Nuclear-targeted EGF receptor enhances proliferation and migration of human anaplastic thyroid cancer cells

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Abstract

Introduction: Epidermal growth factor (EGF) has various important physiological functions, which it exerts by binding to the epidermal growth factor receptor (EGFR). Reports show that EGF expression is strongly correlated with the occurrence and development of many types of tumour. To date, however, the relationship between EGF/EGFR and the occurrence and development of thyroid carcinoma remains unclear.

Material and methods: In the current study, we investigated this phenomenon using human anaplastic thyroid carcinoma cell lines (SUN-80).

Results: The results indicated that EGF triggered the EGFR-mediated intracellular signalling pathway, including signal transducers and activators of transcription 1/3/5 (STAT1/3/5) and protein kinase B (AKT) in a time- and dose-dependent manner. In addition, results from EGF-induced EGFR internalization and co-localization analyses showed that clathrin, Rab5/7, and EEA1 play critical roles in the intracellular trafficking of EGF/EGFR. Interestingly, EGF triggered EGFR translocation into the nucleus, while nuclear-localized EGFR affected cell cycle distribution, thereby significantly promoting the ratio of S phase. Overall, these findings indicated that nuclear EGFR exerts biological activity and physiological functions, including changing cell cycle, which in turn promotes proliferation and migration of SUN-80 cells.

Conclusion: These findings lay a foundation for further explorations seeking to understand the biological effects of the EGF/EGFR system on the occurrence and development of thyroid cancer.

Key words: cell behaviour; clathrin; EGF; EGFR; thyroid cancer

Introduction

Epidermal growth factor (EGF) is a 6045-dalton polypeptide that plays various important physiological roles in organisms. EGF exerts its functions by binding to the epidermal growth factor receptor (EGFR), a transmembrane glycoprotein that is localized in the nucleus of many tissues and cell lines. EGFR, which belongs to a large family of receptor tyrosine kinases and possesses intrinsic tyrosine kinase activity, comprises extracellular, transmembrane, and intracellular domains, the latter made up of a cytoplasmic tyrosine kinase. Previous studies have shown that EGF binds to EGFR to activate the tyrosine kinase domain, which in turn phosphorylates downstream intracellular signalling pathways, including the protein kinase B (Akt), mitogen-activated protein kinase (MAPK), signal transducer and activator (s) of transcription (STAT), and NF-xB pathways [1]. The activated signalling molecules translocate into cell nuclei where they mediate EGF’s biological functions.

Previous studies have demonstrated that EGFR is localized in the nucleus of a variety of systems and is not only strongly associated with high tissue proliferation but also mediates survival signalling [2]. Both EGF and EGFR form complexes with chromatin [3], while EGF can induce both DNA synthesis and its mitogenic effect [4]. Lin et al. [2] reported that EGF/EGFR could activate gene expression by binding to specific DNA sequences, whereas numerous studies have demonstrated that EGF is closely related to the development of thyroid carcinoma. For example, Mitsiades et al. [5] found significant expression and phosphorylation of EGFR in thyroid carcinoma cell lines, suggesting that EGFR may be a therapeutic target in human thyroid carcinoma. On the other hand, Lee et al. [6] demonstrated that EGFR tyrosine kinase inhibitors (TKIs) significantly inhibited cellular growth and induced apoptosis in the anaplastic thyroid carcinoma (ATC) cell lines. To date, however, the relationship between EGF and occurrence and development of thyroid carcinoma cell
lines remains unclear. It is worth noting that the cell characteristics and cell behaviour are closely associated with the biological activities of growth factors.

In this study, we used SNU-80, a rare anaplastic thyroid cancer cell line obtained from Korean thyroid carcinoma patients, to investigate cellular behaviour of EGF. Our results demonstrated that EGF/EGFR was localized in the nucleus, where it regulated proliferation or migration of thyroid carcinoma cells. Taken together, these findings have great significance in the field of medicine because they lay a foundation for future development of therapies for the treatment of thyroid cancer.

Material and methods

Antibodies, reagents, and cell lines

Anti-phospho-specific STAT5/3, ERK1/2 and AKT, as well as non-phospho-specific STAT5/3, ERK1/2, and AKT, and anti-Ki67 (D3B5) were obtained from Cell Signalling Technology (Boston, USA). EGF was acquired from Peprotech (Rocky Hill, NJ, USA), while anti-EGFR (phospho Y1068) (ab40815) and Total-EGFR (ab52894) antibodies, as well as goat anti-Rabbit IgG H & L (FITC), were purchased from Abcam (UK). Cell culture media, RPMI 1640, and foetal bovine serum (FBS) were obtained from Gibco (Grand Island, USA), while Polyvinylidene fluoride (PVDF) membranes were purchased from Millipore (Bedford, MA, USA). Alexa Fluor 555, Alexa Fluor 488, and secondary antibodies were acquired from Sigma-Aldrich (St. Louis, USA), while enhanced chemiluminescence (ECL), cell lysis buffer (RIPA), and BCA kits were purchased from Pierce (Rockford, USA). All other reagents were obtained from Sigma-Aldrich (USA) unless otherwise stated.

The human anaplastic thyroid carcinoma cell line (SUN-80) was cultured in RPMI 1640 medium supplemented with 10% FBS, 1% streptomycin, and 1% penicillin (Biofluids, Rockville, MD, USA) and maintained in a 100% humidified atmosphere of 5% CO₂ and a temperature of 37°C.

Determination of intra-cellular EGF/EGFR trafficking

SUN-80 cells were cultured in a serum-starved medium for 10 h, then subjected to EGF stimulation using 5 nM EGF for 30 min. The cells were washed 3 times with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (PFA) for 15 min, washed again 3 times with PBS, then observed under a confocal laser scanning microscope (CLSM, Olympus FV1000). Image analysis was performed by using the FV10-ASW 1.7 Viewer and ImageJ software.

Indirect immunofluorescence assay

SUN-80 cells were first starved for 10 h by culturing them in a serum-free medium then stimulated with 5 nM EGF. Next, the cells were washed 3 times with PBS, fixed in 4% paraformaldehyde (PFA) for 15 min, then blocked with 5% bovine serum albumin (BSA) for 1 h. The cells were washed 3 times with PBS, incubated with anti-EGF or anti-Ki67 antibodies, washed again, and incubated with FITC-conjugated secondary antibody at 37°C for 1 h in the dark. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI), washed 3 times with PBS, then observed under a CLSM (Olympus FV1000). Image analysis was performed using FV10-ASW 1.7 Viewer and ImageJ software.

Colocalization analysis

Analysis of EGFR colocalization was performed using Rab5/7 or EEA1, as described by Vanlandingham et al. [7], Lopez-Alcalá et al. [8], and Gosney et al. [9]. Briefly, analysis was carried out using ImageJ software and the colocalization plug-in to generate a binary image of colocalized pixels from 2 separate channels. ImageJ was used to automate the channel thresholding, and colocalization was established for pixels whose intensities were higher than the threshold and for which the ratio of intensity was greater than 50%. The data were expressed as the ratio of integrated intensity from the 2 images, and were presented as averages of 3 separate experiments, with a total of 30 cells measured per experiment.

Western blot analysis

Cells were first stimulated with varying concentrations of EGF (3, 5, 10, 20, and 40 nM) for 30 min, or with 5 nM EGF for different time points (2, 10, 30, 40, and 60 min). Next, total proteins were extracted from the cells using RIPA buffer, and protein concentrations determined using the BCA kit. Equal protein concentrations (20 μg per lane) for each sample were separated on 4% to 10% SDS-PAGE gels, transferred to 0.2 μm PVDF membranes, and the membranes blocked for 1 h with 5% non-fat milk at 37°C. The membranes were washed 3 times then incubated overnight with phospho- or total-EGFR, STAT5/3/1, ERK1/2, and AKT antibodies, as well as goat anti-Rabbit IgG H & L (FITC), which were purchased from Abcam (UK). Densitometric analysis of the immunoreactive protein bands was performed using Quantity One® software (developed by BioRad Technical Service Department, USA; LSG. TechServ. US@BioRad.com).

Analysis of the cell cycle

Cells were first subjected to EGF stimulation and collected by centrifugation for 5 min at 1000 rpm. Prior to EGF treatment, the cells were treated with Pitstop (20 μm), a specific inhibitor that blocks clathrin-mediated endocytosis. The cells were then fixed in 70% ethanol, stored at 4°C for 30 min, and resuspended in propidium iodide (PI) buffer (20 μg/mL RNase A and 50 μg/mL PI) with a 30-min incubation at room temperature in the dark. DNA content in the cells was analysed by fluorescence-activated cell sorter analysis (FACS) (BD, San Jose, CA, USA), with the percentages of cells of the cell cycle at each stage estimated according to the manufacturer’s instructions (BD Biosciences).

Migration assay

Cell migration was analysed using Transwell chambers (Corning, New York, NY), according to the manufacturer’s instructions. Briefly, cells on the upper surfaces of Transwell chambers were removed using cotton swabs, following a 24-hour incubation, and those located on the lower surfaces were fixed in methanol for 10 minutes. Subsequently, the cells were stained with 0.1% crystal violet, then photographed and counted in 5 randomly selected fields under a microscope (Olympus, IX71, Japan).

Cell proliferation assays

The effect of EGF or clathrin inhibitor on cell proliferation was measured using an MTT assay. Briefly, cells were seeded in 96-well plates, and incubated with MTT solution (150 μL, 0.5 mg/mL) at 37°C for 5 h. The medium was aspirated from each well and replaced with 150 μL of DMSO followed by a 15-min incubation at 37°C. Absorbance was then measured at 570 nm using a microplate reader (Thermo Scientific, Multiskan FC, Pittsburgh P K, United States).

Statistical analysis

Statistical analyses were performed using the Statistical Analysis System (SAS) software (SAS version 9.0, Institute Inc., Cary, NC, USA), and all data are presented as means ± standard deviations (SD) of the means. Differences between groups were determined using analysis of variance (ANOVA) with Tukey’s HSD test. p < 0.05 were considered statistically significant.
EGFR-mediated EGF endocytosis
Internalised EGF and EGFR were in the form of EGF-EGFR complex. Previous studies have described multiple pathways of endocytosis into cells, including those that act in clathrin-dependent, caveolin-dependent, and caveolin- and clathrin-independent manners [10]. To further elucidate the mechanism through which EGFR mediates endocytosis of EGF, we performed colocalization assays using CLSM, and detected colocalization signals (yellow) of EGFR (green) and clathrin (red) via confocal microscopy after EGF treatment (Fig. 3A).
We also detected colocalization signals between EGFR with Rab5/7 or EEA1 (Fig. 3B), owing to the important role they play in intracellular transportation of EGF/EGFR. Taken together, these findings indicated that clathrin, Rab5/7, and EEA1 play critical roles in the intracellular trafficking of EGF/EGFR.

Nuclear localization of EGFR in SNU-80 Cells
Previous studies have shown that nuclear EGFR not only plays important physiological functions but is also strongly associated with high proliferation of tissues [2–4]. Although numerous studies have demonstrated the close relationship between EGF and thyroid carcinoma, the association between changes in cellular behaviour of EGF/EGFR with the occurrence and development of thyroid carcinoma remains unclear [5, 6]. To determine whether EGF is translocated to the nucleus and elucidate its potential function, we first treated cells with 5 nM EGF for 2, 10, 30, 40, and 60 min, then observed internalization of EGF in SNU-80 cells. The results showed that small amounts of EGF (green signal) were translocated to the cell nuclei at 2 min (Fig. 4A). EGF’s internalization and nuclear localization increased with time, and nuclear fluorescence signals were found to peak at 30 min. On the other hand, cytoplasmic and nuclear fluorescence signals declined slightly at 60 min. These results indicated that
EGFR internalization was time dependent. However, nuclear fluorescence signals significantly decreased when the cells were treated with clathrin inhibitor (Fig. 4B). Collectively, these results indicate that EGFR is translocated to the nucleus, possibly through the clathrin-mediated pathway.

**Effect of nuclear EGFR on cell cycle distribution in SNU-80 cells**

To determine the function of EGFR localized in the nucleus, we performed cell cycle analysis after stimulating the cells with EGF. The results showed that the percentage of cells in the G1 and S phases was 53.26 and 33.06%, respectively (Fig. 5). However, stimulation of cells using clathrin inhibitor resulted in an increase in cells in the G1 phase (69.59%) and a decrease in those in the S phases (17.75%), because it blocked EGFR from entering the cell nucleus and inhibited its nuclear actions. These results indicate that nuclear EGFR affects cell cycle distribution in SNU-80 cells, while EGFR localization in the nucleus causes the cell cycle to be subsequently...
distributed in the S phase, thereby promoting cell proliferation.

**Nuclear localization of EGFR improves proliferation and migration of SNU-80 cells**

We further explored EGF’s cellular behaviour in SNU-80 cells using MTT assays and found that proliferation of these cells improved after EGF stimulation. However, treatment with clathrin inhibitor significantly suppressed cell proliferation (Fig. 6A). Results from CLSM analyses revealed that Ki67, a proliferation marker in human tumour cells, was significantly downregulated in the cells after treatment with a clathrin inhibitor (Fig. 6B). Transwell chamber assay showed that treatment of SNU-80 cells with EGF improved their migratory capacity (Fig. 6C), although this phenomenon was reversed by exposure to the clathrin inhibitor. These results indicate that EGF promotes cell proliferation and migration. As expected, our results indicate that nuclear localization of EGFR promotes proliferation and migration of SNU-80 cells.

**Discussions**

Epidermal growth factor receptor, a member of the receptor tyrosine kinase family, mediates many essential physiological processes in both cancerous and normal cells. Previous studies have shown that it plays a central biological role in many human cancers, regulating processes such as tumourigenesis and malignancy [11–13]. Additional evidence has demonstrated that EGF is closely related to tumour development, as shown by frequent mutations, activation, and over-expression [14, 15]. To date, however, the relationship between cellular functions and changes of EGF/EGFR and the occurrence and development of thyroid carcinoma remains unclear. It is worth noting that cellular characteristics and behaviour are closely associated with biological activities of growth factors. The results of the present study demonstrate that clathrin, Rab5/7, and EEA1 play critical roles in regulating intracellular trafficking of EGF/EGFR, while EGFR plays important physiological roles in the nucleus of SNU 80 cells. Notably, this EGFR
localization in the nucleus significantly improved proliferation or migration of thyroid carcinoma cell lines.

Previous studies have shown that EGFR is not only widely distributed in the nucleus of a range of cancerous cells and tissues but can also be localized in the inner nuclear membrane [16–19]. EGFR’s most well-known role is its ability to act as a plasma membrane-bound receptor. Specifically, EGF binds to and mediates phosphorylation of EGFR, which in turn recruits and activates downstream signalling molecules to regulate gene expression and exert protein function [20]. In the present study, we found that EGFR is mainly localized in the cell membranes. EGFR phosphorylation is associated with activation of many signalling pathways,
Figure 5. Effect of nuclear epidermal growth factor receptor (EGFR) on cell cycle distribution in SNU-80 cells. **A.** The cells were pre-incubated with clathrin inhibitor and then treated with 5 nM EGF for 30 min; **B.** The cells were stimulated with 5 nM EGF for 30 min. The cells were fixed in 70% ethanol and stored at 4°C for 30 min, then the cells were suspended in propidium iodide (PI) buffer (20 µg/mL RNase A and 50 µg/mL PI) for 30 min in the dark at room temperature; **C.** The cell cycle distributions were analysed by fluorescence-activated cell sorter analysis (FACS) (BD, San Jose, CA, USA), and the percentages of cells of the cell cycle were estimated according to the manufacturer’s instructions (BD Biosciences) in each stage. Data presented are means ± standard deviation (SD) from 3 independent experiments. *p < 0.05 and **p < 0.01

Figure 6. Effect of nuclear epidermal growth factor receptor (EGFR) on proliferation and migration in SNU-80 cells. **A.** SNU-80 cells were treated with EGF or clathrin inhibitor. The effect of EGF or clathrin inhibitor on cell proliferation were assessed by MTT assay. Data presented are means ± standard deviation (SD) (n = 5). All experiments were performed in triplicate. Viability of untreated cells was set to 100%; **B.** The cells were fixed with 4% PFA then blocked with 3% bovine serum albumin (BSA) for 1 h. After washing the cells 3 times with cold phosphate-buffered saline (PBS), they were incubated with anti-Ki67 antibody overnight at 4°C. Subsequently, the cells were treated with Alexa 488-conjugated secondary antibody for 1 h at 37°C in the dark. The cells were observed by confocal laser scanning microscope (CLSM). Scale bar =10 µm; **C.** Transwell assays were used to measure cell migration of SNU-80 cells. The SNU-80 cells were re-plated in serum-free medium after stimulation with EGF or clathrin inhibitor, then layered onto the top chambers and invaded the bottom chamber containing serum-supplemented medium for 24 h at 37°C. The cells that located on the lower surfaces were fixed in methanol for 10 minutes, then the cells were stained with 0.1% crystal violet, photographed, and counted in 5 randomly selected fields. Data presented are means ± SD from 3 independent experiments. *p < 0.05 and **p < 0.01
of which the MAPK/ERK pathway plays an important role in many cellular processes. For example, Emamian et al. [21] reported that hyperphosphorylation of AKT was part of the pathological process among various kinds of human tumours. The results of the present study indicated that EGF activated phosphorylation of EGFR, STAT1/3/5, AKT, and ERK1/2 in a dose- and time-dependent manner.

Previous studies have demonstrated that multiple pathways play a role in cell endocytosis. For example, clathrin-mediated endocytosis was shown to stimulate internalization of many receptors, which play critical roles in growth control, cell differentiation, cellular homeostasis, and synaptic transmission [22, 23]. Other evidence has shown that clathrin-mediated endocytosis is the major pathway regulating EGFR internalization. Particularly, EGFR was internalized via clathrin-coated pits and subsequently routed to early endosomes [24]. The results of the present study show that EGF/EGFR could enter into the cytoplasm through clathrin-mediated endocytosis. Clathrin, Rab5/7, and EEA1 contribute to intracellular trafficking of EGF/EGFR (Fig. 3). Studies have shown that as well as clathrin-dependent endocytosis, clathrin-independent endocytosis also plays critical roles in internalization [25, 26]. For instance, EGFR was found to enter the cytoplasm through micropinocytosis and caveolin-mediated endocytosis [25]. Therefore, the action of other unknown factors cannot be excluded.

Furthermore, the results of the current study demonstrate that EGFR could internalize and be translocated into the nuclei of SUN-80 cells after EGF stimulation in a time-dependent manner. It is possible that EGFR is translocated into the nucleus through the clathrin-mediated pathway. Previous studies have reported that nuclear EGFR is not only a prognostic indicator for poor clinical outcomes but also might mediate physiological processes that aggravate activity of tumour cells [12, 18, 19]. Other evidence has shown that EGFR elevates cell proliferation and enhances tumour cell motility [27], while Andl et al. [28] found that exposure to EGFR promoted cell proliferation and migration in oesophageal keratinocytes. To determine the functions of EGFR localized in the nucleus, we assessed the cell cycle after stimulating SNU-80 cells with EGF and found that nuclear EGFR affected the cell cycle distribution. Moreover, the cell cycle was mainly distributed in the S phase when EGFR localized in the nucleus, a phenomenon that promoted cell proliferation. Additionally, localization of EGFR in the nucleus upregulated Ki67 expression and improved migration in SNU-80 cells.

In conclusion, EGF effectively activated EGFR-mediated intracellular signalling of proteins, such as STAT1/3/5, AKT, and ERK1/2, in thyroid cancer cells. Furthermore, clathrin treatment caused EGFR to internalize into thyroid cancer cells, while EGF was successfully translocated into the nuclei of SUN80 cells. Furthermore, localization of EGFR in cell nuclei resulted in various biological activities and physiological functions, including changing cell cycle and promotion of proliferation and migration of thyroid cancer cells. Overall, these findings have great significance in the field of medicine because they lay a foundation for future development of treatment therapies for thyroid cancer. However, the exact mechanisms underlying EGFR’s effect in SNU-80 cells require further exploration.

Conflict of interest
The authors declare that no conflicts of interest exist.

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References


