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Original Article

Frequency of Epstein - Barr virus infection as detected by messenger RNA for EBNA 1 in histologically proven gastric adenocarcinoma in patients presenting to a tertiary care center in South India

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Abstract

Background: Epstein–Barr virus (EBV)-associated gastric carcinoma is a relatively uncommon entity detected in approximately 10% of gastric adenocarcinoma. **Objective:** The purpose of this study is to estimate the frequency of EBV-associated gastric carcinoma and also to assess the nature of presentation, any significant difference between this subgroup and EBV-negative gastric adenocarcinomas with respect to age and sex predilection, lymph nodal status, site of presentation. **Materials and Methods:** We prospectively analyzed 100 cases of gastric adenocarcinoma who underwent either a partial or total gastrectomy during the period from March 2010 to August 2011. The tumour and the corresponding normal gastric tissue from the same patient were analyzed for the presence of Epstein–Barr nuclear antigen 1 (EBNA1) messenger ribonucleic acid (mRNA) by real-time polymerase chain reaction (PCR). **Result:** EBV was detected in 6% cases of gastric adenocarcinoma. All the positive patients were males. The majority of cases involved the proximal stomach and there was variable lymph nodal involvement. **Conclusion:** Our study endorses that there is an association between EBV infection and gastric adenocarcinoma in the Indian population. There was no significant difference between this subgroup and EBV-negative gastric adenocarcinomas with respect to age and sex predilection, lymph nodal status and site of presentation. Short-term follow-up of this subgroup of patients seems to indicate a good overall prognosis after appropriate treatment. However, a larger study with long-term follow-up is needed to further establish the role of EBV in gastric adenocarcinoma in this study population.

Key words: *EBV, gastric adenocarcinoma, India, PCR*

Introduction

Gastric carcinoma was the most common cancer worldwide in the 1980s and is now surpassed only by lung cancer as the leading cause of cancer deaths.[1] There is a substantial geographic variation in the incidence of gastric carcinoma internationally, with higher rates in Japan and some parts of South America and lower rates in Western Europe and the United States.[1]

Carcinoma of the stomach is closely associated with age, occurring predominantly between the fifth and seventh decades of life and in people in the lower socioeconomic groups.[2] The incidence also increases with age, peaking in the seventh decade.[3] Stomach cancer is universally more common in men than women.[2] In the developed countries, the tumour is moving proximally, with a rising incidence of adenocarcinoma reported in the cardiac region of the stomach. Ninety-five percent of all malignant gastric neoplasm is adenocarcinomas. Other histological types include squamous cell carcinoma, adenoacanthoma, carcinoid tumours, GI stromal tumours and lymphoma.[2,3]

There are several studies looking at the microbial/viral aetiology for carcinoma of stomach. The role of *Helicobacter pylori* in the causation of the gastric carcinoma has been well established.[4] Recently, Epstein–Barr virus (EBV) has been accepted as an infective agent causing gastric carcinoma (GC). EBV infection in gastric carcinoma was first reported by Burke *et al*. in 1990 using polymerase chain reaction (PCR).[5] EBV-associated gastric carcinoma consists of monoclonal growth of EBV-infected epithelial cells, which express several EBV-latent genes. Sequential events in the gastric mucosa could be traced from EBV infection of the pit cells to fully developed carcinomas by EBV-encoded small ribonucleic acid (RNA) (EBER) *in situ* hybridization.[6]

EBV is detected in the tissue of about 10% of gastric carcinoma cases throughout the world. In each case, 100%

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of carcinoma cells are infected with EBV.[7] EBV-related gastric carcinoma is demonstrated by EBV-encoded small RNA *in situ* hybridization, monoclonality of EBV deoxyribonucleic acid (DNA) and elevated antibodies.[8] Analysis of EBV in carcinoma biopsies indicates that carcinoma is formed by the proliferation of a single EBV-infected cell. These findings suggest that EBV plays an important role in the development of EBV-positive gastric carcinomas. The EBV genes were expressed as EBV nuclear antigen 1 (EBNA1), two small non-poly adenylated RNAs known as EBV-encoded RNA 1 (EBER1) and EBER2, and the transcripts from the BamHI-A region (BARF0); in addition, some cases also express a small amount of latent membrane protein 2A (LMP2A).[9] There is limited data from India regarding EBV and its presence in carcinoma stomach. This study is aimed to investigate the frequency of EBV infection in a population with carcinoma stomach by detecting the mRNA expression of EBNA1. This study is based on the hypothesis that if EBV was detected only in the tumour tissue and not in the control tissue, the probability of the individual developing adenocarcinoma due to EBV infection is most likely.

Materials and Methods

Subjects

The study reported here was a prospective, case control study. The study was approved by the Institutional Review Board. All patients with histologically proven adenocarcinoma of the stomach admitted under one Surgical Unit of a tertiary care centre in South India and who underwent a surgical resection (curative or palliative) were recruited after an informed consent. All patients who presented with Linitis plastica were excluded from the study, as it is not possible to collect control tissue from grossly normal-appearing stomach in linitis plastica specimen.

The tumour tissue as evident to the naked eye on dissecting the gastrectomy specimen by a senior surgeon was taken as the case sample and the control sample was gastric tissue distal most from the tumour site.

Sample collection and processing of sample

The gastric carcinoma tissue and normal tissue were collected in two separate tubes containing 3 ml of RNA later (Qiagen, Hilden, Germany). These tubes were immediately transferred to the clinical virology department. The gastric carcinoma and normal tissue tissues were cut in to approximately 30 mg and stored in aliquots of 1.7 ml in sterile tubes containing 300 µl of RNA later and kept overnight at 2–8°C in a refrigerator. The RNA later was then discarded from each tube and remaining tissue was stored at -70°C until testing.

mRNA extraction from gastric carcinoma tissues

mRNA was extracted from gastric carcinoma tissue and normal tissue using RNA easy mini kit (Qiagen, Hilden, Germany). Extraction was done as per the manufacturer's instructions. In brief, tissues were first disrupted by grinding in a mortar and pestle. The ground tissue was then homogenized using a needle and syringe after addition of 0.6 ml of lysis buffer. The contents was mixed and centrifuged for 3 minutes at 10,000 rpm. The supernatant was transferred to a new tube and equal volume of 70% ethanol was added, mixed by pipetting without centrifugation and immediately 700 µl of this suspension was transferred to an RNeasy spin column. Following two washing steps, the RNA was eluted in 50 µl of RNase free water. The mRNA extract thus obtained was used for further analysis.

The RNA concentration was estimated by EPOCH micro-plate spectrophotometery (BioTek, Winooski, Vermont, USA) and subsequently subjected to DNAse I treatment to remove any contaminating DNA. The following steps were carried out to remove the DNA from the reaction tube. A concentration of 500 ng of RNA was subjected to DNAse treatment. The volume of RNA was made to 8 µl with distilled water for DNase treatment.

Reverse transcription

Complementary DNA (cDNA) synthesis was performed in a reaction volume of 20 µl containing 20 picomoles of 2 µl random primers (InVitrogen, USA), 20 units of 0.5 µl RNase RNaseOUT inhibito (InVitrogen, USA), 4 µl of 5 × first strand buffer (InVitrogen, USA), 2 µl of dithiothreitol (DTT), 0.8 µl of high-quality deoxynucleotide triphosphates (dNTPs), 0.2 µl of Moloney Murine Leukemia virus (M-MLV) reverse transcriptase and 10 µl of treated RNA. Reverse transcription was done in a thermal cycler (Veriti, Applied bio system, USA), at 37°C for 60 minutes and the reaction was terminated at 95°C for 5 minutes.

EBV real-time PCR

EBV qualitative real-time PCR was based on the Taqman principle targeting the EBNA1 region. EBV real-time PCR was done using 10 µL of cDNA mixed together in a reaction mixture containing 12.5 µl of QuantiTect Multiplex PCR NoROX master mix (Hamburg, Germany) and 0.075 µl (7.5 picomoles) of forward and reverse EBNA1 primers, 0.05 µl (5 picomoles) of EBNA1 probe labelled with Carboxyfluorescein (FAM) reporter at 5' end and Carboxy-tetramethyl-rhodamine (TAMRA) quencher at 3' end. The thermal cycling conditions used for the real-time PCR were as follows; 95°C for 15 minutes, 95°C for 45 seconds and 60°C for 75 seconds for 50 cycles. The DNA was amplified and detected using real time

PCR (Rotor gene RG-3000 and RG-6000 Corbett Research, Sydney, Australia).

β glucosidase real-time PCR

β glucosidase real-time PCR was done using 10 µl of cDNA mixed together in a reaction mixture containing 12.5 µl of QuantiTect Multiplex PCR NoROX master mix (Hamburg, Germany) and 0.075 µl (7.5 picomoles) of forward and reverse β glucosidase primers, 0.05 µl (5 picomoles) of β glucosidase probe labelled with 6 FAM reporter at 5' end and TAM quencher at 3' end. The thermal cycling conditions used for the real-time PCR were as follows: 95°C for 15 minutes, 95°C for 45 seconds and 60°C for 75 seconds for 50 cycles. The reaction was amplified and detected using real time PCR (Rotor gene RG-3000/6000 Corbett Research, Sydney Australia).

The house keeping gene amplification was used to check the DNA integrity and to detect out the relative quantity of EBNA1 mRNA in case both tumour and control tissues became positive.

Results

A total of 100 patients were enrolled in this study. Among these, 73 (73%) were men and 27 (27%) were women. On basis of mRNA for EBNA1, it was found that six patients had EBV in the tumour tissue giving a prevalence of EBV infection in adenocarcinoma stomach in our population of 6%. All the positive patients were males in the age-group more than 50 years of age. All our patients were from East and South India. This is in accordance with the out-patient distribution of our patients who normally are from South and East India. Majority of the EBV-positive gastric carcinomas were located in the upper and middle part of the stomach. Half (50%) of the EBV-positive gastric adenocarcinomas were moderately differentiated while the other half was poorly differentiated on histological grading. Three of the six EBV-positive gastric adenocarcinoma (50%) presented with stage III disease, and the other three (50%) had stage II disease. In our study, we found that there was significant involvement of nodes in half the patients, the other half had less than 50% nodal involvement. Data on the six EBV-positive cases is shown in Table 1.

Discussion

EBV is a ubiquitous herpes virus.[9] Most people in the adult age-group have serological evidence of previous viral infection. EBV is associated with infectious mononucleosis and human cancers, including nasopharyngeal carcinoma, some lymphomas and also gastric carcinomas.[10] EBV infection in gastric carcinoma was initially reported by Burke *et al*. in 1990.[5] Although EBV was first reported in lympho epithelioma-like gastric carcinoma, the virus was also found in conventional adenocarcinomas.[11]

Gastric carcinoma is a common problem in our population. Our hospital is a tertiary referral centre where we perform on an average about 100–125 gastrectomies (total and subtotal) annually for carcinoma stomach. In our study, we found EBV mRNA positivity among 6 cases out of 100 gastrectomy specimens which gave a frequency of 6%. In a meta-analysis where in pooled data from 15 international populations with consistent laboratory testing for EBV in 5,081 cases of carcinoma stomach were studied found that 9% of gastric cancers contain EBV.[12]

In four out of our six patients, only the tumour tissue was positive for EBV, and the corresponding control tissue sample was negative. In one of the two patients where both tumour and control tissue were positive, the expressed level of EBNA1 was very high compared to the control tissue (118 times). However, in another patient, both the tumour and control tissues were positive for EBV with almost equal amount of EBNA1 expression and in fact with control tissue having slightly higher level (0.4 times). This could have been because of the possible latent period from the time of infection to expression as cancer and, therefore, the possibility of metachronous lesions at varying stages of progression. A study by Arikawa *et al.,*[13] and Matsunuo *et al.,*[14] demonstrates different EBV clones in independent foci of the same stomach, indicating that

Table 1: The clinical, histopathological and anatomical site of the lesion of the 6 EBNA1 positive patients

Patient no.	EBV EBNA1 mRNA		Histology	Differentiation	Type	Anatomic site	Stage
	Tumour tissue	Control tissue					
1	Positive	Negative	Adenocarcinoma	Poorly differentiated	Diffuse	Previous gastro-jejunostomy site	III
2	Positive	Negative	Adenocarcinoma	Poorly differentiated	Diffuse	Fundus	III
3*	Positive	Positive	Adenocarcinoma	Moderately differentiated	Intestinal	Antrum	II
4	Positive	Negative	Adenocarcinoma	Moderately differentiated	Intestinal	Body	II
5**	Positive	Positive	Adenocarcinoma	Moderately differentiated	Intestinal	Body	III
6	Positive	Negative	Adenocarcinoma	Poorly differentiated	Diffuse	Fundus	II

*Control tissue showed 0.4 times higher EBNA1 expression level than the tumour tissue. **Tumour tissue showed 118 times higher EBNA1 expression level than the control tissue

EBV-associated gastric adenocarcinoma develops at multiple sites independently. We found in our study that all the EBV-positive gastric adenocarcinoma patients were in the age group more than 50 years, four of them in the age-group between 51–60 years and two of them in the age-group between 61–70 years. However, literature shows that most of the EBV-associated carcinomas are seen in the younger population.[15] and the data from our study does not corroborate this.

EBV-associated gastric carcinoma is found to be slightly higher in males.[15] In our study, all the patients with EBV-associated gastric carcinoma were males, i.e. 100%.

We also found in our study that three of the cases (50%) had moderately differentiated histology and the other three (50%) had poorly differentiated. Uozaki *et al.,*[16] and a study from Japan and Korea,[17] found similar findings with EBV-associated gastric carcinoma being both moderately and poorly differentiated. They also state that more than 80% of lymphoepithelioma-like gastric carcinoma is infected with EBV, but all our cases were adenocarcinomas. In this study, we found EBV-related gastric carcinoma has a preferential location in the upper and middle part of the stomach. In four (67%) of our patients, the tumour was located in the body and fundus. This finding is also similarly reported in literature.

A study conducted in Netherlands by van Beek *et al.,*[15] showed that EBV-positive gastric carcinomas had less lymph node involvement and most of these carcinomas occurred in the younger age-group. However, in our study, the EBV-positive patients were all in the age-group more than 50 years and the majority of them had significant lymph node involvement. One of our patients had a gastric stump malignancy, been operated several years earlier for benign gastric disease. Uozaki *et al.,*[16] stated that the remnant stomach is a risk factor for EBV-associated gastric malignancy. They found that EBV involvement occurs in 27–42% in the remnant which is much higher compared with the 9–10% prevalence that occurs in *de novo* gastric cancer.

We followed up all the patients with EBV-positive gastric cancer during the period of this study (4 months to 14 months). One of the patients died in the immediate post-operative period due to post-operative complications. All the remaining patients had received post-operative adjuvant chemotherapy (Docitaxel and Oxaliplatin based). Three patients had completed chemotherapy and had follow-up in the Surgery Outpatient Department and were all asymptomatic. Imaging studies done during the subsequent follow-ups showed stable disease with no evidence of local recurrence or metastases. The other two patients were followed by telephonic conversation and were asymptomatic and doing well. There is evidence in the current literature that EBV-associated gastric carcinoma has a favourable prognosis compared to EBV-negative gastric carcinoma.[18,19]

There are few data available on the prevalence of EBV infection with relation to carcinoma from India. A study published in 2008 showed a significant association ($P < 0.001$) of EBV DNA in gastric carcinoma (GC) than those with non-ulcer dyspepsia (NUD) (GC versus NUD—82.3% versus 373.3%).[20] There are reports which showed more association of EBV DNA with gastric carcinoma than NUD patients or patient with peptic ulcer and the frequency is similar to the one reported from other countries.[21-23]

Our data also reiterate the findings observed from the above studies that there is an association between EBV and gastric adenocarcinoma and the frequency is also similar.

We, however, could not find any statistical difference in the nature of presentation, age and sex predilection, lymph nodal status, site of presentation compared to EBV-negative gastric carcinoma. A larger study with long-term follow-up is needed to further establish the role of EBV in gastric adenocarcinoma in this study population.

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