Original Research *Highlight article*

Epstein-Barr virus genotype-1 and Mediterranean + strain in gastric cancer biopsies of Ghanaian patients

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Impact Statement

Epstein-Barr virus (EBV) genotypes and strains have been associated with gastric cancer (GC), and the malignancy contributes significantly to cancer morbidity and mortality. With GC on the rise in Ghana, it is imperative to know the contribution of the EBV and its genotypes and/or strains. This current study has established that EBV Mediterranean + genotype-1 is the predominant strain in GC biopsies, and EBV viral load does not necessarily contribute to progression of the cancer.

Abstract

Gastric cancer (GC) prevalence is on the increase in Ghana, and Epstein-Barr virus (EBV) is one of the factors that have been implicated in the etiology of the cancer. It is therefore important to know the contribution of EBV genotype and strains that are associated with GC. In this study, we aimed at genotyping EBV and determining predominant strains in GC biopsies in Ghanaian patients. Genomic DNA was extracted from 55 GC biopsies (cases) and 63 normal gastric tissues (controls) were amplified by polymerase chain reaction (PCR) using specific primers for EBV detection and genotyping followed by PCR fragments sequencing. Epstein-Barr virus positivity were 67.3% and 49.2% in the GC and normal biopsies, respectively. Both cases and controls had the Mediterranean + strain of EBV. The predominant genotype of the virus in the GC cases was genotype-1 (75.7%) compared to 66.7% of genotype-2 among the control group. Infection was associated with GC in the study population (OR = 2.11, $P = 0.014$, 95% CI: 1.19 – 3.75), and EBV genotype-1 significantly increased the risk of GC (OR = 5.88, $P < 0.0001$, 95% CI: 3.18–10.88). The mean EBV load in the cases (3.507 ± 0.574) was significantly higher than in the controls (2.256 ± 0.756) ($P < 0.0001$). We conclude that EBV, especially Mediterranean + genotype-1, was the predominant strain in GC biopsies and GC type or progression is independent of the viral load.

Keywords: Epstein-Barr virus, genotyping, Mediterranean +, gastric cancer, EBV epidemiology, Ghanaian patients

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Introduction

Gastric cancer (GC) is one of the most common cancers and third leading cause of cancer-related deaths worldwide.[1] In Africa, GC is ranked as the twelfth most common cancer with an estimated incidence and mortality rates of 4/100,000 and 3.8/100,000, respectively.[2] The 2018 Globocan report ranks GC as the sixth most common cancer incidence and fifth in terms of cancer associated mortalities in Ghana.[3] Although aetiological factors of GC are not immediately known, studies have associated hereditary, diet, and environmental factors including infections with the cancer, and both host and pathogen virulent factors are considered crucial for infection.[4,5]

Epstein-Barr virus (EBV) is a known human oncovirus which belongs to the group of gamma-herpes viruses and has been strongly implicated in epithelial cancers[6]; however, genetic diversity is crucial in the cancer's development.[7] EBV-encoded microRNAs and also EBV-induced cellular microRNAs play important roles in EBV infection.[8] The virus expresses four distinct latency programs, with each program characterized by the expression of certain EBV latent proteins. Based on the latency program, diseases such as GC, Burkitt lymphoma, nasopharyngeal cancer, diffuse B-cell lymphoma, Hodgkin lymphoma and AIDS-associated B-Cell lymphoma have been associated with the infection.[9] In GC, the mechanism employed by the virus to herald tumorigenesis is by inducing the formation of blood vessels, disrupting the cell cycle and causing promoter hypermethylation in tumor suppressor genes.[10] Promoter hypermethylation in *APC, p14, p16 TP73, CXXC4 and TIMP2* -genes have been observed in EBV-associated gastric cancer (EBVaGC).[11,12]

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The main EBV protein involved in cell transformation is LMP-1, and the protein activates several downstream signaling pathways that play a contributory role in many phenotypic consequences such as upregulation of anti-apoptotic genes and cytokines,[13] and down regulation of tumor suppressors like p53, resulting in cell proliferation and survival.[14,15] Two genotypes and seven strains of the virus have been identified and are geographically distinct,[16] with EBV genotype-2 being the most common genotype in Africa.[17] The greater proportion of tumor cases reported from Asia, Europe and North America were linked to EBV genotype-1, whereas EBV type-2 is commonly associated with tumors found in Papua New Guinea and Central Africa.[17] EBV Genotype-2 was reported in Ghanaian patients diagnosed with nasopharyngeal cancer.[18]

There is therefore little information on the genotypes and strain of the virus and GC. With the growing incidence of GC in Ghana,[3] and paucity of information on possible involvement of EBV in the development of GC, we aimed to detect and characterize EBV genotypes to identify prevalent strains in gastric biopsies received for pathological diagnosis at the Pathology Department of the national referral hospital, Korle Bu Teaching Hospital, Accra, Ghana. This has thrown more light on the specific virulence factors that may contribute to the risk of developing GC in Ghana, and may ultimately guide in its management.

Materials and methods

Study site

Samples used in this study were obtained from the Pathology Department of Korle-Bu Teaching Hospital (KBTH) in the Greater Accra Region of Ghana, and laboratory analysis carried out at the Department of Biochemistry, Cell and Molecular Biology, University of Ghana. Summary of data acquisition and processing is shown in Figure 1.

Ethical approval

Ethical approval was obtained from the Ethics Committee for Basic and Applied Sciences, University of Ghana, Legon (Reference number: ECBAS 021/19-20). All methods were carried out in accordance with relevant guidelines and regulations.

Sample selection and sectioning

Classification of primary tumor was by histology (Supplemental Figure S1). After initial assessment of records of all the reported cases available at the Pathology Department within the study period (January, 2015 to December 2019), 55 cases (41 adenocarcinomas, 12 gastrointestinal stromal tumors and 2 lymphomas) from both gastrectomy and endoscopy were identified. Biopsies diagnosed by pathologist as normal within the same period were used as control. The cases were matched by sex and age, to their respective controls. Formalin-fixed paraffin-embedded (FFPE) tissue blocks of the identified cases and controls were retrieved from the tissue bank and sectioned using rotary microtome. Four sections of 5 μm thickness each of FFPE sample was taken and placed in labeled Eppendorf tubes for

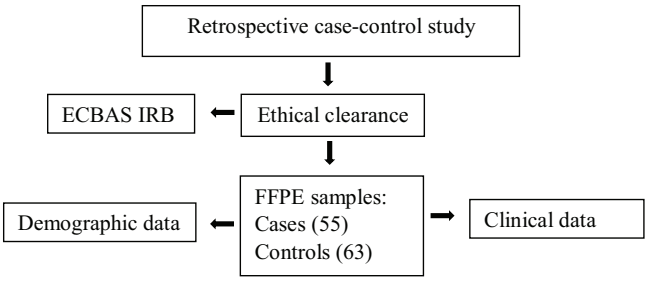

Figure 1. Flowchart on the study design.
ECBAS: Ethics Committee for Basic and Applied Sciences; IRB: Institutional Review Board, FFPE: formalin-fixed paraffin embedded.

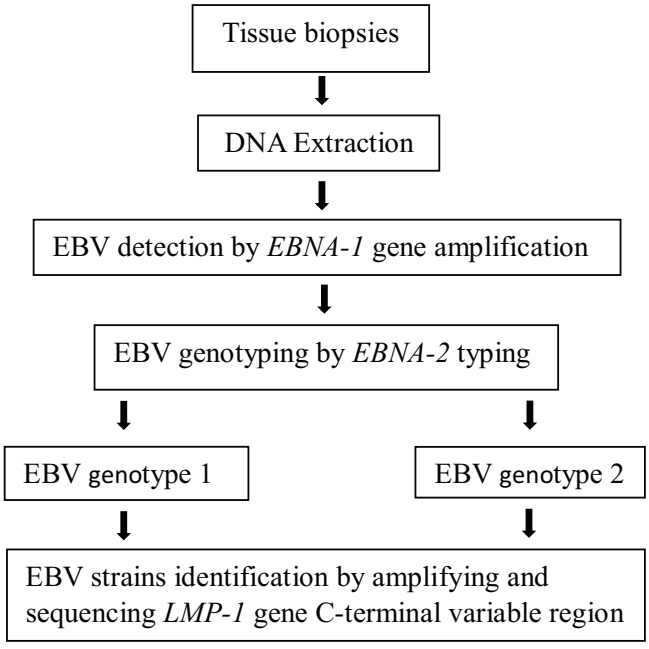

Figure 2. Flowchart on identification and characterization of EBV genotypes and strains.

DNA extraction. Steps involved in EBV detection, genotyping, and strain identification is shown in Figure 2.

DNA extraction

Genomic DNA was extracted from the gastric biopsies using QIAamp DNA FFPE Tissue kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. The concentrations and purities of the eluted DNA was determined using a Nanodrop (Thermo Fischer Scientific, USA), and stored at -20°C until ready for use.

Positive controls

pJET-EBNA-1, pJET-EBNA-2 and pJET-LMP-1 plasmids (Wright Laboratory, University of Sussex) containing EBV DNA were used as positive controls for EBV detection, EBV genotypes-1 and -2 characterization, and EBV LMP1 for strain classification, respectively. Also, pJET-Beta plasmid (Wright Laboratory, University of Sussex) was used as positive control for beta globulin detection.

Table 1. List of primers used for amplification of the genes.

Gene target	Primer name	Primer sequence	Product (bp)
EBNA-1	QP1 (forward)	GCCGGT GTGTTCGTATATGG	213
	QP2 (reverse)	CAAAACCTCAGCAAATATATGAG	
EBNA-2	First round		
	EBNA-2F (forward)	TGGAAACCCGTCACTCTC	801
	EBNA-2I (reverse)	TAATGGCATAGGTGGAATG	
	Second round		
	EBNA-2C (common forward)	AGGGATGCCTGGACACAAGA	
	EBNA-2G (type-1 reverse)	GCCTCGGTTGTGACAGAG	250
	EBNA-2B (type-2 reverse)	TTGAAGAGTATGTCCTAAGG	300
LMP-1	LMP1 (forward)	CGGAACCAGAAGAACCCA	506
	LMP1 (reverse)	TCCCGCACCCTCAACAAG	
B-globin	B-globlin (forward)	(ACACAACTGTGTTCACTAGC)	119
	B-globlin (reverse)	(CAACTTCATCCACGTTCACC)	

EBNA-1: detection of EBV; EBNA-2: genotyping; LMP-1: strains identification; B-globin: internal control.[7,18,19]

EBV detection by *EBNA-1* PCR amplification

The presence of EBV in the extracted DNA samples were detected by EBNA-1 PCR using the primers (QP1 and QP2) that have been reported previously (Table 1). The total reaction volume was 12.5 µL and constituted 6.25 µL of 2X One-Taq master mix (New England Bio Labs, Hertfordshire, UK), 0.12 µL each of the forward and reverse primers, 1 µL of the DNA sample and 5.01 µL of nuclease free water. The thermocycler (Prime Thermal cycler, Bibby Scientific Limited, UK) was programmed such that the samples were initially denatured at 94°C for 30 s, followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 57.2°C for 1 min, extension at 68°C for 1 min and final extension at 68°C for 5 min. The amplicons were then separated on 1.5% agarose (Merck, Germany) gel stained with ethidium bromide (Sigma Aldrich, USA) and bands visualized under UV light for the presence of the amplified DNA, using the Amersham Gel Imager 600 (GE Healthcare UK Limited). Amplified human beta globulin gene in the samples under the same conditions was used as internal control.

Genotyping of EBV by *EBNA-2* nested PCR amplification

EBV genotypes were determined by *EBNA-2* nested PCR using primers previously reported (Table 1). The first-round amplification was a 12.5 µL total reaction consisting of 6.25 µL of 2X One-Taq master mix, 0.12 µL each of the first-round forward and reverse primers (Table 1), 1 µL of the DNA and 5.01 µL of nuclease free water. The PCR reaction was carried out at an initial denaturation of 94°C for 2 min, followed by 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 52.9°C for 1 min 30 s, extension at 72°C for 4 min and final extension at 72°C for 10 min. Also, the second-round amplification was a 12.5-µL final reaction mixture consisting of 6.25 µL of 2X One-Taq master mix, 0.12 µL each of the common forward, second round type-1 and type-2 reverse primers, 1 µL of the amplicon from the first amplification and 4.89 µL of nuclease free water. The PCR reaction was carried out at an initial denaturation of 94°C for 2 min, followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 54.9°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 10 min. The amplicons were then separated on 1.5% agarose gel stained with ethidium bromide and bands visualized under UV light for the presence of the amplified DNA, using the Amersham Gel Imager 600. EBV genotypes were identified by a 250 bp band size for EBV genotype-1 and 300 bp band size for EBV genotype-2.

EBV strain identification by sequencing *LMP-1* gene 3´end variable region

EBV strains identification was carried out by amplifying the *LMP1* gene 3´end variable region using primers previously reported (Table 1), with modifications to the cycling conditions. The total reaction volume was 25 µL and constituted 12.5 µL of 2X One-Taq master mix, 0.25 µL each of the forward and reverse primers, 5 µL of the DNA sample and 7 µL of nuclease free water. The PCR reaction was carried out at an initial denaturation of 95°C for 1 min, followed by 35 cycles consisting of denaturation at 95°C for 1 min, annealing at 53.2°C for 1 min 30 s, extension at 72°C for 3 min and final extension at 72°C for 5 min. The PCR product (7 µL) was separated on 1.5% agarose gel with ethidium bromide staining and bands visualized under UV light, using the Amersham Gel Imager 600. *LMP-1* positive samples were identified by a 506 bp band size and remaining amplicons were shipped for commercial sequencing at Inqaba Biotec, South Africa.

EBV burden determination in GC and normal gastric biopsies by ENBA-1 real-time PCR

EBV DNA load in the tumor biopsies were determined by relative quantification method using real-time PCR (QauntStudio5 PCR System, Applied Biosystem), by amplifying *EBNA-1* gene. PCR reaction volume of 10 µL containing the following components: 5 µL 1X Sybr Green SuperMix, template DNA, 0.3 µL of 0.1 µM each of target primer (QP1 and QP2) (Table 1) and 3.4 µL of nuclease-free water. Each reaction was normalized by amplification of *Beta globin* gene. The cycling conditions consisted of an initial denaturation step 1 min at 95°C, and then 40 cycles of 95°C for 15 s (denaturation), 55°C for 30 s (annealing), and 60°C for 30 s (reannealing and extension). All the samples were analyzed in triplicates and after amplification, melting curve analyses

was constructed to confirm that only specific products were amplified.

Construction of standard curve for EBV DNA load quantification

To infer the unknown concentration of EBV load in the various FFPE samples, a calibration curve was constructed using a known concentration of EBV DNA. pJET plasmid containing *EBNA-1* gene was spectrophotometrically quantified and EBV copies were calculated using a formula. A 10-fold serial dilution of the stock pJET-EBNA-1 of concentration 10^{11} copies/μL was prepared after which working dilutions of 10^5 to 10^{10} were made. The serially diluted pJET-EBNA-1 plasmids were run in triplicates to generate cycling threshold (C_T) values for each dilution. A calibration curve was then constructed by plotting the C_T values against the log concentration of each dilution containing the viral DNA.

Statistical analyses

Statistical analysis was done using Stata Statistical Software Version 15 (StataCorp LLC) and GraphPad Prism version 8.4 (GraphPad Inc, USA). Descriptive data were presented as mean ± standard deviation, and significance of mean difference determined using Student t-test and one-way analysis of variance (ANOVA) after satisfying test for normality by performing Gaussian test in GraphPad Prism. Categorical data were presented as percentages and analyzed using Fisher's exact test and odds ratio (OR). *P*-values less than 0.05 were considered statistically significant.

Results

Demographic characteristics and EBV infection prevalence of study participants

The mean age of the patients whose biopsies were included in the study was 58.1 years. Males accounted for 63.3% and mean age (57.9 ± 13.83 years) compared to their female counterparts (58.5 ± 14.33 years) was not statistically significant ($P < 0.764$), however, gastric cancer cases were significantly higher in males than females (OR = 2.97; $P < 0.0001$). Out of the total 55 gastric cancer biopsies screened using *EBNA-1* gene (Supplemental Figure s2), 37 (67.3%) were positive for the *EBNA-1* gene while 31/63 (49.2%) were found in the controls. Association of EBV infection with gender (Supplemental Table S1) was not statistically significant ($\chi^2 = 0.058$, $P = 0.809$).

Association of EBV genotypes with gastric cancer

Amplification of *EBNA-2* genes in gastric and non-gastric cancer biopsies is shown in Supplemental Figure S3. Out of the 37 EBV positive gastric cancer biopsies screened, 28 (75.7%) were positive for EBV genotype-1 while 3/37 (8.1%) were positive for EBV genotype-2. A total of 6 (20.0%) out of the 30 EBV positive gastric control biopsies were EBV genotype-1, while EBV genotype-2 accounted for 20 (66.7%), and genotypes-1 and -2 co-infection was reported in 4 (13.3%) of the control samples. In all, a total of 7 (6 cases and 1 control) *EBNA-1* positive samples could not be typed by *EBNA-2* amplification. The association between EBV genotype and risk of gastric cancer development is shown in Supplemental Table S2. There was a significant association between EBV genotype-1 and risk of gastric cancer development ($\chi^2 = 25.95$, $P < 0.0001$).

Identified EBV strain in gastric biopsies

The amplified 3´end variable region of EBV *LMP-1* gene in the GC and normal biopsies is shown in Supplemental Figure S4. The sequences from the 37 amplicons (20 cases and 17 controls) of the EBV *LMP-1* gene were aligned with reference strains in NCBI database using the Basic Local Alignment Search Tool (BLAST) (Supplemental Figure S5). All the sequences were identified as EBV Mediterranean + strain.

Phylogenetic tree based on EBV *LMP-1* gene 3´ end variable region

The diversity in the 37 sequences against the known strains of EBV are shown in Figure 3. The results are presented as a circular phylogenetic tree. The analysis was done with the maximum likelihood method with bootstrapping of 1000 trials.

EBV load in gastric cancer and control biopsies

Comparison of the mean EBV load between gastric cancer biopsies ($n = 38$) and controls ($n = 33$) is shown in Figure 4. The log EBV DNA copies/ml for the gastric cancer biopsies (3.507 ± 0.574), was significantly higher than that of the control biopsies (2.256 ± 0.756) ($P < 0.0001$).

Gastric cancers and EBV DNA load

Comparison of the mean EBV load between gastric adenocarcinoma ($n = 25$) and gastrointestinal stromal tumor ($n = 10$) is shown in Figure 5. The mean EBV load of gastric adenocarcinoma (3.538 ± 0.5545) and that of gastrointestinal stromal tumor (3.378 ± 0.6377) showed no statistically significantly difference ($P = 0.4656$).

EBV DNA load and gastric adenocarcinoma grades

The mean values of EBV load in well differentiated ($n = 5$), moderately differentiated ($n = 8$) and poorly differentiated ($n = 11$) cancers are presented in Figure 6. Difference in EBV load between the GC grades was not significantly significant ($P = 0.7181$).

Discussion

Our present study showed that EBV DNA load were significantly higher in GC than the non-GC biopsies. Similarly, the percentage of EBV genotype-1 in the cases were higher than in the controls. Also, all the EBV sequences were identified as the Mediterranean + strain of the virus.

Gastric cancer is the most frequent malignancy that develops in one layer of the stomach and gradually invades into the outer layers,[19] and grows at a slow pace over many years without showing symptoms.[3] In Ghana, the cancer ranks sixth as the most common cancer incidence in both sexes, and it is the fifth cause of cancer-associated mortalities.[3]

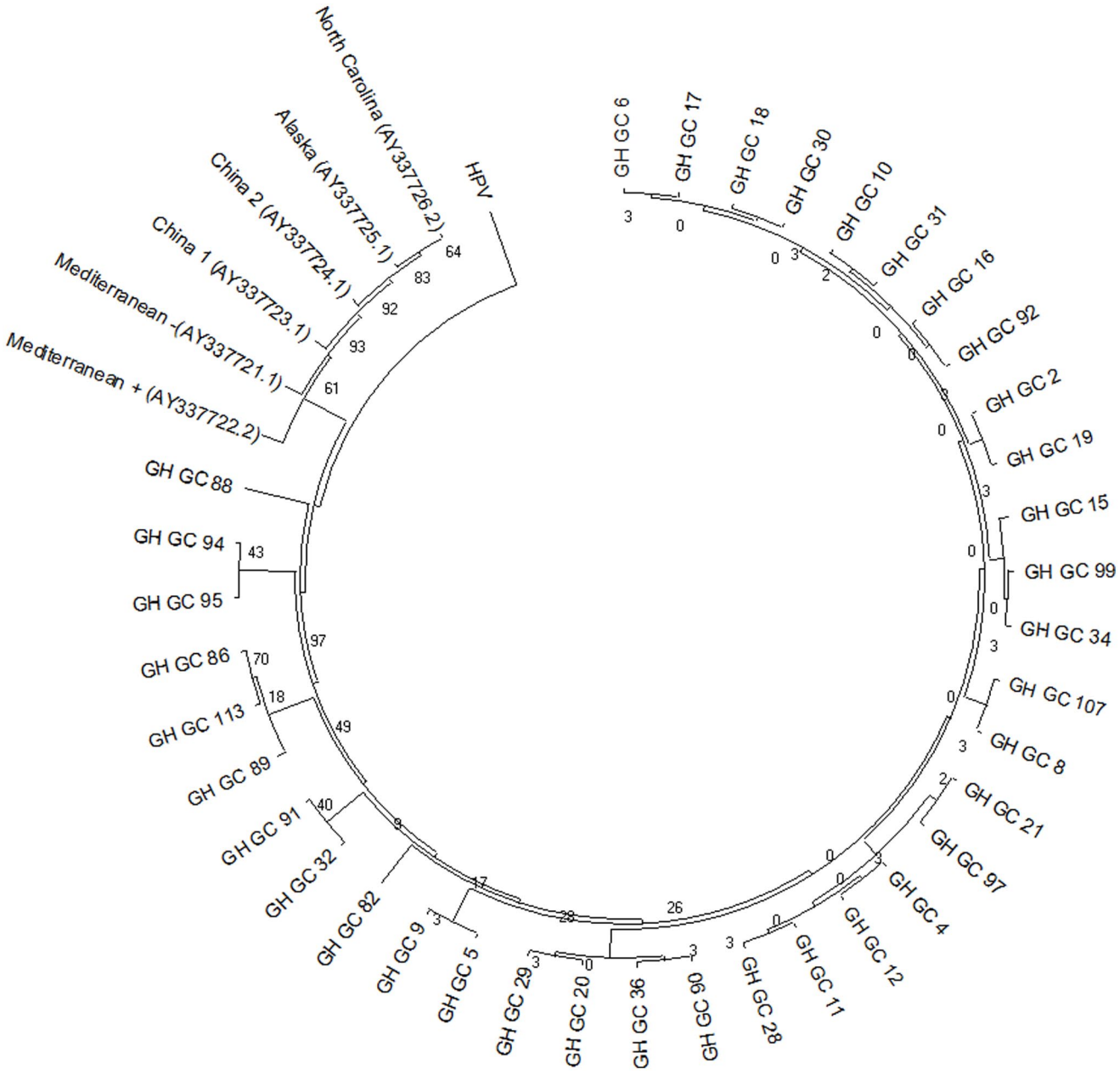

Figure 3. Phylogenetic analysis of EBV *LMP-1* gene C-terminal variable region from EBV positive GC and control biopsies. Known strains of EBV from GenBank was included, and HPV was used as an outgroup.

In this current study, the mean age at diagnosis of these GC patients was 58 years, and the result is in accordance with a previous study.[20] Also, males were twice as much affected with GC than females in the current study and this supports global GC incidence for male: female ratio of 2:1.[3,21] The scientific basis for this observation has not yet been elucidated. However, several studies have attributed the variations in sex distribution of GC to genetic differences such as susceptibility of germline mutation carriers of the *CDH1* gene,[22–24] and environmental risk factors such as excessive intake of alcohol, cigarette smoking and high intake of salt.[25,26]

A crucial environmental agent associated with GC are pathogenic factors such as *Helicobacter pylori* and EBV infections.[27] EBV-associated GC has been reported in various parts of the world with varied prevalence which can be as high as between 50% to 80% of all GC cases, with methylation in tumor suppressor genes, angiogenesis and cell cycle disruption being some of the mechanisms employed by the virus to herald tumorigenesis.[28] The global adult population infected with EBV is more than 90%.[29] Primary infection usually occurs at an early stage of life and infection is asymptomatic in children, while producing infectious mononucleosis (IM) in young adults.[30] EBV upon infection, establishes a lifelong latency in the form of episomes found in the nucleus of host cells,[30] and only a small subset of the 90 coding regions of the virus is expressed in the latency phase. The expression include six nuclear proteins (EBNA-1, -2, -3A, -3B, -3C and -LP) and three membrane proteins (LMP-1, -2A and -2B).[31,32] The replication of the virus is held in check by specific EBV cytotoxic T-cells, and individuals

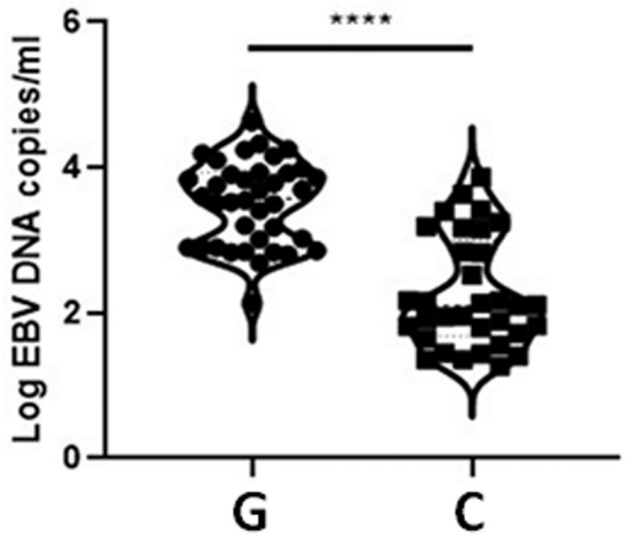

Figure 4. Comparison of EBV DNA load (Log EBV DNA copies/ml) in the gastric cancer and non-gastric cancer biopsies.
G: gastric cancer and C: control biopsies. ***$P < 0.0001$.

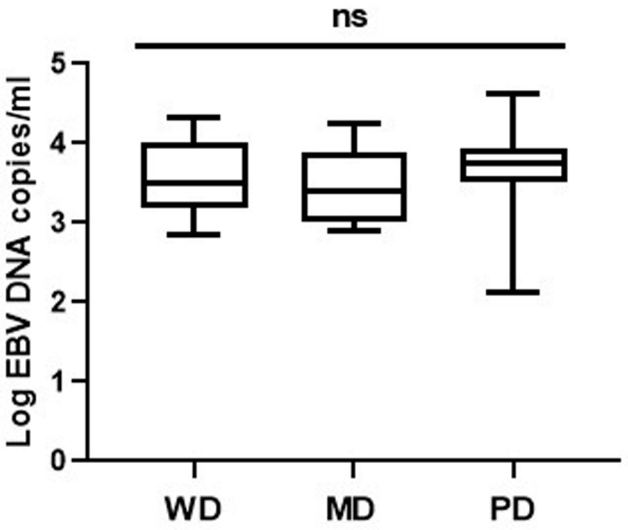

Figure 6. Comparison of EBV DNA load (Log EBV DNA copies/ml) in graded gastric adenocarcinoma.
WD: well differentiated; MD: moderately differentiated; PD: poorly differentiated; ns: no significant difference.

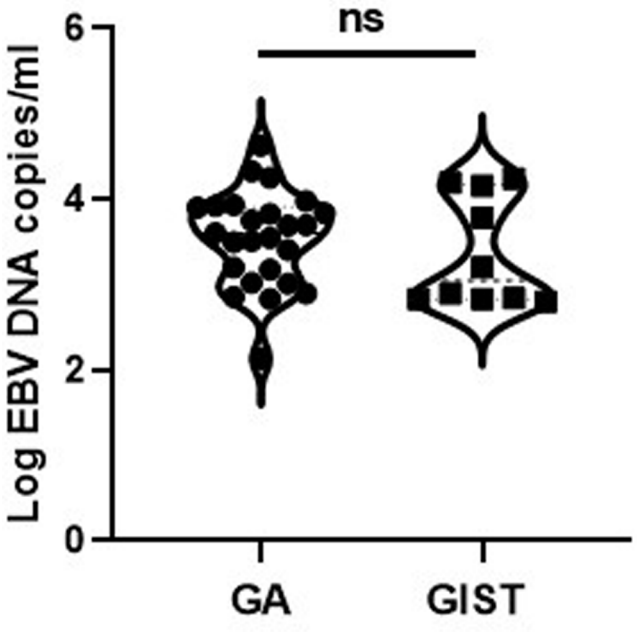

Figure 5. Comparison of EBV DNA load (Log EBV DNA copies/ml) in gastric adenocarcinoma and gastrointestinal stromal tumor.
GA: gastric adenocarcinoma; GIST: gastrointestinal stromal tumor and ns: no significant difference.

are usually without symptoms of EBV-associated diseases. However, the onset of the disease occurs primarily due to failure to properly regulate latency or lytic infection, usually in immunocompromised individuals.[33]

In this study, EBV in the GC biopsies and normal biopsies were detected by *EBNA-1* PCR amplification and highest frequency of the infection was found in the GC biopsies compared to the normal tissues. The infection prevalence agreed with a similar study done in Portugal, where EBV prevalence in gastric tumor was higher than normal tissues surrounding the tumor.[34] This study showed equal chance for both males and females in having EBV infection, and this contradicts a similar study where it was reported that females were twice at risk of having EBV infection than their male counterparts.[18]

The two genotypes of EBV reported have been the subject of study in recent years,[18,29] and the differences in the genotypes are due to variations in the EBNA-2 gene.[35] Studies have indicated high prevalence of EBV genotype-1 in greater number of tumors reported from Asia, Europe and North America, whereas EBV genotype-2 was associated with tumors found in Africa.[17,36,37] Our laboratory first reported EBV genotype-2 as the prevalent genotype among nasopharyngeal cancer patients in Ghana.[18] The current study reports EBV genotype-1 in gastric cancer biopsies obtained from Ghanaian patients. This contradicts the general assertion that the predominant EBV genotype in African population is EBV genotype-2. EBV genotype-1 is known to be more efficient in transforming epithelial cells into malignant tumors than EBV genotype-2.[6] Perhaps, that could explain the high prevalence of genotype-1 of the virus among EBV-infected GC patients.

Our previous report of EBV genotype-2 in nasopharyngeal cases compared to current finding in GC suggests that the EBV genotypes found in the study population is disease specific and not limited to geographic variants. The different strains of EBV are based on sequence variations within the C-terminus region of the *LMP-1* gene.[7] The *LMP-1* gene has been shown to be an essential EBV gene since its expression causes morphological changes in B-cells and epithelial cells.[31] Within the 3′of the *LMP-1* gene, the frequently observed variations are the 15 bp deletion, 30 bp deletion, 69 bp deletion, and 33 bp repeat sequence.[38,39] We report for the first time the presence of Mediterranean + EBV strain in Ghanaian gastric cancer patients. The strain is characterized by 30 bp deletion in the *LMP-1* gene. This data are consistent with several studies that have reported on 30 bp deletion within the *LMP-1* gene and have been reported as the most widely observed variation globally.[40–42] The 30 bp deletion arises as a result of wrong paring of two 9-nucleotide repeats that

codes two glycine triplets (GGCGGCGGT).[7] Several lines of evidence have shown that EBVaGC has a strong correlation with the 30 bp deleted variant.[43,44] However, in the current study, both EBV infected GC biopsies and normal biopsies had the 30 bp deleted variants, suggesting that the deletion in the Ghanaian population may not be associated with the disease phenotype but may be geographic or race specific.

Although all the sequences were Mediterranean + EBV, several subgroups within this strain were observed, and this was mainly due to nucleotide changes within the 3′ end of the different sequences compared to the reference Mediterranean + strain of the virus. These changes in the order of nucleotides may have an effect on *LMP-1* protein folding and its function; a reason for possible difference in virulence among the cases and controls. Comparison of EBV burden among GC samples and normal samples showed a significantly high viral load among the cases than the controls, and the trend of viral burden supports the findings of Ryan *et al*.[45] It has been shown that in EBV-associated malignancies, the virus actively replicates in tumor cells, maintaining significantly higher levels of viral DNA in circulation.[46,47] EBV after primary infection establishes latency in both B-cells and epithelial cells,[48] suggesting that even in asymptomatic individuals, there may be quantifiable levels of EBV DNA. This may explain why in the control subjects, there were appreciably high levels of EBV DNA. Interestingly, there was no significant difference in the mean viral load between gastric adenocarcinoma and GIST, suggesting that EBV replication rate is independent of stomach cell types. Also, different grades of GC showed no significant difference in the mean viral loads, for which there is no clear explanation.

Some limitations of this study were the small sample size and the single study site. Larger sample size and cross-sectional multicenter study approach is required to establish the prevailing circulating strains in the population and associate the changes in nucleotide sequences of the *LMP-1* gene with disease phenotypes.

In summary, the 30 bp Mediterranean + EBV genotype-1 variant was found to be predominant in patients with gastric cancer among the study population. However, the relationship of this genotype with the host remains to be properly characterized. Therefore, further studies need to be conducted to characterize hosts relationship's susceptibility to EBV genotype-1 to enhance our understanding of EBVaGC.

AUTHORS' CONTRIBUTIONS

E.A.T. and O.Q. conceived the idea, J.A.A. and E.A.T conducted experiment(s), E.A.T., A.D.A. B.P. and O.Q. supervised the work. E.A.T., J.A.A. and O.Q analyzed the data, J.A.A, prepared first draft of manuscript, E.A.T., A.D.A., B.P. and O.Q. edited the manuscript. All authors reviewed the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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DATA AVAILABILITY

The nucleotide sequences of the *LMP-1* gene have been deposited in the GenBank of the NCBI with accessions numbers OP209190 to OP209226.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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