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Original Article

Tumor necrosis factor α promotes HEp-2 cell proliferation via toll-like receptor 4 and NF-κB signaling pathways

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Abstract: Laryngeal carcinoma is a serious, life-threatening disease. Tumor necrosis factor α (TNF-α), a proinflammatory cytokine, has complex effects on the proliferation and growth of cancer cells. Previously, we treated a laryngeal cancer cell line (HEp-2) with TNF-α and demonstrated that this treatment suppressed polycystin-2, a transient receptor potential cation channel expression and ATP-induced Ca^{2+} release but increased HEp-2 cell proliferation and growth. However, the mechanisms and signaling pathways underlying the TNF-α effects on the HEp-2 cells were unclear. Therefore, we here used RNA-seq techniques to examine the effect of TNF-α on the gene transcript expression profile in these cells. We found that TNF-α treatment (100 ng/mL, 24 h) upregulated 2,811 genes and downregulated 1,128 genes. The *IRAK1* gene encoding an effector protein downstream of toll-like receptor 4 (TLR4) was ranked 19th in the upregulated differentially expressed genes. In a gene ontology (GO) analysis, 168 GO terms were identified in the biological process domain for the upregulated differentially expressed genes, and cell cycle and DNA replication functions were enriched. In a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, TNF-α treatment enhanced the NF-κB pathway in HEp-2 cells. Moreover, both the transcript and protein expression levels of TLR4 as well as the expression of genes encoding downstream TLR4 effectors were significantly increased in TNF-α-treated HEp-2 cells. We concluded that TNF-α increased HEp-2 cell proliferation and growth likely via enhancing TLR4- and NF-κB-associated signaling pathways and that TNF-α may play an important role in the development of laryngeal cancer.

Keywords: Tumor necrosis factor α, human laryngeal squamous cell carcinoma, RNA-seq, proliferation, toll-like receptor 4, NF-κB

Introduction

Laryngeal carcinoma is one of the most common malignant tumors of the head and neck. Unfortunately, the diagnosis for approximately 60% of patients with laryngeal carcinoma does not occur until stage III or IV, and the 5-year survival rate has decreased over the past 40 years although the overall incidence is declining [1]. In addition, although treatment of laryngeal carcinoma with surgery and chemoradiotherapy has been vastly improved in recent years [2, 3], the many adverse effects of these treatments, including partial or complete loss of swallowing and vocal function, negatively impact the quality of life for these patients [2-4]. The complex biological processes involved in the occurrence and progression of laryngeal carcinoma is not completely known. Thus, elucidating the cellular and molecular mechanisms associated with this carcinoma may aid in the development of new treatment strategies that arrest the disease while maintaining maximal laryngeal function.

Tumor necrosis factor α (TNF-α) is currently the strongest antitumor cytokine. It plays key roles in inhibiting tumor progress, such as through cell apoptosis or killing tumor cells and in hemorrhagic necrosis as well as in increasing immunity through various signaling pathways [5]. However, TNF-α can also act as a tumor promot-

er under some conditions [5, 6]. Our previous research has shown that TNF-α treatment enhances proliferation and growth of HEp-2 cells, derived from an epidermoid carcinoma of the larynx, by suppressing the expression of the protein encode by the *PKD2* gene, polycystin 2, a transient receptor potential cation channel, also known as TRPP2, and TRPP2-mediated Ca^{2+} signaling [7]. In addition, TNF-α is important in cytokine network regulation. We found that TNF-α treatment downregulated phosphorylated eukaryotic translation initiation factor 2α and phosphorylated protein kinase R-like endoplasmic reticulum kinase expression levels in HEp-2 cells [7].

In the present study, we used Gene Ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) database pathway analyses to determine the mechanisms and signaling pathways underlying the TNF-α effect on HEp-2 cells and to provide a plausible mechanism for the role of TNF-α in the development of laryngeal cancer.

Materials and methods

Materials

Toll-like receptor 4 (TLR4) and β-tubulin antibodies were purchased from Santa Cruz (USA). Nonidet P-40, sodium deoxycholate, sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA), and TNF-α were purchased from Sigma (USA). Horseradish peroxidase-conjugated secondary antibody was purchased from Promega (USA). The ECL plus western blotting detection system was obtained from Thermo Fisher Scientific (USA).

Cell culture

The laryngeal carcinoma HEp-2 cell line (a cell line considered to have originated from a human laryngeal carcinoma, but was contaminated with a HeLa cell from cervical cancer) was purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's Medium supplemented with 10% fetal bovine serum, streptomycin (100 μg/mL) and penicillin (100 U/mL) in humidified atmosphere of 5% CO_2 at 37°C. HEp-2 cells were seeded on 6-well plate and cultured in Dulbecco's modified Eagle's medium with or without TNF-α (100 ng/mL) or phosphate-buffered saline for 24 h at 37°C. The HEp-2 cells were then detached by trypsin, washed twice with cold phosphate-buffered saline, and used in following experiments.

Differential expression analysis

The preprocessing tool Trimmomatic (v0.36) was used to remove adapters and low-quality reads from the HEp-2 cell RNA-seq data in raw fastq files [8]. The reads were then mapped to the Ensembl hg19 reference genome using the alignment program HISAT2 2.0.5 [9]. For this process, a maximum of two mismatches were allowed, and all other parameters were used with the default settings. The mapped data were assembled into potential transcripts and quantified using StringTie v1.3.1c. All default parameter settings were used for the quantification based on the human annotation file of Ensembl GRCh37. After applying StringTie, a matrix of expression values in FPKM was obtained. A value of 1 was added to each raw FPKM value before the ensuing data analysis [9]. All statistical analyses were conducted using the Limma package in R software (http://www.r-project.org/). The upregulated differentially expressed genes (DEGs) were obtained using a threshold fold change greater than 1.5. Clustering analysis of DEGs was performed and heat maps were obtained using R software.

GO Terms and KEGG pathways analysis

GO enrichment analysis (with P values < 0.05) was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID 6.8; https://david.ncifcrf.gov/). An assessment of the enrichment for the GO term annotation category biological process was conducted to obtain a set of genes or a subgraph of biological networks [10].

KEGG pathways were manipulated with the online biological tool KEGG Mapper 83.0 (http://www.kegg.jp/kegg/mapper.html).

Construction of protein-to-protein networks

The online STRING search tool (https://string-db.org/) for the retrieval of interacting genes was used to construct protein interactions [11]. The protein-to-protein (PPI) network for DEGs was constructed with a minimum required interaction score set at > 0.4.

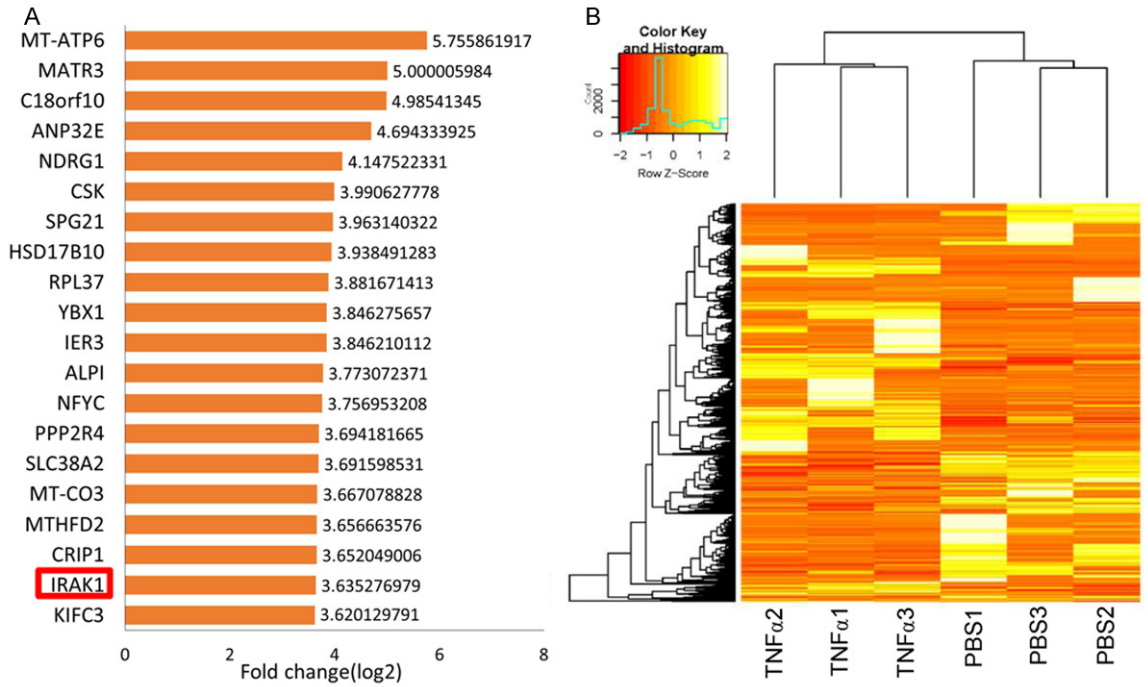

Figure 1. Differentially expressed genes and hierarchical clustering analysis in HEp-2 cells. A. The top 20 upregulated differentially expressed genes. B. Heat map showing the expression of the differentially expressed genes and hierarchical clustering analysis following TNF-α (100 ng/mL) and phosphate-buffered saline (PBS) treatment in HEp-2 cells.

Western blotting

Immunoblotting was performed as described in our previous study [7]. Briefly, whole-cell lysates from HEp-2 cells with or without TNF-α (100 ng/mL) treatment were extracted with a detergent extraction buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 1 mM Na_2EDTA, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, and leupeptin. Subsequently, the samples were loaded into each well of a 10% SDS-PAGE gel. For immunoblots, polyvinylidene difluoride membranes carrying transferred proteins were incubated at 4°C overnight with the primary antibody anti-TLR4 (1:200). Immunodetection was accomplished using a horseradish peroxidase-conjugated secondary antibody (1:1,000). The immunosignals were visualized using an ECL plus detection system. The optical density of each blot was normalized to GAPDH analyzed within the same lane and represented as the relative optical density.

Statistical analysis

SigmaPlot software was used to perform the statistical analyses. All data are expressed as means ± SEM. Two-tailed, unpaired Student's *t*-test was used to estimate the significance. A value of $P < 0.05$ was considered statistically significant.

Results

Identification of DEGs

Cytokines can affect biological behaviors, including cell proliferation. We used TNF-α (100 ng/mL) to treat HEp-2 cells for 24 h and compared the change in gene transcript expression between the TNF-α-treated and control groups. Next-generation sequencing techniques were used to obtain RNA-seq data and identify significant DEGs. Our results showed that 2,811 genes were upregulated, while 1,128 genes were downregulated. We examined the top 20 upregulated DEGs, and found that interleukin-1 receptor-associated kinase-1 (*IRAK1*), a gene encoding a downstream effector of TLR4, was ranked 19th (**Figure 1A**, red circle). Using a heat map showing the expression of the DEGs, our hierarchical clustering analysis indicated that TNF-α treatment was distinguished from the control group by the clustering of the DEGs (**Figure 1B**). Thus, the data could be rea-

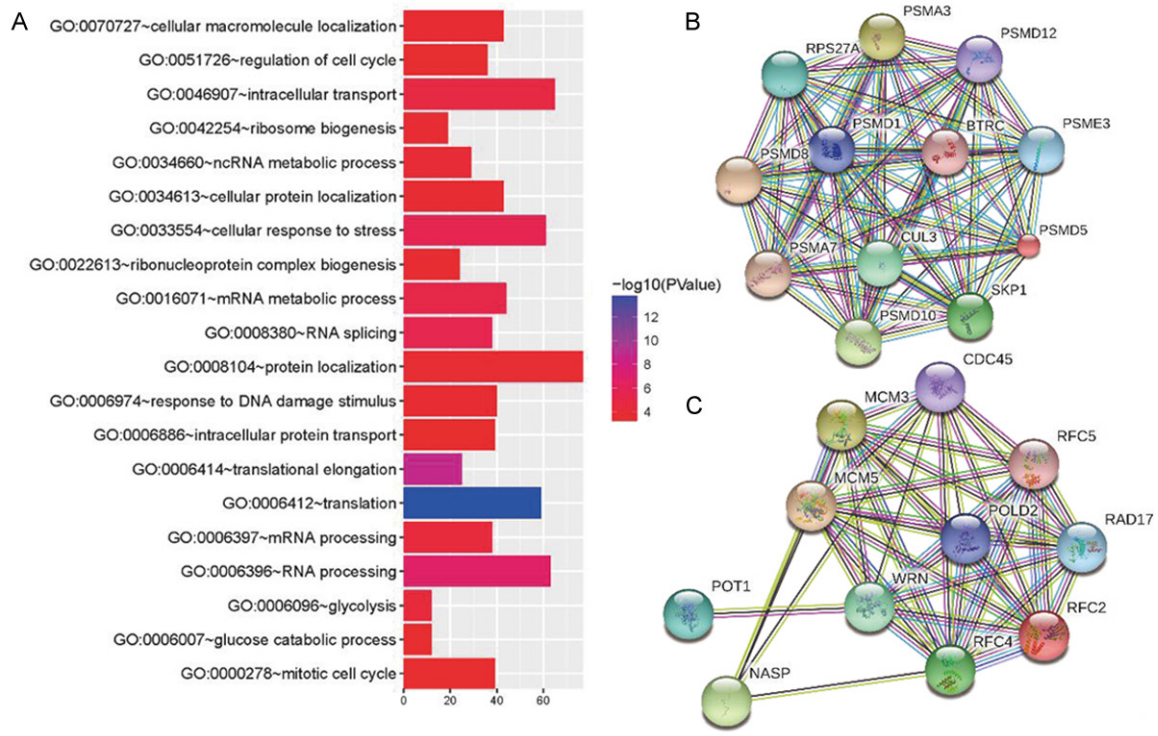

Figure 2. Gene ontology (GO) enrichment analysis and protein-to-protein interaction networks in HEp-2 cells. A. The top 20 identified GO terms in the biological process category for the upregulated differentially expressed genes. B, C. Protein-to-protein interaction networks of cell cycle- and DNA replication-related genes in the upregulated differentially expressed genes.

sonably partitioned and applied to further analyses.

GO analysis of upregulated DEGs

Biological process, cellular component, and molecular function are three GO term annotation categories used in GO analyses to classify the functions and roles of DEGs. Using these three categories, we conducted a GO analysis of the upregulated DEGs and identified 168 GO terms with *P* values less than 0.05. The top 20 ranked GO terms described under biological process are shown in **Figure 2A**. We previously demonstrated that TNF-α treatment significantly enhanced the HEp-2 cell cycle and proliferation [7]. In the present GO analysis of biological process for the upregulated DEGs, we found that cell cycle and DNA replication functions were enriched. Next, we used these enriched genes to construct a PPI network with the STRING search tool (**Figure 2B**, **2C**). We found that *BTRC* and *POLD2* were in the centers of the cell cycle (**Figure 2B**) and DNA replication (**Figure 2C**) networks, respectively. The heat map also showed that many key genes in the PPI networks were markedly upregulated, including *BTRC*, *SKP1*, and *RPS27A*, in the cell cycle, and *POLD2*, *MCM3*, *MCM5, RFC2, RFC4,* and *RFC5* in DNA replication (Supplementary Figure 1). These results suggested that TNF-α treatment accelerated the HEp-2 cell cycle as well as proliferation likely via upregulating these key genes.

TNF-α-induced activation of MAPK, PI3K-Akt and NF-κB pathways

We used KEGG signaling pathway enrichment analysis and found that NF-κB, MAPK, and PI3K-Akt pathways, which are associated with cell survival, cell cycle, and proliferation, were upregulated (**Figure 3** and Supplementary Figures 2 and 3). NF-κB is also downstream from the MAPK and PI3K-Akt signaling pathways.

TNF-α enhances the TLR4 signaling pathway

In the cell cycle PPI network, several genes, including *BTRC*, *SKP1,* and *RPS27A*, are highly associated with TLR4 and the NF-κB pathway [12-14]. Therefore, we used a heat map to show

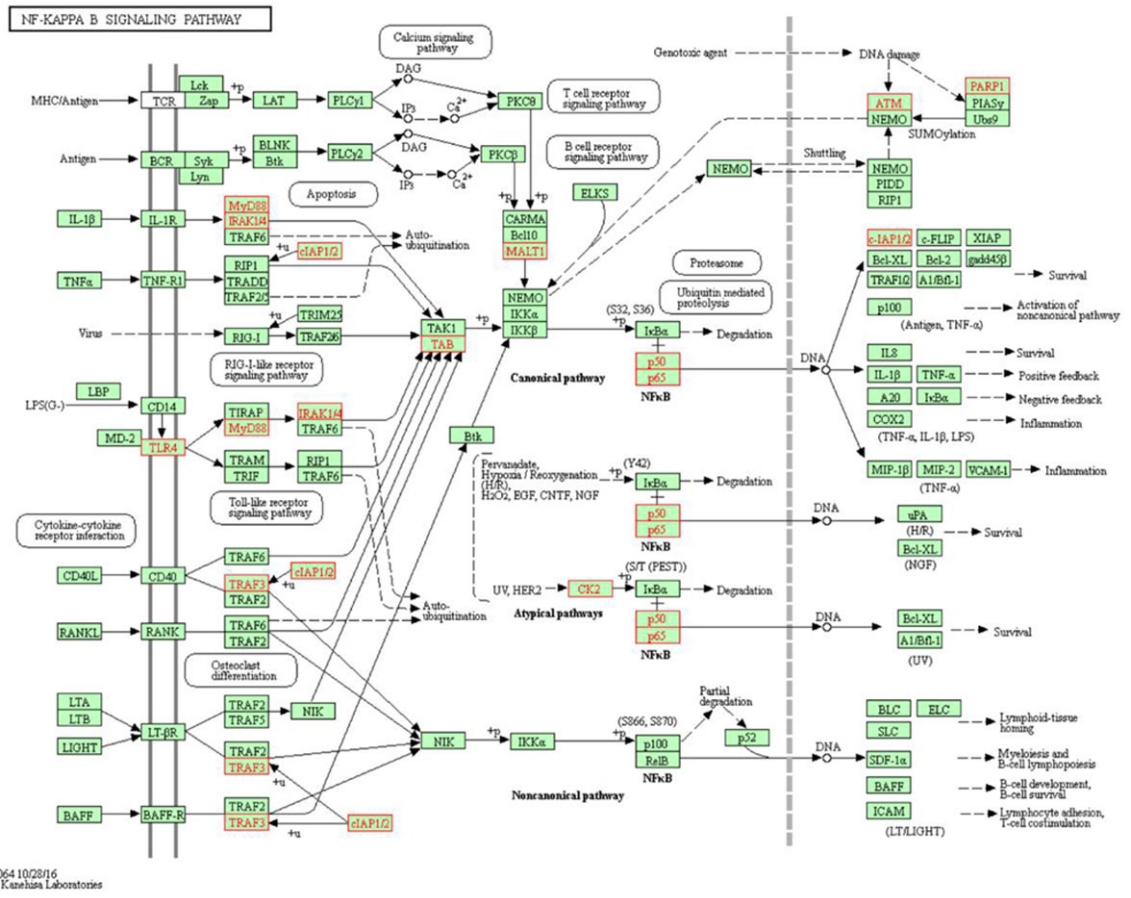

Figure 3. TNF-α-induced changes in the NF-κB signaling pathway in HEp-2 cells. HEp-2 cells were treated with TNF-α (100 ng/mL) for 24 h. Upregulated genes are shown in red.

the expression profiles of the TLR and NF-κB families. The expression levels of TLR4 and NF-κB1 were significantly increased (**Figure 4A**). More importantly, genes for effectors downstream of TLR4, including *MYD88*, *IRAK1* and *TAB*, were all significantly upregulated in the TNF-α-treated group (**Figure 4A**), and as mentioned above, *IRAK1* was ranked the top 19th upregulated DEG (**Figure 1A**) [14].

To confirm our findings, we used immunoblotting to identify the TLR4 protein expression level in the TNF-α-treated group. Our results indicated that TNF-α (1,000 ng/mL) treatment for 24 h significantly increased TLR4 protein expression (**Figure 4B**).

Discussion

In the present study, we used RNA-seq techniques to determine the effects of TNF-α treatment on HEp-2 cells. Our primary findings were as follows: (1) TNF-α treatment induced 2,811 upregulated and 1,128 downregulated genes in HEp-2 cells, and the gene encoding a downstream effector of TLR4, *IRAK1*, was ranked 19 in the upregulated DEGs; (2) Functional analyses showed that TNF-α treatment enhanced the expression of many key genes, including *BTRC*, *SKP1*, and *RPS27A* in the HEp-2 cell cycle as well as *POLD2*, *MCM3*, *MCM5*, *RFC2*, *RFC4*, and *RFC5* in HEp-2 cell DNA replication; (3) TNF-α treatment enhanced the NF-κB, MAPK, and PI3K-Akt signaling pathways in HEp-2 cells; (4) The expression levels of genes encoding downstream effectors of TLR4 and TLR4-related genes were significantly increased in HEp-2 cells treated with TNF-α. Taken together, our results indicated that TNF-α treatment enhanced the expression of many key genes involved the cell cycle and DNA replication of HEp-2 cells and increased TLR4- and NF-κB-associated signaling pathways, which may underlie the enhanced HEp-2 cell proliferation and growth.

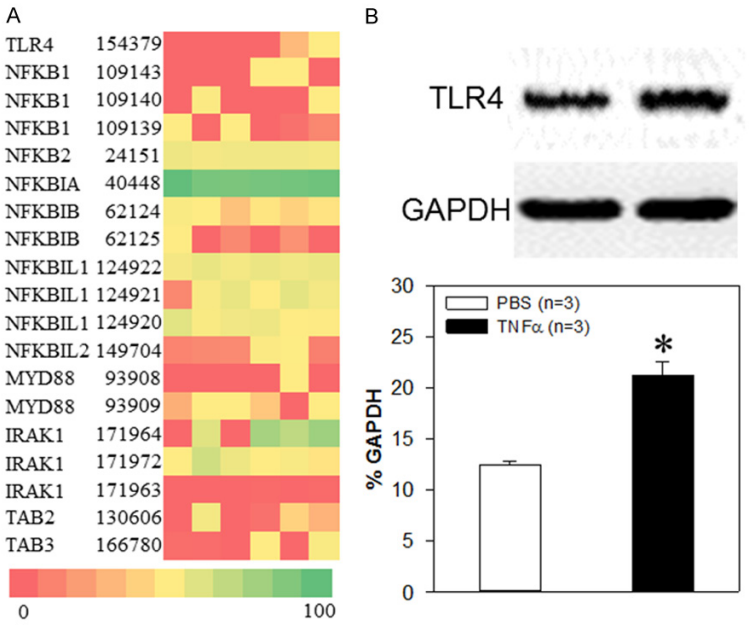

Figure 4. Transcript expression profiles of toll-like receptor 4 (TLR4)- and NF-κB-associated signaling pathways and the effect of TNF-α on TLR4 protein expression in HEp-2 cells. A. Heat map showing transcript expression of TLR4- and NF-κB-associated signaling pathways. B. Representative immunoblot images (upper) and summary data (lower) showing TLR4 protein expression levels in HEp-2 cells pretreated with TNF-α (100 ng/mL) or phosphate-buffered saline (PBS, control) for 24 h. GAPDH was used as a loading control. Values are shown as the mean ± SEM (n = 3); *$P < 0.05$, control vs. TNF-α treatment.

Previously, we used cell counting and flow cytometry cell cycle assays to demonstrate that TNF-α, a proinflammatory cytokine, enhances HEp-2 cell proliferation and growth via suppressing TRPP2 (also known as polycystin-2, PKD2) expression and TRPP2-mediated Ca^{2+} signaling. However, the TNF-α-induced alterations in the protein network were unclear. Therefore, here we used RNA-seq techniques to identify and analyze gene transcript expression profiles altered by TNF-α in HEp-2 cells. Our results first confirmed that *PKD2* expression was decreased (fold change = 0.205). We also found that 2,811 genes were upregulated, including many cell cycle- and DNA replication-related genes, which have strong interactions. Moreover, cell cycle- and proliferation-related signaling pathways, including MAPK and PI3K-Akt pathways, were enriched. These results confirmed our previous findings for the effects of TNF-α on HEp-2 cell proliferation and growth.

Toll-like receptors, including at least 11 members in mammals, play a pivotal role in the innate immune response, and the expression levels of these receptors may reflect the sensitivity of immune cells to infections. Toll-like receptors and interleukin 1 (IL-1) receptors have similar cytoplasmic domains that use the same signaling pathways. The activation of TRLs may trigger their downstream cascades, including myeloid differentiation primary response 88 (MYD88), IL-1 receptor-associated kinases (IRAKs) and TNF receptor-activated factor 6, to eventually activate the NF-κB pathway, which would then induce cell survival [15, 16]. The binding of lipopolysaccharide to TLR4 triggers human head and neck squamous cell carcinoma, adipocytes, and monocytes to produce cytokines, for example TNF-α and IL-6, which play dominant roles in the inflammatory response [13, 14, 17]. However, TNF-α treatment was previously shown to suppress TLR4 expression and NF-κB pathway signaling in human monocytes [17, 18]. Here, we used RNA-seq sequencing techniques and found that eight members of the TLR family were expressed in HEp-2 cells. But, dissimilar to the results of these aforementioned studies, TNF-α treatment for 12 h significantly enhanced TLR4 transcript and protein expression. The expression levels of the genes encoding protein effectors downstream of TLR4, including *MYD88*, *IRAK1* and *TAB*, were also all increased. The PI3K-Akt and NF-κB pathways generally participate in cell survival regulation [14], and our results showed that both the PI3K-Akt and NF-κB pathways were upregulated. Therefore, our data indicated for the first time that TNF-α has an effect in HEp2 cells opposite to that previously shown in human monocytes.

In conclusion, we demonstrated that TNF-α treatment upregulated many key genes involved in the cell cycle and DNA replication. Moreover, TLR4, NF-κB, MAPK, and PI3K-Akt signaling pathways were also increased, and these increases may underpin the enhanced TNF-α-induced proliferation and growth of HEp-2 cells. Our findings indicate that TNF-α

may play a pivotal role in the development of laryngeal cancer.

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Disclosure of conflict of interest

None.

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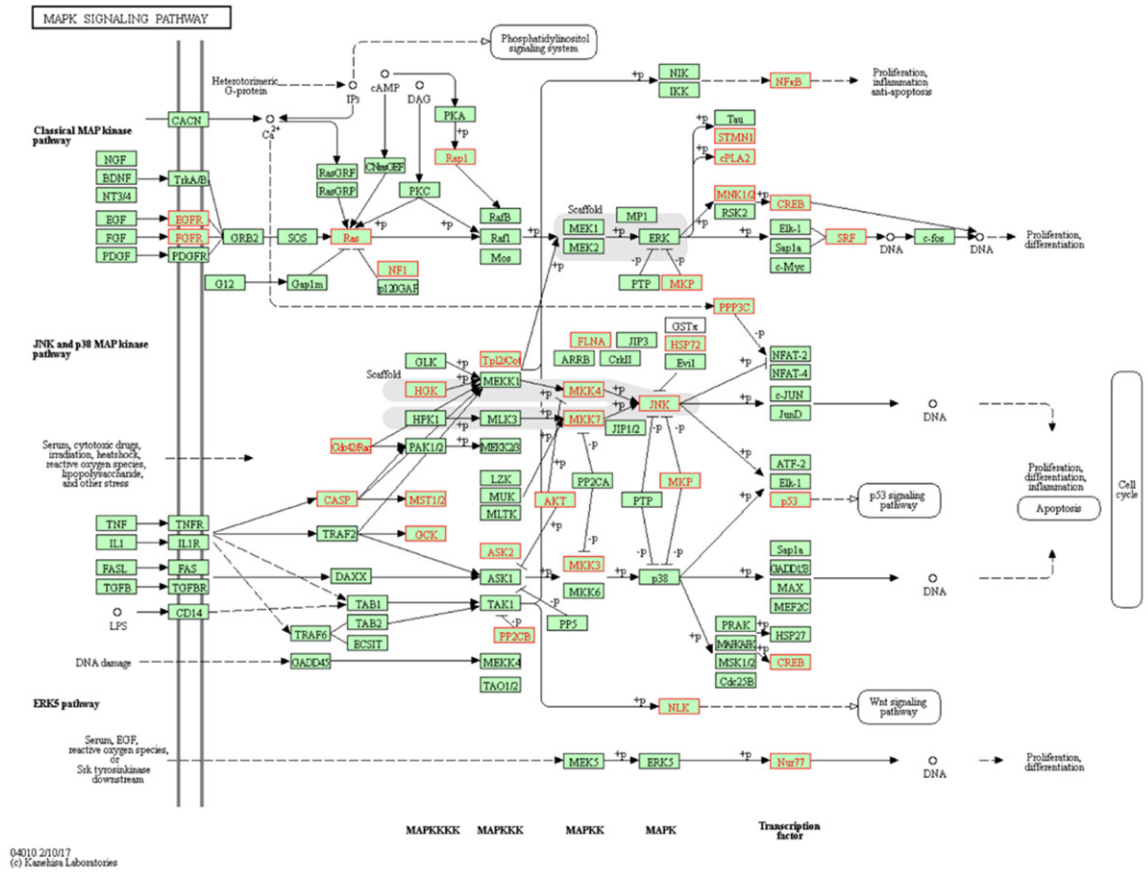
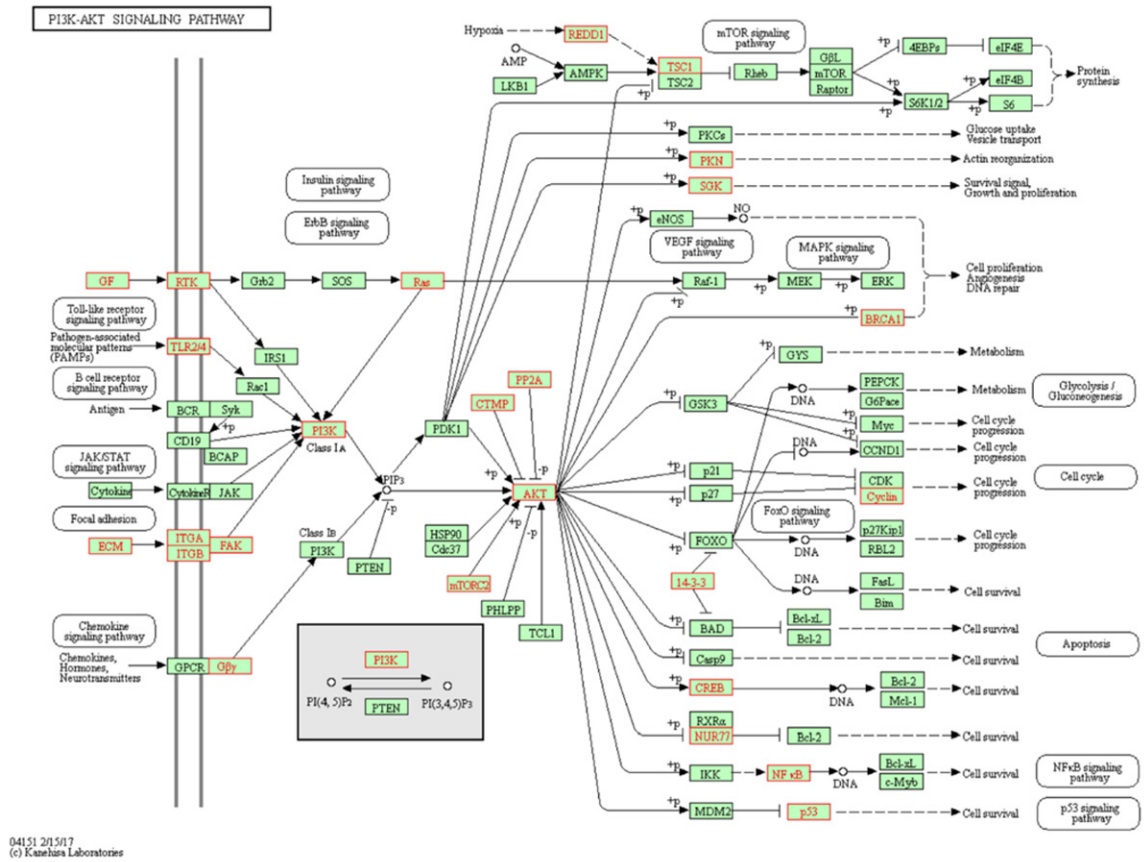
Supplementary Figure 1. Transcript expression profiles of cell cycle- (A) and DNA replication (B) -associated genes in HEp-2 cells.



Supplementary Figure 2. TNF-α-induced changes in the MAPK signaling pathway of HEp-2 cells. HEp-2 cells were treated with TNF-α (100 ng/mL) for 24 h. Upregulated genes are shown in red.



Supplementary Figure 3. TNF-α-induced changes in the PI3K-Akt signaling pathway of HEp-2 cells. HEp-2 cells were treated with TNF-α (100 ng/mL) for 24 h. Upregulated genes are shown in red.