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ORIGINAL ARTICLE

Determination of EBV genotypes and EBNA1 oncogene variants among breast cancer patients in Peshawar, Pakistan

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ABSTRACT

Background: Epstein-Barr virus (EBV) is a common DNA virus linked to several human cancers, including breast cancer (BC). Specific EBV oncogenes, like EBNA1, are also implicated in the process of oncogenesis. This study aimed to investigate the prevalence of different EBV genotypes and EBNA1 oncogene variants in BC patients in Peshawar, Pakistan.

Methodology: This study was conducted in the Institute of zoological sciences, University of Peshawar, Khyber Pakhtunkhwa. Tumor biopsies and demographic data were collected from 75 BC patients. DNA was extracted from the samples and EBV genotypes were detected using polymerase chain reaction (PCR) with specific primers. The EBNA1 oncogene was also amplified *via* PCR and sequenced using Sanger's sequencing method. The resulting sequences were analyzed to determine EBNA1 variants. All data were analyzed using descriptive statistics, primarily percentages and chi square test where applicable.

Results: EBV was detected in 40% of the study participants. All positive samples were identified as EBV genotype-1. The most prevalent EBNA1 type found in these patients was the prototype P-Threonine. The study population was predominantly post-menopausal (63%), with most tumors being malignant (93%) and of grade II (69%). Among the malignant cases, 63% were hormone receptor (HR) positive, 11% were HER2-positive, and 26% were triple-negative breast cancer (TNBC).

Conclusion: EBV type-1 and EBNA1 P-Threonine are present in breast cancer patients in Peshawar, Pakistan. This study provides valuable insight into the molecular characteristics of EBV in breast cancer within this specific population.

Key words: Breast Cancer, Epstein Barr Virus, Genotypes, Nuclear Antigen

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Introduction

Epstein Barr virus (EBV) is the first cancer causing human virus identified, infecting more than 90% of the human population (1). It primarily causes infectious mononucleosis and is linked with numerous malignancies like Burkitt lymphoma, ovarian cancer, nasopharyngeal carcinoma, gastric cancer, and breast cancer (2, 3, 4). EBV was first discovered in 1964 in Burkitt lymphoma (5). EBV belongs to the family *Herpesviridae* and is a dsDNA virus. It comprises two major genotypes, EBV genotype-I and EBV

genotype-II, distinguished by variations in the EBNA2 and EBNA3C genes (6,7). EBV genotype-I is more prevalent in Asia and Europe, whereas EBV genotype-II is commonly reported in Africa (3).

EBV encodes several oncogenes, including latent membrane proteins (LMPs), Epstein–Barr nuclear antigens (EBNAs), and viral microRNAs (8) and are essential for immune evasion, inhibiting apoptosis, and promotes cell survival (3). Among the nuclear proteins, EBNA1 is the only one consistently expressed in all EBV-associated malignancies (9). In the latent stage, EBNA1 is responsible for EBV genome replication and possesses oncogenic potential (10). It regulates key cellular pathways and thus plays a critical role in oncogenesis (11). EBNA1 is also considered an important therapeutic target for the treatment of EBV-related cancers (12). Several EBNA1 types have been reported, including two prototypes: P-Alanine (P-Ala) and P-Threonine (P-Thr), as well as three variants: V-Valine, V-Leucine, and V-Proline (13).

In Pakistan, BC is reported as the most common malignancy among females, with a reported prevalence of 31.3% (14). Although BC is widespread in established countries as well (15) In Pakistan, one in every nine women is at risk. Every year, more than 83,000 new cases are reported nationwide (16,17,18). Multiple risk factors contribute to the high burden of BC in Pakistan (19) but the contribution of infectious agents such as EBV remains largely unexplored. The role of EBV in BC is not yet fully understood (3). It is necessary to screen BC patients for EBV due to its oncogenic potential. Moreover, EBV-EBNA1 variants have not been investigated in Pakistani populations which is vital oncoprotein responsible for maintaining EBV genome in the host cell (10). Identifying the prevalent variant among study population will help in understanding the viral and host interaction in future studies. Therefore, the aim of this study is to characterize EBV genotypes and EBV-EBNA1 variants in BC patients in Pakistan.

Methods

The study was conducted in Peshawar, Khyber Pakhtunkhwa after the approval of Research Ethics Board University of Peshawar via letter number REB 04/04 dated 15-04-2025. Female BC patients admitted to the Surgical-B ward of Khyber Teaching Hospital (KTH), Peshawar, Pakistan, were included in this study irrespective of their age.

Tissue samples were collected from patients during mastectomy with the assistance of surgeons and stored in 70% ethanol for further use. Clinical data were collected from patients using a predesigned questionnaire. Histological information was obtained from the patients' histopathology reports.

DNA was isolated from the collected tissue samples through the GeneJet DNA Isolation Kit (ThermoFisher, USA) using Proteinase-k and Digestion solution. PCR for EBV detection and genotyping was performed using protocol as described previously (7). The 1st round Primers used for genotyping were Outer forward: 5´-GAGAAGGGG AGC GTG TGT TGT-3´and Outer reverse: 5´- GCT CGT TTT TGA CGT CGG C -3´ while the primer sequence for the 2nd round was: Inner forward: 5´-TCA TAG AGG TGA TTG ATG TT-3´ and Inner reverse: 5´- ATG TTT CCG ATG TGG CTT AT -3´. PCR conditions for both 1st and 2nd round were: Initial denaturation at 95°C for 3min followed by 30 cycles of denaturation (94°C, 25sec), annealing (54.6°C, 25sec) and extension (72°C, 15sec). The final extension was 72°C for 5 minutes.

PCR for EBNA1 was carried out using primers (OF: 5´-GAAGTCGTGAAAGAGCCA-3´ and OR: 5´-ATCACCTCCTTCATCTCC-3´) for the first-round reaction. The nested reaction was performed using primers (IF: 5´-CAGTAGTCAGTCATCATCATCC-3´ and IR: 5´-CACCTCCTTCATCTCCGT-3´). PCR conditions were followed as described by Tabatabaie et al (20). The product size of EBNA1 second round product is 743bp.PCR amplicons were resolved by agarose gel electrophoresis and visualized under UV illumination using a gel documentation system.The amplified product of EBNA1 gene was sequenced using unidirectional Sanger sequencing method.

All data was analyzed in terms of percentages. Pearson chi-square test was applied using SPSS v-25. P-value <0.05 was considered as statistically significant. Sequenced data were analyzed using the BioEdit tool. Sequences were aligned with the reference sequence B95.8 obtained from NCBI through multiple sequence alignment (MSA). Nucleotide positions 109,408–109,410 were targeted to detect EBNA1 variants.

Results

In this study, 75 BC patients were included. The patients were stratified into three age groups: Group-I (≤30 years, *n*=7), Group-II (31–45 years, *n*=24), and Group-III (>45 years, *n*=44). Among these patients, 65 were married and 10 were unmarried. A total of 28 patients were premenopausal, while 47 were postmenopausal.

Overall, 93% of the patients had malignant BC, whereas 7% had benign tumors. Malignant cases were further categorized by tumor grade: 1 patient had grade-I, 48 had grade-II, and 21 had grade-III disease. Regarding molecular BC subtypes, 44 malignant cases were hormone receptor (HR) positive (estrogen receptor [ER]-positive, progesterone receptor [PR]-positive, or both), 8 were Her2/Neu-positive, and 18 were classified as triple negative breast cancer (TNBC) (Table-1).

Of the total value, EBV (EBNA 3C) was detected in 40%, all of which were classified as genotype-I. EBV genotype-II was not detected in any sample (Figure-1).

Table-1: Demographics of the BC patients (N=75)

Demographic Characteristics		BC(N=75) n (%)
Age groups (years)	Group-I (≤30)	7 (9)
	Group-II (31-45)	24 (32)
	Group-III (≥45)	44 (59)
Marital status	Married	65 (87)
	Un-married	10 (13)
Menstrual cycle	Pre-menopausal	28 (37)
	Post-menopausal	47 (63)
Type of BC disease	Malignant	70 (93)
	Benign	5 (7)
Tumor grade	I	1 (1)
	II	48 (69)
	III	21 (30)
Receptor status	HR+	44 (63)
	HER2+	8 (11)
	TNBC	18 (26)

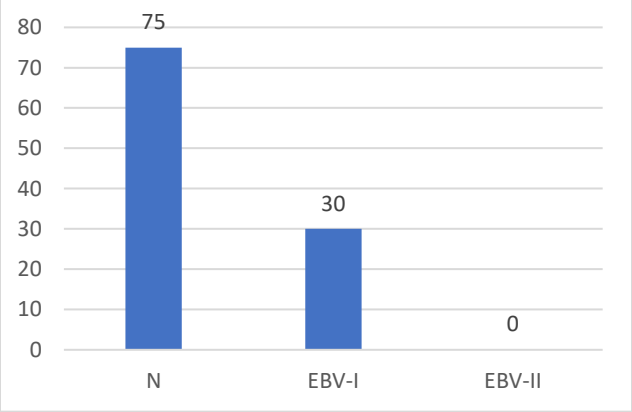


Figure-1: Frequency of EBV positive cases and its genotype distribution in breast cancer patients (N=30)

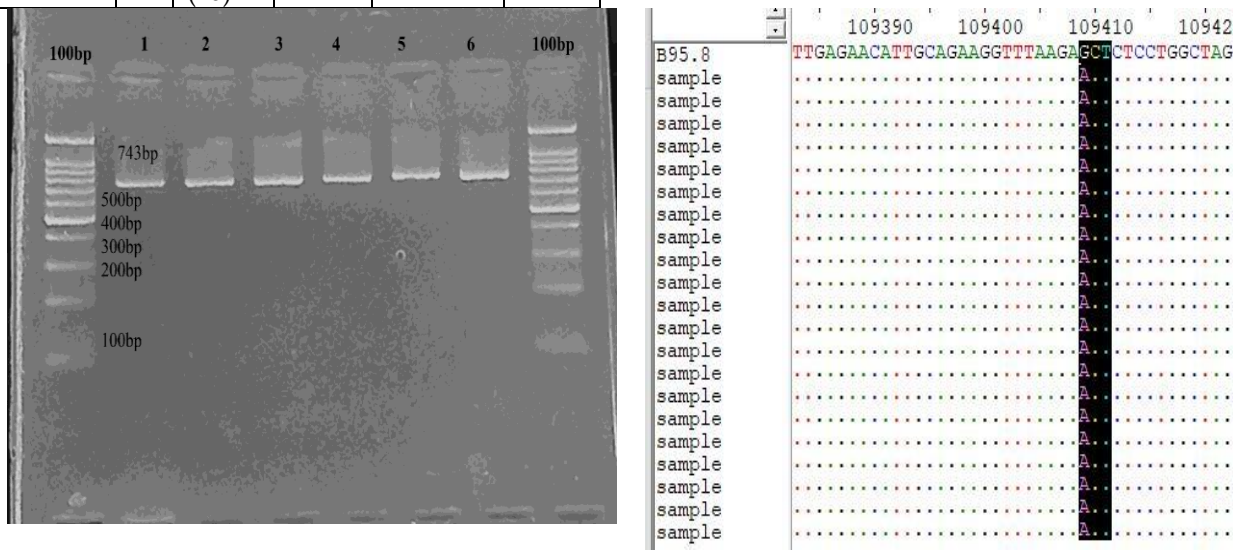
The malignant patients were 70, of which 65 were of ductal carcinoma while 05 were of

lobular carcinoma. All the EBV positive cases were of ductal carcinoma. Of these, all were of EBV type-I. On the other hand, lobular carcinoma and benign patients were not positive for EBV. Statistically the result was significant (P<0.05) (Table-2).

Table-2: EBV frequency in various BC types

BC- type	N	EBV Genotype n (%)		EBV negative	P-value
		Type I	Type II		
Ductal carcinoma	65	30 (46%)	-	35 (57)	0.02
Lobular carcinoma	5	-	-	5 (100)	
Benign	5	-	-	5 (100)	
Total	75	30 (40)	-	45 (60)	

The EBNA1 gene of EBV, amplified by PCR (Figure-2a), was sequenced and analyzed using multiple sequence alignment. When compared with the reference sequence, all samples showed a substitution at the targeted nucleotide position. At nucleotide position 109408, a change observed was G>A resulting in the P-threonine prototype (Figure-2b).



(a) **(b)**

Figure-2: Agarose Gel picture and Multiple sequence alignment (a) Gel image showing EBNA1 positivity (743bp) in breast biopsies compared with 100bp DNA Ladder marker (b) Multiple sequence alignment of EBNA1: Highlighted nucleotides position (109,408-109,410) shows presence of P-threonine (ACT) in the study population in comparison to reference sequence B95.8

Discussion

BC is currently one of the principal causes of death among women worldwide (21). It is vital to understand the role of infectious agents such as EBV in the development of BC, as EBV is an oncogenic virus previously associated with several human malignancies (22) To our knowledge, no nationwide BC study focusing on the viral etiology of BC, particularly EBV, has been published. EBV is classified as a Group 1 carcinogen due to its established association with various human

cancers (23). Moreover, no study to date has focused specifically on the association of the EBV oncogene EBNA1 with BC.

In this study, most patients were above 45 years of age. Previous reports indicate that BC risk increases with advancing age, partly due to prolonged exposure to endogenous hormones (19). Age is therefore considered a key risk factor for BC susceptibility (24). Most patients in this cohort were diagnosed with grade II tumors, followed by grade III. This pattern may be explained by the fact that sampling was conducted after neoadjuvant therapy, which may have downgraded some tumors from grade III to grade II. Based on molecular subtypes of BC, the majority of patients were HR-positive, an important factor for endocrine therapy, as these receptors are targeted during treatment (25). Hormone receptor positivity has also been reported as the most common molecular subtype in previous studies (26). Conversely, TNBC cases were fewer in number, despite being the most aggressive subtype, requiring therapies beyond endocrine treatment (25).

Previous studies suggests that EBV is associated with aggressive BC subtypes, including HR+ and TNBC (27). However, it is also reported that no significant association is present between hormone receptors, HER2/neu prognostic markers, and EBV expression (26). In our study, most patients were postmenopausal, reflecting the older age of the cohort. BC risk is known to increase with late menopause due to prolonged estrogen exposure (28). On the other hand, some studies suggest that menopause itself is not directly responsible for BC onset, but rather the accumulation of mutations with age contributes to disease development (29).

In this study, EBV DNA was detected in 40% of malignant breast tumor samples, suggesting a potential role of EBV in BC development. In our cohort, only EBV type I was detected, indicating that it is the predominant circulating genotype in this region. Similar findings of EBV type I prevalence have been reported in other Asian countries (30). EBV positivity has also been linked with higher-grade breast tumors (31). Thus, our finding of 40% EBV positivity supports the possible etiologic role of EBV in BC. Moreover, EBV-positive tumors presenting with more aggressive phenotypes could serve as potential therapeutic targets (32).

All EBV-positive BC cases in this study were also positive for the EBNA1 oncogene. EBV oncogenes have previously been detected in breast cancer (33), nasopharyngeal carcinoma (34), cervical and ovarian cancers (20), and Hodgkin lymphoma (35) These oncogenes interact with various host proteins, disrupting biological and signaling pathways that promote oncogenesis (36,37.38). In this study, all EBNA1 sequences from malignant breast tumors carried threonine at position 487 of the EBNA1 protein.

It is suggested to further investigate how tissue specific factors, such as cellular interactions and immune responses, influence the selection and persistence of EBNA1 variants. P-Thr was also reported in nasopharyngeal carcinoma, suggesting that this prototype may be considered as a viral screening marker for NPC (34). Based on our findings, EBV may have a potential role in BC development. Further studies are necessary to elucidate the role of EBV oncogenes in BC progression.

Conclusion

EBV genotype-I is the predominant genotype in breast cancer patients. Significant association was observed between EBV

positivity and breast cancer types. Moreover, EBNA1-P-Threonine is the most prevalent prototype among malignant breast cancer patients in Peshawar, Pakistan.

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