EGF promotes HIF-1α expression in colorectal cancer cells and tumor metastasis by regulating phosphorylation of STAT3

F.-L. ZHAO, C.-F. QIN

Department of Hernia and Abdominal Wall Surgery, Beijing ChaoYang Hospital, Capital Medical University, Beijing, China.

Abstract. – OBJECTIVE: Hypoxia-inducible factor 1α (HIF-1α) functions importantly in the development of colorectal cancer. HIF-1α is induced by some cytokines and growth factors and is also regulated by another kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathways. Meanwhile, inhibiting HIF-1α expression can inhibit the development of colorectal cancer. The aim of this study was to explore the effect of epidermal growth factor (EGF) on the activation of signal transducer and activator of transcription 3 (STAT3) in human colorectal cancer cells SW480. In addition, the underlying mechanism of the STAT3 signaling pathway in regulating HIF-1α and further affecting tumorigenesis and metastasis was investigated.

MATERIALS AND METHODS: Immunofluorescence and Western blotting were used to detect the activation of STAT3 by EGF in human colorectal cancer cells SW480. SW480 cells were transfected with STAT3 siRNA or treated with STAT3 inhibitor Niclosamide, and then stimulated with EGF to change the expressions of STAT3 and p-STAT3. The expression level of HIF-1α mRNA in SW480 cells was detected by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). In addition, transwell assay and tumor formation experiments were performed to validate whether STAT3 and HIF-1α affected SW480 through EGF.

RESULTS: STAT3 was not activated in SW480 cells in vitro. EGF induced STAT3 activation and enhanced its phosphorylation level, so that it shuttled into the nucleus. Phosphorylated activation of STAT3 was a necessary condition for EGF to induce HIF-1α up-regulation. Both HIF-1α and EGF-induced phosphorylation of STAT3 could significantly promote the proliferation and metastasis of SW480, and enhance tumorigenesis.

CONCLUSIONS: In SW480 cells, EGF regulated HIF-1α through the STAT3 phosphorylation pathway, eventually promoting the occurrence and metastasis of colorectal cancer.

Key Words: STAT3, HIF-1α, Colorectal cancer, EGF.

Introduction

Colorectal cancer is the third most common type of cancer that causes human death. Although colorectal cancer can be treated by surgical resection, radiotherapy and chemotherapy, the prognosis of patients is still poor. In recent years, with the development of small molecule targeted drugs, the survival time of patients with colorectal cancer has been significantly prolonged. Meanwhile, the quality of their life has been improved. Clinical studies have shown that the effective rate of EGFR tyrosine kinase inhibitors (TKIs) therapy in patients with a positive mutation in colorectal cancer receptor (EGFR) is 70%-80% (TKIs). However, the effective rate is only about 10%-20% in patients with wild-type EGFR, suggesting that TKIs treatment still has certain limitations. Even in patients with effective EGFR-TKI initial treatment, acquired resistance will eventually occur with the prolongation of the treatment.

Signal transducer and activator of transcription 3 (STAT3) is a key protein involved in signal transduction and transcriptional activation. Studies have showed that the sustained activation and expression of STAT3 are associated with the occurrence, development and prognosis of multiple malignant tumors. STAT3 can regulate various abnormalities involved in the transcription factor of tumor signaling pathways, such as anti-apoptotic gene B-cell lymphoma 2 (Bcl-2) and cell cycle control genes of Myc and cyclin D1. This may eventually induce tumor cell proliferation and inhibit cell apoptosis. Therefore, STAT3 is recognized as one of the most important oncogenes. Hypoxia-inducible factor 1α (HIF-1α) is the initiating factor for the changes of tumor biological characteristics under hypoxic conditions. Its expression and stability are closely related to the biological characteristics and clinical features of tumors. Previous studies have observed that...
the expression of HIF-1α is associated with the prognosis, recurrence, metastasis and tumor aggressiveness of colorectal cancer9. STAT3, which is a costimulatory factor of HIF-1α, can activate downstream target genes of HIF-1α (such as vascular endothelial growth factor) and drive HIF-1α-dependent tumorigenesis under hypoxic condition10-12. However, the effects of STAT3 and HIF-1α expression in colorectal cancer induced by EGF remain elusive. The aim of this study was to explore the inducing effect of EGF on STAT3 and HIF-1α in colorectal cancer cells, thereby providing new suggestions for the treatment of colorectal cancer.

Materials and Methods

Cell Culture

Human colorectal cancer cell line SW480 was purchased from the Cell Bank of Chinese Academy of Sciences' Type Culture Collection Committee (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) complete medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 100 IU/mL penicillin, 100 μg/mL streptomycin at 37°C in a 5% CO₂ incubator. Antibodies against EGF, EGFR, HIF-1α, STAT3 and p-STAT3 were purchased from Abcam (Cambridge, MA, USA). STAT3 inhibitor Niclosamide and HIF-1α inhibitor 2-Methoxyestradiol were purchased from Selleck (Houston, TX, USA).

Immunofluorescence Assay

SW480 cells were cultured in slides for 24 h. After stretching and attaching to the slides, the cells were washed with phosphate-buffered saline (PBS) and cultured with medium containing 0.1% FBS overnight. On the next day, the medium was replaced by complete medium supplemented with 100 ng/mL EGF and 10% FBS. After 1 h, the cells were rinsed with PBS and fixed with 4% paraformaldehyde for 10 min. Then, they were treated with 0.25% Triton X-100 for permeabilization and blocked with 5% FBS for 1 h. Subsequently, the cells were incubated with primary antibody against STAT3 or p-STAT3 for 2 h. After propidium iodide (PI) cell nuclear staining, the cells were observed under a fluorescence microscope and photographed.

Transfection of STAT3 siRNA

STAT3 siRNA sequence was 5'-CCUGAGUUGAAUUAUCAGCUU-3'. When the confluency of SW480 cells was up to 80%-90%, they were transfected with STAT3 siRNA according to the instructions of Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). Silencing efficiency was verified by Western blot after 72 h of transfection.

Western Blot Assay

After the treatment, the nuclear protein/ cytoplasmic protein and total protein in cells were extracted according to the kit instructions. Briefly, 50 μg of protein sample from each group was separated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skim milk for 30 min, the membrane was incubated with primary antibody against STAT3 or p-STAT3 (diluted 1:1000) at 4°C overnight. β-actin was used as an internal reference. Then, the membranes were washed with Tris-Buffered Saline and Tween 20 (TBST) and incubated with the corresponding secondary antibody at room temperature for 2 h. Protein bands were observed by enhanced chemiluminescence (ECL) (Thermo-Fisher Scientific, Waltham, MA, USA).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Cells were collected, and total RNA was extracted in one step using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary Deoxyribonucleic Acid (cDNA) was synthesized from 500 ng of total RNA, and the template was synthesized according to the instructions of RT-PCR kit (TaKaRa, Otsu, Shiga, Japan). Primer sequences used in this study were as follows: HIF-1α (f: 5'-CTTTCTTGGAAACGAGTGAAAGG-3'; r: 5'-TGAGTAATTCTTCACCCTGCAG-3'), β-actin (f: 5'-AAATCGTGCGTGACATTAA-3'; r: 5'-CTCGTCATACTCCTGCTTG-3'). QRT-PCR results were obtained from an ABI 7900 instrument (Applied Biosystems, Foster City, CA, USA).

Transwell Assay

Cells in the experimental group or the control group were re-suspended in 600 μL of serum-free medium and cultured in the upper chamber of transwell. Meanwhile, 600 μL of complete medium was added to the lower chamber. After incubation at 37°C for 24 hours, cells in the
upper chamber were gently removed with a cotton swab. The cells on the surface of the lower chamber were stained with 0.1% crystal violet and photographed under a microscope.

**Statistical Analysis**

Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA) and GraphPad 6 software (La Jolla, CA, USA) were used for all statistical analyses. Data were expressed by mean ± SD. One-way analysis of variance (ANOVA) was used to compare the differences among different groups, followed by Post-Hoc Test (Least Significant Difference). \( p < 0.05 \) was considered statistically significant.

**Results**

**EGF Promoted STAT3 Activation in Colorectal Cancer Cells SW480**

To determine whether EGF could promote STAT3 activation in vitro, SW480 cells were first treated with EGF. Subsequently, immunofluorescence staining was performed to detect STAT3 activation. Under fluorescence microscopy, we found that activated STAT3 (p-STAT3) in untreated SW480 cells was mainly located in the cytoplasm, and the level of activation was very low. After EGF stimulation, STAT3 in SW480 cells partially shuttled into the nucleus and was activated at a high level (Figure 1A). Meanwhile, no significant change was found in STAT3 expression in the cytoplasm of SW480 cells after EGF stimulation (Figure 1B). Western blot analysis demonstrated that p-STAT3 was lowly expressed in untreated SW480 nuclei, whereas was highly expressed in the nucleus after EGF treatment (Figure 1C). However, STAT3 was highly expressed in the cytoplasm before and after EGF stimulation, showing no significant difference (Figure 1D). Thus, EGF could increase the level of nuclear p-STAT3 and phosphorylation of STAT3.

![Figure 1](image-url)

**Phosphorylation of STAT3 Induced by EGF Enhanced HIF-1α Expression**

To further investigate the effect of STAT3 on SW480, STAT3-siRNA was transfected in cells to
knockdown STAT3. Western blot analysis showed that the protein expression of STAT3 in cells transfected with STAT3-siRNA was significantly decreased when compared with that of the negative control group (p<0.05). This indicated that STAT3-siRNA could significantly block STAT3 expression. After EGF stimulation, si-STAT3 exerted no effect on STAT3 expression, but significantly promoted p-STAT3 expression (Figure 2A). On the other hand, qRT-PCR results revealed that the mRNA expression of HIF-1α in cells transfected with si-STAT3 was significantly lower than that of the negative control group (p<0.05). Although EGF remarkably increased HIF-1α mRNA expression, this effect could be attenuated by si-STAT3 transfection (Figure 2B). Besides, SW480 cells were treated with STAT3 inhibitor Niclosamide. Western blot indicated that Niclosamide had no significant effect on STAT3 expression. However, it markedly inhibited the phosphorylation of
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STAT3. After treatment with Niclosamide, the activation of STAT3 by EGF was also significantly weakened \((p<0.05)\) (Figure 2C). In addition, the effect of EGF on up-regulating the mRNA expression of HIF-1α was remarkably attenuated by Niclosamide \((p<0.05)\) (Figure 2D). The above results proved that p-STAT3 had a function in regulating HIF-1α expression in SW480 cells.

**EGF-Induced Proliferation and Invasion of Colorectal Cancer was Depended on STAT3 and HIF-1α.**

Previous results have shown that EGF promotes the proliferation of SW480 cells. However, after STAT3 knock-down, EGF-induced cell proliferation was significantly attenuated (Figure 3A). After treatment with STAT3 inhibitor Niclosamide, the proliferation of EGF-induced SW480 cells was also weakened (Figure 3B). 2-Methoxyestradiol is one of the specific inhibitors of HIF-1α. After inhibition of HIF-1α by 2-Methoxyestradiol, EGF-induced SW480 cell proliferation was remarkably attenuated as well (Figure 3C). Subsequently, we explored whether the effect of EGF on cell migration was dependent on STAT3 using transwell assay. The results showed that STAT3-siRNA transfection could significantly inhibit the promoting effect of EGF on the invasion of SW480 cells (Figure 3D). At the same time, the administration of Niclosamide and 2-Methoxyestradiol in SW480 cells also significantly inhibited the promoting effect of EGF (Figure 3E). These results indicated that EGF promoted the proliferation and invasion of SW480 cells through the phosphorylation of STAT3 and activation of HIF-1α.
The Promoting Effect of STAT3 and HIF-1α on the Tumorigenesis of Colorectal Cancer was Associated with EGF-EGFR In vivo

To further explore the effect of EGF on tumorigenesis in vivo, we implanted SW480 cells subcutaneously in nude mice. When tumors were formed to the same size, mice in different groups were injected through tail veins with PBS, Niclosamide, and 2-Methoxyestradiol, respectively. It was found that Niclosamide and 2-Methoxyestradiol significantly inhibited tumor formation and the proliferation of SW480 cells (Figure 4A). Meanwhile, the tumor size and weight were significantly less than those of control PBS mice (Figure 4B and 4C). Then the tumors were removed, and the mRNA and protein expressions were detected. QRT-PCR (Figure 4D) and Western blot (Figure 4E) showed that both the mRNA and protein expressions...
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of EGF and EGFR in tumor tissues treated with Niclosamide and 2-Methoxyestradiol were significantly decreased. Therefore, the inhibition of STAT3 phosphorylation and HIF-1α expression could suppress the tumorigenesis of colorectal cancer. Moreover, the tumor-promoting effects of STAT3 and HIF-1α were closely related to EGF.

Discussion

Studies have shown that abnormal activation of STAT3 exists in many malignancies, including leukemia, breast and prostate cancer. Phosphorylation occurs when STAT3 is activated, which then forms a dimer and shuttles into the nucleus to combine with a homologous DNA-binding region. This may induce transcriptional activation. STAT3 tyrosine phosphorylation has been observed in colorectal cancer. Among the seven members of the STAT family, STAT3 and STAT5 have been most widely researched. STAT3 locates in the cytoplasm and can be stimulated by cytokines or growth factors. Under the action of tyrosine kinases, especially just another kinase (JAK) kinases, tyrosine residues of the carboxyl-terminal domain are phosphorylated to activate STAT3. Phosphorylated STAT3 forms homologous or heterologous dimers and shuttles into the nucleus to recognize and bind to the response element of target gene-specific promoter. The inhibitor Niclosamide can suppress DNA replication and selectively reduce the phosphorylation level of STAT3. However, it has no significant inhibitory effect on the activation of other homologs, such as STAT1 and STAT5. RNA interference (RNAi) technology belongs to post-transcriptional gene silencing and it is the most commonly used tool for the research of gene function. Therefore, siRNA and inhibitor Niclosamide act at different levels. STAT3 functions through phosphorylation and de-phosphorylation. The use of siRNA alone cannot fully explain the function of STAT3. Besides, the inhibitor Niclosamide can only inhibit the phosphorylation active form of STAT3. Consequently, the above two ways were combined to inhibit STAT3 expression and phosphorylation, to further explain the regulation of phosphorylated STAT3 on HIF-1α expression.

EGF is an important cytokine that promotes cell proliferation, adhesion and metastasis. After several trials and experiments, we determined that 10 ng/mL of EGF could significantly enhance the invasion and metastasis of SW480 cells. HIF-1α is a transcription factor widely found in mammals and humans under hypoxic condition. During the growth of malignant tumors, excessive tissue hyperplasia can induce local severe hypoxia, eventually leading to an imbalance between energy supply and energy consumption. HIF-1α enhances energy supply by up-regulating the expression of EGF and glycolytic enzymes, indicating that HIF-1α is closely related to tumorigenesis. In this study, we also revealed a positive correlation between HIF-1α and EGF. Our findings suggested a promoting effect of EGF on tumor proliferation. Studies have shown that STAT3 is able to regulate a variety of HIF-1α-related abnormal tumor signaling pathways involved, thereby inducing tumor cell proliferation and blocking cell apoptosis. Therefore, STAT3 gene is recognized as one of the most important oncogenes. Studies have also indicated that STAT3 can directly bind to the promoter of HIF-1α, thereby regulating the expression of HIF-1α. In this study, we found that there was a correlation between STAT3 and EGF expression. Meanwhile, phosphorylated STAT3 could enhance the invasive and migratory abilities of colorectal cancer cells. Thus, blocking the STAT3 signaling pathway could inhibit multiple signaling pathway-mediated tumorigenesis and prevent tumor growth and metastasis. Meanwhile, pSTAT3 and HIF-1α might be effective targets for inhibiting the expression of EGF in colorectal cancer.

Conclusions

In the present study, we found that EGF enhanced the phosphorylation level of STAT3 in colorectal cancer cell line SW480 and elevated the expression of HIF-1α. Furthermore, it ultimately promoted tumorigenesis and tumor invasive ability of colorectal cancer cells.

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Conflict of Interests

The authors declare that they have no conflict of interest.
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