Effect of tyrosine kinase inhibitors on cell migration and epithelial-to-mesenchymal transition in Asian head and neck cancer cell lines

Aye Myat Thwe | Peter Mossey | Ian R. Ellis

Abstract

Background: We investigated the role of epidermal growth factor (EGF) and transforming growth factor α (TGFα) on Asian head and neck cancer patient cell lines; in terms of epithelial-to-mesenchymal transition (EMT) and cell migration to determine whether these changes could be reversed using tyrosine kinase inhibitors (Gefitinib and Erlotinib).

Methods: Cell migration, protrusion and EMT were assessed using both Scatter assay and Scratch assay. Protein expression and localisation were evaluated using immunofluorescence, SDS-PAGE and Western blotting techniques to identify the involvement of phosphorylated MAPK (Thr202/Tyr204), phosphorylated EGFR (Y1068) and phosphorylated AKT (Ser473) protein expression.

Results: EGF and TGFα induced an EMT-like phenotypical change, cellular protrusion and cell migration while Gefitinib and Erlotinib blocked these morphological changes and cell migration. We also examined the effect of EGF/TGFα± tyrosine kinase inhibitors on phosphorylation sites Y1068 of epidermal growth factor receptor (EGFR). Y1068 was phosphorylated in all test conditions, and all tested concentrations of inhibitors did not inhibit Y1068 phosphorylation. EGF and TGFα increased phosphorylation of MAPK (Thr202/Tyr204) residues compared with serum-free control while a one-hour pre-treatment with tyrosine kinase inhibitor(s) before addition of growth factors completely blocked this phosphorylation. Phosphorylation of Akt Ser 473 was also induced by EGF and TGFα, and a one-hour pre-treatment with the tyrosine kinase inhibitor(s) reduced this phosphorylation.

Conclusion: These data suggest that Gefitinib and Erlotinib prevent activation of downstream signalling proteins MAPK (Thr202/Tyr204) and Akt (Ser473) thereby blocking phenotypic change and cell migration. This study supports the potential therapeutic value of Gefitinib and Erlotinib in targeting head and neck cancer.

Keywords
Asian cell lines, cell migration, cell motility, epidermal growth factor receptor, epithelial-to-mesenchymal transition
1 | INTRODUCTION

Head and neck cancer is the 6th most common cancer in the world. Alterations to epidermal growth factor receptor (EGFR) such as overexpression and mutation are major mechanisms believed to play an important role in head and neck carcinogenesis. Currently, the only FDA-approved EGFR target drug in head and neck cancer is Cetuximab, a monoclonal antibody therapy which targets EGFR overexpression by binding the extracellular domain and inhibiting ligands from binding. Mutations such as EGFR VIII, EGFR tyrosine kinase domain and EGFR-hetero dimerisation/co-expression with other ErbB receptors are often neglected in terms of therapeutic drug design. EGFR mutations are more common in Asian patients than Caucasians. Patients with an EGFR tyrosine kinase mutations are also resistant to Cetuximab treatment while they do respond to tyrosine kinase inhibitors such as Gefitinib and Erlotinib. Cetuximab resistance was also found in patients with EGFR VIII mutation while addition of tyrosine kinase inhibitors can overcome this resistance. A series of studies also reported that HER2 activation was related with resistance to Cetuximab. In contrast, targeting both EGFR and HER2 with tyrosine kinase inhibitors overcame this resistance. These studies suggest that EGFR mutation, EGFRVIII and receptor dimerisation/co-expression are often related to Cetuximab resistance, and that tyrosine kinase inhibitors have a potential to alleviate this. Gefitinib and Erlotinib are first-generation tyrosine kinase inhibitors which are currently used in non-small cell lung cancer treatment. Tyrosine kinase inhibitors bind to the intracellular domain of EGFR by competing with Adenosine 5’ triphosphate and inhibiting EGFR phosphorylation. None of them have FDA approval for use with head and neck cancer patients. The aim of the project was to study the effect of Gefitinib and Erlotinib on EMT-like phenotypical change, cellular projection and cell migration (Figure 1).

2 | MATERIAL AND METHODS

2.1 Cell lines and culture

The cancer cell lines used in the project were chosen based upon ethnicity rather than location of tumours. TYS (oral adeno squamous cell carcinoma), HSG (epithelial ductal cell line derived from irradiated human salivary gland) and AZA1 (HSG cells treated with 5-Azacytidine) are gifts from Dr Koji Harada and Prof Mitsunobu Sato, University of Tokushima, Japan and are from Asian patients. HaCaT (Normal adult keratinocyte-Caucasian origin) was a gift from Prof. S.L. Schor (Late) DDS, University of Dundee, UK. All cells were cultured at 37℃ and 5% carbon dioxide in minimum essential medium (MEM) supplemented with 10% Foetal calf serum and 200 mM L-glutamine.

2.2 Reagent, protein, inhibitors and antibodies

Secondary antibodies used were anti-rabbit IgG, HRP-linked antibody (7074 Cell signalling technology) and Goat anti-rabbit Ig

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**FIGURE 1** EGFR signalling pathway. Ligands such as EGF and TGFα (green dot) bind to EGFR (light blue cell surface receptor) eliciting several phosphorylation events (yellow dots). Activated pathways follow a series of reported actions leading to alterations in the nucleus. These can be in proliferation, migration, resistance to apoptosis, EMT and angiogenesis. Inhibitors (in red) are known to interact with the pathway.
G(H+L), F(ab”)2 Fragment (Alexa Fluor® 488 conjugate - 150077 Abcam). Tyrosine kinase inhibitors used were Gefitinib (4765) and Erlotinib (5083 - Cell signalling technology).

2.3 Cell scattering and immunofluorescence

Scatter assay and Immunofluorescence techniques were performed as described earlier. Cells were passaged and plated at 1 x 10\(^5\) cells per 60 mm dishes and grown up until they reached about 30%-40% confluence. The cells were washed with Hanks Balanced Salt solution (HBSS) twice and changed into serum-free media, and this was incubated overnight and next day the media were changed into test conditions with different concentrations of EGF/TGF\(\alpha\)± inhibitors. Inhibitor only and serum-free dishes were used as controls. The cells were assessed for individual/single cell migration, EMT-like morphology and cell protrusion for up to 48 h. Photographs were taken at an appropriate time interval at either 100X or 200X magnification using an inverted microscope (IX70) (Olympus). For immunofluorescence, the cells were washed with phosphate-buffered saline (PBS) and fixed with ice-cold methanol for 20 min and then washed with PBS for 5 min x 3 times. 0.2% (v/v) triton X-100 in PBS was added for 5 min and then washed again with PBS for 3 times (5 min each on an orbital shaker). Small areas of the dishes were ringed with an Immuno-pen (DAKO) and blocked with 5% (v/v) Normal goat serum/PBST (PBS + 0.1 Tween 20) for 1 h. The cells were then washed with PBS, and the area was incubated with the following primary antibodies: anti-Phospho P44/42 MAPK Erk1/2 (Thr202/tyr204)(1:200), AKT473(1:200) diluted in 5% normal goat serum in PBS T at 4℃ overnight. The area was washed with PBS for 5 min (3 times) and incubated with secondary antibody Alexa Flour (1:1000) for 1–2 h. The section was rinsed gently with PBS and then again washed with PBS for 5 min (3 times). Sections then had a coverslip with a drop of mounting medium (Sigma Aldrich) added and were allowed to dry. Images were taken with Olympus IX70 inverted fluorescent microscope using 100X or 200X. Images were then analysed by ImageJ software (NIH, Bethesda).

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**FIGURE 2** Cell Scatter Assay: Four cell types were plated as described in the Material and Methods (TYS, HSG, AZA1 and HaCaT). Column (A) serum free for 48 h, (B) EGF (50 ng/ml) for 48 h, (C) TGF\(\alpha\) (50 ng/ml) for 48 h, (D) One-hour pre-incubation with Gefitinib (5 µM) before incubation with EGF (50 ng/ml) for 48 h, (E) One-hour pre-incubation with Gefitinib (5 µM) before incubation with TGF\(\alpha\) (50 ng/ml) for 48 h, (F) One-hour pre-incubation with Erlotinib (5 µM) before incubation with EGF (50 ng/ml) for 48 h, (G) One-hour pre-incubation with Erlotinib (5 µM) before incubation with TGF\(\alpha\) (50 ng/ml) for 48 h. Images illustrate the scattering as cells remain in colony in the absence SF (red arrow). Partial scattering of the colonies where the cells exhibit an elongated phenotype (yellow arrows) and complete scattering where some loss of cell-to-cell junction and single cells with an elongated migratory phenotype (green arrows). (All images captured using an Olympus SC35 digital camera at x100 magnification (A, D-G) and x40 magnification (B and C).
2.4 | Scratch assay

2D migration assay was performed as described earlier. Cells plated onto 35 mm dishes were checked for confluence and healthiness. When the cells reached 90%–100% confluence, the medium was replaced with SF-MEM overnight. A scratch line was then made through the centre of culture dish with a 1 mm pipette tip. The dishes were rinsed with SF-MEM twice and incubated with (EGF/TGFα± inhibitors) for 24 h. SF-MEM and inhibitors were used as control. Photographs were taken at the scratch line with camera using an Olympus IX70 at either 100X or 200X magnification.

2.5 | Cell lysis, protein estimation assay, SDA page and western blot

Cell lysis was performed as described earlier. Cells were grown on 60 mm Tissue Culture dishes until 80% confluency was achieved, and they were then serum-starved for 24 h. The medium was changed into test conditions (EGF/TGFα± inhibitors) for 5 min and 24 h. The cells were then lysed with ice-cold lysis RIPA buffer, (prepared with a tablet of protease and phosphatase inhibitors into 10 ml RIPA buffer), 0.5 ml per dish for 10 min and cell lysates were collected into Eppendorf tubes and stored at −20°C. Protein estimation assay was carried out to quantify the protein using Pierce BCA protein Assay Kit (23225). A syringe with a needle was used to break down the gelation of lysate samples. For SDS-PAGE, cell lysates were spun at 13,000 rpm for 5 min. Suitable amount of sample was mixed with appropriate volume of loading buffer including 5% (v/v) 2-mercapto-ethanol. The sample was heated at 95°C in a water bath for 5 min then centrifuged briefly. Samples were loaded onto wells which fractionate proteins between 30 and 150 kDa Biorad TGX precast gels and run electrophoresis. After SDS page, samples were transferred onto PVDF (Biorad) and blots were incubated with antibodies phospho EGFR Tyr 1068 (1:1000), anti-phospho p44/42 MAPK (Erk1/2) (Thr202/Tyr204 1:2000), anti-phospho-akt (Ser473 1:2000) and goat anti-rabbit secondary antibody (1:2000). Blots were developed using BioRad Clarity Western ECL Substrate and analysed using Biorad GelDoc system.

FIGURE 3 Scratch Assay. Four cell type were plated as described in the Material and Methods (TYS, HSG, AZA1 and HaCaT). Column (A) serum free for 48 h, (B) EGF (50 ng/ml) for 48 h, (C) TGFα (50 ng/ml) for 48 h, (D) One-hour pre-incubation with Gefitinib (5 µM) before incubation with EGF (50 ng/ml) for 48 h, (E) One-hour pre-incubation with Gefitinib (5 µM) before incubation with TGFα (50 ng/ml) for 48 h, (F) One-hour pre-incubation with Erlotinib (5 µM) before incubation with EGF (50 ng/ml) for 48 h, (G) One-hour pre-incubation with Erlotinib (5 µM) before incubation with TGFα (50 ng/ml) for 48 h. Images have shown variable migratory behaviour of different cell lines. All cell lines responded to 10 ng/ml of EGF and TGFα over the 48-hour treatment. (All images were captured using an Olympus SC35 digital camera at x100 magnification (TYS, HSG and HaCaT cell lines) or x40 magnification (AZA10). Columns D-G show that the inhibitors block the growth factor-stimulated gap closure.
3 RESULTS

3.1 EGF and TGFα induce EMT-like phenotype and cell scattering/single cell migration and phosphorylation of EGFR, Akt and MAP kinase

Different concentrations of EGF and TGFα were used to investigate the effect of EGFR ligands on EMT-like morphology change, cellular protrusion and single cell migration. After a number of pilot experiments, 50 ng/ml of each growth factor was chosen as the most active test condition (Data not shown). The cells for all four cell types at the start of the experiment were in clusters or colonies and displayed an epithelial characteristic before addition of growth factors (Figure 2A). Incubation with 50 ng/ml of (EGF and TGFα) for 48 h induced consistent morphological changes in all four cell lines used in this project. However, the intensity of morphological changes was different with each cell line. The control cell line HaCaT showed minimal changes compared with the other cell lines. Incubation with 50 ng/ml of either EGF or TGFα for 48 h induces loosely connected cells with elongated cells with cellular projections (Figure 2B,C). The TYS cell line exhibited more scattering than HaCaT cells with a mixture of rounded and elongated cells with cellular projections. The HSG and AZA1 cells exhibited the most prominent results for cell scattering with more rounded cell than elongated cells with cellular projections (Figure 2B,C).

3.2 One-hour pre-treatment with Gefitinib or Erlotinib inhibited EMT-like morphological changes, cellular protrusion and cell scattering/single cell migration

Different concentration of Gefitinib and Erlotinib was used as preliminary experiments (data not shown). A one-hour pre-treatment of either Gefitinib (5 μM) or Erlotinib (5 μM) before addition of EGF and TGFα completely inhibited an EMT-like morphological change and single cell migration in all the cell lines (Figure 2D,E,F,G).

3.3 EGF and TGFα induce collective cell migration

Incubation with 50 ng/ml of (EGF and TGFα) for 24 h induced collective cell migration in the scratch/wound assay in HSG, TYS and HaCaT cell lines (Figure 3B,C). Cellular bridge formation and gap closure of the wound were found in HSG, TYS and HaCaT cell lines. However, AZA1 cells were not stimulated to migrate across the wound. There was no cellular bridge formation or closure of scratch line in AZA1 cell line in response to EGF and TGFα (Figure 3B,C).

3.4 One-hour pre-treatment Gefitinib/Erlotinib inhibited the EGF and TGFα-induced collective cell migration

One-hour pre-treatment with Gefitinib (5 μM) and Erlotinib (5 μM) before addition of EGF and TGFα was able to inhibit the effect of collective migration in HSG, TYS and HaCaT cell lines (Figure 3D,E,F,G).

3.5 EGF and TGFα induce phosphorylation of MAPK (Thr202/Tyr204) residue while Tyrosine kinase inhibitor blocked its phosphorylation

All the cell lines were analysed for phosphorylation of MAPK at Thr202/Tyr204 using two techniques (Western blot and immunofluorescence), and this would allow us to investigate both the amount of phosphorylation and the location of the phosphorylated protein. Immunofluorescence (Figure 4) indicated that phosphorylation of MAPK at Thr202/Tyr204 was increased in EGF and TGFα-treated dishes compared with serum free in HSG, AZA and TYS cell lines. The tyrosine kinase inhibitor (Gefitinib and Erlotinib)-treated dishes blocked this phosphorylation. In HaCaT cell line, MAPK phosphorylation was not seen in all test conditions. Using Western blot, the phosphorylation of MAPK was increased in EGF and TGFα treated dishes compared with serum free control (Figure 5B-Lane1,2,3). In contrast, one-hour pre-treatment with Gefitinib (5 μM) and Erlotinib (5 μM) before introduction of EGF and TGFα completely blocked the phosphorylation of MAPK at these residues 202/204 (Figure 5B-Lane4,5,6,7).

3.6 EGF tyrosine residue 1068 was phosphorylated in all conditions (EGF/TGFα± inhibitors)

All the cell lines treated with (EGF/TGFα± inhibitors) for 24 h were analysed for EGRF-1068 phosphorylation. EGFR-1068 was phosphorylated in all conditions including serum free (Figure 5A). This suggested that even in serum-free conditions, EGFR was able to self-phosphorylate.

3.7 AKT Ser473 phosphorylation

All the cell lines treated with (EGF/TGFα± inhibitors) were analysed for phosphorylation of AKTSer473 by using two techniques (Western blot and immunofluorescence). In Western blotting (Figure 5C), AKT Ser473 phosphorylation was increased in EGF and TGFα-treated dishes of HSG and HaCaT cell line. In TYS cell line, the intensity of AKT Ser473 phosphorylation between control and growth factor-treated dishes was similar. Gefitinib and Erlotinib completely inhibit Akt phosphorylation in HaCaT cell line and reduce...
phosphorylation in TYS and HSG cell line. AKT Ser473 phosphorylation was not found in AZA1 cell line.

Immunofluorescence showed that phosphorylated AKT Ser473 expression was faintly seen in all the cell lines, and we considered it to be too low to be able to interpret the data (data not shown).

4 | DISCUSSION

Invasion and metastasis are one of the important hallmarks of cancer. Cell migration is pivotal in invasion and the metastasis process of cancer. In order for the cells to migrate, it has been proposed that the cells need to undergo epithelial-to-mesenchymal transition (EMT). EMT is the process in which the epithelial cells lose their cuboidal shape, cell adhesion and apical-basal polarity and acquire elongated spindle-shaped fibroblast-like phenotype, front to back leading edge polarity and motile. EMT is a reversible process, and conversion via MET is also frequently reported in cancer.14

Growth factors such as epidermal growth factor (EGF), transforming growth factor(TGFα), fibroblast growth factor(FGF), hepatocyte growth factor(HGF), bone morphogenesis proteins (BMPs) and extracellular matrix macromolecules such as, collagen, hyaluronic acid (HA), as well as chronic inflammation and hypoxia are known to induce EMT. A number of signalling pathways are involved in both initiation and regulation of EMT.15 TGFα, EGFR mRNAs and protein levels are elevated in head and neck cancer patients.16 EGF and TGFα ligands bind to EGFR and cause structural changes to receptor and eventually leading to the phosphorylation of six tyrosine residues (Y1068, Y1148, Y1173, Y1086, Y992 and Y1045) which have kinase activity.17 This is then followed by activation of various signalling pathways such as ERK/ MAPK, PI3K/AKT, mTOR, PLC-γ1-PKC, JNK and STAT. These pathways are inter-connected (cross talk) with each other, and this then leads to various cellular responses such as cell proliferation, differentiation, cell migration, invasion and metastasis, angiogenesis and inhibition of apoptosis.18 Overactivation of EGFR signalling is therefore postulated to lead to head and neck cancer in a number of cases.

In this study, we have investigated the role of EGF and TGFα-induced EMT and cell migration in Asian head and neck cancer cell lines. Incubation with EGF and TGFα for 48 h induced the cells to scatter from colonies into individual cells with mixture of rounded and elongated shape cells with protrusion at the edge of some cells. Several studies have reported similar changes when cells are incubated with EGF and TGFα.19,20 When scattering the cells, often have a rounded and/or elongated cell phenotype which we report here.
The cells respond to EGF and TGFα in a way which can be classified as undergoing EMT and following an amoeboid and mesenchymal phenotype of single cell migration.21

One study has reported that amoeboid shape migration requires Rho-Rock signalling while the mesenchymal phenotype is Rac dependent with filamentous (F) actin-rich protrusions which does not require Rho or ROCK.22 Our results, both the HSG and AZA1 cell lines, are more amoeboid-shaped cells than mesenchymal, and the Rho-Rock pathway may well be involved and will require further investigation. HaCaT cell migration is more mesenchymal than amoeboid, and we hypothesise that Rac might be involved. Finally, the TYS cell line produced a mixture of amoeboid and mesenchymal morphology, and both Rho-Rock and Rac might be involved. However, these are still to be investigated.

Cellular projection which is found at the leading edge of cells can be either: lamellipodia (flat broad membrane protrusion), or filopodia (finger-like protrusion), or podosomes, or invadopodia. Theoretically, lamellipodia and filopodia are found in normal cells while invadopodia are found in cancer cells. Podosomes are found in highly invasive normal cells.23,24 Since TYS, HSG and AZA1 are transformed cells, it would be the best to define as invadopodia (Figure 2B,C). Cellular projection of HaCaT can be either podosomes or filopodia or lamellipodia (Figure 2B,C).

In this project, we also found that the addition of EGF and TGFα induced collective cell migration in scratch assay. This is similar to other published data.25 Collective cell migration can occur via two-dimensional monolayer or three-dimensional tissue scaffold. Collective migration is not only found in cancer but also present in wound healing and tissue regeneration. Collective cells movement follows three basic principles in that cells remain connected and move in the following ways: (a) cell-to-cell adhesion is maintained and cells are moving as a groups. (b) Cell polarity and actin cytoskeleton cause actin filaments to generate traction. (c) The cells have ability to modify the tissue along their migratory road.26,27 There are two types of cells in collective cell migration. First is leader cells/pioneer cell which leads. Second is follower cells or rear cells. Leader cells and follower cells are differed in both morphology and gene expression. Leader cells are often mesenchymal shape with less ordered while follower cells are tightly pack rosettes shape.26,27 In
this study, leader cells and follower cells were not differentiated between and remain an area for further study.

EMT has often been reported to be related resistant to TKIs.\(^{28}\) It is important to note how the cells used in this project responded to TKIs. We found that EGFR residue 1068 was phosphorylated in growth factors and inhibitors-treated dishes. A report with a human epidermoid cancer cell line which has high EGFR overexpression, tyrosine kinase inhibitors blocked all ligand phosphorylation sites except Y992 and Y1068.\(^{27}\) (Guo et al., 2003). This matched with our results. Phosphorylation of tyrosine residue Y1068 has been suggested to be a predictive biomarker for response to tyrosine kinase inhibitors treatment in lung cancer patient with wildtype EGFR.\(^{29}\)

Our results for the head and neck cell lines used here also respond to the tyrosine kinase inhibitors.

One-hour pre-treatment with Gefitinib and Erlotinib inhibited EMT, single and collective cell migration. MAPK 202/204 was phosphorylated in response to EGF and TGF-\(\alpha\) treatment while Gefitinib and Erlotinib inhibited this. Phosphorylation of AKT473 was seen in EGF and TGF-\(\alpha\)-treated dishes while Gefitinib and Erlotinib lowered this phosphorylation. A similar report has been shown in the literature.\(^{30}\)

As briefly discussed in introduction, Cetuximab resistance is often reported in patients with EGFR mutation.\(^{4,6}\) This is very common in Asian patients. It is therefore important to investigate the role of tyrosine kinase inhibitors on Asian head and neck cancer patients.

In conclusion, the tyrosine kinase inhibitor Gefitinib and Erlotinib have ability to inhibit EGF and TGF-\(\alpha\)-induced EMT, single cell migration and collective cell migration. A one-hour pre-treatment with Gefitinib and Erlotinib downregulates MAPK 202/204 and AKT473 expression. All the data in this project suggest that both Gefitinib and Erlotinib working well in cell lines of Asian head and neck origin. It would be of interest to increase the number of cell lines in future studies from other Asian ethnic backgrounds. These data would suggest some therapies may work better in some regions of the world and would suggest multi-centred international trials for the efficacy of treatments would be a more inclusive practice.

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**CONFLICT OF INTEREST**

The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

**AUTHOR CONTRIBUTIONS**

Aye Myat Thwe: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing-original draft. Peter Mossey: Conceptualization; Project administration; Supervision; Writing-review & editing. Ian R. Ellis: Conceptualization; Investigation; Methodology; Project administration; Supervision; Validation; Writing-review & editing.

**ETHICAL APPROVAL**

The data in this article did not require ethical approval.

**ORCID**

Ian R. Ellis [https://orcid.org/0000-0001-8035-6012](https://orcid.org/0000-0001-8035-6012)

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