of the product of one-stage PCR under the same condition but with different primers, namely the EBV-1 and EBV-2 type specific inner primers: Ap1(5'-TCT TGA TAG GGA TCC GCT AGG ATA-3') and Ap2(5'-ACC GTG GTT CTG GAC TAT CTG GAT C-3') for EBV-1, and Bp1(5'-CAT GGT AGC CTT AGG ACA TA-3') and Bp2(5'-AGA CTT AGT TGA TGC CCT AG-3') for EBV-2 in two sets of amplification tubes (3).

4. Agarose gel electrophoresis

Ten µl of the PCR product was electrophoresed in 2% agarose gel containing ethidium bromide. The 596 bp product of the common primers, and the 497 bp and 150 bp products of the type-specific primers were visualized under U.V light. The 100 bp DNA ladder (Fermentus) was used as molecular size marker (figure 1).



A) 1st PCR round.
a) Lines 1 &14: 100 bp DNA ladder.
b) Lines 2-11: samples.
c) Lines 12 &13: positive and negative PCR control.



B) 2nd PCR round.
a) Line 1: 100 bp DNA ladder.
b) Lines 2 & 3: positive and negative PCR control.
c) Lines 4 - 12: samples.

Figure 1 A, B. Agarose gel electrophoresis of the products of EBV nested- PCR tests.

5. Data processing

Data were processed by SPSS statistical software program version 11.5. The correlations were subjected to χ^2 (Pearson, chi-square) and Fischer's exact test. Statistical significance was set as a *P*-value less than 0.05.

RESULTS

The characteristics of studied subjects including age, gender, EBV DNA and type of thymic tumor are shown in Tables 1 and 2. A total of 41 individuals, including 16 thymus tumor patients (7 females and 9 males) and 25 controls (9 females and 16 males), were analyzed (Table 2).

Table 1. Demographic data of thymic epithelial tumor cases and EBV DNA status.

No. cases	Gender	Age (yrs.)	Histologic subtype	EBV DNA
1	M	38	Lymphocyte predominant (invasive)	Negative
2	M	76	Epithelial type (invasive)	Negative
3	F	36	Lymphocyte predominant (invasive)	Negative
4	M	16	Lymphocyte predominant	Negative
5	M	48	Epithelial type (questionable invasive)	Negative
6	F	22	Lymphocyte predominant	Positive
7	M	19	Mixed Lymphocyte, epithelial (invasive)	Negative
8	F	38	Lymphocyte predominant	Negative
9	F	69	Mixed Lymphocyte and epithelial	Positive
10	F	34	Lymphocyte predominant	Positive
11	F	19	Lymphoepithelial mixed	Negative
12	M	70	Epithelial type	Negative
13	F	25	Lymphocyte predominant	Negative
14	M	22	Lymphocyte predominant	Negative
15	M	43	Mixed Lymphocyte and epithelial	Positive
16	M	17	Thymic lymphoepithelial- like carcinoma	Positive

The mean ages of both groups were 37 ± 19.81 (S.D.) and 47.64± 19.21 (S.D.) years, respectively (Table 2). The mean age and gender distribution in the study group were comparable with those of the control group (p=0.095 for mean age, p=0.620 for gender; Table 2).

Out of 16 thymic tumor subjects, 8 cases were thymomas of lymphocyte predominant type (2 of them were invasive), 3 cases were thymomas of epithelial type (2 of them were invasive), 4 cases were mixed lymphocyte and epithelial types (1 of them was invasive) and only 1 case was thymic carcinoma which was lymphoepithelial-like type (Table 1).

Statistical differences were observed in the presence of EBV DNA between these two groups (Table 2). The prevalence of EBV DNA was 31.25% while none of controls were positive for EBV DNA. (p=0.006; Table 1). Out of 5 EBV- positive cases, 2 cases were of the lymphocyte predominant type, 2 cases were mixed and the last positive case was the

only case of thymic lymphoepithelial- like carcinoma (Table 1).

In the study group, no significant differences were observed in EBV DNA positivity and gender or positivity and tumor types (gender; p=0.596, tumor types; p=0.590).

Table 2. The Characteristics of study subjects and prevalence of EBV DNA in Thymic Tumor Patients and Controls.

Parameters	Cases (N=16)	Controls (N=25)	P-value
Age (yr ± SD)	37 ± 19.81	47.64± 19.21	0.095 (t-test)
Gender			
Female	7 (43.75) [1]	9 (36)	0.620
Male	9 (56.25)	16(64)	
EBV			
Positive	5 (31.25)	0 (0)	0.006
Negative	11 (68.75)	25 (100)	

[1] Numbers in parentheses are percentages.

DISCUSSION

The association between EBV and thymic epithelial tumor is inconclusive and reports in this regard are not entirely consistant. In addition, methods employed have different sensitivity and specificity which may affect the results. In our study 31.25% of cases were positive for EBV genome, including non-invasive and invasive thymoma, and thymic carcinoma. Most of the previous studies reported the presence of EBV gnome in thymic carcinoma which exhibited a lymphoepithelial-like morphology (5, 11). This was initially reported by Leyveraz et al (5). We had only one case of thymic carcinoma which was of the lymphoepithelial type and positive for EBV DNA. Considering the well documented link between the EBV infection and the development of nasopharyngeal carcinoma (12, 13), we suggest that EBV may also play an etiologic role in lymphoepithelial-like carcinoma of thymus.

McGuire et al. reported a positive association of EBV not only with thymic lymphoepithelial-like carcinoma; but also, with all thymomas and thymic lymphoid hyperplasia (6). We also had 4 cases of thymoma which were positive for EBV (2 lymphocyte predominant types and 2 mixed lymphocyte and epithelial types). The question which may arise is that, "is the EBV positivity in our cases and McGuire's due to the presence of EBV in infiltrating lymphocytes and not in the epithelial cells?" To rule out this possibility it was better to perform in situ hybridization for localization of viruses in different types of cells. But, due to absence of facilities for this method, we used lymphoid tissue of the mediastinum as controls and our controls were totally negative for EBV.

In comparative study, Alebouyeh reported the prevalence of EBV infection of Iranian healthy antibody- positive children to be 70% as compared with 56% positivity in the German group (14). Considering the fact that the incidence of cancer varies markedly by ethnicity and geographic location (15) and reports regarding the negative association of EBV in thymic epithelial tumor of European patients (4, 7, 8) and positive association of EBV in Taiwaness patients (16) as well as our cases, and also with regard to findings of Alebouyeh, the endemic nature of EBV infection is suspected. Influence of the host's ethnicity on EBV infection and non thymic tumor was also reported by Peh SC. (17).

We used nested PCR for detection of EBV DNA which is a sensitive method (18); but, for detection of virus DNA we suggest using in situ hybridization or immunohistochemistry for localization of EBV in the cellular component of the tumor.

CONCLUSION

Our findings support the association of EBV with thymic epithelial tumor in the Iranian population and we suspect that this association may be due to the endemic nature of EBV infection, or ethnicity.

REFERENCES

1. Hsu JL, Glaser SL. Epstein-barr virus-associated malignancies: epidemiologic patterns and etiologic implications. ***Crit Rev Oncol Hematol*** 2000; 34 (1): 27-53.
2. Yukio Shimosato, Kiyoshi Mukai. Atlas of Tumor Pathology, tumor of mediatinum. AFIP, third series, page 157, Thymic carcinoma.
3. Durmaz R, Aydin A, Koroglu M, Aker H, Ozercan IH, Atik E, et al. Detection and genotyping of Epstein-Barr virus by polymerase chain reaction in tissues obtained from cases with Hodgkin's disease in Turkey. ***Acta Virol*** 1998; 42 (6): 375- 81.
4. Christopher DM, Fletcher. Diagnostic Histopathology of tumor, the thymys. Page: 1271-1274.
5. Leyvraz S, Henle W, Chahinian AP, Perlmann C, Klein G, Gordon RE, et al. Association of Epstein-Barr virus with thymic carcinoma. ***N Engl J Med*** 1985; 312 (20): 1296- 9.
6. McGuire LJ, Huang DP, Teoh R, Arnold M, Wong K, Lee JC. Epstein-Barr virus genome in thymoma and thymic lymphoid hyperplasia. ***Am J Pathol*** 1988; 131 (3): 385- 90.
7. Borisch B, Kirchner T, Marx A, Muller-Hermelink HK. Absence of the Epstein-Barr virus genome in the normal thymus, thymic epithelial tumors, thymic lymphoid hyperplasia in a European population. ***Virchows Arch B Cell Pathol Incl Mol Pathol*** 1990; 59 (6): 359- 65.
8. Inghirami G, Chilosi M, Knowles DM. Western thymomas lack Epstein-Barr virus by Southern blotting analysis and by polymerase chain reaction. ***Am J Pathol*** 1990; 136 (6): 1429- 36.
9. Impraim CC, Saiki RK, Erlich HA, Teplitz RL. Analysis of DNA extracted from formalin-fixed, paraffin-embedded tissues by enzymatic amplification and hybridization with sequence-specific oligonucleotides. ***Biochem Biophys Res Commun*** 1987; 142 (3): 710- 6.
10. Saiki RK, Bugawan TL, Horn GT, Mullis KB, Erlich HA. Analysis of enzymatically amplified beta-globin and HLA-DQ alpha DNA with allele-specific oligonucleotide probes. ***Nature*** 1986; 324 (6093): 163- 6.
11. Matsuno Y, Mukai K, Uhara H, Akao I, Furuya S, Sato Y, et al. Detection of Epstein-Barr virus DNA in a Japanese case of lymphoepithelioma-like thymic carcinoma. ***Jpn J Cancer Res*** 1992; 83 (2): 127- 30.
12. Tao Y, Deng X, Hu Z, Tang M, Gu H, Yi W, et al. EB virus encoded latent membrane protein 1 modulates the phosphorylation of epidermal growth factor receptor in nasopharyngeal carcinoma cell line. ***Zhonghua Zhong Liu Za Zhi*** 2002; 24 (3): 226- 9.
13. Dolcetti R, Menezes J. Epstein-Barr virus and undifferentiated nasopharyngeal carcinoma: new immunobiological and molecular insights on a long-standing etiopathogenic association. ***Adv Cancer Res*** 2003; 87: 127-57.
14. Alebouyeh M, Peller P, Goetz O, Ameri MA. Comparative study of the prevalence of Epstein-Barr virus infections in Iran and Germany. ***Monatsschr Kinderheilkd*** 1984; 132 (11): 850- 1.
15. Gadgeel SM, Kalemkerian GP. Racial differences in lung cancer. ***Cancer Metastasis Rev*** 2003; 22(1): 39-46
16. Chen PC, Pan CC, Yang AH, Wang LS, Chiang H. Detection of Epstein-Barr virus genome within thymic epithelial tumours in Taiwanese patients by nested PCR, PCR in situ hybridization, and RNA in situ hybridization. ***J Pathol*** 2002; 197 (5): 684- 8.
17. Peh SC. Host ethnicity influences non-Hodgkin's lymphoma subtype frequency and Epstein-Barr virus association rate: the experience of a multi-ethnic patient population in Malaysia. ***Histopathology*** 2001; 38 (5): 458- 65.
18. Haque T, Crawford DH. PCR amplification is more sensitive than tissue culture methods for Epstein-Barr virus detection in clinical material. ***J Gen Virol*** 1997; 78 (Pt 12): 3357- 60.