Original Article

ADAM17 and NF-κB p65 form a positive feedback loop that facilitates human esophageal squamous cell carcinoma cell viability

Lin Gao1,2*, Hongbin Liu3,4*, Rong Xu5,6, Jianwei Qiu5,6, Xiao Peng1,2, Yanmei Yang5,6, Dongmei Zhang1,2, Junbo Qian5,6

1Medical Research Center, Affiliated Hospital 2 of Nantong University, Nantong 226001, China; 2Medical Research Center, The First People’s Hospital of Nantong, Nantong 226001, China; 3Department of Pathology, Affiliated Hospital 2 of Nantong University, Nantong 226001, China; 4Department of Pathology, The First People’s Hospital of Nantong, Nantong 226001, China; 5Department of Gastroenterology, Affiliated Hospital 2 of Nantong University, Nantong 226001, China; 6Department of Gastroenterology, The First People’s Hospital of Nantong, Nantong 226001, China. *Equal contributors and co-first authors.

Received February 9, 2020; Accepted May 10, 2021; Epub July 15, 2021; Published July 30, 2021

Abstract: A Disintegrin and metalloproteinase 17 (ADAM17) was proposed to cooperate with NF-κB p65, promoting tumorigenesis and progression of several human cancers. However, the role of ADAM17 remains unknown in human esophageal squamous cell carcinoma (ESCC). In this study, gene expression analyses and cell viability assays suggested that knockdown of ADAM17 suppressed ESCC cell viability. Gene expression analyses and ChIP-qPCR revealed that NF-κB p65 positively regulated ADAM17 expression by binding to the ADAM17 promoter. Rescue experiments showed that overexpression of ADAM17 in NF-κB p65-depleted ESCC cells restored cell viability. In addition, western blot analyses and ChIP-qPCR indicated that ADAM17 was responsible for the persistent activation of NF-κB p65 and contributed to ADAM17 expression in ESCC cells. In conclusion, we propose that ADAM17-activated NF-κB p65 signaling positively regulates ADAM17 expression, and facilitates ESCC cell viability.

Keywords: ADAM17, NF-κB p65, ESCC, cell viability

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most frequently diagnosed digestive cancers, and the sixth most common cause of cancer-related death worldwide [1]. Although advances have been made in treatments for ESCC, the poor prognosis remains largely disappointing, with a 5-year survival rate less than 15% [2]. Therefore, it is necessary to explore the molecular mechanisms of ESCC pathogenesis and develop targeted therapy.

A Disintegrin and metalloproteinase 17 (ADAM17), also termed tumor necrosis factor-α converting enzyme (TACE), is an ADAM family member that cleaves tumor necrosis factor-α (TNF-α) and causes proteolytic release of several other cell-surface protein [3-5]. Multiple studies show that ADAM17 plays crucial roles in several human tumors, such as glioma, lung cancer, hepatocellular carcinoma and ovarian cancer [6-9]. Despite proved high expression in ESCC [10, 11], the underlying molecular mechanisms of ADAM17 in ESCC progression remain unclear.

NF-κB p65 subunit, also known as RelA, is an important member of NF-κB family that has been extensively studied in human cancers over recent years. Several studies reveal that NF-κB p65 is implicated in ESCC cell viability [12-14]. However, the regulatory mechanisms are not fully understood.

Emerging research revealed that the NF-κB p65 signaling pathway might cause the constitutive expression of ADAM17 [15, 16]. In contrast, ADAM17 was put forward as a contributor to NF-κB p65 signaling [15]. However, the complex
ADAM17 facilitates ESCC cell viability

Table 1. Primers for qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5′-3′)</th>
<th>Reverse (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>CATGTACGGTTGCTATCCAGGC</td>
<td>CTCCTTAAATGTCAGGCTAGAT</td>
</tr>
<tr>
<td>ADAM17</td>
<td>GGTGGGATAGAAGAAGAAGTGA</td>
<td>CGACAGGTGTCGTGTTCCAGATA</td>
</tr>
<tr>
<td>PCNA</td>
<td>GAGGAGGAGCTGTTACCATTAGGA</td>
<td>ACTGAAGGACCGTGAAGAGAGA</td>
</tr>
<tr>
<td>KI67</td>
<td>AAGAAGAAGACATTACGCCAGTACT</td>
<td>CGTCCAGCTAGTCTAGGAAAGTA</td>
</tr>
<tr>
<td>BIM</td>
<td>TCCCTACAGACACAGGCACAGAG</td>
<td>CCTACCTCGGTGATTGCCTTC</td>
</tr>
<tr>
<td>TRAIL</td>
<td>GAGCCTGGAAGCACATGCAAGA</td>
<td>TGACCGAGGGTCCACTTGACTTG</td>
</tr>
<tr>
<td>BCL2</td>
<td>CTGGGAGAAGACGGTACGAATAC</td>
<td>CCTCGGAGACGGCTTATAGGAT</td>
</tr>
<tr>
<td>MCL1</td>
<td>AGAGGAGGAGGACCAGGTGTA</td>
<td>AGACCTGCCATGGCTTTTG</td>
</tr>
</tbody>
</table>

interrelationships between NF-κB p65 and ADAM17 in ESCC remain unknown.

In this study, a putative NF-κB p65-binding site existing in the ADAM17 promoter was found by the JASPAR website. Thus, we hypothesized that ADAM17 and NF-κB p65 might form a positive feedback loop and facilitate cell viability of human ESCC cells.

Materials and methods

Cell culture

Human ESCC cell lines TE-1 and Eca109 were purchased from American Type Culture Collection (ATCC). All cell lines were maintained in RPMI 1640 medium (Gibco), supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin, and incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Transfection

All the plasmids were purchased from Oligobio Co., Ltd (Beijing, China). All the siRNAs were designed and generated by TsingKe Biological Technology (Beijing, China). The plasmids or siRNAs were transfected by using Lipofectamine 2000 reagent (Invitrogen) with the recommended protocol. The sequence of ADAM17 siRNA was sense, 5′-CUAUAGACCCAUAGA-3′ antisense, 5′-ACUUCUGAUCACUCUUGTT-3′. The sequence of NF-κB p65 siRNA was sense, 5′-GACAUGUGAUGUGUUUCATT-3′ and antisense, 5′-UGAAAUAACCUAUGUUTT-3′.

Cell viability assay

For cell counting kit-8 (CCK-8) assay, ESCC cells were inoculated in a 96-well plate. Cells at 50% confluence were transfected with siRNAs or plasmids, followed by incubation for 24 h, 48 h, or 72 h. As previously described [17], cells were subjected to a treatment with CCK-8 (Dojindo Laboratories, Kumamoto, Japan). The absorbance was measured at 450 nm by using a Synergy H1 Microplate Reader (Bio-Tek).

RNA extraction and qRT-PCR

In brief, the total RNA from cultured cells was extracted with TRIzol reagent (Invitrogen). After reverse transcription (TaKaRa, Japan), 2× QuantNova SYBR Green PCR Master Mix (QIAGEN) was applied to analyze the relative levels of mRNAs. β-actin was used as the internal control. The fold change relative to controls was calculated by using 2−ΔΔCt method. The primer sequences are listed in Table 1.

Protein extraction and western blot

To obtain total protein, cells were lysed in RIPA buffer (Beyotime) containing 0.5 mM PMSF (Sigma) and 1× phosphatase inhibitor (Roche). Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) was applied for nuclear protein extraction with the recommended protocol. β-actin and H3 were used as internal controls for the total and nuclear proteins, respectively. The following antibodies were used: anti-β-actin antibody (Proteintech, 66009-1-lg, 1:5000), anti-H3 antibody (Proteintech, 17168-1-AP, 1:2000), anti-ADAM17 antibody (Abcam, ab2051, 1:1000), anti-NF-κB p65 antibody (Wanleibio, WL01980, 1:500), anti-P-NF-κB p65 antibody (Wanleibio, WL02169, 1:500), anti-PCNA antibody (CST, #13110, 1:1000), HRP Goat Anti-Mouse IgG (Jackson, 115-035-003, 1:10000), HRP Goat Anti-Rabbit IgG (Jackson, 111-035-003, 1:10000). The grey levels were analyzed by using Image J software.
ADAM17 facilitates ESCC cell viability

Dual-luciferase reporter assay

Dual-luciferase reporter assay was performed to examine the promoter activity of ADAM17. An ADAM17 promoter located from -1600 to -1 bp upstream of the transcriptional start site (TSS) was cloned into pGL3-Basic vector and named pGL3-Basic-ADAM17. Briefly, cells were seeded in a 96-well plate. Cells at 50% confluence were co-transfected with pGL3-Basic-ADAM17 (500 ng), or its corresponding negative control vector, and pRL-TK (25 ng), and meanwhile co-transfected with si-NF-κB p65 (50 nM) or a scrambled control. A dual-luciferase reporter assay system (Promega) was applied to assess the relative luciferase activity.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was carried out by using Pierce™ Agarose ChIP Kit (Thermo, 26156). The ChIP-grade anti-NF-κB p65 antibody was purchased from CST (#6956, 1:50). Immunoprecipitated DNA was analyzed by real-time PCR with 2× QuantiTNG SYBR Green PCR Master Mix (QIAGEN). The putative NF-κB-binding sites were speculated on by the JASPAR website and the primers were generated by TsingKe Biological Technology (Beijing, China). The results from three independent experiments were averaged and plotted as percentage of input. Primer sequences are listed in Table 2.

Statistical analysis

Statistical analyses, including Student’s t-test and one-way ANOVA, were performed by using GraphPad Prism 8.0 (GraphPad Software). The experiments were repeated three times independently. Data are presented as mean ± SEM of three independent experiments. $P < 0.05$, $P < 0.01$ and $P < 0.001$ represented different degrees of statistical significance.

Table 2. Primers for ChIP-qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAM17</td>
<td>AAACAGCAACATGTACCCAACGG</td>
<td>ACTTGGTCCATTAGCTTAGACTGGG</td>
</tr>
<tr>
<td>c-Myc</td>
<td>CTAAGTGATCAGACACGGTCAGG</td>
<td>TGTCCCTGGTGCAAGTAGGTTT</td>
</tr>
<tr>
<td>CyclinD1</td>
<td>CCAGGCTAGAGGACGAGATGAAGG</td>
<td>GCGTCTTGCAAGTGCGCAGGGGG</td>
</tr>
<tr>
<td>MMP2</td>
<td>GTAGGCAAAGTGACCTTCTCAGT</td>
<td>GTAAGCACCTAGATACCTCTCT</td>
</tr>
</tbody>
</table>

Results

Knockdown of ADAM17 inhibits ESCC cell viability

To investigate the role of ADAM17 in human ESCC cell viability, TE-1 and Eca109 cells were transfected with siRNAs to either ADAM17 or a scrambled control. We performed qRT-PCR to analyze cell viability-related genes’ mRNA expression in these ESCC cells. At the transcriptional level, knockdown of ADAM17 caused a distinct increase in the transcription of pro-apoptosis genes BIM and TRAIL, and a significant decrease in the transcriptional level of proliferation-related gene PCNA, and anti-apoptosis genes BCL2 and MCL1, but no obvious change in another proliferation-related gene, Ki67 (Figure 1A). We then examined PCNA at the protein level and observed a similar alteration (Figure 1B). To confirm the effect of ADAM17 on ESCC cell viability, we performed a CCK-8 assay. As shown in Figure 1C, knockdown of ADAM17 significantly suppressed TE-1 and Eca109 cell viability.

NF-κB p65 signaling pathway is involved in constitutive expression of ADAM17 in ESCC cells

On the basis of the investigation at the JASPAR website, the predicted NF-κB p65-binding site is located from -847 to -838 bp upstream of the TSS of ADAM17 promoter (Figure 2A). TE-1 and Eca109 cells were co-transfected with a luciferase reporter plasmid containing a human ADAM17 promoter fragment (termed pGL3-Basic-ADAM17), the pRL-TK plasmid and si-NF-κB p65. As shown in Figure 2B, transfection of the pGL3-Basic-ADAM17 generated markedly higher luciferase activity than that of pGL3-Basic vector control, while silencing of NF-κB p65 showed a significantly lower luciferase activity compared with transfection of a scrambled control, indicating that high transcriptional...
ADAM17 facilitates ESCC cell viability

To further confirm this opinion, we depleted NF-κB p65 of TE-1 and Eca109 cells by using siRNA, followed by detection of ADAM17 expression. As seen in Figure 2C, 2D, qRT-PCR and western blot showed that knockdown of NF-κB p65 significantly down-regulated ADAM17 expression at the transcriptional level and the protein level, respectively. ChIP-qPCR showed that the enrichment of NF-κB p65 at the promoter of ADAM17 was significantly decreased in response to the knockdown of NF-κB p65 (Figure 2E), indicating that NF-κB p65 directly regulates ADAM17 transcription in ESCC cells.

**Overexpression of ADAM17 rescues NF-κB p65-depleted ESCC cell viability**

To further confirm the involvement of ADAM17 in NF-κB p65-modulated ESCC cell viability, we performed rescue experiments by overexpressing...
ADAM17 facilitates ESCC cell viability

Figure 2. Knockdown of NF-κB p65 down-regulates ADAM17 expression in ESCC cells. A. The predicted NF-κB p65-binding site in the ADAM17 promoter. B. Relative luciferase activity was analyzed after 72 h post-transfection; n = 10; *** P < 0.001, compared with the pGL3-Basic (500 ng) + pRL-TK (25 ng) + si-Scramble (50 nM) group; ### P < 0.001, compared with the pGL3-Basic-ADAM17 (500 ng) + pRL-TK (25 ng) + si-Scramble (50 nM) group; one-way ANOVA. C. qRT-PCR quantification of the mRNA levels of ADAM17 after 72 h post-transfection of si-Scramble or si-NF-κB p65 (50 nM). ** P < 0.01; Student’s t-test. D. Western blot analysis of NF-κB p65 and ADAM17 protein expression after 72 h post-transfection of si-Scramble or si-NF-κB p65 (50 nM). E. ChIP-qPCR analysis of the binding of NF-κB p65 to ADAM17 promoter after 72 h post-transfection of si-Scramble or si-NF-κB p65 (50 nM). *** P < 0.001; Student’s t-test.

To determine the role of ADAM17 in NF-κB p65 signaling activation in ESCC cells, we depleted
ADAM17 facilitates ESCC cell viability

ADAM17 in TE-1 and Eca109 cells by using siRNA. The total lysate and the nuclear lysate were extracted after 72 h of transfection, respectively. Western blot indicated that silencing of ADAM17 caused remarkably decreased phosphorylation levels of NF-κB p65 and translocation of NF-κB p65 to the nucleus (Figure 4A).

To examine whether ADAM17-activated NF-κB p65 bound to the promoters of its target genes, we silenced ADAM17 in TE-1 and Eca109 cells and carried out ChIP-qPCR. Our data revealed that NF-κB p65 pulled fewer promoter segments of ADAM17, c-Myc, CyclinD1 and MMP2 in response to depletion of ADAM17 in TE-1 and Eca109 cells (Figure 4B). Moreover, qRT-PCR indicated that knockdown of ADAM17 caused a significant decrease in the transcriptional level of c-Myc, CyclinD1, and MMP2 in ESCC cells (Figure 4C).

Discussion

As a type of esophageal cancer, ESCC affects 0.15% of the population [18]. Despite of the efforts made about the therapy and prevention of ESCC, patients still suffer from poor prognosis. NF-κB signaling pathway plays a crucial role in the development of ESCC, including tumor growth, angiogenesis, and metastasis [19], while the implicated molecules remain largely unclear.

In recent years, multiple studies revealed that NF-κB p65 is involved in the progression of human ESCC [20, 21]. However, the underlying NF-κB p65-dependent proteins remain to be elucidated. As a downstream protein of NF-κB p65 signaling, ADAM17 has been proposed to play essential roles in nasopharyngeal carcinoma and oral squamous cell carcinoma [15, 16]. Moreover, ADAM17 was markedly upregulated in comparison with those in adjacent normal tissues as previously described [10, 11]. Thus, we suspected that a similar molecular process might exist in ESCC cells and play a decisive role in cell viability.

To determine the effect of ADAM17 on ESCC cell viability, we analyzed the expression of apoptosis-related genes, including BIM, TRAIL, BCL2, and MCL1, that were related to ESCC cell viability as previously described [22-25], as well as two proliferation-associated genes PCNA and Ki67, at mRNA levels. Our data indicated a great increase in the expression of BIM and TRAIL, as well as a significant decrease in the expression of BCL2, MCL1, and PCNA at mRNA levels in ADAM17-depleted TE-1 and Eca109 cells (Figure 1A). Likewise, we observed a similar decrease in PCNA protein (Figure 1B). Moreover, CCK-8 assay showed a distinct decrease in TE-1 and Eca109 cell viability, respectively (Figure 1C). Overall, these data suggest that ADAM17 is vital for ESCC cell viability.
To test whether ADAM17 was regulated by NF-κB p65 signaling pathway in ESCC cells, we focused on ADAM17 constitutive expression. Numerous experiments collectively demonstrated the significant role of NF-κB p65 in ADAM17 expression at varying levels (Figure 2). Furthermore, rescue experiments highlighted the important role of ADAM17 in NF-κB...
ADAM17 facilitates ESCC cell viability

Figure 5. Schematic diagram of ADAM17/NF-κB p65 feedback loop in ESCC cell survival. NF-κB p65 directly up-regulates ADAM17 expression, and ADAM17 maintains the constitutive activation of NF-κB p65 by unknown pathways, which contribute to ESCC cell viability.

p65-dependent cell survival (Figure 3). Of note, ADAM17 maturation is essential for its catalytic activity and regulated by the NF-κB signaling pathway in oral squamous cell carcinoma [16]. The molecular mechanisms of ADAM17 maturation in ESCC await future investigation, because of limited resources.

Several studies revealed that NF-κB p65 signaling is constitutively activated in ESCC cells [13, 26, 27], raising a possibility that unknown signals could drive NF-κB p65 to enrich in the nucleus. As shown in the previous studies, ADAM17 releases the soluble form of TNF-α or several other cell-surface proteins from the plasma membrane, followed by activating the NF-κB signaling pathway and facilitating the development of human cancers, such as nasopharyngeal carcinoma, glioma, lung cancer, breast cancer, and leukemia [15, 28-31]. Thus, we supposed that a similar molecular pattern might exist in ESCC cells. To test this hypothesis, we depleted ADAM17 in ESCC cells and performed ChIP-qPCR. We found that depletion of ADAM17 negatively regulated the affinity between NF-κB p65 and ADAM17 promoter in TE-1 and Eca109 cells (Figure 4B). As described in previous studies, c-Myc, CyclinD1, and MMP2 are typical target genes of NF-κB p65 in ESCC cells [32, 33]. To verify ADAM17-activated NF-κB p65 signaling in ESCC cells, ChIP-qPCR was carried out to analyze the enrichment of NF-κB p65 at the promoter of these genes and showed similar results (Figure 4B). Despite these data, how ADAM17 activates NF-κB p65 signaling in ESCC cells remains enigmatic. In the previous studies, as two typical substrates of ADAM17, EGFR and VEGFR2 were stimuli for progression of ESCC [34, 35] and proved to activate NF-κB p65 in other disease models [36, 37]. However, the roles of EGFR and VEGFR2 in ADAM17-activated NF-κB signaling in ESCC remain to be elucidated.

In summary, this study reveals a novel molecular mechanism through which ADAM17 and NF-κB p65 form a positive feedback loop to facilitate ESCC cell viability (Figure 5). This positive feedback loop might be a novel therapeutic target for ESCC.

Acknowledgements

This work was supported by Nantong Science and Technology Project (JC2018001, JC2019-031, JC219011); Medical Research Project
ADAM17 facilitates ESCC cell viability

References


ADAM17 facilitates ESCC cell viability


