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*Original Article*

# Significant up-regulation of Toll-like receptor (TLR) signaling pathway in Epstein-Barr virus-associated gastric cancer

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**Abstract:** Objectives: Epstein-Barr virus (EBV) associated gastric cancer (EBVaGC) represents a distinct subtype of gastric carcinoma clinically characterized by low frequency of lymph node metastasis and better prognosis as compared to the EBV-negative gastric cancer (EBVnGC). Differential expression analysis of the transcriptome from tumor tissues revealed frequent involvement of immune genes which emphasizes the exclusive significance of immune response in EBVaGC patients. Considering the reported over-expression of toll-like receptor (TLR) genes in EBV infection and giving the crucial roles of TLRs in the innate immune system, we assumed that the entire TLR signaling pathway could have been differentially regulated in EBVaGC. Methods: We tested our hypothesis by performing a differential expression analysis of the whole TLR signaling pathway using gene set enrichment test. Results: A self-matched test detected a significant upregulation of the TLR signaling pathway in tumor as compared with non-tumor gastric tissues of EBVaGCs ($P = 4\times10^{-3}$) but no significant differential regulation of the pathway in EBVnGCs. A comparison of tumor gastric tissue in EBVaGCs versus non-tumor gastric tissue in EBVnGCs showed an even more significant upregulation of the pathway with a high enrichment of overexpressed genes ($P = 2.5\times10^{-4}$). Conclusions: Briefly, this study revealed a distinct pattern of activation of the TLR signaling pathway in EBVaGCs which can be seen as a specific feature of molecular pathology in the disease. This feature characterizes the disease as a distinct subtype of gastric cancer in oncogenesis which can be linked to its clinical manifestation and prognosis to motivate improved treatment strategies for both EBVaGC and EBVnGC patients.

**Keywords:** Epstein-Barr virus, gastric cancer, gene expression, Toll-like receptor signaling pathway, gene-set enrichment analysis

## Introduction

As a human lymphotropic herpesvirus, the Epstein-Barr virus (EBV) infects more than 90% of the adult population globally and is also the first virus identified by the World Health Organization (WHO) to cause cancer [1]. Epidemiological studies have shown a very high chronic infection rate of over 95% in the human population [2]. EBV infection has been found to affect the pathogenesis of multiple human diseases from asymptomatic to malignant forms. It is estimated that, among all gastric cancers globally, averagely 8.9% are EBV associated gastric cancer (EBVaGC) [3]. EBVaGC represents a distinct subtype of gastric carcinoma due to its oncogenesis and molecular features [4], which is clinically characterized by low frequency of lymph node metastasis and better prognosis as compared with the EBV-negative gastric cancer (EBVnGC).

Toll-like receptors (TLRs) play crucial roles in the innate immune system by recognizing pathogen-associated molecular patterns derived from various microbes. TLRs recognize EBV to initiate immune responses to the production of inflammatory cytokines and antiviral mediators. TLR7 and TLR9 are both expressed in intracellular vesicles of mainly B cells and TLR9 also in monocytes. TLR7 is able to recognize EBV single-stranded RNA (ssRNA), whereas

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TLR9 recognizes EBV unmethylated 2'-deoxyribo (cytidine-phosphate-guanosine). Both activities play important roles in innate defense against EBV. In a recent gene expression study, Liu et al. [5] reported increased expression of TLR7 and TLR9 in monocytes and B lymphocytes of patients with chronic active EBV infection. Higher levels of TLR-2 and TLR-9 were observed in advanced prostate cancer with EBV infection [6]. In another study, Farina et al. [7] found that EBV lytic infection promotes activation of TLR8 innate immune response in systemic sclerosis monocytes. The overactivation eventually results in excessive inflammation which contributes to the occurrence and development of tumors [8]. Kim et al. [9] performed a global gene expression profiling comparing tumor and non-tumor gastric tissues in EBVaGC and EBVnGC patients. The study identified fewer differentially expressed genes in EBVaGC than EBVnGC patients suggesting higher molecular homogeneity in EBVaGC patients. Most importantly, they found that the most changes in gene expression activity in EBVaGCs occur in immune response genes which again emphasizes the exclusive significance of immune response in EBVaGC patients. Considering the over-expression of TLR7 and TLR9 genes in chronic active EBV infection [5] and active immune response in EBVaGC patients [9], we hypothesize that the entire toll-like receptor (TLR) signaling pathway could have been differentially regulated in EBV associated gastric cancer. To test our hypothesis, this study performed an overall differential expression analysis of the whole TLR signaling pathway using gene set enrichment analysis by comparing tumor and non-tumor gastric tissues both in EBVaGC patients and between EBVaGC (tumor gastric tissue) and EBVnGC (non-tumor gastric tissue) patients. This paper reports the significant findings as well as the impact on results by different analytical strategies.

## Materials and methods

### *Patients and gene expression data*

This study makes use of the genome-wide gene expression data collected from tumor and non-tumor tissues on 14 EBVnGC and 12 EBVaGC patients in the differential gene expression study conducted by Kim et al. [9]. Detailed information about the study samples can be found in the Table S1 of the original article [9]. Gene expression activity was measured by mRNA levels using the Agilent SurePrint G3 Human Gene Expression Microarray covering 26083 genes in the human genome. The raw gene expression data were preprocessed and normalized using the quantile normalization method [10]. The normalized data were deposited as GSE51575 in Gene Expression Omnibus (GEO). From the normalized data, expression values of the 102 member genes of the TLR signaling pathway were extracted for pathway-based gene-set enrichment analysis. Annotation of the TLR signaling pathway was downloaded from the KEGG pathways in the Molecular Signatures Database (MSigDB) at https://www.gsea-msigdb.org/gsea/msigdb/index.jsp.

### *Gene-set enrichment analysis*

Gene-set enrichment analysis was performed using the Rotation Gene Set Tests (ROAST) [11] implemented in the *limma* package [12] of Bioconductor version 3.9 at https://bioconductor.org/packages/release/bioc/html/limma.html. ROAST is a self-contained and statistically rigorous gene set test that introduces residual space rotation for multivariate regression instead of permutation while allowing for gene-wise correlation. ROAST can be used with complex experimental design and with small numbers of replicates. The method has been evaluated as the best-performing approach in a comparative study [13]. The regression model for the design matrix regresses gene expression value on gastric tissues (tumor = 1, non-tumor = 0). ROAST is the only gene-set test that incorporates both up and down regulated patterns simultaneously [14]. The ROAST method estimates an active proportion as the percentage of genes with a log scaled fold change (logFC) of expression levels between the tumor and the non-tumor gastric tissues more than one standard error above zero.

Visualization of the enrichment pattern of the TLR signaling pathway is performed using the barcodeplot() function in the *limma* package. This function plots the positions of 102 TLR signaling pathway genes in a ranked list of the logFCs for all 26083 genes, with the 102 specified genes marked by vertical bars (forming a pat-

tern like a barcode). A worm worm showing the relative density (the enrichment score) of pathway genes in a span about each position is also plotted. The function draws a pale red rectangle for logFC>$\sqrt{2}$ and a pale blue rectangle for logFC<-$\sqrt{2}$.

*Analytical schemes*

The availability of expression data for the 102 genes of the TLR signaling pathway from both tumor and non-tumor tissues of EBVaGC and EBVnGC patients respectively allowed us to perform five pathway-based enrichment tests to test if the entire TLR signaling pathway is differentially regulated: (1) between tumor and non-tumor gastric tissues in EBVaGCs; (2) between tumor and non-tumor gastric tissues in EBVnGCs; (3) in tumor gastric tissue between EBVaGC and EBVnGC patients; (4) in non-tumor gastric tissue between EBVaGC and EBVnGC patients; (5) between tumor gastric tissue of EBVaGC patients and non-tumor gastric tissues in EBVnGC patients. The statistical model for schemes (1) and (2) tested the differential expression between tumor and non-tumor tissues while controlling for individual differences in gene expression with the self-matched design. The statistical model for schemes (3) to (5) tested the differential expression between EBVaGC and EBVnGC patients in gastric tumor (scheme 3), in gastric non-tumor tissue (scheme 4), or in gastric tumor tissue of EBVaGC patients and gastric non-tumor tissue of EBVnGC patients (scheme 5). For each scheme, we tested the statistical significance of the entire TLR signaling pathway using ROAST as well as of each of the 102 member gene of the pathway using an empirical Bayes method implemented in *limma* [12, 15].

All statistical data analysis and graphics were done using the free R software (https://www.r-project.org/).

## Results

*Up-regulated TLR signaling pathway in tumor tissue of EBVaGCs*

We first focused on the differential expression analysis of tumor versus non-tumor gastric tissues in EBVaGC patients using scheme 1 as described Methods. **Figure 1A** displays a volcano plot plotting the negative logarithm of the *p* value for each of probes of the 102 pathway genes (Table S1) on the y axis (with base 10) against the logarithm of gene expression fold change (with base 2) between tumor and non-tumor tissues. The figure reveals a clear pattern of up-regulation (red colored) of gene expression in terms of both statistical significance and effect size. As a result, gene-set enrichment analysis detected a highly significant up-regulation pattern for the pathway with $P = 4\times10^{-3}$ (active proportion 35.27%) and a non-significant pattern of down-regulation with P = 0.996 (active proportion 13.69%). When both patterns combined (active proportion 48.96%), the whole pathway is significantly regulated with $P = 5\times10^{-5}$ (**Table 1**). **Figure 1A** also shows gene-specific expression patterns for TLR7 (square), TLR8 (triangle) and TLR9 (diamond) with TLR8 clearly over-expressed in the gastric tumor tissue.

*No up-regulated TLR signaling pathway in tumor tissue of EBVnGCs*

We next performed a similar differential expression analysis of tumor versus non-tumor gastric tissues but in EBVnGC patients using the analytical scheme 2. This time, the predominant trend of up-regulation as observed in EBVaGC patients disappears (**Figure 1B**). Instead, the volcano plot displays no specific pattern of up or down regulation although some top significant genes are down-regulated (Table S2). Likewise, gene-set enrichment test for the TLR signaling pathway revealed no specifically up or down regulation but an overall significant activation of the whole pathway ($P = 5\times10^{-5}$). Note that, in **Figure 1B**, TLR7, TLR8 and TLR9 are all down-regulated although with low statistical significances. In **Figure 2**, the logarithm of gene expression fold change (with base 2) between tumor and non-tumor tissues in EBVaGC patients (scheme 1) is plotted against that in EBVnGC patients (scheme 2). Again, the figure reveals a clear pattern of overall up-regulation of the TLR signaling pathway in EBVaGC as compared with EBVnGC patients.

*Up-regulated TLR signaling pathway in both tumor and non-tumor gastric tissues of EBVaGCs*

The availability of both tumor and normal gastric tissues from EBVaGC and EBVnGC patients

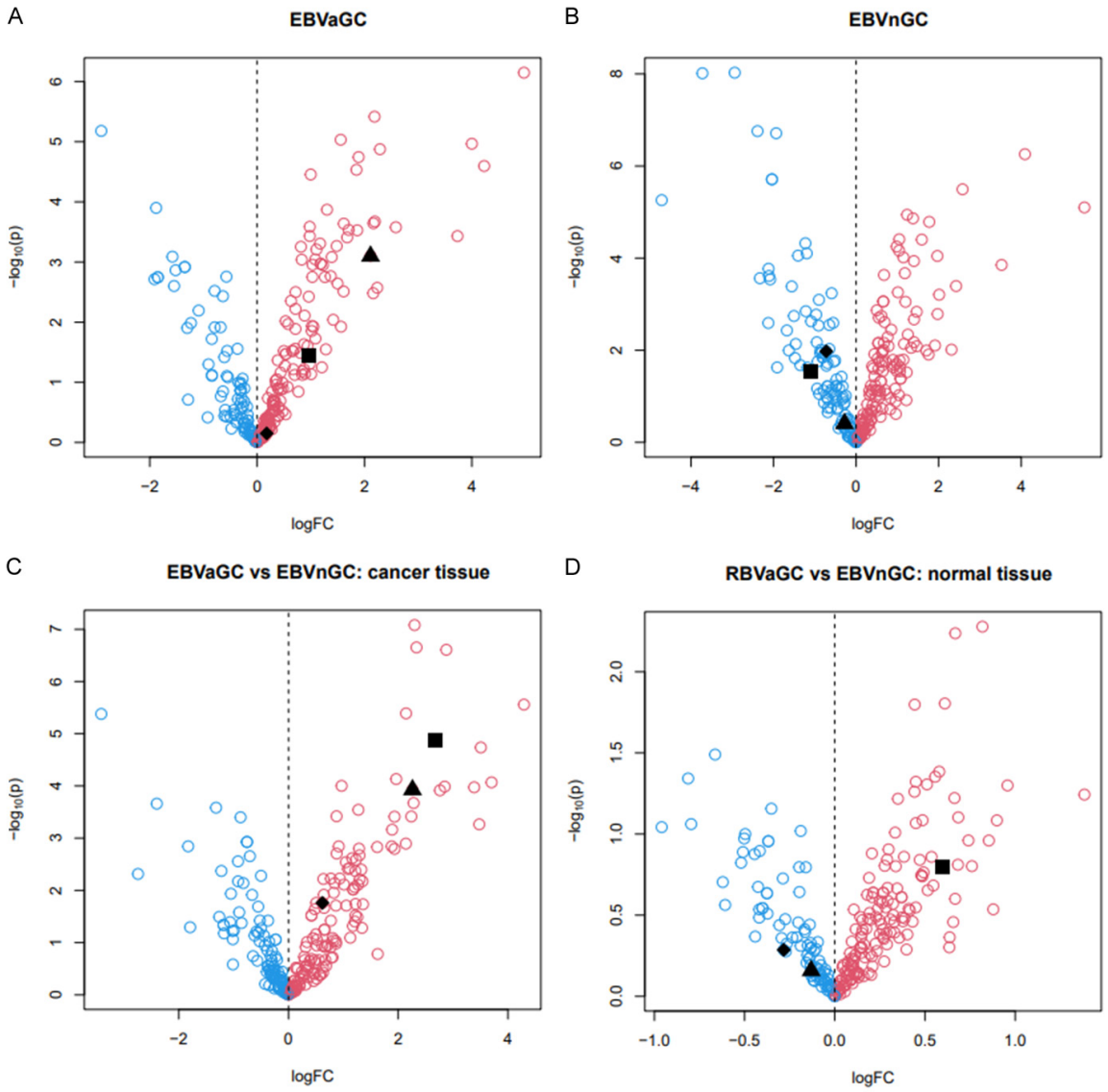


**Figure 1.** Volcano plots plotting the negative log *p* value (base 10) of genes against their fold changes in log scale (base 2, logFC), by comparing the gene expression levels in tumor versus non-tumor gastric tissues of EBVaGCs (A); in tumor versus non-tumor gastric tissues of EBVnGCs (B); in tumor gastric tissues of EBVaGCs versus EBVnGCs (C); in non-tumor gastric tissues of EBVaGCs versus EBVnGCs (D). The red dots represent upregulated genes (lgFC>0), the blue dots represents downregulated genes (lgFC<0).

allowed us to conduct additional analyses to compare the differential regulation of the TLR signaling pathway (1) between the gastric tumor tissues of EBVaGC and EBVnGC patients as described in scheme 3 and (2) between the normal gastric tissues of EBVaGC and EBVnGC patients using scheme 4. Interestingly, our results on single genes of the pathway indicate obvious over-expressed gene activity in both tumor and non-tumor gastric tissues of EBVaGC patients as compared with EBVnGC patients (**Figure 1C** and **1D**) (Tables S3, S4). Note that, TLR7, TLR8 and TLR9 are among the up-regulated genes in the analysis of tumor tissue (**Figure 1C**). However, only TLR7 is activated in the normal gastric tissue of EBVaGC patients with TLR8 and TLR9 genes tended to be suppressed (**Figure 1D**). Gene set enrichment analysis proved the significant up-regulation of the pathway with P = 0.021 in the tumor tissue and P = 0.045 in the non-tumor tissue (**Table 1**).

**Table 1.** Statistics of enrichment tests by ROAST

| | Down-regulated | Up-regulated | Mixed |
|---|---|---|---|
| EBVaGC matched | | | |
| Active proportion (%) | 13.693 | 35.27 | 48.963 |
| *P* value | 0.996 | 0.004 | 0.0005 |
| EBVnGC matched | | | |
| Active proportion (%) | 28.216 | 37.759 | 65.975 |
| *P* value | 0.854 | 0.146 | 0.0005 |
| EBVaGC/EBVnGC: Cancer tissue | | | |
| Active proportion (%) | 16.183 | 32.780 | 48.963 |
| *P* value | 0.979 | 0.021 | 0.0005 |
| EBVaGC/EBVnGC: Normal tissue | | | |
| Active proportion (%) | 5.394 | 11.618 | 17.012 |
| *P* value | 0.955 | 0.045 | 0.342 |
| EBVaGC unmatched | | | |
| Active proportion (%) | 14.938 | 42.323 | 57.261 |
| *P* value | 1.000 | 0.00025 | 0.0005 |

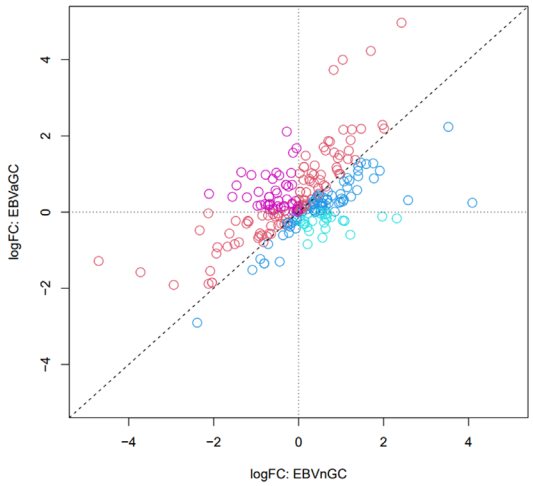


**Figure 2.** Comparison of gene expression fold change (logFC) estimated by self-matched analysis on EBVaGC (Y axis) versus that on EBVnGC (X axis) patients. The warm colored dots are genes with logFC (EBVaGC)>logFC (EBVnGC); The cold colored dots are genes with logFC (EBVaGC)<logFC (EBVnGC).

*Improving the power of association testing*

The significant up-regulation of the TLR signaling pathway in the normal gastric tissue of EBVaGC suggests the self-matched design that compares gene expression in tumor versus non-tumor gastric tissues of same patient could impede the power in statistical testing because the baseline or reference, i.e. gene expression in the normal gastric tissue of EBVaGC patients, are in fact already over-expressed in comparison with that in EBV uninfected normal gastric tissue. Instead, as described in scheme 5, using gene expression in non-tumor gastric tissue of EBVnGC patients as baseline for comparison should help with improving the power of analysis. Indeed, the new strategy of analysis led to improved power in statistical testing (Table S5), which can be seen in the volcano plot for member genes of TLR signaling pathway both in the estimated effect size (logFC) and in statistical significance (**Figure 3A**), and in the improved significance of pathway up-regulation ($P = 2.5\times10^{-4}$) (**Table 1**). Finally, the significant enrichment of up-regulated genes in the pathway is visualized by a barcode plot (**Figure 3B**) where a high enrichment of over-expressed genes to the right side of the figure can be seen and where the highest enrichment score is reached.

## Discussion

The toll-like receptors recognize EBV, organize immune responses and lead to the production of inflammatory cytokines and antiviral mediators. Although different TLR genes may have been involved in the process with specific functionalities and some of them have been more investigated than the others, this study for the first time reveals a general up-regulation pattern of the entire TLR signaling pathway in EBVaGC. As can be seen in **Figure 3A**, of the three reported genes TLR7, TLR8 and TLR9, two are actively activated but not among the most upregulated and one (TLR9) even inactive. Among the topmost significant genes in Table S5, STAT1 and CXCLs have been found to control immune responses in EBVaGCs [4, 16]. Our pathway-based analysis not only reveals an overall pattern of up-regulation of the entire TLR signaling pathway but also points to new pathway member genes that contribute most to the activation of the pathway.

In **Figure 2**, there is a higher number of more upregulated or fewer downregulated genes in the EBVaGCs (147 genes, the warm colored

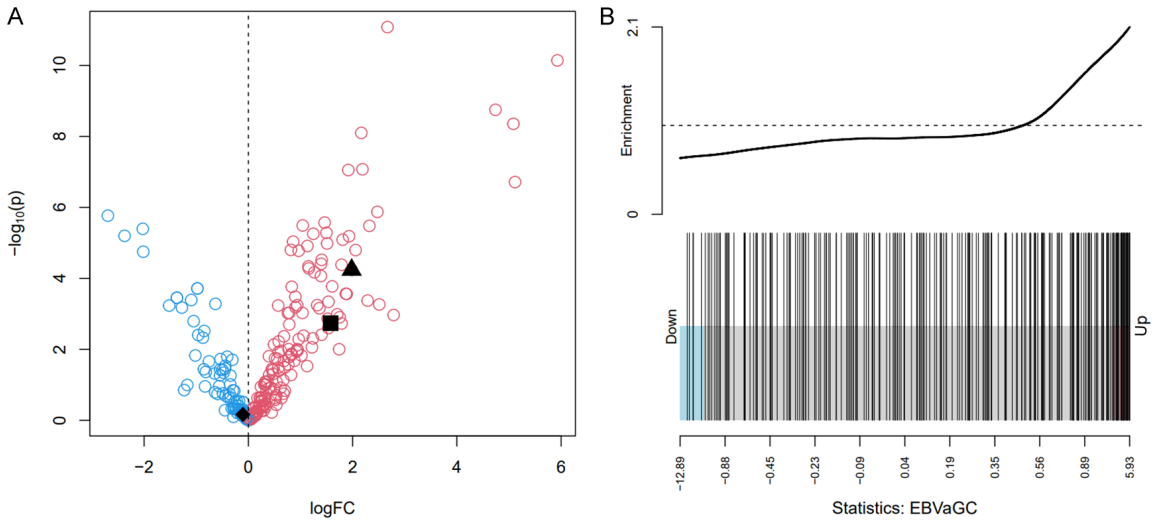


**Figure 3.** Results of differential expression analysis by comparison of tumor gastric tissue of EBVaGC with non-tumor gastric tissue of EBVnGC patients, presented by a volcano plot (A) showing differential expressions (red for up and blue for down regulated genes) and a barcode plot (B) displaying high enrichment of overexpressed genes of the TLR signaling pathway.

dots) than that in EBVnGCs (106 genes, cold colored dots). Also, in the genes exhibiting opposite effects, there are more genes that are upregulated in EBVaGCs but downregulated in EBVnGCs (51 genes, pink dots) than genes that are downregulated in EBVaGCs but upregulated in EBVnGCs (30 genes, light blue dots). In fact, the results in **Figure 1C** obtained from directly comparing tumor tissues of EBVaGC with EBVnGC likewise explicitly displays an unbalanced differential expression pattern by TLR signaling pathway genes. These patterns are in consistence with the significant upregulation of the whole pathway as detected by our gene-set enrichment analysis. However, a correlation analysis on logFC of between EBVaGCs and EBVnGC in **Figure 2** showed a highly significant positive correlation of r = 0.60 ($P<2.2\times10^{-16}$). The significant consistence in the activity of the TLR signaling pathway indicates active involvement of the pathway in both forms of gastric cancer but with different patterns (more activated TLR signaling pathway genes in EBVaGC) that characterize their molecular pathology.

The results displayed in **Figure 1D** reveal an important phenomenon, i.e. even in the non-tumor gastric tissue of the EBVaGC patients, there is also a weak pattern of upregulation of the TLR signaling pathway (P = 0.045, **Table 1**). An important consequence of such a pattern is that the self-matched differential expression analysis in analytical scheme 1 can be low-powered because the baseline is already, to some degree, upregulated. This is confirmed by the results in **Figure 3** which presents a clearer pattern of upregulation with increased statistical significance ($P = 2.5\times10^{-4}$). Our result here serves as a good example of improper use of the self-matched design in biomedical genomics where the baseline needs to be carefully determined to avoid the impact of similar disease pathology as in the diseased tissue.

The volcano plot in **Figure 3A** displays a one arm pattern of increased gene expression activity by the member genes of TLR signaling pathway. Likewise, the barcode plot in **Figure 3B** clearly shows a very high enrichment of upregulated pathway genes. Overall, results in **Figure 3** reveal a distinct pattern of activated function of the pathway in EBVaGCs which can be seen as a specific feature in the molecular pathology of EBVaGCs. This feature characterizes the disease as a distinct subtype of gastric cancer in oncogenesis which can be linked to its clinical manifestation and prognosis to motivate improved treatment strategies for both EBVaGC and EBVnGC patients.

As one of the most common cancers in the world, the prognosis of gastric cancer is still poor although there have been a lot of improve-

ment in cancer treatment approaches. It is therefore urgent to develop novel and more effective treatments to improve the survival rate of gastric cancer patients. In fact, the TLRs have been regarded as a promising target against gastric cancer [17-19]. Here, our identified upregulation of TLR signaling pathway and better prognosis of EBVaGC as compared with EBVnGC might could suggest that the TLRs agonists might be potentially used as therapeutic agents to treat EBVnGC. Given the complexity in the function of TLR signaling pathway in cancer [20], more research is needed to examine the translational value of the TLR signaling pathway in combating gastric cancer.

## Conclusion

This study revealed a distinct pattern of activation of the whole TLR signaling pathway in EBVaGCs which can be seen as a specific feature of molecular pathology in the disease. This feature characterizes the disease as a distinct subtype of gastric cancer in oncogenesis which can be linked to its clinical manifestation and prognosis to motivate improved treatment strategies for both EBVaGC and EBVnGC patients.

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## Disclosure of conflict of interest

None.

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