Research in Molecular Medicine

In Silico Analysis of Dysregulated Genes and Drug Resistance in Epstein-Barr Virus Associated Gastric Cancer

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Citation Sedaghat Anbouhi A, Sazegar H, Rahimi E. In Silico Analysis of Dysregulated Genes and Drug Resistance in Epstein-Barr Virus Associated Gastric Cancer. Research in Molecular Medicine. 2023; 11(3):203-212. https://doi.org/10.32598/rmm.11.3.1346.1

https://doi.org/10.32598/rmm.11.3.1346.1

Article Type:
Research Paper

Article info:
Received: 08 Jun 2024
Revised: 30 Jun 2024
Accepted: 15 Jul 2024

Keywords:
Gastric cancer (GC), Epstein-Barr virus (EBV) associated gastric cancer (EBVaGC), Biomarker, Drug resistance

ABSTRACT

Background: Gastric cancer (GC) poses a significant health challenge worldwide. Recognizing its complex and diverse nature, the cancer genome atlas (TCGA) research network has identified four distinct subtypes of GC. Among these subtypes, Epstein-Barr virus (EBV) associated GC accounts for around 9% of all GC cases. The primary aim of this study was to identify dysregulated genes in EBV-positive samples in contrast to EBV-negative samples, with the secondary goal of assessing their potential utility as diagnostic biomarkers. In addition, the study also aimed to evaluate the correlation between the expression levels of these candidate genes and drug resistance and sensitivity.

Materials and Methods: Differential gene expression analysis was employed to compare gene expression patterns between the EBV-positive and EBV-negative groups within the TCGA-stomach adenocarcinoma (TCGA-STAD) cohort. Gene ontology (GO) analyses were performed to elucidate the biological roles of the candidate genes. GSE13861 was used to confirm the gene expression levels in GC samples compared to the normal samples.

Results: Our findings revealed that 128 genes exhibited up-regulation in EBV-positive samples compared to EBV-negative samples. *CCL1*, *CCL5*, *CXCL1*, *CXCL10*, *CXCL11*, *KLRK1*, and *TBX21* genes were notably enriched in the process of leukocyte migration, which emerged as the hub pathway with the highest degree of interactions among the identified terms. Our analysis indicated that most of these genes could be deemed potential diagnostic biomarkers, as their area under the curve values exceeded 0.9. Additionally, our results demonstrated a correlation between some of these genes and resistance to specific drugs, including panobinostat, L-685458, L-BW242, and sorafenib.

Conclusion: Our study identified several key genes closely linked to EBV status and demonstrated a strong association with drug resistance. These genes hold promise as molecular markers for predicting EBV-positive samples.

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Introduction

Gastric cancer (GC) ranks as the sixth most prevalent cancer globally and stands as the third leading cause of cancer-related fatalities [1]. GC can be classified into four molecular subtypes based on genomic and clinical characteristics: Chromosomal instability, genomic stability, microsatellite instability, and Epstein-Barr virus (EBV) associated GC associated GC (EBVaGC). Among these groups, EBVaGC represents approximately 9% of cases [1]

A defining characteristic of EBVaGC is its lymphoepithelioma-like carcinoma presentation. This type of carcinoma exhibits a diffuse-type histology with significant lymphoid infiltration. EBVaGC is identified by the EBV within neoplastic cells [2]. Infected individuals predominantly comprise males, exhibit a younger age profile, and generally have more favorable prognoses than EBV-negative GC patients [3, 4].

The rise of genomic and transcriptomic profiling technologies alongside the development of selective molecular targeted therapies has underscored the growing significance of biomarkers in the clinical management of cancer patients. Moreover, cancer drug resistance represents a major challenge in modern oncology. Identifying resistance mechanisms and associated biomarkers can catalyze new avenues of investigation in cancer therapy [5]. The aim of this study was threefold: To identify genes that undergo expression changes under the influence of EBV; to identify potent biomarker genes capable of distinguishing EBV-positive samples from EBV-negative samples; and finally, to examine the association between the expression levels of candidate genes and drug resistance using CCLE and dataset.

Materials and Methods

Data resources, preprocessing, and differential gene expression analysis

This study's discovery dataset, TCGA-stomach adenocarcinoma (TCGA-STAD), comprised 375 tumor samples and 32 normal samples. The RNA-seq data in count format was obtained using the "TCGAbiolinks" R package [6]. Count data were utilized for the differential gene expression analysis using the "DESeq2" R package. Genome annotation files were retrieved from Gencode [7] to convert Ensembl IDs to gene symbols and segregate protein-coding genes. EBV information, as outlined in a prior publication involving 263 GC patients, was acquired. Subsequently, we identified the intersection between 375 tumor samples and the 263 samples with EBV information, resulting in 228 tumor samples with precise EBV information. Next, 205 EBV-negative tumor samples and 21 EBV-positive tumor samples were selected for further analyses [8].

To distinguish differentially expressed genes (DEGs) between the EBV-positive and EBV-negative groups, we applied a cutoff of >0.5-|log2FC| >0.5 and adjusted P<0.05. Only genes meeting this filtering criterion were deemed as DEGs. We then visualized these DEGs using a volcano plot, employing the R package (ggplot2). Furthermore, DEG analysis was conducted using TCGA RNA sequencing data obtained from gastric tumor samples and normal tissue samples. This analysis aimed to identify genes exhibiting significant expression level differences between tumor samples and normal samples.

The raw data from the GSE13861 study, comprising 65 primary gastric adenocarcinoma samples and 19 surrounding normal fresh frozen tissues, was downloaded. Initial preprocessing steps on the data involved background light removal, data normalization using the RMA method, and data transformation into logarithmic mode with a base of 2. The resulting expression matrix derived from these preprocessing steps served as the basis for all subsequent analyses conducted in the study.

Functional enrichment analysis

Gene ontology (GO) functional enrichment analysis was conducted using the package (ClueGo). for up-regulated genes. ClueGO is a user-friendly Cytoscape plug-in designed to enhance the biological interpretation of large gene lists. ClueGO initially generates a binary gene-term matrix containing selected terms and their corresponding genes. Using this matrix, ClueGO calculates a term-term similarity matrix employing chance-corrected kappa statistics to assess the association strength among terms. Given the categorical nature of the term-term matrix, the kappa statistic emerged as the most suitable method for analysis. Ultimately, ClueGO constructs a network wherein terms are depicted as nodes linked based on a predefined kappa score threshold [9]. An adjusted P<0.05 was considered statistically significant.

Biomarker analysis

In this study, the diagnostic efficacy of the candidate gene was evaluated through receiver operating characteristic (ROC) analysis, which quantifies the area under the ROC curve. The expression levels of potential genes

were compared between the EBV-positive and EBV-negative groups. Additionally, through ROC analysis, the extent of gene expression overlap between the two groups was assessed.

Drug resistance and sensitivity analysis

The R package "PharmacoGx" was employed to leverage data from the Cancer Cell Line Encyclopedia (CCLE) and dataset [10, 11]. These datasets were utilized to investigate the association between the expression levels of candidate genes and drug resistance or sensitivity. Furthermore, the data were utilized to evaluate the correlation between the expression levels of potential genes and the half-maximal inhibitory concentration (IC_{50}) of various drugs.

Statistics

All preprocessing and data analysis were conducted using R programming (version 4.2.2). GraphPad software (version 9) was employed for graph drawing and visualization. The linear model method was utilized to compute differences in expression, and significance levels between groups were determined through multiple hypothesis testing. A false discovery rate threshold of <0.05 was considered for all analyses.

Results

Comparative gene expression analysis reveals distinct patterns associated with EBV infection phenotype in GC

We conducted a comparative analysis of gene expression to investigate the association between gene expression and EBV infection phenotype. Our study involved the examination of the TCGA-STAD cohort, encompassing 205 tumor samples with EBV-negative status and 21 tumor samples with EBV-positive status. Subsequent analyses were performed based on this categorization. A differential gene expression analysis was executed, comparing EBV-positive samples to EBV-negative samples. Our findings disclosed 1688 differentially expressed genes (DEGs), consisting of 128 up-regulated and 1560 down-regulated genes (Figure 1A).

Functional annotation

To explore the function of these up-regulated genes, we performed GO functional enrichment analysis using the ClueGO plugin in the Cytoscape app. The terms with the most interaction with other terms were selected as hub terms or pathways (Figure 1B). Our enrichment results revealed that our genes were enriched in various biological processes, including chemokine activity, T cell proliferation, cytokine activity, T cell activation, positive regulation of calcium ion transport, regulation of T cell activation, leukocyte migration, and several other terms

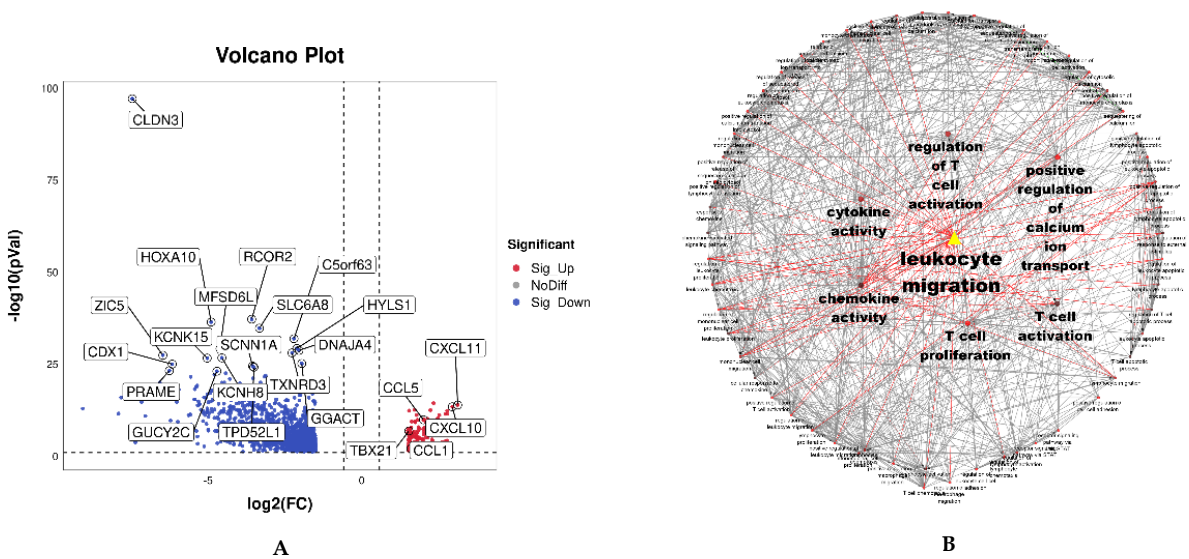

Figure 1. Differential gene expression analysis and identification of Hub pathway

A) A volcano plot illustrates dysregulated genes in EBV-positive samples compared to EBV-negative samples based on TCGA database analysis. The red dots denote up-regulated genes, while the blue dots indicate down-regulated genes, B) A plot displays pathway enrichment analysis using the GO dataset positions terms with high interactions in the center.

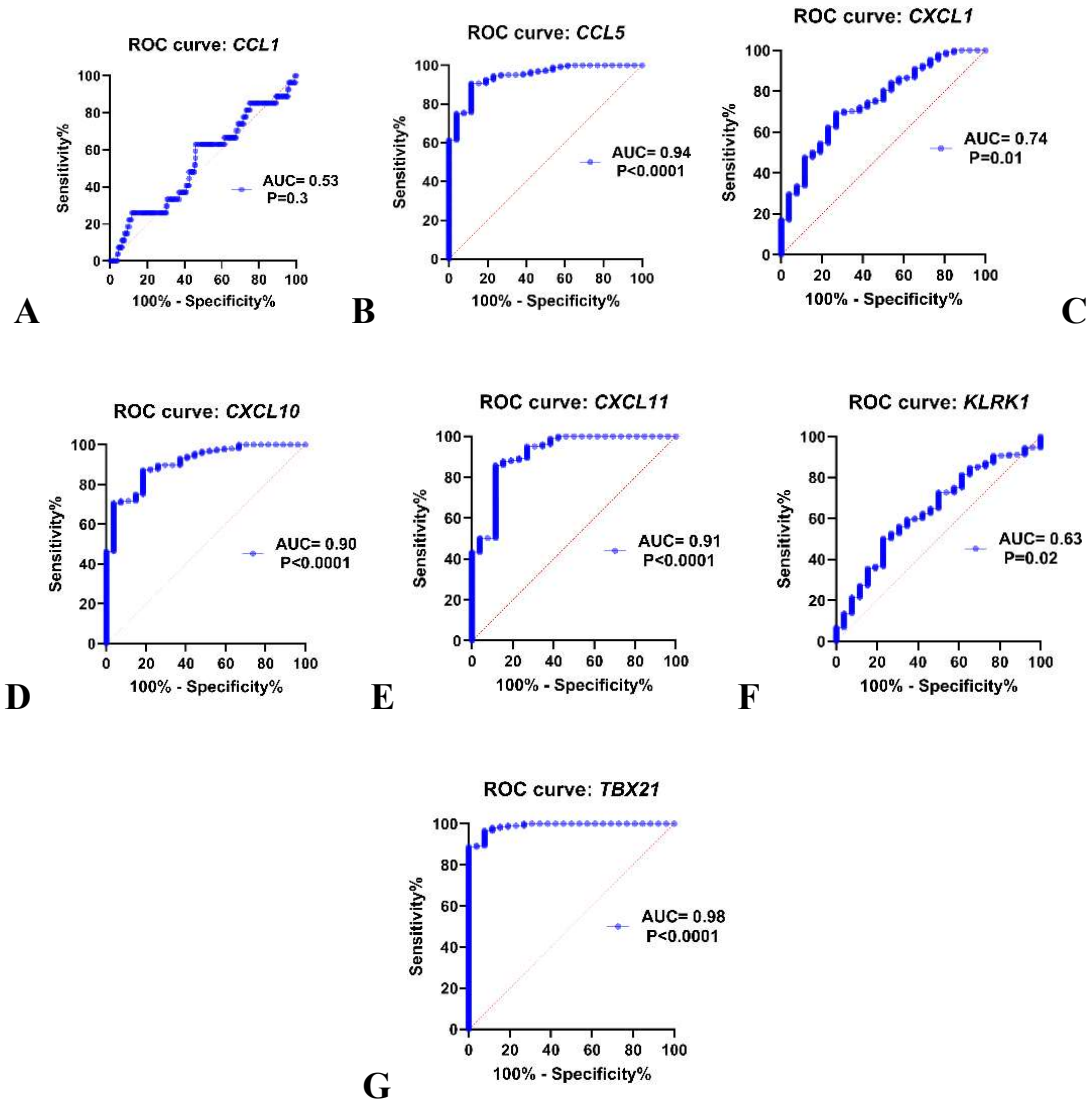

Figure 2. The potential candidate genes as diagnostic markers

Notes: A-G) ROC curves displaying the candidate genes in EBV-positive samples to EBV-negative samples are depicted utilizing TCGA data.

with a P<0.01. Among these GO terms, "leukocyte migration" was identified as the hub term with the highest degree of interaction. The relevant genes associated with this term are *CCL1*, *CCL5*, *CXCL1*, *CXCL10*, *CXCL11*, *KLRK1*, and *TBX21*. These results suggest that our enriched genes could play a vital role in GC with EBV-positive through interaction with other key terms.

Assessment of candidate gene potential as diagnostic markers for discriminating EBV-positive and EBV-negative samples in GC

We assessed the potential of our candidate genes to differentiate between EBV-positive and EBV-negative samples by estimating their expression levels using the ROC metric with the TCGA-STAD normalized gene expression matrix. Our findings indicated that *CCL1* (AUC=0.53, P=0.3), *CCL5* (AUC=0.94, P<0.0001), *CXCL1* (AUC=0.74, P=0.01), *CXCL10* (AUC=0.90, P<0.0001), *CXCL11* (AUC=0.91, P<0.0001), *KLRK1* (AUC=0.63, P=0.02), and *TBX21* (AUC=0.98, P<0.0001) genes hold promise as diagnostic markers in GC. Except for *CCL1*, *KLRK1*, and *CXCL1*, the remaining genes exhibited high efficiency in distinguishing EBV-positive samples from EBV-negative samples (Figure 2A-G). Furthermore, we reported the AUC values for the candidate genes using the normalized matrix

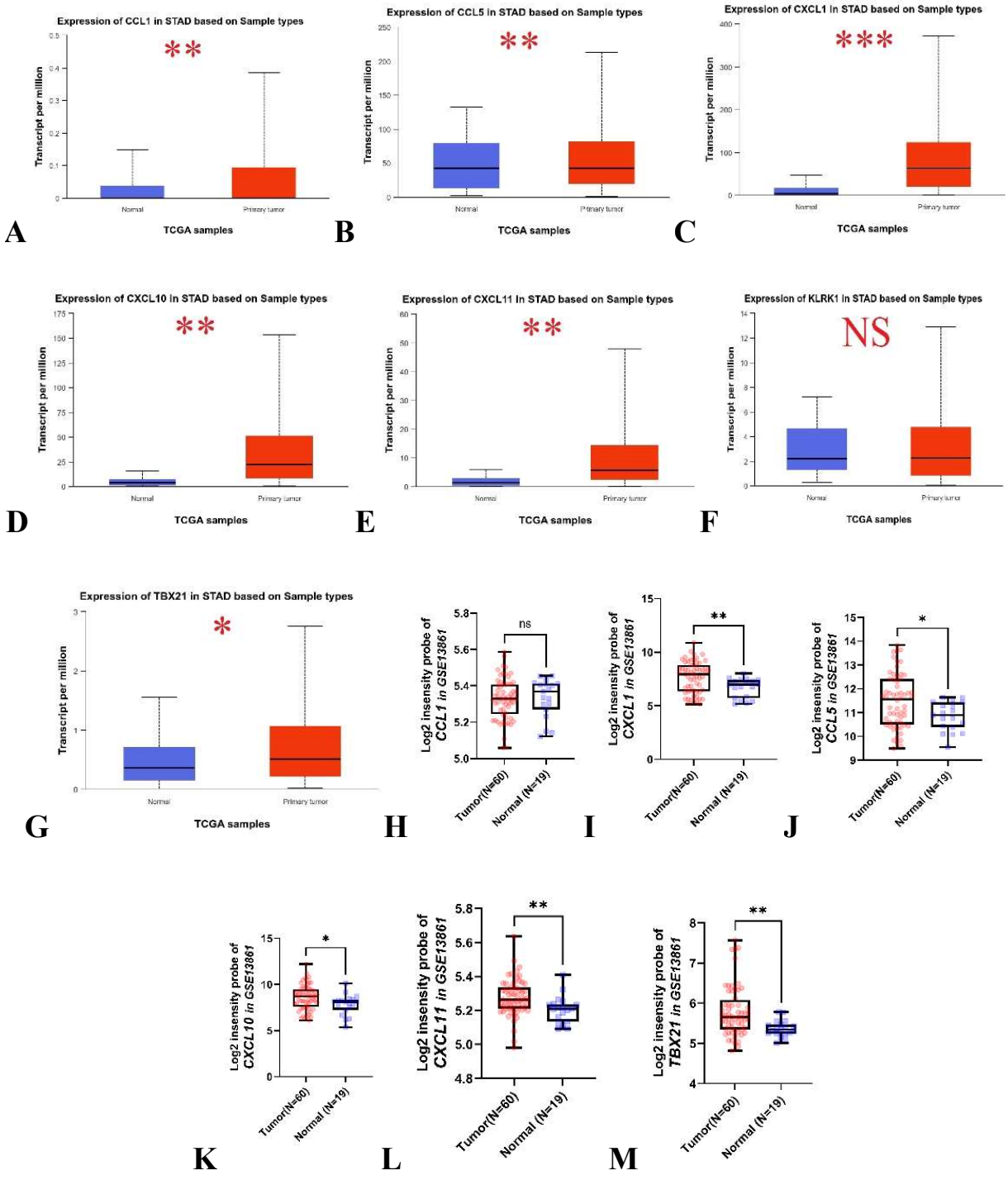

Figure 3. DEGs analysis GC compare to the normal samples

NS: Not significant.

Notes: A-G) Box plots represent the expression levels of candidate genes in tumor samples compared to normal samples. A significance cutoff of P<0.05 was performed. H-M) Confirming the analysis of DEGs in GC compared to normal samples using GSE13861 dataset.

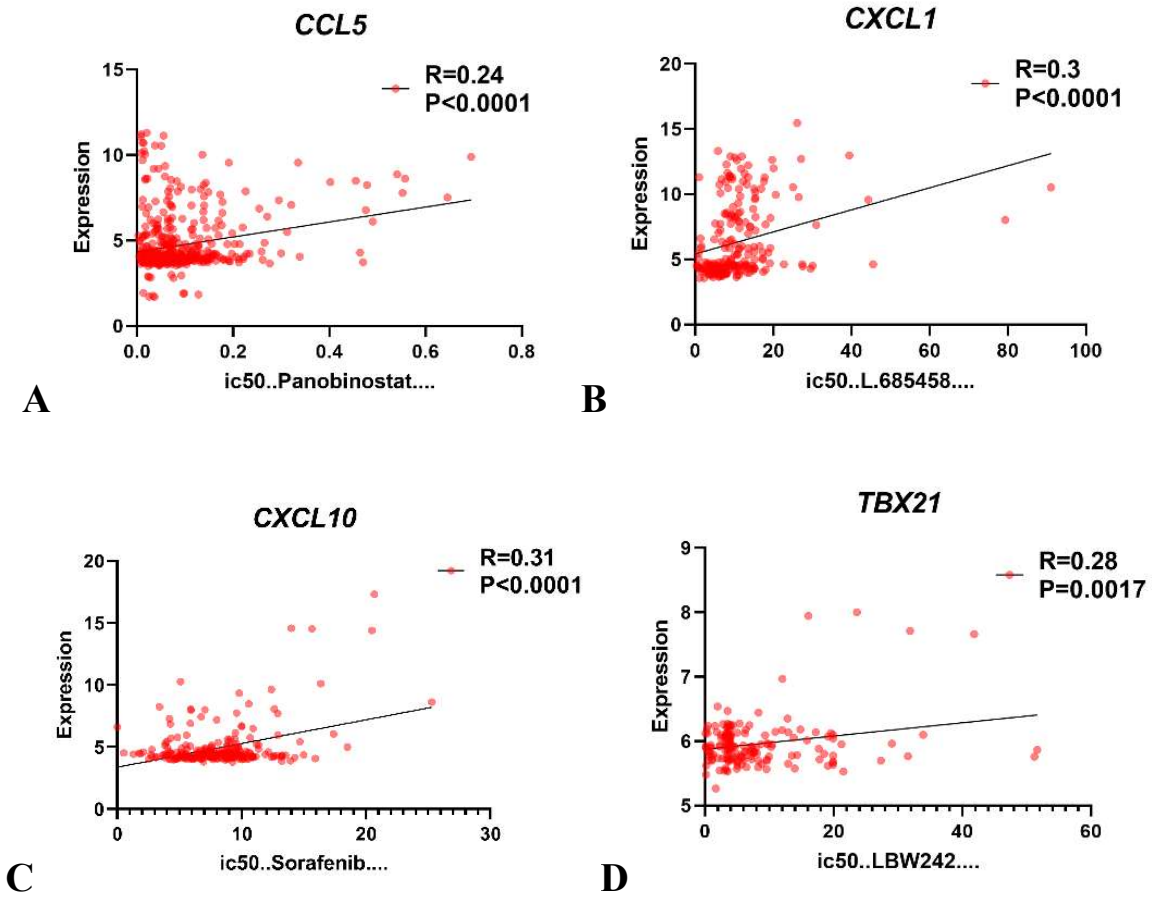

Figure 4. The association of candidate genes with drug resistance

Notes: A-D) The Pearson correlation analysis between IC_{50} values and expression levels of candidate genes. A significance cutoff of P<0.05 performed.

from the GSE13861 cohort, as shown in Supplementary Table 1. So, these results showed that these genes could be considered powerful diagnostic markers.

Gene expression analysis revealing upregulation of key candidates in gastric tumor samples: Validation using TCGA-STAD and GSE13861 Cohorts

The analysis of candidate gene expression levels in tumor samples compared to normal samples was conducted using TCGA-STAD RNA-Seq data. Our findings revealed that, except for KLRK1, the expression levels of the other genes were significantly up-regulated in tumor samples (Figure 3A-G). Moreover, the GSE13861 study was used as a second cohort for validation of the levels of candidate genes in gastric samples compared to the normal samples. Our results showed that the expression levels of *CCL5*, *CXCL1*, *CXCL10*, *CXCL11*, and TBX21 were significantly over-expressed in GC compared to the normal samples, while the levels of *CCL1* and *KLRK1* were not significantly changed (Figure 3H-M). These results suggest that most of these genes could be vital in GC progression.

Exploring correlations between candidate gene expression and drug resistance/sensitivity: Insights from CCLE databases and the pearson correlation analysis

We utilized data from the CCLE databases to establish connections between the expression levels of candidate genes and drug resistance or sensitivity. As outlined in the materials and methods section, we conducted the Pearson correlation test to validate our findings. Our analysis revealed significant correlations between *CCL5*, *CXCL1*, *CXCL10*, and *TBX21* expression levels and resistance to various drugs. Specifically, elevated levels of *CCL5* and

Supplementary table 1. AUC results using GSE13861

Gene Name	AUC	P-value
CCL1	68%	NS
CCL5	89%	<0.01
CXCL1	69%	<0.05
CXCL10	92%	<0.01
CXCL11	88%	<0.01
KLRK1	51%	NS
TBX21	92%	<0.01

NS: not significant

CXCL1 were significantly associated with resistance to panobinostat and L685458 (Figure 4A-B). Furthermore, high expression of *CXCL10* was notably linked to resistance to sorafenib (Figure 4D). Finally, increased *TBX21* expression was correlated with resistance to LBW242 (Figure 4E). These findings highlight the potential correlation between the expression of specific candidate genes and drug resistance or sensitivity. However, for a comprehensive understanding of the roles of these candidate genes, further investigations through in vitro and in vivo studies are imperative.

Discussion

GC ranks among the top causes of cancer-related deaths globally. However, unraveling its molecular and clinical characteristics has proven challenging due to its histological and etiological diversity. Most cases of GC are linked to infectious agents, such as the bacterium Helicobacter pylori and the EBV. The distribution of histological subtypes of GC and the prevalence of H. pylori and EBV-associated GC exhibit variation worldwide [12, 13]. Analysis of gene expression profiles in patients with EBV associated GC (EBVaGC) reveals notable alterations in immune response genes. These changes suggest the potential for enhanced recruitment of reactive immune cells, leading to improved survival outcomes among patients [14].

Chemokines, compact chemoattractant molecules secreted by cells, wield significant influence over immune and inflammatory responses and cell migration, proliferation, and survival. Their pivotal role extends across diverse biological and pathological processes, including cancer [15]. Researchers have identified over 50 distinct chemokines and approximately 20 corresponding chemokine receptors [16]. Chemokines and their corresponding receptors, known as chemokine receptors, can influence several crucial aspects of cancer progression [17, 18]. These include the degree of immunocyte infiltration and phenotype, angiogenesis, tumor cell growth, metastasis, and survival [19-21]. These factors collectively impact the prognosis of the patient [22].

In this study, we utilized TCGA RNA-Seq and clinical data to identify dysregulated genes in EBV-positive samples compared to EBV-negative samples. Our analysis revealed 1688 DEGs that were significantly dysregulated in the context of EBV infection. Using the ClueGO plugin in the Cytoscape app, we identified the leukocyte migration term as a hub term that exhibited interactions with other significantly enriched terms. Our investigation indicated up-regulation of the expression levels of *CCL1*, *CCL5*, *CXCL1*, *CXCL10*, *CXCL11*, and *TBX21* in EBV-positive samples compared to EBV-negative samples.

Among candidate genes, we focus on the genes associated with drug resistance and sensitivity. C–C chemokine ligand 5 (*CCL5*), also referred to as regulated upon activation, normal T cell expressed and secreted, binds to several G-protein–coupled receptors, including *CCR1*, *CCR3*, *CCR4*, *CCR5*, *GPR75*, and *CD44* [23]. A previous study showed that *CCL5* actively participates in the recruitment of various leukocytes to sites of inflammation. *CCL5* is produced by T lymphocytes, macrophages, platelets, synovial fibroblasts, tubular epithelial cells, and tumor cells [24]. Our results also showed that the expression levels of *CCL5* were up-regulated in GC samples compared to the normal samples. Moreover, Wenlong Ma et al. discovered that EBV amplifies the production of numerous angiogenesis-related proteins. Among these proteins, *CCL5* emerges as a novel molecular driver responsible for EBV-induced angiogenesis and tumor progression in nasopharyngeal

carcinoma [25]. Our results showed that the *CCL5* can be utilized as a biomarker in GC, especially for detecting EBV-positive samples compared to EBV-negative samples. Interestingly, a previous study indicated that *CCL5* may serve as a potential biomarker for the early diagnosis of colorectal cancers [26]. Moreover, our results showed that the high level of this gene was related to Panobinostat resistance. Interestingly, Silvia Waldeck et al. showed that *CCL5* could be considered a biomarker to predict drug resistance [27]. So, these findings showed that this gene could play a crucial role in GC and drug resistance biomarkers.

CXCL1, also known as C-X-C motif chemokine ligand 1, belongs to the CXC chemokine subfamily and serves as a ligand for *CXCR2*. Its primary role in the immune system involves attracting neutrophils through chemotaxis. Studies have demonstrated that *CXCL1* plays a significant role in numerous cancer-related processes. The expression levels of *CXCL1* were up-regulated in breast cancer compared to the normal sample. High *CXCL1* expression in breast tumors is positively correlated with lymph node metastasis [28, 29]. *CXCL1* may play a crucial role in the progression of cervical cancer, particularly in pre-cancerous cervical lesions. The expression of this chemokine, along with other *CXCR2* ligands such as *CXCL7*/neutrophil-activating protein 2 (NAP-2) and CXCL8/IL-8, is significantly increased in cervical intraepithelial neoplasia grades 1 (CIN1) and CIN2 [30]. Our results also showed that the high levels of *CXCL1* were up-regulated in gastric samples compared to the normal samples. A recent study showed that *CXCL1* plays a pivotal role in the GC microenvironment, potentially contributing significantly to cancer progression by fostering invasion and metastasis, which are predominant drivers of cancer advancement [31]. Our findings indicate that this gene could be a good diagnostic marker for distinguishing EBV-positive samples from EBV-negative samples. Furthermore, our results showed that high levels of *CXCL1* were associated with resistance to L685458.

CXCL10, also called interferon-(IFN-) γ-induced protein 10 (IP-10), is a member of the CXC chemokine subfamily. It features a single variable amino acid situated between two of the four highly conserved cysteine residues [32]. Recent studies have validated the stromal origin of *CXCL10* in human tumors, with its overexpression observed in human pancreatic cancer. This overexpression is correlated with poor survival among patients with pancreatic adenocarcinoma [33]. Our findings indicate significantly elevated levels of this gene in gastric samples compared to normal samples.

Interestingly, our results also suggest that *CXCL10* could be a diagnostic marker for distinguishing EBV-positive samples from EBV-negative samples. Xiao-Jing Qin et al. demonstrated that *CXCL10* might have a potential role in detecting human papillary thyroid cancer [34]. Furthermore, our findings revealed that the high levels of these genes were correlated with drug resistance. Interestingly, Xiuming Wu et al. found that *CXCL10* mediates breast cancer tamoxifen resistance and promotes estrogen dependence [35]. Therefore, our results suggest that this gene could play a vital role in the progression of cancer cells, possibly through drug resistance mechanisms.

TBX21 belongs to a phylogenetically conserved gene family, and the Tbx21 protein serves as a Th1 cell-specific transcription factor. It regulates the expression of the characteristic Th1 cytokine, interferon-gamma (IFN-γ) [36, 37]. Recently, an elevated occurrence of *TBX21* has been associated with cancer development [38]. A recent study showed that the expression of *TBX21* was notably correlated with the prognosis of skin cutaneous melanoma (SKCM) patients and was intricately involved in numerous immunological pathways influencing tumor occurrence and development [39]. Furthermore, research has demonstrated that *TBX21* could play a vital role in other diseases, such as Alzheimer disease [40]. Our findings revealed a significant increase in the expression levels of *TBX21* in gastric tumor samples.

Interestingly, *TBX21* emerges as a potent biomarker for distinguishing EBV-positive and EBV-negative samples. Our findings also indicate that elevated *TBX21* expression correlates with resistance to LBW242. All the results of this study provide a comprehensive understanding of the in silico analysis of candidate genes that play a main role in drug resistance in GC. However, some limitations associated with in silico studies should be acknowledged. Therefore, further studies are recommended to evaluate the findings via in vivo approaches.

Conclusion

A previous study indicated that most EBVaGC patients demonstrated resistance to current chemotherapy options. In this study, we investigated the genes that were differentially expressed in EBV-positive samples compared to EBV-negative samples. Our findings highlighted that these candidate genes could play a crucial role in drug resistance. Thus, our results suggest that considering these genes as therapeutic targets could impede GC progression and alleviate resistance to certain drugs.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

Funding

This research did not receive any grant from funding agencies in the public, commercial, or non-profit sectors.

Authors contribution's

Conceptualization, methodology, software, validation, investigation resources and writing: Tabassom Sedaghat Anbouhi; Supervision, and visualization: Hossein Sazegar; Formal analysis: Ebrahim Rahimi; Project administration: Ebrahim Rahimi; and Hossein Sazegar.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgements

The authors thank Genius Gene Center, Mashhad, Iran for assisting in laboratory work and data analysis

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