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Integrative Identification of Epstein–Barr Virus–Associated Mutations and Epigenetic Alterations in Gastric Cancer

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BACKGROUND & AIMS: The mechanisms by which Epstein–Barr virus (EBV) contributes to the development of gastric cancer are unclear. We investigated EBV-associated genomic and epigenomic variations in gastric cancer cells and tumors. **METHODS:** We performed whole-genome, transcriptome, and epigenome sequence analyses of a gastric adenocarcinoma cell line (AGS cells), before and after EBV infection. We then looked for alterations in gastric tumor samples, with (n = 34) or without (n = 100) EBV infection, collected from patients at the Prince of Wales Hospital, Chinese University of Hong Kong (from 1998 through 2004), or the First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China (from 1999 through 2006). **RESULTS:** Transcriptome analysis showed that infected cells expressed 9 EBV genes previously detected in EBV-associated gastric tumors and 71 EBV genes not previously reported in gastric tumors. Ten viral genes that had not been reported previously in gastric cancer but were expressed most highly in EBV-infected cells also were expressed in primary EBV-positive gastric tumors. Whole-genome sequence analysis identified 45 EBV-associated nonsynonymous mutations. These mutations, in genes such as *AKT2*, *CCNA1*, *MAP3K4*, and *TGFBR1*, were associated significantly with EBV-positive gastric tumors, compared with EBV-negative tumors. An activating mutation in *AKT2* was associated with reduced survival times of patients with EBV-positive gastric cancer ($P = .006$); this mutation was found to dysregulate mitogen-activated protein kinase signaling. Integrated epigenome and transcriptome analyses identified 216 genes transcriptionally down-regulated by EBV-associated hypermethylation; methylation of *ACSS1*, *FAM3B*, *IHH*, and *TRABD* increased significantly in EBV-positive tumors. Overexpression of Indian hedgehog (*IHH*) and TraB domain containing (*TRABD*) increased proliferation and colony formation of gastric cancer cells, whereas knockdown of these genes reduced these activities. We found 5 signaling pathways (axon guidance, focal adhesion formation, interactions among cytokines and receptors, mitogen-activated protein kinase signaling, and actin cytoskeleton regulation) to be affected commonly by EBV-associated genomic and epigenomic alterations. **CONCLUSIONS:** By using genomic, transcriptome, and epigenomic comparisons of EBV infected vs noninfected gastric cancer cells and tumor samples, we identified alterations in genes, gene expression, and methylation that affect different signaling networks. These might be involved in EBV-associated gastric carcinogenesis.

Keywords: Genome Sequencing; Transcriptome; Methylation; AKT2.

Epstein–Barr virus (EBV) is a human herpes virus that infects more than 90% of the world population before adolescence. This oncogenic virus has been identified in epithelial malignancies including gastric cancer.[1] EBV-associated gastric cancer accounts for 8%–10% of all gastric cancer cases and is estimated to occur in more than 90,000 patients annually.[2] EBV-associated (EBV(+)) gastric cancer represents a distinct subtype of gastric cancer, with unique clinicopathologic features as compared with EBV-negative (EBV(-)) gastric cancer. However, the molecular genetic changes that account for the malignant behavior of EBV-associated gastric cancer remain largely unclear.

Clonal EBV is present in nearly all neoplastic cells and thus suggests a causal role in gastric carcinogenesis. In healthy individuals, EBV infection of gastric epithelial cells is a rare event. Even if EBV infects gastric epithelial cells, EBV usually is cytotoxic and induces cell death. However, once triggered, EBV infection will evolve into a persistent latent infection, which initiates progression into gastric cancer. Previous studies on EBV-associated gastric cancer by us[3]

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Abbreviations used in this paper: **AGS-hygro, AGS cells with hygro vector producing hygromycin-resistance; AP-1, activator protein-1; 5-Aza, 5-Aza-2'deoxycytidine; CCNA1, cyclin A1; DNMT, DNA methyltransferase; ERK, extracellular signal–regulated kinase; EBV, Epstein–Barr virus; EBV(-), Epstein–Barr virus negative; EBV(+), Epstein–Barr virus positive; LMP2A, latent membrane protein 2A; MAPK, mitogen-activated protein kinase; p-AKT, phosphorylated AKT; qPCR, quantitative polymerase chain reaction; RPKM, reads per kilobase per million; RT, reverse transcription; SNV, single-nucleotide variant; indel, small insertion and deletion; SV, structural variation; TGFBR1, transforming growth factor β receptor 1.**

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and others[4] have focused mostly on aberrant host gene methylation, which is a consequence of increased activity of DNA methyltransferases caused by EBV gene expression such as latent membrane protein 2A (LMP2A). Other studies also have investigated host genetic abnormalities including gene mutation,[5] microsatellite instability,[6] and cytogenetics[7] in EBV-associated gastric cancer. These findings collectively infer that EBV infection affects host cells at both epigenomic and genomic levels during gastric carcinogenesis.

However, systematic and integrative analyses concerning the impact of EBV on host cell alterations have not been performed to date. The AGS–EBV cell model with stable EBV infection has been applied successfully to study the effects of EBV infection in gastric cancer by us[3,8] and others.[9,10] Successful identification of EBV-associated methylated genes in gastric cancer using the AGS–EBV cell model highlights the feasibility of studying EBV-associated aberrations in gastric cancer using this cell model. The purpose of this study was to systematically elucidate the molecular genetic characteristics of EBV-associated gastric cancer by cataloguing the genomic and epigenomic alterations detected by whole-genome sequencing, transcriptome sequencing, and epigenome analysis in AGS–EBV cells as compared with the parental EBV-negative AGS cells, with an emphasis on identifying EBV-associated genomic/epigenomic events and aberrant molecular pathways. The identified important molecular abnormalities were verified further in primary EBV(+) gastric cancers.

Materials and Methods

AGS–EBV Cell Model

The AGS–EBV cell model stably infected with a recombinant EBV strain (added with a hygromycin-resistance gene for selective maintenance of EBV-positive cells during culture) was a gift from Dr Shannon C. Kenney (University of Wisconsin School of Medicine and Public Health).[3] The uninfected AGS cells, and AGS cells stably transfected with the empty pRI-GFP/Hygro vector producing hygromycin-resistance (AGS-hygro), were used as controls in this study.

Human Gastric Samples

Gastric cancer samples were collected in the Prince of Wales Hospital, The Chinese University of Hong Kong from 1998 to 2004, and the First Affiliated Hospital of Sun Yat-sen University in Guangzhou from 1999 to 2006. The presence of EBV was determined by in situ hybridization analysis of EBV-encoded small RNA, and quantitative polymerase chain reaction (qPCR) examination of *BamH1* W and *EBNA1* regions at the DNA level as described previously.[1] Gastric cancer samples with positive results for both in situ hybridization and qPCR examination were considered EBV-positive (n = 34), whereas those with negative results for both were considered EBV-negative (n = 100). Informed consent was provided by all participants, and this study was approved by both the ethics committee of the Chinese University of Hong Kong and the Clinical Research Ethics Committee of Sun Yat-sen University.

Other details and additional experimental procedures are provided in the Supplementary Materials and Methods.

Results

EBV Copies and Viral Gene Expression in AGS–EBV Shown by Whole Genome and Transcriptome Sequencing

Whole-genome sequencing reads were mapped to both the human reference genome (UCSC hg19) and the EBV reference genome (NC_007605). Whole-genome sequencing of the AGS–EBV and AGS cells showed a sequencing depth of 59-fold in AGS–EBV, and 42-fold in AGS for the human genome. A total of 91.59% and 91.57% of the whole genome region in AGS–EBV and AGS, respectively, were covered with more than 10 reads. Moreover, an 897-fold sequencing depth covering 91.38% of the whole EBV genome was obtained in AGS–EBV cells only (Supplementary Figure 1*A*). Therefore, approximately 15 EBV episomes in 1 AGS–EBV cell could be inferred (897-fold EBV/59-fold human = 15.2), consistent with the findings by others.[11]

In an attempt to uncover the EBV gene expression status in gastric cancer cells, 154.09 Mb reads of the AGS–EBV transcriptome were mapped to the EBV genome, with sequencing reads distributed across the entire EBV genome (Figure 1*A*). Visualization of transcriptome sequencing coverage across the EBV genome showed an EBV transcription profile in AGS–EBV cells with active regions similar to those identified in type I latency Burkitt's lymphoma cells (Supplementary Figure 1*B*).[12] Robust viral gene expression was yielded in AGS–EBV cells, with a median expression level of all genes being 255.4 reads per kilobase per million (RPKM) (Figure 1*B*). Transcriptome analysis of AGS–EBV identified the expression of 9 EBV genes (*BARF0*, *BARF1*, *BcLF1*, *BHRF1*, *BLLF1*, *BRLF1*, *BZLF1*, *EBNA1*, and *LMP2A*) previously detected in EBV(+) gastric tumors, and 71 EBV genes not reported previously in gastric cancer. The expression levels of these 71 genes are higher than that of *LMP2A* (27.0 RPKM), which could be well validated by reverse-transcription (RT)-PCR (Figure 1*B* and Supplementary Tables 1 and 2).

EBV Gene Expression Identified in AGS–EBV Is Verified in EBV(+) Gastric Cancer Cell Lines and Primary Gastric Cancer Tissues

The top 11 EBV genes (*BNLF2a*, *BNLF2b*, *BHRF1*, *BFRF1*, *BFRF2*, *BFRF3*, *BKRF4*, *BMRF2*, *BKRF3*, *BMRF1*, and *BFRF1A*) were verified in AGS–EBV and 2 other EBV(+) gastric cancer cell lines with natural EBV infection (SNU719 and YCCEL1) by RT-PCR. The expression of all 11 genes was detected in the 3 EBV(+) gastric cancer cell lines, but not in EBV(-) AGS cells (Figure 1*B*). Notably, *BHRF1*, a viral oncogene detected in EBV(+) gastric cancer,[13,14] was the third most highly transcribed EBV gene in AGS–EBV (5103.9 RPKM). The other 10 genes have not been examined previously in primary gastric cancer, including the DNA replication or repair enzyme *BKRF3*, capsid or tegument coding genes (*BFRF1*, *BFRF3*, and *BKRF4*), the gene facilitating viral attachment to cells (*BMRF2*), 2 uncharacterized EBV genes (*BFRF2* and *BFRF1A*), and 3 lytic genes (*BNLF2a*, *BNLF2b*, and *BMRF1*). We performed immunofluorescence