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Lack of Association between EBV DNA and Squamous Cell Carcinoma of the Esophagus in Iranian Patients

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Abstract

The aim of this study was to determine whether an association exists between Epstein Barr Virus (EBV) and esophageal squamous cell carcinoma (ESCC) among Iranian patients. The incidence of the cancer in Iranians is high. EBV has been implicated in a number of human malignancies, many of epithelial origin. The etiology of ESCC appears to involve genetic and environmental factors. Tumerous and histologically normal paraffin-embedded esophageal tissue of 30 patients, and 6 cancerous and 4 non-cancerous freshly frozen tissue specimens of ESCC patients were studied. The samples were evaluated for the presence of EBV by PCR and Southern blot hybridization. The EBV genome was detected in only one non-cancerous specimen. It was concluded that EBV is probably not associated with ESCC among Iranian patients. This is consistent with the results of studies on several but not all other populations. The implications of the geographical variation are discussed.

Keywords: *Esophagus, Squamous cell carcinoma, Epstein Barr virus, Iranian patients, PCR, Southern blot hybridization*

Introduction

Esophageal cancer has a very high mortality rate and was ranked as the ninth most common malignancy worldwide based on data of 1985 (Pisani *et al.,* 1993). There has been a recent increase in the incidence of the adenocarcinoma form of the cancer in Western populations, but the squamous cell carcinoma form remains predominant worldwide (Lam, 2000). One of the most interesting features of the cancer is the

extreme variation of its incidence rate in different regions of the world, ranging from a few to approximately 150 per 100,000 individuals (Munoz, 1993). One of the high risk areas is the "Asian esophageal cancer belt" which includes the northern provinces of Iran south of the Caspian Sea. Incidence in parts of these provinces approaches 150 per 100,000 individuals. Most Iranian patients suffer from the squamous cell type of esophageal cancer.

Many genes have been implicated in the occurrence of esophageal cancer, perhaps most significantly cyclin Dl, p53, p21 and the Tylosis gene (Mandard *et al.,* 2000**;** Risk *et al.,* 1999). The geographically localized nature of the disease strongly suggests environmental factors are operating to increase ESCC incidence in certain areas. Epidemiological studies have implicated alcohol, tobacco, dietary deficiencies, thermal injuries and environmental radiation (Lam, 2000**;** Azin *et al.*, 1998; Baumforth *et al*., 1999). These factors may act directly or possibly render esophageal tissue more susceptible to other environmental carcinogens such an nitrosyl compounds or viruses. The viruses most often considered in relation to esophageal cancer are the human papilloma virus and the Epstein Barr virus (EBV). In the present study, we attempt to assess the possible relevance of EBV in the occurrence of esophageal squamous cell carcinoma among Iranian patients.

EBV is a herpesvirus with which most of the world's population is infected. Epithelial cells of the oropharynx are primary sites of infection and viral replication. The virus usually establishes latent infection in the subsequently infected B cells and these cells probably serve as a reservoir for later infections of epithelial cells (Baumforth *et al.,* 1999). EBV is associated with mononucleosis and a number of human malignancies including Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, T cell lymphomas and gastric carcinoma (Baumforth *et al.,* 1999).

The viral genome codes various proteins whose activities may be relevant to carcinogenesis such as proteins which down regulate the immune response (Rousset *et al.,* 1992), inhibit apoptosis (Oudejans *et al.,* 1995) and associate with retinoblastoma and p53 proteins (Szekely *et al.,* 1993). The LMP1 viral protein can transform rodent fibroblast cell lines (Wang *et al.,* 1985). B cells and esophageal

epithelial cells both express CD21, a purported receptor for EBV (Wang *et al.,* 1987; Wang *et al.,* 1990).

The possible association of EBV with cancer of the esophagus has been investigated in various low risk and high risk populations during the past few years. The nature of these studies and their results are summarized in Table 1. In short, EBV was not found in the cancerous samples of most of the studies. The virus was identified in Taiwanese and also in cancerous tissues of European origin. In one of the studies on European subjects, EBV was detected at the same frequency in cancerous and non-cancerous subjects and the association with cancer was therefore deemed to be probably insignificant (Morgan *et al.,* 1997).

Table 1 – Summary of studies on association of EBV with cancer of the esophagus

Country	Sample source	Differentiation	Method	EBV target	Ruslts according to sample type No. samples tested/%samples EBV+ve				Ref. *
					T	N/T	N	Cell line	
China									
1	Cell lines		Southern blot					2 ESCC/0%	1
2			ISH	EBNA-1	74 ESCC/ 0%				2
3	Paraffin - embedded tissue	9W, 31M, 11P	PCR ISH	IR1 EBER-1	51 ESCC/ 0% (same result by 2 methods)	42/0% (same result by 2 methods)			3
Taiwan									
1	Frozen tissue (PCR) Paraffin- embedded tissue (ISH+IHC)		PCR/Southern blot ISH IHC	Bam HIW EBER-1, EBER-2 LMP-1	31 ESCC/ 35% 31 ESCC/ 35% 31 ESCC/ (0%)	31/23% 31/13% 31/0%			4
Japan									
1	Paraffin- embedded tissue	8W, 10M, 11P	PCR ISH IHC	IR1 EBERs LMP1	29 ESCC/ 0% 1 undifferentiated esophageal carcinomal/100% (same results by 3 methods)				5
2	Surgical specimens, cell lines		PCR/Southern blot	EBNA-1	41 ESCC/ 0%			12/0%	6
3	Paraffin- embedded tissue	9W, 12M, 5P	PCR/Dot blot	EBNA-1	27 Carcinomas/7%		12/0 %		7
4	Paraffin- embedded tissue	P	ISH PCR	EBER-1 IR1	1 LELEC/ 0% (same result by both methods)				8
5	Frozen tissue (PCR) Paraffin- embedded tissue (ISH) Cell lines	ESCC: 19W, 38M, 13P	PCR ISH	IR1 EBERs	70 ESCC/ 20% (PCR);0%(ISH) 1 undifferentiated carcinoma/0% (PCR) 2 adenocarcinoma/0% (PCR) 2 blastoid-squamous carcinoma/50% (PCR);50%ISH (signal only in lymphocytes) 1 malignant melanoma/0% (PCR) 1 adenoid cystic carcinoma/0% (PCR)			30 ESCC/ 0% (PCR) 2 adenocarcinomas / 0% (PCR)	9
Sweden									
1	Biopsies		PCR ISH IHC	EBNA-1 EBERs BZLF	10 ESCC/ 20% (PCR) 10 ESCC/0% (ISH+IHC)				10
France									
1	Paraffin- embedded tissue Frozen tissue		PCR/Southern blot		14 ESCC/21%				11
Italy									
1	Paraffin- embedded tissue Frozen tissue		PCR/Southern blot	BAMHIW	20 ESCC/10%				11
USA									
1	Paraffin- embedded tissue Frozen tissue		PCR/Southern blot	BAMHIW	18 ESCC/0%				11
UK									
1	Frozen tissue		PCR/Southern blot	EBNA-1	17 Adenocarcinomas/47%		17/ 47 %		12
Iran									
1	Paraffin- embedded tissue Frozen tissue		PCR/Southern blot	BAMHIW	8 ESCC/0%				11
2	Paraffin- embedded tissue Frozen tissue		PCR/Southern blot	IR1	34 ESCC/0%	32/3%			This articl e

W= well differentiated, M= moderately differentiated, P= poorly differentiated, ISH= in situ hybridization, IHC= immunohistochemistry, T= esophageal tumor tissue, N/T= histologically normal epithelial tissue of cancer patients, N= normal epithelial tissue of non-cancerous individuals

* 1.Wong *et al.,* 1992; 2.Lam *et al.,* 1995, 3.Wang J. *et al.,* 1999; wang L.S. *et al.,* 1999; 5. Mori *et al.,* 1994; Misobuchi *et al.,* 1997; 7. Kurshid *et al.,* 1998; 8. Sashiyama *et al.,* 1999; 9. Hong *et al.,* 2000; 10. Lewensohn-Fuchs *et al.,* 1994; 11. Jenkins *et al.,* 1996; 12. Morgan *et al.,* 1997.

Materials and Method

Samples: Esophageal squamous cell tumor tissue specimens of thirty patients and histologically normal esophageal epithelial tissue of the same patients were examined. The paired samples were obtained from formalin-fixed paraffin-embedded tissue blocks kept at the archives of the Pathology Department of Imam Khomeini Hospital. Information regarding the tissue blocks was obtained from hospital records and histological features were confirmed by microscopic examination of newly prepared H+E stained sections. Of the tumor samples, 4 were well differentiated, 14 were moderately differentiated and 12 were poorly differentiated. Sixteen of the patients were women and 14 were men. Their mean age was 55.3 years. Additionally, ten samples were obtained from ten esophageal squamous cancer cell (ESCC) patients during surgery at the same hospital. Examination of stained frozen sections of these samples indicated that six were cancerous and four were non-neoplastic. LCLPI4, an EBV infected lymphoblastoid cell line and Louckes, a non-infected cell line were used, respectively, as control EBV-positve (EBV+ve) and EBV-negative (EBV-ve) cells. These were purchased from the Cell Culture Collection of the Pasteur Institute of Iran.

DNA extraction: DNA was extracted from one or two 6 μM sections of paraffin embedded tissue by modifications of published procedures (Wright & Manos, 1990). Briefly, sections were treated with xylene and ethanol to remove paraffin, then incubated at 63^{o}C for one hour in 100-200 μl digestion buffer containing 200 μg/ml proteinase K (Sigma), 10mM Tris-HCl (pH 8), 50 mM KCl, 0.1% Triton X-100. The samples were incubated at 95^{o}C for 10 minutes and briefly microfuged. Supernatants were phenol-chloroform extracted, ethanol precipitated and dissolved in 50 μl water. The surgical tissue samples were initially used by others in the laboratory to obtain RNA by a protocol involving acid phenol extraction. For the purposes of this study, DNA was recovered from the phenol phase by first adding an equal volume of 1 M Tris base (pH=10.5). The DNA in the aqueous phase was precipitated with ethanol and dissolved in water. DNA from cell lines was prepared according to standard procedures.

PCR: In order to detect the EBV genome, PCR amplification of a portion of the IR1 repeated sequence was performed. The primers used (M1 and M2) define a 121 bp fragment and have been previously used by many authors (Mori *et al.,* 1994; Sashiyama *et al.,* 1999). Amplification reactions were performed in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 uM of each of the four deoxynucleotide triphosphates, 0.5 uM of each of the primers, 1 U Taq DNA polymerase (Cynagen, Iran) and 4 µl DNA template solution in a final volume of 20 µl. A reaction containing EBV+ve control DNA and another containing EBV-ve negative control DNA was included in every experiment. After an initial four minute incubation at 95^{o}C, amplification was achieved during 40 cycles of 1 minute at 95^{o}C, 1 minute at 58^{o}C, and 1 minute at 72^{o}C, followed by a 10 minutes at 72^{o}C. The template potential of every DNA sample was confirmed in a PCR reaction using β globin primers which amplified a 240 bp fragment. Eight µl of each reaction mixture was electrophoresed on 2% agarose gels and PCR products were visualized by ethidium bromide staining.

The sensitivity of the EBV-specific PCR amplification was assessed by using diluted samples of EBV positive LCLP14 cell line DNA as template. The DNA was diluted with water or with EBV negative Louckes cell line DNA.

Southern blot analysis: To confirm the electrophoretic results and possibly increase sensitivity of detection, Southern blot analysis was performed. All electrophoresed PCR products were transferred from agarose gels to membranes exactly as described (Jenkins *et al.,* 1996). The membranes were hybridized with dioxygenin-ddUTP (Boehringer Manneheim) 3' end-labeled M3 oligonucleotide probe (5'-TCTCCTAAGCCCAACACT-3'), the sequence of which lies within the DNA fragment amplified by the M1 and M2 primers. The probe was labeled according to the company's instructions. Hybridization was performed for 6 hrs at 47^{o}C in 1% Blotto, 0.1% N-lauryl sarcosyl, 2% SDS and 5X SSC. Washings were performed as described (Jenkins *et al.,* 1996). Visual detection was achieved with immunologic and enzymatic reactions using anti-dioxygenin antibody conjugated to alkaline phosphatas (Boehringer Manneheim) according to the company's instructions.

Results

Sensitivity: EBV DNA was detected by PCR in the presence of 0.1pg or more DNA of the EBV infected cell line LCLP14 (Fig. 1). This corresponds to over a million-fold dilution of the LCLP14 DNA and represents a sensitivity comparable to that reported by others (Jenkins *et al.,* 1996).

PCR and Southern blotting: A positive EBV signal was not detected by PCR nor by Southern blot hybridization in the DNA of any of the tumerous ESCC tissue samples studied. Amongst the same number of non-tumerous esophageal tissue samples of ESCC patients, a positive EBV signal was detected in one by PCR (Fig. 2a). The PCR product of only this sample hybridized with an EBV specific probe after Southern blotting (Fig. 2b). The β-globin gene of all the DNA samples was successfully amplified with the globin specific primers. Additionally, the EBV primers always amplified the EBV IR1 sequence in the positive control LCLPI4 DNA included in every experiment and the PCR product always produced a signal after probe hybridization of Southern blots. The negative control DNA never produced a PCR product nor a hybridization signal.

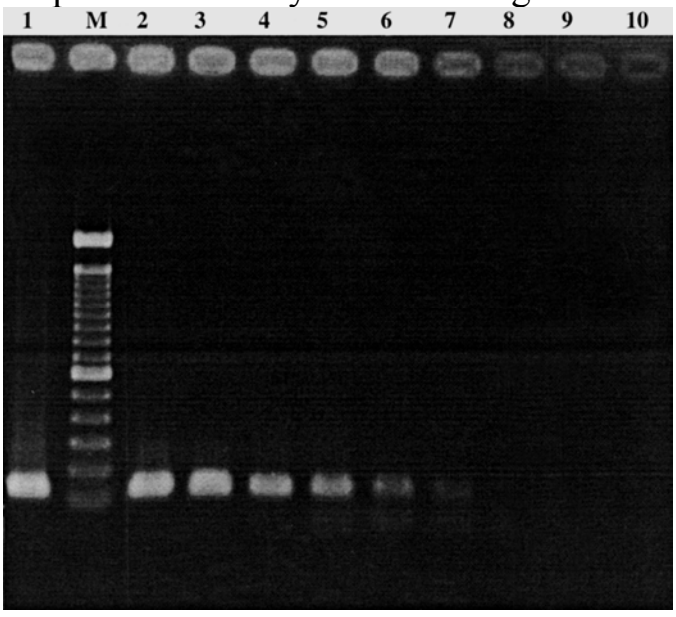

Figure 1 - Sensitivity of detection of EBV DNA by PCR. PCR was performed in the presence of decreasing amounts of LCLP14 (EBV+ve) DNA: 600 ng (lane 1), 40 ng (lane 2), 4 ng (lane 3), 400 pg (lane 4), 40 pg (lane 5), 4 pg (lane 6), 0.4 pg (lane 7), 0 pg (lane 8). Lane M contains 100 bp ladder size marker. Lanes 9 and 10 are empty.

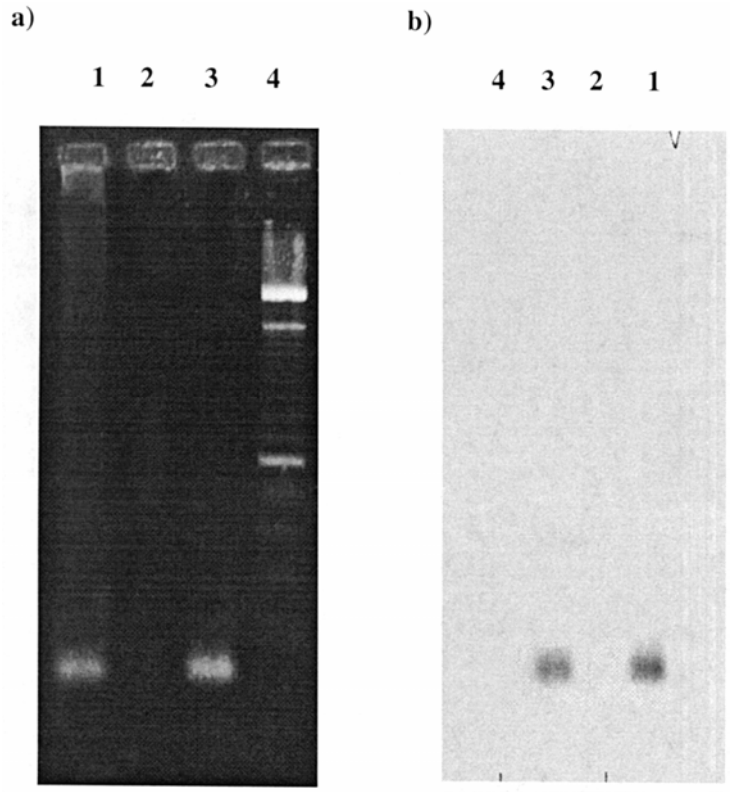

Figure 2 - PCR and Southern blot hybridization of single EBV positive esophageal tissue sample. a. Electrophoretic profile of PCR products. DNA template in lane 1: from a non-tumerous tissue sample lane 2: from tumerous sample of same patient lane 3: from LCLP14 cells. Lane 4 contains 100 bp ladder size marker. b. Southern blot after hybridization with labeled M3 and color detection. DNA templates are same as in a.

Discussion

The results suggest that there is no significant correlation between persistent EBV infection and ESCC among Iranian patients. It is likely that the single EBV positive response which was found in a histologically non-cancerous esophageal tissue sample was due to infiltrating EBV positive lymphocytes. EBV infected lymphocytes were the probable cause of positive PCR responses in some previously reported studies on ESCC (Mori *et al.,* 1994; Hong *et al.,* 2000; Lewensohn-Fuchs *et al.,* 1994). As the EBV genome is present in latent form in the lymphocytes of many populations and as we detected the viral genome in only one of our samples, it can be concluded that our protocol generally precluded lymphocyte

contamination. The conclusion on Iranian patients is consistent with that of many studies on Chinese (Wong *et al.,* 1992; Lam *et al.*, 1995; Wang J. *et al.,* 1999), Japanese (Mori *et al.,* 1994; Mizobuchi *et al.,* 1997), and US (Jenkins *et al.,* 1996) ESCC patients. A previous study on eight Iranian ESCC patients also found none to be EBV positive (Jenkins *et al.,* 1996). Nevertheless, a notable fraction of samples of Taiwanese (Wang L.S. *et al.,* 1999), French (Jenkins *et al.,* 1996) and Italian (Jenkins *et al.,* 1996) origins was reported to be EBV positive. The geographical variation between EBV infection and ESCC remains to be explained.

It is interesting that whereas virtually all Burkitt's lymphomas found in the high incidence regions of equatorial Africa and Papua New Guinea are EBV positive, only 15% of sporadic tumors from other regions of the world carry the virus (Baumforth *et al.,* 1999). Similarly, squamous cell nasopharyngeal carcinomas arising in areas where the carcinoma is endemic are mostly EBV positive whereas sporadic cases in other areas are commonly EBV negative (Baumforth *et al.,* 1999; Pathmanathan *et al.,* 1995). The association of EBV with gastric adenocarcinomas also shows geographical variation, in this case a higher association found in regions of low incidence (Shibata & Weiss, 1992; Gulley *et al.,* 1996). Geographical variation in the association of EBV with carcinomas of the thymus and salivary gland has also been reported (Fujii *et al.,* 1993; Imai *et al.,* 1994). On the other hand, the association of EBV with some tumors is not geographically biased (d'Amore *et al.,* 1996; Borisch *et al.,*1993, Niedobitek *et al.,* 1991).

If one were to forgo consideration of use of different detection protocols and sample sizes, an explanation for the geographical difference in the association between EBV and some pathologies must be sought in more biologically relevant factors. In light of different transforming potentials of EBV types 1 and 2, variation in the distribution of these two types and even some subtypes throughout the world can be considered (Young *et al.,* 1987; Sixbery *et al.,* 1989; Lung *et al.,* 1992). Finally, there exists a possibility of a "hit and run" role for EBV in some diseases (Baumforth *et al.,* 1999). In this case variation in persistence of the virus in patients of certain regions may be due to non-viral related factors.

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