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Epidermal Growth Factor Receptor Regulates Matrix Metalloproteinase-2 Activity in MDA-MB-231 Human Breast Cancer Cells

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Authors' contributions

The work was carried out by both authors who have been listed according to their contributions. Both authors have read and approved the final manuscript.

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ABSTRACT

Aims and Study Design: Overexpression of epidermal growth factor receptor (EGFR) and its phosphorylated form (p-EGFR) in breast carcinomas has been associated with an increase in invasive potential and decreased survival. EGFR mediated signal transduction has been reported to be involved in regulation of matrix metalloproteinases (MMPs). Elevated MMP-2 expression and activity shows significant correlation with increased invasive potential in breast cancer. As MMP-2 plays a crucial role in tumour invasion, the role of EGFR in regulation of MMP-2 expression and activity in breast cancer was studied using the human breast adenocarcinoma cell line MDA-MB-231 as a model.
Methodology: MDA-MB-231 cells were cultured on 1 µg/ml epidermal growth factor (EGF) coated culture dishes for 24 hours. Control cells were cultured without EGF. MMP-2 activity was assayed by gelatin zymography. Expression of EGFR, MMP-2, focal adhesion kinase (FAK) and mitogen activated protein kinase (p38MAPK) and phosphorylation of EGFR were assayed by Western blot.
Results: When MDA-MB- 231 cells were cultured on EGF, increased activation of MMP-2 and an overall increase in MMP-2 expression and activity was observed. The observed upregulation of MMP-2 was appreciably inhibited if cells were pre-treated with anti-EGFR antibody, thus blocking

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EGFR. Phosphorylation of EGFR and expression of p38MAPK and FAK were appreciably increased upon culture of cells on EGF.

Conclusion: In MDA-MB-231 breast cancer cells, EGFR-EGF interactions promote activation of MMP-2 and an increase in MMP-2 expression and activity via EGFR mediated signal transduction cascades involving FAK and p38MAPK. As elevated MMP-2 expression and activity correlate with tumour aggressiveness, tumour cell EGF interaction via EGFR might increase the invasive potential of breast cancer cells.

Keywords: Epidermal growth factor receptor (EGFR); breast cancer; matrix metalloproteinase-2 (MMP-2); mitogen activated protein kinase (MAPK); focal adhesion kinase (FAK).

1. INTRODUCTION

Epidermal Growth Factor Receptor (EGFR) belongs to the ErbB family of tyrosine kinase receptors and consists of extracellular, transmembrane and intracellular tyrosine kinase domains [1-4]. A number of ligands including epidermal growth factor (EGF), heparin-binding EGF, amphiregulin, betacellulin, epiregulin and transforming growth factor–α (TGF-α) can interact with EGF. Binding of EGFR to its cognate ligand EGF induces receptor dimerization and autophosphorylation of intracytoplasmic tyrosine residues creating binding sites for adaptor proteins and promoting activation of intracellular signalling cascades which modulate gene expression, cell proliferation, migration, angiogenesis and apoptosis [1-5]. Two major routes for EGFR mediated signalling include the Ras-Raf/ mitogen activated protein kinase (MAPK)/ extracellular signal regulated kinase (ERK) pathway and phosphatidylinositol 3' kinase (PI3K)/ Akt pathway [1-5]. EGFR mediated signalling has also been associated with tumour invasion and metastasis. EGFR is overexpressed in a number of solid tumours and tumour cell lines, including breast and ovarian cancers, squamous cell carcinomas of the head and neck, and melanomas and overexpression of EGFR has been associated with poor prognosis of these cancers [1,2,4,6,7]. Expression of EGFR and its phosphorylated form (p-EGFR) in breast carcinomas has been associated with an increase in invasive potential and decreased survival [4,7,8]. pEGFR expression has been shown to be related to angiogenesis and invasiveness via pAkt pathway [4,7,8]. In FGFR1-driven breast cancer, upregulation of EGFR mediated signalling has been correlated with tumour stroma remodelling and tumour recurrence [9].

Matrix metalloproteinases (MMPs), a family of zinc containing endopeptidases, are crucial in ECM remodeling and play vital roles in wound healing, cell migration, angiogenesis and apoptosis as well as in tumour growth and metastasis [10-13]. Several studies indicate that MMP-2 (gelatinase A/ 72 kDa Type IV collagenase) is an important mediator of tumour invasion and metastasis [10,13-16]. Pro-MMP-2 (72 kDa) is activated via proteolytic cleavage of the pro-domain by a cell membrane associated activation complex to generate active MMP-2 (68-62 kDa) [10,12,13,17,18]. Increased MMP-2 activation and elevated MMP-2 expression and activity show a significant correlation with increased metastatic potential in breast, lung, thyroid, oral, stomach and colon carcinomas [10,13-18]. MMP-2 disrupts the integrity of the basement membrane by cleaving type IV collagen, is involved in the extensive degradation of matrix proteins during invasion and metastasis and plays important roles in intravasation and extravasation [10,13-18].

Interaction of EGFR with its ligands has been reported to modulate MMP expression [6,19-21]. EGFR stimulation of the ERK/MAPK pathway has been reported to activate several MMP genes, including MMP-1, MMP-3, MMP-7, MMP-9 and MT1-MMP in ovarian carcinoma [6]. The promoter regions of MMP-1, MMP-3, MMP-7 and MMP-9 contain an AP-1 consensus sequence and these MMPs are transcriptionally upregulated upon signal transduction through MAPK cascades [22]. EGFR induces MMP-9 and MMP-1 expression in bladder cancers and regulates MMP expression and function in fibroblasts via signalling through MAPK and AP-1 [19,20]. EGFR regulates lung morphogenesis by promoting MMP-2 activation which appears to be a major downstream target of signalling through EGFR [21]. In the metastatic breast cancer cell line SKBR-3, interaction of EGFR with EGF or amphiregulin leads to an increase in MMP-9 expression [23].

Tyrosine phosphorylation and activation of focal adhesion kinase (FAK), a non-receptor protein tyrosine kinase, occurs upon receptor ligand

interaction and leads to activation of downstream signalling cascades involving MAPK, ERK, PI3K and JNK [24-26]. FAK is thus involved in the activation of signalling molecules, including MAPK, which are associated with EGFR mediated signalling. Elevated expression of FAK has been clinically associated with tumour cell invasion in a number of cancers including breast cancer and FAK is believed to play a crucial role in metastasis by regulating MMP expression and cytoskeletal remodeling [26-28].

In the present study, considering the pivotal role played by MMP-2 in promoting tumour invasion and metastasis, we focused on the role of EGFR in regulation of MMP-2 expression and activity in breast cancer using the metastatic breast adenocarcinoma cell line MDA-MB-231 as a model.

2. MATERIALS AND METHODS

2.1 Antibodies and Reagents

Epidermal Growth Factor (human, recombinant, expressed in *E. coli*) was purchased from Sigma-Aldrich (USA). Primary antibodies (anti-EGFR, anti-p-EGFR, anti-MMP-2, anti-FAK, anti-p38 MAPK) and secondary antibodies (alkaline phosphatase coupled) were purchased from Santa Cruz Biotechnology Inc. (USA). Leibovitz's L-15 medium and foetal bovine serum (FBS) were purchased from HiMedia (India). Nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indoyl phosphate (NBT-BCIP) were purchased from Bangalore Genei (India). Other fine chemicals were bought from Merck (USA) and Sigma-Aldrich (USA).

2.2 Cell Line

The human breast adenocarcinoma cell line MDA-MB-231 was obtained from National Centre for Cell Science (NCCS), Pune, India. Cells were cultured and maintained in L-15 medium containing 10% FBS at 37℃ in a CO_2 incubator (3% CO_2).

2.3 Treatment of Cells with EGF

Varying concentrations of EGF (0.5 and 1 µg/ml) were used to coat culture petri dishes. MDA-MB-231 cells were cultured in serum free culture medium (SFCM) on EGF coated culture petri dishes. Control MDA-MB-231 cells were cultured in SFCM in culture dishes without EGF coating. Cells were collected after 24 hrs and extracted in cell extraction buffer (37.5 mM Tris-HCl, 75 mM NaCl, 0.5% Triton X-100, 1% protease inhibitors) by homogenization at 4℃. Protein concentrations of the resultant extracts were determined by modified Lowry's method [29].

2.4 Gelatin Zymography

Cell extracts were incubated with sample buffer containing 2.5% SDS (without β-mercaptoethanol) at 37℃ for 30 mins. Gelatin zymography was performed on 10% SDS-PAGE co-polymerized with 0.1% gelatin. Gels were incubated in 2.5% Triton X-100 for 30 mins to remove SDS, incubated for 40 hrs in reaction buffer at 37ºC and stained with 0.25% Coomassie Brilliant Blue. Bands were visualized by destaining the gel with water [30]. Relative intensity of the bands was quantified using Image J Launcher.

2.5 Western Blot

Cell extracts were suspended in Laemmli's buffer containing β-mercaptoethanol for 10 mins at 90℃, run on 7.5% or 10% SDS-PAGE as appropriate and proteins were transferred on to nitrocellulose membranes by Western blot. The membranes were blocked with 1% BSA and incubated with anti-MMP-2/ anti-EGFR/ anti-p-EGFR/ anti-p38MAPK/ anti-FAK antibodies (1:1000 dilution) for 1.5 hrs at 37℃, washed with Tris buffered saline with Tween-20 (TBS-T), incubated with corresponding alkaline phosphatase coupled secondary antibodies (1:2000 dilution) for 1.5 hrs at 37℃ and again washed extensively with TBS-T. Bands were visualized using NBT-BCIP as substrate.

2.6 Study of Effects of EGFR Inhibition

MDA-MB-231 cells were incubated with anti-EGFR monoclonal antibody (1:1000 dilution) in SFCM for 1 hr and then cultured on 1 µg/ml EGF coated culture petri dishes for 24 hrs. Cells were collected and extracted as described. The effect of EGFR inhibition on modulation of MMP-2 expression and activity were assayed by zymography and Western blot.

3. RESULTS

3.1 Assay of MMP-2 Activity in MDA-MB-231 Cells Cultured on EGF

Gelatin zymography showed that culture of MDA-MB-231 cells on 1 µg/ml EGF for 24 hrs (Fig. 1

lane 3) led to activation of MMP-2 (68 kDa). Quantification of relative intensity of bands of gelatinase activity showed that MMP-2 activity was significantly increased (3.17 fold and 7.30 fold for 72 kDa and 68 kDa forms respectively) on culture of MDA-MB-231 cells on 1 µg/ml EGF compared to control MDA-MB-231 cells cultured without EGF (Fig. 1 lane 1). When MDA-MB-231 cells were cultured on 0.5 µg/ml EGF for 24 hrs (Fig. 1 lane 2), activation of MMP-2 was far less and increase in MMP-2 activity was less significant (1.25 fold and 2.30 fold for 72 kDa and 68 kDa forms respectively) compared to control cells. On plotting MMP-2 activity (as fold intensities) against concentrations of EGF, it was found that MMP-2 activity increased with increasing concentrations of EGF in a dose dependent manner.

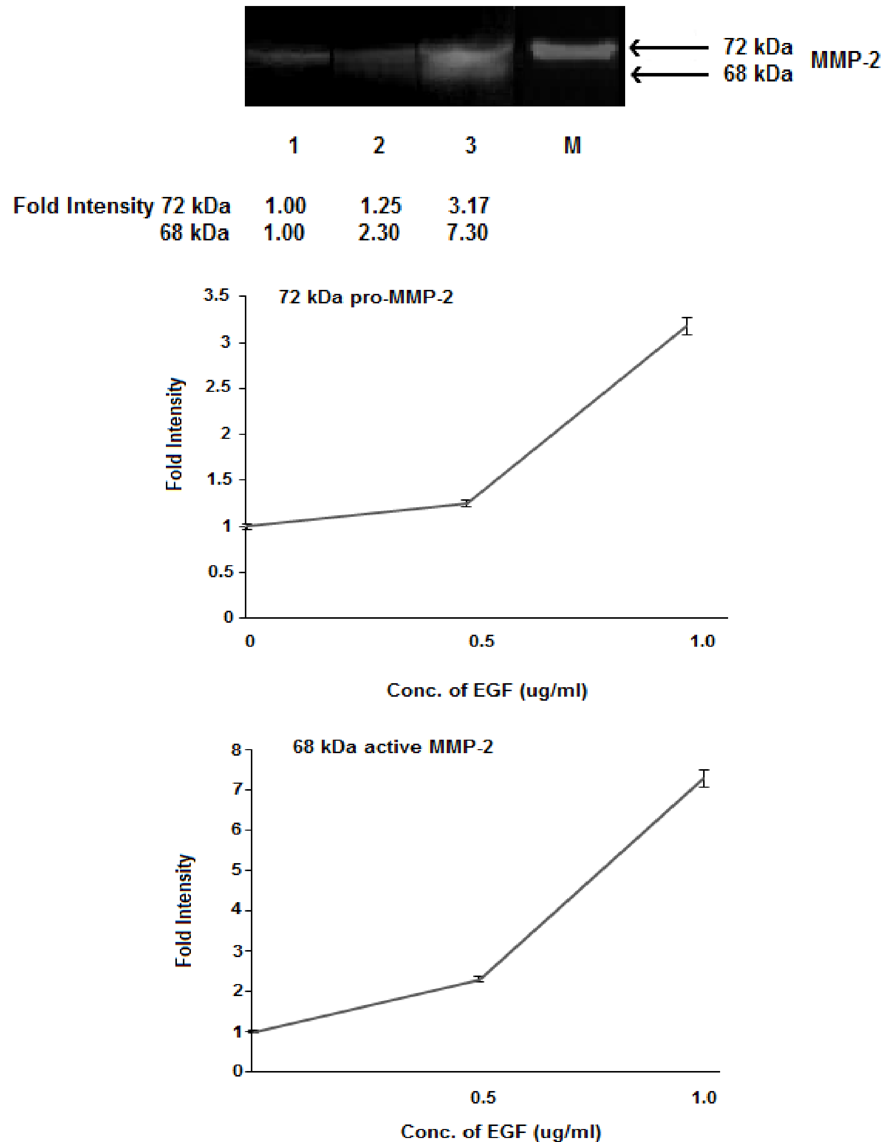

Fig. 1. Assay of MMP-2 activity in MDA-MB-231 cells cultured on EGF

Cell extracts of MDA-MB-231 cells cultured without EGF (lane 1), on 0.5 µg/ml EGF (lane 2) and on 1 µg/ml EGF (lane 3) for 24 hrs were subjected to gelatin zymography on 10% SDS-PAGE co-polymerised with 0.1% gelatin. After incubation in appropriate reaction buffers, the zymogram was stained with Coomassie Brilliant Blue. Bands were clearly visualized by destaining the gel in water. Lane M: MMP-2 marker. Relative intensity of bands was quantified using Image J Launcher (taking intensity of bands in control cells as 1.00). Fold intensities of MMP-2 activity were plotted against increasing concentrations of EGF

3.2 Assay of MMP-2 Expression in MDA-MB-231 Cells Cultured on EGF

Western blot (Fig. 2) showed expression of activated MMP-2 (68 kDa) in cell extracts of MDA-MB-231 cells cultured on 1 µg/ml EGF for 24 hrs (lane 2). Expression of pro-MMP-2 (72 kDa) was also increased compared to control MDA-MB-231 cells cultured without EGF (lane 1).

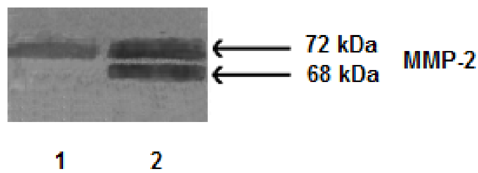

Fig. 2. Assay of MMP-2 expression in MDA-MB-231 cells cultured on EGF

Cell extracts of MDA-MB-231 cells cultured without EGF (lane 1) and on 1 µg/ml EGF (lane 2) for 24 hrs were subjected to Western blot. Membranes were incubated with anti-MMP-2 antibodies followed by incubation with alkaline phosphatase coupled secondary antibodies. Bands were visualized using NBT-BCIP as substrate

3.3 Effect of EGFR Inhibition on MMP-2 Activity and Expression

Gelatin zymography and quantification of relative intensity of bands of gelatinase activity showed that upregulation of MMP-2 activity was significantly more (2.34 fold and 3.48 fold for 72 kDa and 68 kDa forms respectively) in MDA-MB-231 cells cultured on 1 µg/ml EGF without antibody treatment (Fig. 3A lane 1) compared to MDA-MB-231 cells treated with anti-EGFR antibody prior to culture on 1 µg/ml EGF for 24 hrs (Fig. 3A lane 2). When MDA-MB-231 cells were treated with anti-EGFR antibody prior to culture on 1 µg/ml EGF for 24 hrs (Fig. 3B lane 2), upregulation of MMP-2 expression was appreciably less compared to MDA-MB-231 cells cultured on EGF without antibody treatment (Fig. 3B lane 1).

3.4 Assay of EGFR Expression and Phosphorylation in MDA-MB-231 Cells Cultured on EGF

Western blot showed a slight increase in EGFR expression (Fig. 4A) and an appreciable increase in EGFR phosphorylation (Fig. 4B) when MDA-MB-231 cells were cultured on 1 µg/ml EGF for 24 hrs (lane 2) compared to control cells cultured without EGF (lane 1).

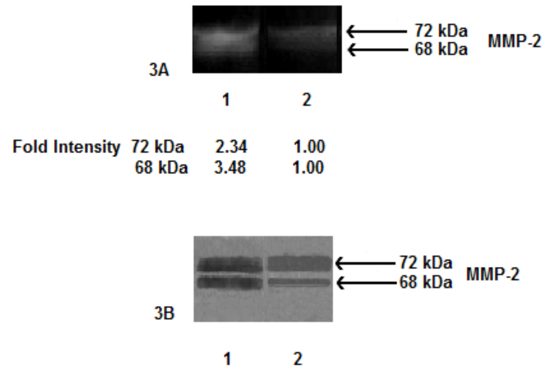

Fig. 3. Effect of EGFR inhibition on MMP-2 activity and expression

Fig. 3A: Cell extracts of MDA-MB-231 cells cultured for 24 hrs on 1 µg/ml EGF (lane 1) and on 1 µg/ml EGF after incubation with anti-EGFR antibody (lane 2) were subjected to gelatin zymography on 10% SDS-PAGE co-polymerised with 0.1% gelatin. After incubation in appropriate reaction buffers, the zymogram was stained with Coomassie Brilliant Blue. Bands were clearly visualized by destaining the gel in water. Relative intensity of bands was quantified using Image J Launcher (taking intensity of bands in cells treated with anti-EGFR antibody as 1.00). Fig. 3B: Cell extracts of MDA-MB-231 cells cultured for 24 hrs on 1 µg/ml EGF (lane 1) and on 1 µg/ml EGF after incubation with anti-EGFR antibody (lane 2) were subjected to Western blot. Membranes were incubated with anti-MMP-2 antibodies followed by incubation with alkaline phosphatase coupled secondary antibodies. Bands were visualized using NBT-BCIP as substrate

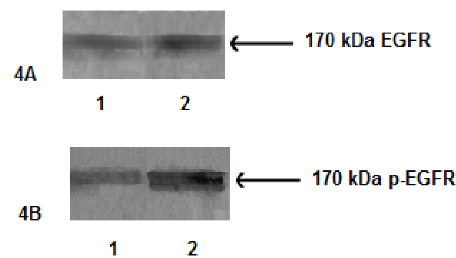

Fig. 4. Assay of EGFR expression and phosphorylation in MDA-MB-231 cells cultured on EGF

Cell extracts of MDA-MB-231 cells cultured without EGF (lane 1) and on 1 µg/ml EGF (lane 2) for 24 hrs were subjected to Western blot. Membranes were incubated with anti-EGFR antibodies (Fig. 4A) or anti-p-EGFR antibodies (Fig. 4B) followed by incubation with corresponding alkaline phosphatase coupled secondary antibodies. Bands were visualized using NBT-BCIP as substrate

3.5 Assay of p38 MAPK and FAK Expression in MDA-MB-231 Cells Cultured on EGF

Western blot showed an appreciable increase in expression of p38 MAPK (Fig. 5A) and FAK (Fig. 5B) when MDA-MB-231 cells were cultured on 1 µg/ml EGF for 24 hrs (lane 2) compared to control cells cultured without EGF (lane 1).

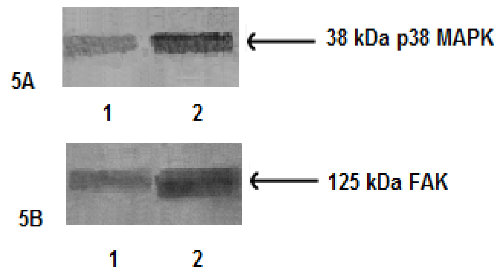

Fig. 5. Assay of p38 MAPK and FAK expression in MDA-MB-231 cells cultured on EGF

Cell extracts of MDA-MB-231 cells cultured without EGF (lane 1) and on 1 µg/ml EGF (lane 2) for 24 hrs were subjected to Western blot. Membranes were incubated with anti- p38MAPK antibodies (Fig. 5A) or anti- FAK antibodies (Fig. 5B) followed by incubation with corresponding alkaline phosphatase coupled secondary antibodies. Bands were visualized using NBT-BCIP as substrate

4. DISCUSSION

Culture of MDA-MB-231 breast cancer cells on EGF (1 µg/ml) coated culture dishes for 24 hrs promoted MMP-2 activation and led to an appreciable increase in MMP-2 expression and activity compared to control MDA-MB-231 cells cultured in absence of EGF. As MDA-MB-231 cells grown on culture dishes coated with a lower concentration of EGF (0.5 µg/ml) did not show a comparable increase in MMP-2 activity, cells cultured on 1 µg/ml EGF coated culture dishes were used for assaying further parameters. The crucial role of EGFR in upregulation of MMP-2 expression and activity was further elucidated by incubating MDA-MB-231 cells with anti-EGFR monoclonal antibody (to block EGFR) prior to culture of cells on EGF coated culture dishes. When MDA-MB-231 cells were treated with anti-EGFR antibody prior to culture on 1 µg/ml EGF, upregulation of MMP-2 expression and activity in cell extracts was appreciable hindered. These results seem to indicate that interaction of tumour cell surface EGFR with its ligand EGF plays an important role in modulation of MMP-2 activity in MDA-MB-231 cells. MMP-2 is an important mediator of tumour invasion and metastasis. Increased MMP-2 activation and elevated MMP-2 activity correspond with increased metastatic potential in a number of human cancers including breast cancers [10,13-18]. Thus, the observed increase in MMP-2 activation and upregulation of MMP-2 expression and activity upon tumour cell EGF interaction via EGFR could promote increased invasion and metastasis in breast cancer.

EGFR expression was partially increased and phosphorylation of EGFR was appreciably increased on culture of MDA-MB-231 cells on EGF coated culture dishes, indicating the activation of EGFR mediated signal transduction cascades upon tumour cell EGF interaction. Our observations indicated that the p38MAPK pathway is stimulated in MDA-MB-231 cells upon interaction of EGFR with its ligand EGF. MAPK is an important component of EGFR mediated signal transduction cascades and EGFR mediated signalling through MAPK has been reported to regulate MMP expression and function [2,4,6,20,22,31]. Studies have indicated that signalling through p38MAPK is involved in regulation of MMP-2 in MDA-MB-231 cells and hindering p38MAPK mediated signal transduction causes downregulation of MMP-2 and suppression of cell migration and invasion [32,33]. EGFR mediated signal transduction cascades involving p38MAPK may therefore play an important role in modulation of MMP-2 expression and activity in MDA-MB-231 breast cancer cells and the resultant upregulation of MMP-2 upon tumour cell EGF interaction could promote metastasis and tumour invasion. Also hyperactivation of MAPKs is characteristic of many cancers and signal transduction through MAPK regulates cell proliferation and migration [22]. EGFR mediated signal transduction through p38MAPK may thus play multiple roles in promoting tumour invasion. This would correlate well with the observations that aberrant EGFR expression and activity are associated with increased cell motility, invasion and a poor clinical prognosis in breast cancer.

Although several studies indicate that FAK mediated signalling is involved in modulation of MMP-2 activity in breast cancer cells [28,34,35], there are few previous reports of involvement of FAK in EGFR mediated signalling cascades in breast cancer [36]. Our experiments indicate that expression of FAK is appreciably increased on

EGFR-EGF interaction in MDA-MB-231 breast cancer cells and strongly indicates an important role for FAK in EGFR mediated signal transduction cascades. Integrins are a family of heterodimeric transmembrane receptors which mediate cellular adhesion to extracellular matrix proteins and modulate intracellular signal transduction cascades [37,38]. Studies indicate that integrin mediated signal transduction for upregulation of MMP-2 activity in breast cancer occurs through FAK [28,35]. FAK may also thus be involved in cross talk between various receptor mediated signalling cascades for upregulation of MMP-2 expression and activity in breast cancer.

5. CONCLUSION

Thus, in MDA-MB-231 breast cancer cells, interactions of EGFR with its ligand EGF promote MMP-2 activation and an increase in MMP-2 expression and activity. EGFR mediated signal transduction cascades involving FAK and p38MAPK appear to be involved in regulation of MMP-2. As elevated MMP-2 expression and activity show appreciable correlation with increased metastasis and tumour invasion, tumour cell EGF interaction via EGFR might increase the invasive potential of breast cancer cells. Our studies on EGFR mediated regulation of MMP-2 expression and activity thus provide a more comprehensive insight into tumour invasion and metastasis in breast cancer by indicating that the observed increase in invasive potential in breast carcinomas due to increased expression and phosphorylation of EGFR could be associated with an EGFR mediated increase in MMP-2 expression and activity. Future studies targeting EGFR-ligand interactions and EGFR mediated signal transduction cascades may be of use in clinical management of breast cancers.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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