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Expression Analysis of *BIRC3* as One Target Gene of Transcription Factor NF-κB for Esophageal Cancer

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Abstract: Esophageal cancer (ESCA) is one of the highest lethal malignancy tumors worldwide. Baculoviral IAP repeat-containing protein 3 (*BIRC3*) is the main inhibitor of apoptosis in many malignancies. The aim of this study was to clarify how *BIRC3* acts in ESCA cells. Through TNMplot and GEPIA2 analysis, *BIRC3* was found abundantly expressed in ESCA cells. The quantitative RT-PCR assay confirmed *BIRC3* was pronouncedly induced in all used ESCA cell lines. In addition, proinflammatory cytokines TNFα and IL-1β were shown to have promotion effects on *BIRC3* expression in ESCA cells. These promotive effects were blocked when the function of NF-κB was inhibited by bay 11-7082, which indicates the expression of the *BIRC3* gene was regulated via the NF-κB transcription pathway in ESCA. Moreover, bioinformatics analysis showed that the *BIRC3* gene had many NF-κB binding cis-elements. Chromatin immunoprecipitation was then performed and it was found that NF-κB directly interacts with cis-elements of the *BIRC3* gene. In conclusion, our data proved that the high expression level of *BIRC3* maintained the survival of ESCA cells. *BIRC3* was up-regulated by proinflammatory cytokine TNFα and IL-1β through the NF-κB signaling pathway, and this may be helpful for esophageal cancer prevention and therapy.

Keywords: *BIRC3*; esophageal cancer; cytokine; tumor necrosis factor α (TNFα); transcription factor

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1. Introduction

Due to high cancer-related deaths, esophageal cancer (ESCA) is the sixth most common malignancy tumor worldwide. Two major histological ESCA are squamous cell carcinoma (ESCC) and adenocarcinoma (EAC) [1]. Although the portion of ESCA in cancer patients has declined in recent years, the total number of ESCA patients still increased every year, especially in elderly patients over 70 years old [2]. China is one of the countries suffering from a rapidly increasing cancer burden. In 2017, China had the world's highest number of esophageal cancer incident cases (235,000) and deaths (213,000) [3]. ESCA has become the fourth most common malignant cancer causing death in China [4]. The risk factors of ESCA in China are still not certain, but drinking beverages at high temperatures, low intake of fruit and vegetables, poor nutrition, and tobacco and alcohol use are considered as the main causes. Based on these strong risk factors, many precaution strategies have been taken to prevent ESCA. However, primary and secondary prevention of ESCA, such as changing daily routines and lifestyle, and the detection of precancerous and early cancerous lesions, are not as effective as expected [5]. Further information, particularly molecular mechanisms underlying ESCA progression, needs to be investigated for improving therapeutic outcomes and survival of patients.

Recently, thanks to high-throughput sequencing, a variety of gene-encoding proteins regulating cancer-related processes have been identified in relation to ESCA [6]. Genes involved in apoptosis, angiogenesis, or in oncogenes tumor suppressor processes were considered as important factors regulating ESCA forming. Among them, inhibitors of the apoptosis protein (IAP) family, regulating caspases, apoptosis, immunity, mitogenic

kinase signaling, and cell proliferation, are the most studied molecular and therapeutic targets. Baculoviral IAP repeat containing 3 (*BIRC3*), also known as cellular IAP2 (cIAP2), which is a typical IAP protein, was a pro-oncogenic protein, associated with cancer evasion from apoptosis [7]. Previous studies have demonstrated that the high expression of *BIRC3* correlated with many kinds of cancers, such as endometrial cancer, pancreatic cancer, and glioblastoma [8–10]; meanwhile, the overexpression of *BIRC3* encourages tumor progression in different cancers [11]. The depletion of the *BIRC3* gene can significantly suppress tumor initiation and progression [12]. Lower *BIRC3* expression levels were associated with a favorable outcome and a *BIRC3* level increase paralleled cancer recurrence and therapeutic resistance [13]. *BIRC3* has become a potential therapeutic target in multiple cancers due to its multidirectional effects and complicated functions. However, the roles of *BIRC3* in ESCA have only been investigated to a limited extent.

NF-κB (nuclear factor kappa B) signaling has been implicated in many hallmarks of cancer development, such as self-sufficiency in growth signals, insensitivity to growth inhibitors, and evasion of apoptosis [14]. In mammals, NF-κB contains five homologous subunits, including RelA/p65, c-Rel, RelB, p50/NF-κB1, and p52/NF-κB2. Previous studies suggest that both *BIRC2* and *BIRC3* contribute to the inhibition of apoptosis through an E3 ubiquitin-dependent manner [15]. The RING domain of *BIRC3* is necessary for the interaction with the E3 ubiquitin ligase that leads to proteasomal degradation of MAP3K14, which is the central kinase of the NF-κB non-canonical pathway. Thus, *BIRC3* is a biological negative regulator of non-canonical NF-κB signaling [16], which is alongside the canonical NF-κB signaling stimulated by tumor necrosis factor (TNF). On the other hand, *BIRC3* was reportedly required for the canonical activation of NF-κB and MAPK signaling pathways by members of the TNF receptor (TNFR) superfamily [17]. NF-κB signaling is a crucial signaling axis, with a variety of key molecules aberrantly expressed or genetically altered in cancer patients [18]. It is still not clear whether *BIRC3* regulates apoptosis in ESCA through the NF-κB pathway.

Meantime, *BIRC3* is also involved in the control of inflammasome activation. Recent work has shown that *BIRC3* can be induced by the inflammatory cytokine Interleukin-1β (IL-1β) in breast cancer cells, which resulted in a chemoresistance to doxorubicin [19]. It needs to be illustrated whether the functions of *BIRC3* in esophageal cancer depend on the inflammatory cytokine. Here, the expression profiles of *BIRC3* in five kinds of ESCA cells treated with TNF-α and IL-1β were analyzed to reveal the roles of *BIRC3* in ESCA. Moreover, bioinformatics analyses of *BIRC3* and chromatin immunoprecipitation (ChIP) in ESCA cells were performed to identify the *BIRC3* acting manner in ESCA. The results provide proof that *BIRC3* functions as a tumor stimulator in ESCA through the NF-κB pathway and it may be a therapeutic target for ESCA prognosis.

2. Materials and Methods

The expression profile of *BIRC3* across various human cancer types was examined through TNMplot (https://tnmplot.com/analysis/ (accessed on 21 August 2022)) [9]. On the homepage, select '*BIRC3*' from the gene list. The boxplots produced showed the expression level of *BIRC3* with left bars representing normal samples and right bars representing tumor samples. Gene expression correlation analysis of *BIRC3* in normal and esophageal cancer tissues was analyzed using the GEPIA2 web server (http://gepia2.cancer-pku.cn/#index (accessed on 21 August 2022)), setting the parameter as: "log scale: Yes", "cancer name: ESCA", "Matched Normal data: Match TCGA normal and GTEx data". The prognostic value of the *BIRC3* gene in esophageal cancer was analyzed using the Kaplan–Meier Plotter (http://kmplot.com/analysis/ (accessed on 21 August 2022)). The survival rates of patients with high and low levels of *BIRC3* were shown using a Kaplan–Meier survival plot.

For *BIRC3* gene expression analysis, the esophageal cancer cell lines KYSE150, KYSE510, KYSE450, TE3, and SHEEC cell lines from China ESCA patients were gifted by Professor Xu Yanli from Shantou University. The epithelial cell lines 293T and THLE3 purchased

from BLUEFBIO Biotech (BLUEFBIO Biotech, Shanghai, China) were used as controls. Esophageal cancer cells were grown in RPMI1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (TransGen Biotech, Beijing, China), 1% penicillin and streptomycin (both from Beyotime Biotech, Shanghai, China) with 5% carbon dioxide at 37 °C. For NF-κB activation, 15 ng/mL, 30 ng/mL, and 60 ng/mL TNFα (PeproTech, Rocky Hill, NJ, USA) were added respectively and cultured for 1 h. For NF-κB inhibition, cells were pre-incubated with the downer 50 μmol/L Bay 11-7082 (Beyotime Biotech) for 30 min, then 30 ng/mL TNFα were added. After being cultured for 1 h, cells were collected for analysis. For the IL-1β experiment, cells were cultured with 10 ng/mL, 20 ng/mL, and 40 ng/mL human IL-1β (PeproTech), respectively, for 1 h. For the IL-1β effects but without NF-κB stimulation experiment, cells were incubated with 50 μmol/L Bay 11-7082 for 30 min and then cultured for 1 h with a medium containing 20 ng/mL IL-1β. Control cells were cultured in the same way but without TNFα, Bay 11-7082, or IL-1β. Cells were collected by centrifugation. The total RNA was extracted from collected cells using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) followed by isopropyl alcohol precipitation. RNA quality and concentration were assessed using NanoDrop (Applied Biosystem, Foster City, CA, USA). PrimeScript™ RT reagent Kit (Takara, Tokyo, Japan) was applied for cDNA synthesis following the manufacturer's instructions. Gene relative expression was measured using TB Green Fast qPCR Mix (Takara) in a Real-Time Light Cycler 96 (Roche, Basel, Switzerland), using the PCR program with initial denaturing at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. Relative mRNA expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ equation. Cell lines 293T and THLE3 were used as the control group, respectively. Values for expression of each gene were normalized to the housekeeping gene *GAPDH* (GeneBank No: 2597). Specific primers used for PCR analysis were: *GAPDH* forward, 5′-ATTTGGTCGTATTGGGCG-3′, reverse, 5′-CTCGCTCCTGGAAGATGG-3′, and *BIRC3* forward 5′-ATTTGATGAAAAGCGCCAACAC-3′, reverse, 5′-AACCCCAGCAGGAAAAGTGG-3′. Each PCR was carried out in triplicate and shown as mean ± SD.

The *BIRC3*-related genes were identified through GeneMANIA (http://genemania.org/ (accessed on 21 August 2022)). Setting parameter of GeneMANIA is: "organism: Homo sapiens", "network: automatically selected weighting method", all networks including physical interactions, co-expression, predicted, co-localization, pathway, genetic interactions, and shared protein domains were chosen. Further, the gene structure of *BIRC3* was analyzed through UCSC software (http://genome.ucsc.edu/ (accessed on 21 August 2022)), and the cis-elements of the *BIRC3* gene binding to NF-κB transcript factor (TF) were shown in a table.

To detect the relationship between the NF-κB transcript factor and the *BIRC3* gene, ChIP was performed as described before [20]. Antibody for RelA TF (p65, Abcam, Cambridge, UK) was used for the affinity purification and complete rabbit IgG (Abcam) for the negative control. In addition, an antibody for acetylate histones H3K27Ac (Abcam) was applied to detect the acetylate histones signals. In brief, SHEEC cells treated with 30 ng/mL TNFα or 20 ng/mL IL-1β were applied for chromatin isolation, and were sheared on ice with a Branson 450 sonicator (Branson Ultrasonics, Danbury, CT, USA) for 3 cycles of 15 s pulses of half-maximal power with 30 s cooling time between pulses. Protein A-Agarose beads (Santa Cruz Bio-technology, Santa Cruz, CA, USA) were used for affinity purification, and samples were treated with proteinase K (Invitrogen). After precipitation, DNA was diluted in 100 μL water, and purified with a PCR products purification kit (Solarbio, Beijing, China). *BIRC3* gene fragments were amplified with specific primers (*BIRC3*-84F, 5′-GCTGGAGTTCCCCTAAGTCC-3′, *BIRC3*-84R, 5′-TATCTGTACCAGGCGTTCCG-3′ and *BIRC3*-117F, 5′-GAACGCCTGGTACAGATAGGG-3′, *BIRC3*-117R, 5′-CCCAGTCTTTTCAAGCGACAC-3′), using the purified DNA as template. As input control, 8% volume of the supernatant from all pre-immune samples was set in parallel and re-suspended in 1000 μL of water for subsequent PCR.

All data in this study were calculated with IBM SPSS Statistics software (Version 13, IBM SPSS, Chicago, IL, USA). Significances between the groups were identified using t test or ANOVA followed by Dunnett's multiple comparison test.

3. Results

3.1. In Silico Analysis of Gene Expression of BIRC3 in Tumors

The gene expression of *BIRC3* in tumors and normal tissues was analyzed through the TNMplot web server. During 22 surveyed human cancers, the expression of *BIRC3* is significantly higher in nine kinds of tumors (Figure 1A), including esophageal cancer. The further GEPIA2 analysis of the *BIRC3* expression profile in esophageal cancer confirmed that *BIRC3* had a much higher expression level than normal tissues (Figure 1B). Kaplan–Meier analysis revealed that a high expression level of *BIRC3* was correlated with a good survival rate in esophageal squamous cell carcinoma cancer ($p = 0.035$; Figure 1C), but there is no significant correlation between the expression level of *BIRC3* and the survival rate of patients for esophageal adenocarcinoma cancer ($p = 0.2$; Figure 1D). This observation suggests that *BIRC3* acts in esophageal cancer, but its role seems to be comprehensive.

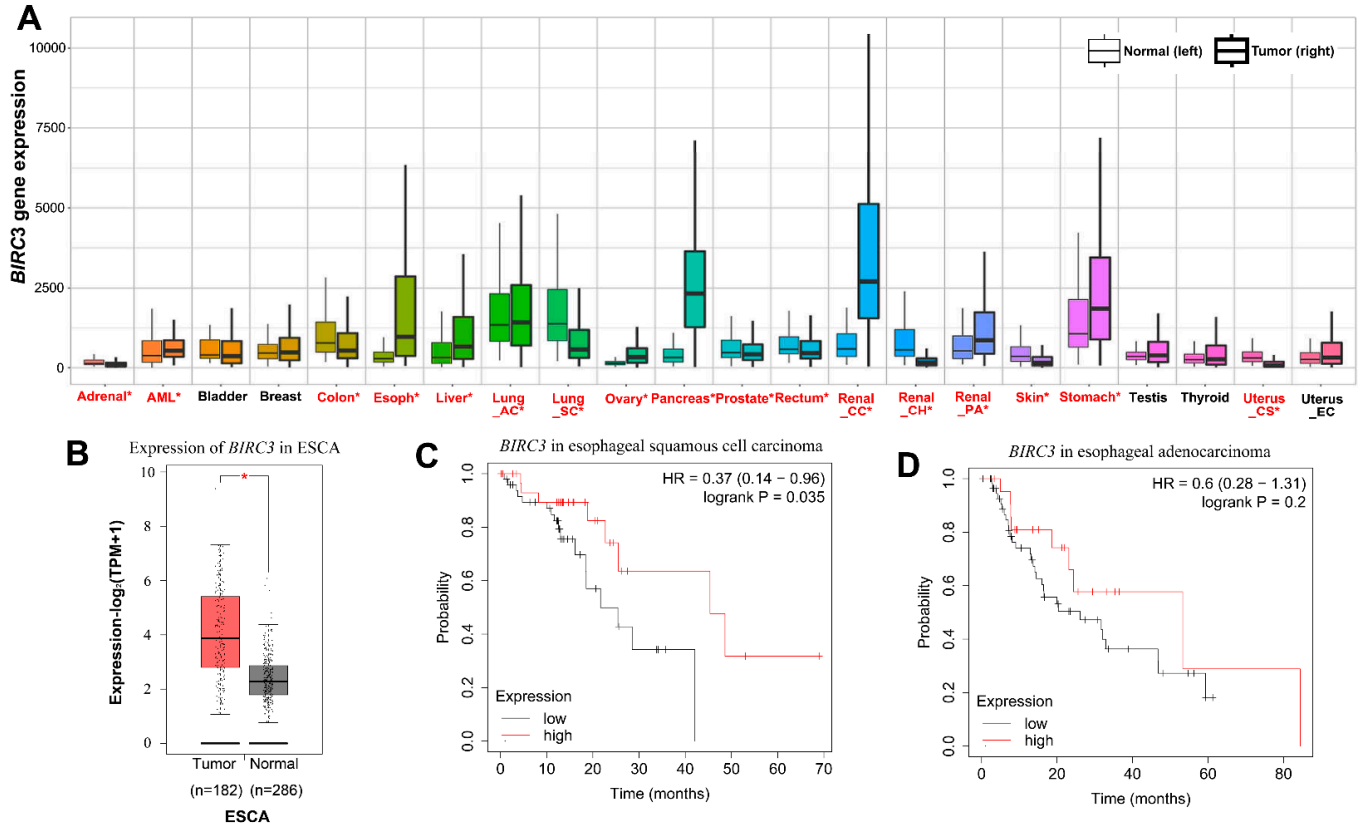

Figure 1. In silico analysis of expression levels of *BIRC3* in normal and tumor tissues: (**A**) TNMplot analysis of *BIRC3* expression profiles. The cancers with significant differences in gene expression of *BIRC3* are marked with red color and asterisk. (**B**) Expression levels of *BIRC3* in esophageal cancer. The analysis was performed by the multiple gene comparison program of the GEPIA2 database. (**C**,**D**) Kaplan–Meier Plotter analysis of *BIRC3* in esophageal squamous cell carcinoma and esophageal adenocarcinoma, respectively. The red line indicates the overall survival rate with high levels of *BIRC3*, and the black line shows the low levels of *BIRC3* in esophageal cancer. In silico analysis of expression levels of *BIRC3* in normal and tumor tissues.

3.2. BIRC3-Induced Expression in Esophageal Cancer Cells

To analyze the *BIRC3* gene expressions in esophageal cancer cells, quantitative real-time PCR analyses were performed. Five esophageal cancer cell lines were applied. Expres-

sions of *BIRC3* were detectable in all these cells. Compared with the control cells 293T and THLE3, *BIRC3* was significantly induced to be expressed in all used esophageal cancer cells (Figure 2). Among the detected five kinds of esophageal cancer cell lines, KYSE150 cells have the lowest expression of *BIRC3* with about 7.9 and 2.8 times more than the controls 293T and THLE3, respectively. While the gene inducement of *BIRC3* in KYSE450 cells was most prominent, which was 1106.2 times and 6950.5 times more than the controls. The transcripts analysis confirmed the prediction of the GEPIA2 database (Figure 1B). The above finding suggests that *BIRC3* is induced in esophageal cancer cells to maintain survival.

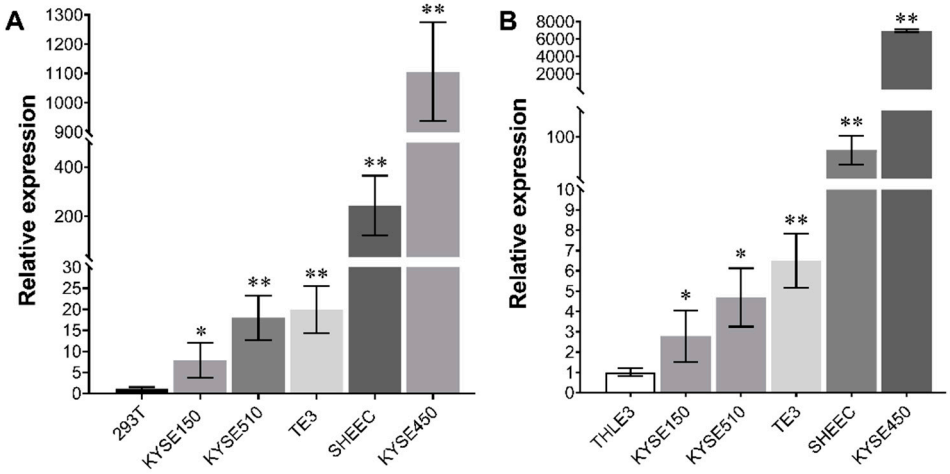

Figure 2. Gene expressions of *BIRC3* in human esophageal cancer cells. Cell lines 293T cells and THLE3 served as controls, respectively. Bars indicate mean ± SD. * and ** shows significant difference between groups $p < 0.05$ and $p < 0.01$, analyzed according to one-way ANOVA, respectively. Gene relative expressions of *BIRC3* in ESCA compared with the control cells 293T (**A**) and THLE3 (**B**).

3.3. TNFα Encourages Expression of the BIRC3 *Gene in Esophageal Cancer Cells*

Previous reports showed that *BIRC3* could negatively regulate NF-κB signaling activation [21]. TNFα is a common stimulator of NF-κB signaling. To validate whether the activation of NF-κB could affect the expression of *BIRC3*, mRNA levels in five kinds of esophageal cancer cells treated with three gradient concentrations of TNFα were measured by qPCR analysis. Figure 3A showed that after TNFα-stimulation, expressions of *BIRC3* in five kinds of cells increased significantly. Among the three concentrations, 30 ng/mL TNFα had the best-induced effects on *BIRC3* gene expression in five kinds of esophageal cancer cells. At the same time, the expression of *BIRC3* in SHEEC cells treated with different TNFα had the highest increase, enhanced more than 9.0-fold.

Further, after NF-κB was inhibited with Bay 11-7082, the effects of TNFα stimulation on *BIRC3* expression declined noticeably (Figure 3B). In KYSE510 and SHEEC cell lines, the increased degrees of mRNA of *BIRC3* levels from 4.2-fold and 9.0-fold to 1.2-fold and 1.3-fold, respectively. While in KYSE150, KYSE450, and TE3, the expression of *BIRC3* even decreased to 68.9%, 52.5%, and 70.6%, respectively. The results implicated that *BIRC3* induced by TNFα was through the NF-κB pathway.

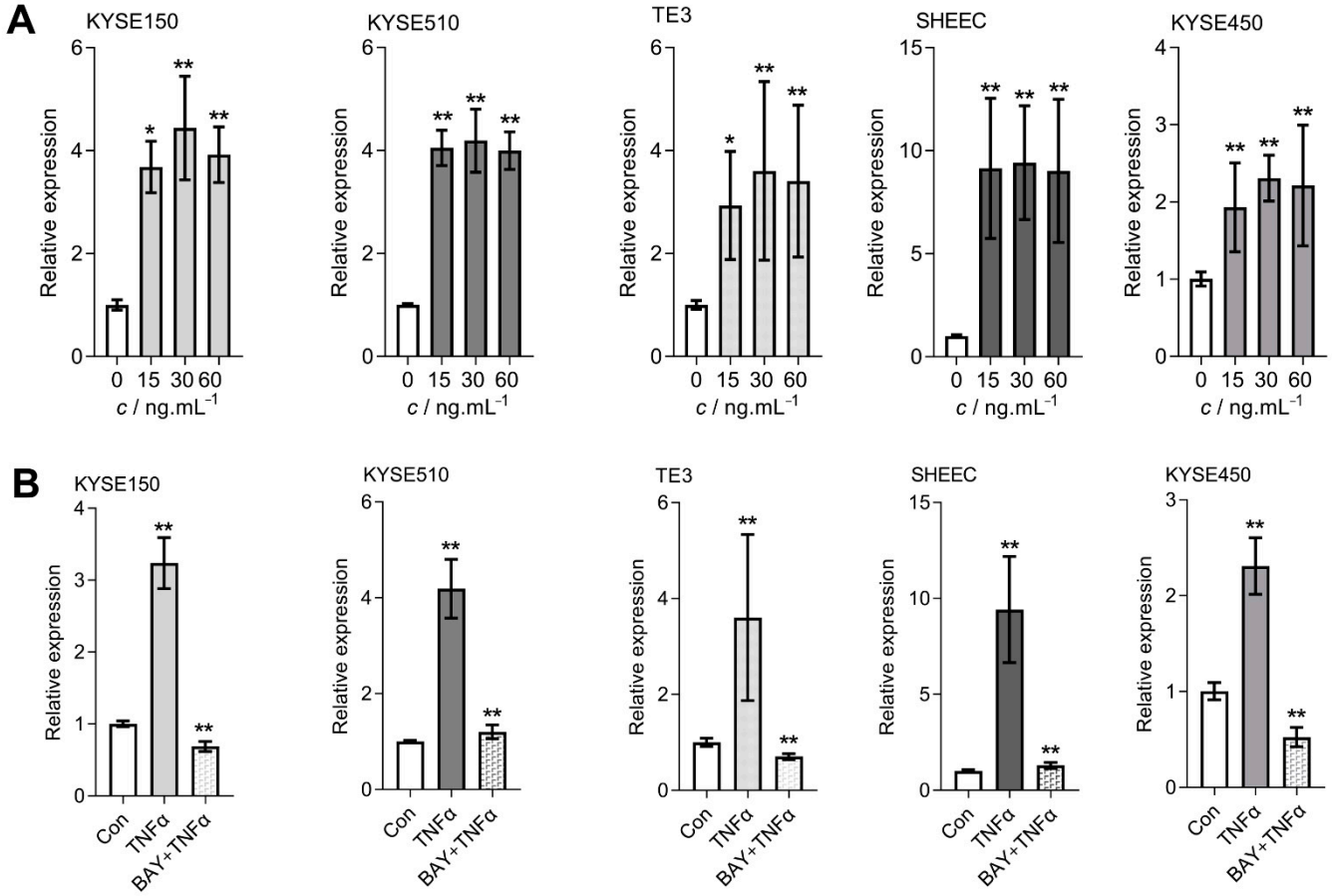

Figure 3. *BIRC3* up-regulated by TNFα. Relative expression of *BIRC3* gene in different concentrations of TNFα-treated cells (**A**) and 50 μmol/L Bay 11-7082 and 30 ng/mL TNFα combined treated cells (**B**). The mRNA relative expression of *BIRC3* was normalized to that of the housekeeping gene *GAPDH*. The data were presented as the means ± SD, * and ** indicate $p < 0.05$ and $p < 0.01$, analyzed according to one-way ANOVA, respectively.

3.4. IL-1β Encourages the Expression of the BIRC3 Gene in Esophageal Cancer Cells

Relative expression of the *BIRC3* gene in KYSE150, KYSE510, TE3, and SHEEC cells treated by IL-1β was determined by qPCR. As shown in Figure 4A, IL-1β up-regulated the expression of the *BIRC3* gene in four kinds of esophageal cancer cells. However, various concentrations of IL-1β had different effects on *BIRC3* expression in different esophageal cancer cell lines. In KYSE150, a higher concentration of IL-1β (40 ng/mL) did not affect the mRNA level of *BIRC3* (not significantly, $p > 0.05$), while a lower concentration of IL-1β (10 ng/mL and 20 ng/mL) could promote *BIRC3* expression to 1.44-fold and 1.64-fold, respectively. The effects of IL-1β on mRNA levels of *BIRC3* in KYSE510 and TE3 were similar, whereby the low concentration of IL-1β (10 ng/mL) had no effect on *BIRC3* expression, and medium concentration (20 ng/mL) of IL-1β increased mRNA of *BIRC3* to its highest level, which was 4.27-fold and 2.64-fold, respectively. The *BIRC3* expression in SHEEC was up-regulated by IL-1β, and encouraged mostly by 20 ng/mL IL-1β promoted to 19.28-fold more than the untreated control.

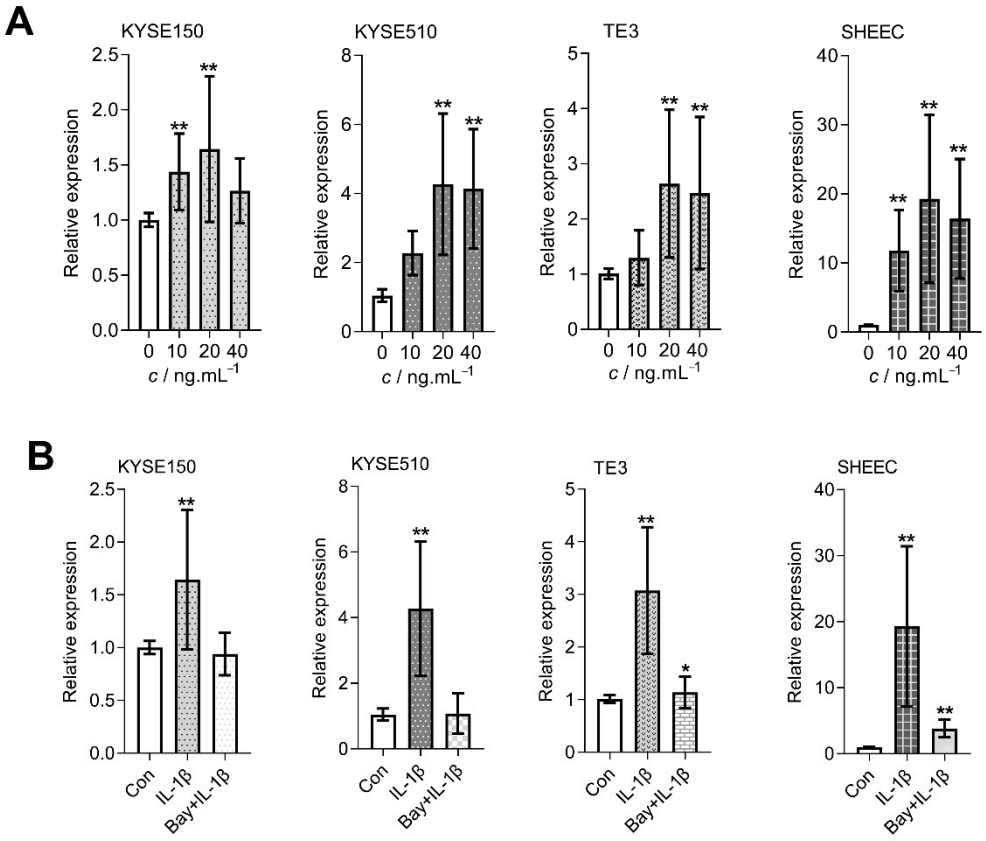

Figure 4. The mRNA of *BIRC3* stimulates by IL-1β. Relative expression of *BIRC3* gene in different concentrations of IL-1β-treated cells (**A**) and 50 μmol/L Bay 11-7082 and 20 ng/mL IL-1β combined treated cells (**B**). The mRNA relative expression of *BIRC3* was normalized to that of *GAPDH*. The data were shown as the means ± SD, * and ** indicate $p < 0.05$ and $p < 0.01$, analyzed according to one-way ANOVA, respectively.

We also examined the mRNA levels of *BIRC3* in KYSE150, KYSE510, TE3, and SHEEC cells treated with 50 μmol/L Bay 11-7082, and 20 ng/mL IL-1β combination. The results showed the inducing effect of IL-1β on *BIRC3* gene expression was inhibited by Bay 11-7082 (Figure 4B). When NF-κB was suppressed, and even IL-1β was added, in KYSE150 and KYSE510, the mRNA level of *BIRC3* had no changes, and in TE3 and SHEEC cell lines, the expression of *BIRC3* declined from 3.1-fold and 19.3-fold to 1.1-fold and 3.8-fold, respectively. The results showed that the *BIRC3* gene was up-regulated by IL-1β, but also depended on the NF-κB transcription factor.

3.5. BIRC3 Regulated by the NF-κB Transcription Factor

The related genes of *BIRC3* were predicted via GeneMANIA. The twenty best-matched genes are listed in Table S1. Among them, four transcription factors (TFs) were predicted, including NF-κB TF. In addition, several genes involved in the NF-κB regulation pathway were found, such as TRAF1, TRAF2, TRAF3, and IKBKB (Figure S1). To further reveal the relationship between *BIRC3* and NF-κB, the NF-κB/RelA binding sites at 1500 bp upstream of *BIRC3* (chr11: 102,315,950–102,339,394, and GRCh38/hg38) were analyzed by UCSC (Table 1). The results showed that there are eight NF-κB/RelA bound sites in the *BIRC3* promoter region. Among them, two in the sense strand and six in the reverse strand. This indicates that *BIRC3* is regulated through the NF-κB transcription pathway.

Table 1. Analysis of NF-κB bound sites of *BIRC3* promoter in UCSC database.

Factor Name	Position (Strand)	Core Match	Matrix Match	Sequence	Matrix Identifier
c-Rel	99 (−)	0.785	0.830	GTAAAtgccg	V$CREL_01
NF-κB (p65)	149 (−)	1.000	0.983	GGAAAtcccc	V$NFKAPPAB65_01
NF-κB (p65)	162 (+)	0.867	0.876	tgggtTTGCC	V$NFKAPPAB65_01
c-Rel	199 (+)	0.758	0.811	tggttATTAC	V$CREL_01
NF-κB (p65)	213 (−)	1.000	0.839	ggagtTCCCC	V$NFKAPPAB50_01
c-Rel	235 (−)	1.000	0.879	GGAAAgcacc	V$CREL_01
c-Rel	275 (−)	0.952	0.788	GGAACgcctg	V$CREL_01
c-Rel	353 (−)	1.000	0.849	GGAAAaggcc	V$CREL_01

The special binding of the NF-κB to the *BIRC3* gene was assessed via chromatin affinity purification. ChIP with a RelA TF (p65) antibody was performed on a protein extract derived from SHEEC cells (Figure 5A). The NF-κB transcript factor was shown to be able to bind to the promoter of the *BIRC3* gene of both 84 bp and 117 bp intron fragments. At the same time, the acetylate histones H3K27Ac (H3) were also found interacting with the *BIRC3* gene fragments 84 bp and 117 bp (Figure 5B). When SHEEC cells were treated with 30 ng/mL TNFα, the interaction between the NF-κB and *BIRC3* gene 84 bp and 117 bp fragments increased 15.8- and 16.9-fold compared with negative controls, respectively (Figure 5C,D). In addition, the target 84 bp and 117 bp of the *BIRC3* promoter DNA sequence was respectively 18.9- and 15.7-fold enriched after affinity purification with the p65 antibody in SHEEC cells after treatment by IL-1β (Figure 5E,F). These indicated that *BIRC3* is regulated by NF-κB TF.

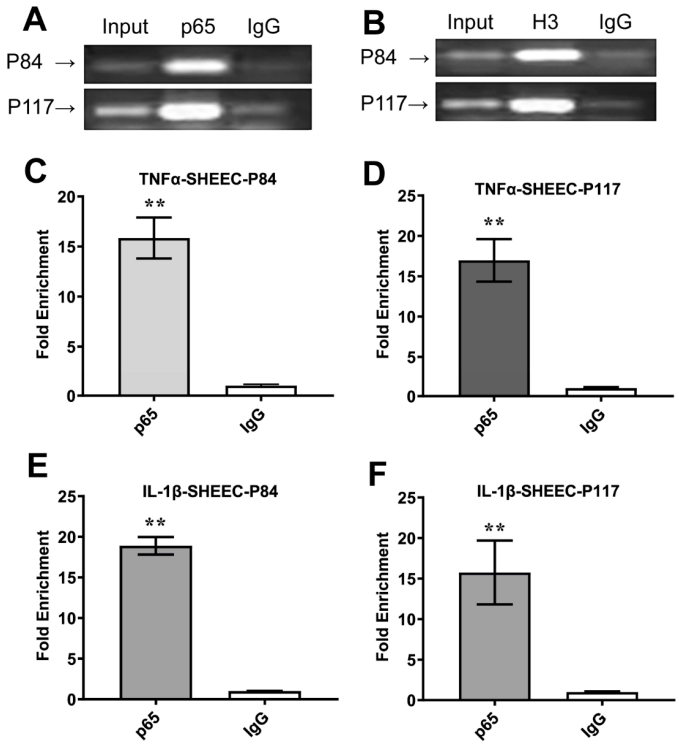

Figure 5. ChIP analysis of NF-κB TF binding to the *BIRC3* gene. In vivo NF-κB (**A**) and acetylate histones H3K27Ac (**B**) binding to the *BIRC3* gene in SHEEC cell lines. Diluted samples from ChIP inputs were analyzed by PCR in parallel (lane 1), and lane 3 showed the negative control in the presence of rabbits IgG. H3 indicated the addition of acetylate histones H3K27Ac. (**C**,**D**) and (**E**,**F**), the fold enrichment of *BIRC3* genes interacted with NF-κB compared with IgG in SHEEC cell lines treated by 30 ng/mL TNFα and 20 ng/mL IL-1β, respectively. P84 and P117 indicated the length of *BIRC3* fragments. ** shows $p < 0.01$.

4. Discussion

ESCA has emerged as a major health issue in Asian countries, especially in China, due to its unclear pathogenesis and high death rate. *BIRC3* (cIAP2) is a member of the inhibitor apoptosis (IAP) protein family, which controls survival and cell death through regulating caspases and apoptosis [22]. Commonly, *BIRC3* acts as a pro-oncogenic protein, which helps cancer cells to avoid undergoing apoptosis. In different kinds of tumors, high expression of *BIRC3* was detected to contribute to osteosarcoma growth and to reduce apoptosis [9]. In this study, TNMplot analysis showed *BIRC3* increased in many kinds of cancers (Figure 1A), which implied that *BIRC3* is an important ingredient in the process of cancer. GEPIA2 bioinformatics analysis (Figure 1B) also showed *BIRC3* to be positively associated with esophageal cancer growth. As early as 2004, cIAP2 (*BIRC3*) was demonstrated to have a higher expression in esophageal cancer tissue than in normal mucosa; although, the differences were not significant [23]. Now, 18 years later, the esophageal cancer cell lines can be well classified. Here, in this study, the expression of *BIRC3* was promoted obviously in all five kinds of esophageal cancer cells (Figure 2). It confirmed that *BIRC3* plays a major role in ESCA pathogenesis. However, the high expression of *BIRC3* was positively related to the survival rate of ESCA patients (Figure 1C,D). *BIRC3* duplications were detected in (Chronic lymphocytic leukemia) CLL patients [24], and genetic inactivation or down-regulation of *BIRC3* had unfavorable clinical contribution in cancer patients. All indicated that the genetic modulation of *BIRC3* will have comprehensive effects when designing ESCA therapeutic approaches.

BIRC3 plays a role in protecting cells from apoptosis when exposed to proinflammatory cytokines. It has been demonstrated that *BIRC3* protein expression increased noticeably in primary nasal epithelial cells when exposed to IFN-γ or TNF-α [25]. Our discovery also revealed that *BIRC3* was up-regulated by cytokines such as TNFα and IL-1β (Figures 3 and 4), which confirmed that *BIRC3* was the key mediator in inflammatory and immunity processes. Previous studies have shown that the TNFα-initiated apoptosis in tumors was suppressed by NF-κB via activating a group of gene targets, such as inhibitor-of-apoptosis (IAP) proteins c-IAP1 and c-IAP2 (*BIRC3*) [26]. Proinflammatory cytokines TNFα and IL-1β both act in apoptosis promotion and activation of transcription factor NF-κB. Here, in all ESCA cell lines, *BIRC3* was noticeably stimulated by TNFα, and suppressed by the NF-κB inhibitor bay 11-7082 (Figure 3). It is evidenced that TNFα-induced killing is somehow inhibited by the *BIRC3* protein, which is activated through NF-κB activation.

The NF-κB signaling pathway was evidenced as an important regulatory mechanism in esophageal cancer. The ESCC could intervene by modulating genes involved in the NF-κB signaling pathway. As referred before, overexpressed FSTL1, which was an upstream stimulator of NF-κB, abolished the promoting effect of lncRNA on cell invasion, and further resulted in the low survival of ESCC patients [27]. *BIRC3* was a downstream regulator in the NF-κB pathway. As referred previously, *BIRC3* was one of the genes of the regulatory complex in the noncanonical NF-κB pathway [21]. Transcription factors regulated downstream genes often by occupying their promoter sites. Here, so many subunits of NF-κB transcription complexes, such as p65 (RelA) and c-Rel subunits, were found in the *BIRC3* promoter (Table 1). Most importantly, as expected, NF-κB TF appeared to interact with *BIRC3* gene fragments (including upstream and intron sequences). Finally, ChIP results confirm that *BIRC3* is regulated by NF-κB (Figure 5). An enrichment of acetylated histones H3 was found around the *BIRC3* promoter (Figure 5B), which suggests that NF-κB-enhanced expression of *BIRC3* may occur by promoting histone acetylation in ESCA, just like that in breast cancer [28]. Activated NF-κB and up-regulated IAPs (*BIRC2* and *BIRC3*) in ovarian cancer cells might mediate chemotherapy resistance [29]. Here, the stimulated NF-κB pathway could increase the expression of *BIRC3* in ESCA and may provide a clinical therapeutic basement. Nevertheless, there must be other possible mutual accommodation between *BIRC3* and NF-κB, and the increased NF-κB level partly resulted from the expression of the *BIRC3* elevated level in ESCA. Further mechanisms of *BIRC3*/NF-κB signaling in ESCA will undoubtedly need to be revealed.

Based on the above findings, as well as the previous literature [30], a putative model of how the molecular mechanism of *BIRC3* modulates esophageal tumor cell apoptosis via the NF-κB pathway was proposed (Figure 6). cIAPs are crucial regulators of canonical and non-canonical NF-κB activation pathways. There is a positive feedback loop between *BIRC3* and NF-κB [31], the expression of *BIRC3* was induced by NF-κB (Figures 3 and 4), and *BIRC3* promotes NF-κB activation. In the presence of proinflammatory cytokines, NF-κB was activated to suppress apoptosis through a canonical and non-canonical pathway [14]. Previous studies have shown that in the case of TNFα, TRAF2 and *BIRC3* were activated via the NF-κB pathway to suppress apoptosis via inhibiting caspase-8 [26]. As one of the coding products of the canonical NF-κB pathway stimulated by both TNFα and IL-1β, the increased expression of *BIRC3* could be inhibited when NF-κB was suppressed. The NF-κB inhibitor verified this notion. When the phosphorylation of IκBα was blocked by bay 11-7082, it caused the failure of NF-κB stimulation, and the gene expression of *BIRC3* immediately declined to a constitutive expression normal level (Figures 3 and 4). *BIRC3* is also famous due to its function of proteasome degradation of MAP3K14, the major driver of activation of the non-canonical NF-κB pathway [32]. This study demonstrates that *BIRC3* is a promoter of the NF-κB regulatory mechanism in ESCA to help cells withstand apoptosis. Therefore, further studies, in particular, to suppress both *BIRC3* and NF-κB in ESCA patients, can yet be regarded as a new attempt in treatment.

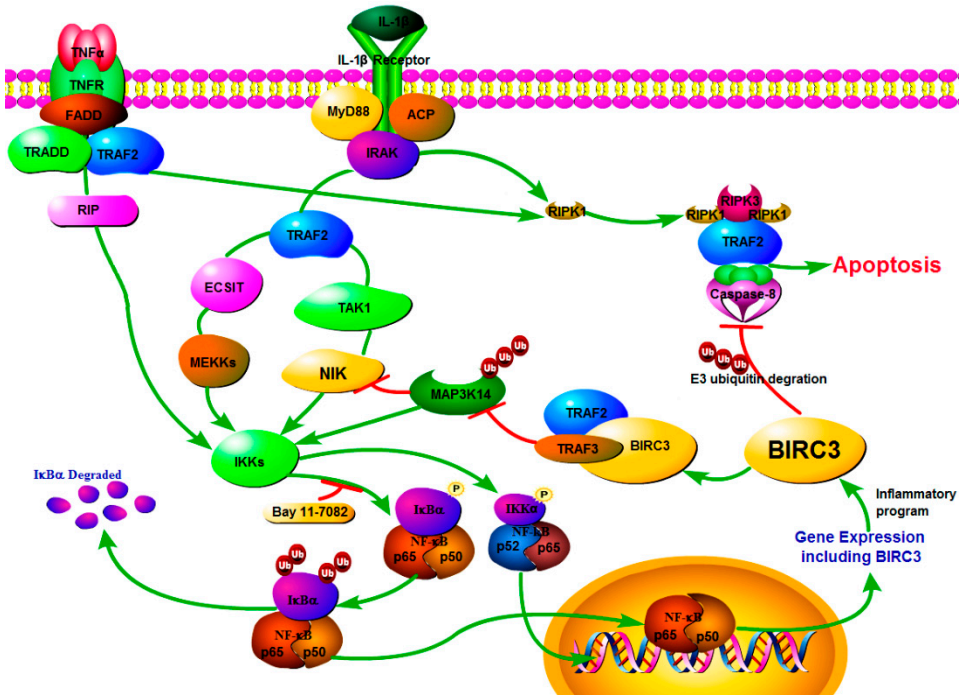

Figure 6. Putative *BIRC3* roles in the NF-κB pathway regulated by TNFα and IL-1β in esophageal cancer cells. The pathway map was constructed by Pathway Builder Tool 2.0.

5. Conclusions

In summary, our findings have shed some light on the regulation of *BIRC3* in ESCA. *BIRC3* was significantly induced in ESCA cell lines. Proinflammatory cytokine TNFα and IL-1β had promotive effects on *BIRC3* expression via the NF-κB transcription pathway in ESCA. In particular, an in-depth disclosure of *BIRC3* up-regulated through the NF-κB pathway in ESCA was uncovered. NF-κB directly interacts with CIS-elements of the *BIRC3* gene to regulate its expression. Our data provided targeted *BIRC3*/NF-κB/as a relevant therapeutic agent of ESCA. Combined inhibition of *BIRC3* and NF-κB may have clinical potential for ESCA treatment.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pr10091673/s1, Figure S1: The relationship of *BIRC3* and its related protein predicted through GeneMANIA software, Table S1: *BIRC3*-related genes predicted by online servers of GeneMANIA.

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