Oral Oncology (2009) 45, 156–163

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/oraloncology

ORAL ONCOLOGY

Epiregulin promotes migration and invasion of salivary adenoid cystic carcinoma cell line SACC-83 through activation of ERK and Akt

Kai Hu [a], Sheng-lin Li [a], Ye-hua Gan [a], Cun-yu Wang [b], Guang-yan Yu [a,*]

[a] *Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology, 22 Zhongguancun Nandajie, Haidian District, Beijing 100081, PR China*
[b] *Laboratory of Molecular Signaling, Division of Oral Biology and Medicine, UCLA School of Dentistry, Los Angeles, CA 90095, USA*

Received 26 March 2008; received in revised form 16 April 2008; accepted 19 April 2008
Available online 11 July 2008

KEYWORDS
Salivary adenoid cystic carcinoma;
Metastasis;
Epiregulin;
ERK1/2;
Akt

Summary Hematogenous metastasis is one of the most important factors determining the outcome of the patients with salivary adenoid cystic carcinoma (SACC). In the present study, we examined expression profile of genes in SACC cell lines to look for molecules responsible for its unique metastatic trait. A transcriptomic microarray analysis between the lower lung-metastatic rate cell line SACC-83 and the higher lung-metastatic rate cell line SACC-LM were performed, and eight genes, showed by microarray to be highly expressed in SACC-LM, were picked for validation by quantitative real-time PCR. Among the genes, the expression of epiregulin, a novel member of epidermal growth factor family, was 350-folds higher in SACC-LM than in SACC-83. Accordingly, we examined the effects of epiregulin on migration and invasion in SACC-83 as well as its targeted downstream molecules, and found that epiregulin could promote in vitro migration and invasion in SACC-83. Furthermore, epiregulin not only induced activation of both ERK1/2 and Akt, but also expression of COX-2. In addition, all these effects could be partially blocked by U0126, a specific inhibitor of mitogen-activated protein kinase kinase (MEK or MAPKK), or LY294002, a specific inhibitor of phosphatidylinositol 3-kinase (PI3K). Conclusively, the results suggest that epiregulin may play an important role in lung metastasis of SACC.

Introduction

Salivary adenoid cystic carcinoma (SACC) is an uncommon malignant tumor characterized with neurotropic, invasive growth and dormant distant metastasis.[1,2] Despite it has a slow

* Corresponding author. Tel.: +86 10 62191099; fax: +86 10 62173402.
E-mail address: gyyu@263.net (G.-y. Yu).

1368-8375/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.oraloncology.2008.04.009

growing pattern, SACC shows intriguingly a notable biologic ability to develop micrometastases in the very early phase.[3]

Previously, we established a human cell line of salivary adenoid cystic carcinoma, SACC-83, from a patient pathologically diagnosed as salivary adenoid cystic carcinoma in sublingual gland.[4] SACC-83 has a relatively lower lung-metastatic rate that only 33.3% of the nude mice showed lung metastases three months after injection of the tumor cells into the tail vein of nude mice. Further, we generated a derived cell line SACC-LM with higher lung-metastatic rate from SACC-83 by repeating of culture and injection of the lung metastatic tumor cells into the tail vain of nude mice. The lung-metastatic rate was finally 85.0% for SACC-LM while only 33.3% for SACC-83. Recently, it is believed only rare cellular variants from large-scale cellular heterogeneity within tumor populations could acquire augmented metastatic abilities.[5,6] Therefore, SACC-LM cells may represent the rare augmented metastatic cells in SACC-83 populations as they were selected from SACC-83. We hypothesized that genes differentially expressed in the two SACC cell lines may be responsible for the difference of their lung-metastatic potential. Transcriptomic microarray analysis may help screen differentially expressed genes in the two cell lines.

Epiregulin is a novel member of epidermal growth factor (EGF) family.[7] High levels of epiregulin mRNA were detected in several human cancer cell lines, suggesting epiregulin may be involved in cancer progression.[8] In addition, epiregulin has broader specificity than other EGF-like ligands. Noticeably, it conducts a more potent mitogenic signal than does EGF, regardless of binding to various receptor combinations with lower affinity.[9,10]

Like many other receptor tyrosine kinases, epidermal growth factor receptor (EGFR, also known as ErbB) dependent signaling transduction is initiated by ligand binding and mediates multiple signaling cascades. Two well studied downstream molecules of receptor tyrosine kinases are extracellular signal-regulated kinases 1/2 (ERK1/2) in mitogen-activated protein kinase (MAPK) cascades and phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT). ErbB1 not only transiently stimulates the Ras-MAPK pathway, but also couples to the Ras-PI3K-AKT pathway, whereas ErbB3 directly activates the PI3K/AKT pathway.[11] Activated ERK1/2 plays a principal role in growth factor-MAPK cascades induced cell proliferation and migration.[12] Besides, AKT signaling pathways regulates diverse cellular processes, including cell proliferation and survival, tissue invasion and angiogenesis.[13,14]

In the present study, a transcriptomic microarray analysis between SACC-83 and SACC-LM was performed to examine expression profile of genes. Eight genes which may be related to lung metastasis were picked for quantitative real-time PCR validation and epiregulin was found to be most dramatically increased in SACC-LM. Further, the effects of epiregulin on cell migration and invasion in vitro as well as its targeted downstream molecules were examined.

Materials and methods

Cell culture

SACC-83 and SACC-LM, previously established in our laboratory, were cultured in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (FBS; Hyclone), 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

Microarray analysis

Methods for RNA extraction, labeling and hybridization for DNA microarray analysis of the cell lines have been described previously.[15] Briefly, total RNAs of SACC-83 and SACC-LM were extracted with Trizol reagents and cleaned with an RNeasy kit (Qiagen). Twenty microgram aliquots of total RNAs were transcribed to first strand complementary DNA (cDNA) using SuperScript II reverse transcriptase (Invitrogen) with an oligo-dT primer that has a T7 RNA polymerase site on the 5′ end, and subsequent second-strand synthesis was carried out to obtain double-strand CDNA. Then, the cDNAs were used in an in vitro transcription reaction in the presence of biotinylated nucleotides to generate single stranded RNAs as recommended by Affymetrix. The biotin-labeled RNAs were fragmented and used for hybridization to Affymetrix human U133 genechips. Data were analyzed using Affymetrix Genechip software.

Quantitative real-time PCR

Quantitative real-time PCR analysis was performed in the SYBR® Green I assay using a 7500 Real Time PCR System (Applied Biosystems). Two microgramme of total RNA was applied to the SuperScript III reverse transcriptase reaction system (Invitrogen). First-strand cDNA was synthesized in a 20 μl reaction mix with 200 U of SuperScript III reverse transcriptase. cDNA equivalent to approximately 25 ng of starting RNA was used in each PCR reaction. Human epiregulin, COX-2, PTPRZ, ASK1, IL-20Rα, ICAM-4, AQP3, S100p and GAPDH (as control) were amplified with Power SYBR® Green PCR Master Mix (Applied Biosystems) under ABI Prism 7500 Relative Quantification (ddCT) System. All primers were used at 250 nM in each PCR reaction. Amplification conditions were 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 60 °C for 1 min, followed by a dissociation curve stage to check amplification specificity. Threshold cycle (CT) values for specific and control genes were shown automatically by SDS software. The changes of relative expressions for specific genes between groups were analyzed using the ΔΔCT Method.[16]

The primers of epiregulin, COX-2, PTPRZ, S100p and GAPDH were designed as previously described.[17–21] The primers of ASK1, IL-20Rα, ICAM-4, AQP3 were designed using Primer Premier 5.0 Software according to corresponding mRNA sequences from the National Center for Biotechnology Information (NCBI). All primer sequences were as follows. Epiregulin: sense/antisense, 5′-ATCCTGGCATGTGCTAGGGT-3′/5′-GTGCTCCAGAGGTCAGCCAT-3′; Cox-2: sense/antisense, 5′-TGAGCATCTACGGTTTGCTG-3′/5′-TGCTTGTCTGGAACAACTGC-3′; PTPRZ: sense/antisense, 5′-TTAGAGGACAGTACATCCCCTAGAGTT-3′/5′-TGCTCCGACATCATCTGAAATT-3′; ASK-1: sense/antisense, 5′-TTCACACAAAACGGATGTAACATT-3′/5′-CCTAAACAGTTATGGTCACATTTTGG-3′; IL-20Rα: sense/antisense, 5′-TGATGGGCTATTCCATCTACCG-3′/5′-TCCGAGATATTGAGGGTGATAAAG-3′; ICAM-4: sense/antisense, 5′-ACCAGCTGCTCGACGTGA-3′/5′-GCCTCAGGGT-

CACCA-3′; AQP3: sense/antisense, 5′-CCCATCGTGTCCCCACTC-3′/5′-GCCGATCATCAGCTGGTACA-3′; S100p: sense/antisense, 5′-TGCAGAGTGGAAAAGACAAGGAT-3′/5′-CCACCTGGGCATCTCCATT-3′; GAPDH: sense/antisense, 5′-CCATGGAGAAGGCTGGG-3′/5′-CAAAGTTGTCATGGATGACC-3′.

In vitro wound closure assay

Confluent monolayers of overnight serum starved SACC cells were scratched with a yellow pipette tip to create wounded areas with 400—600 μm distance. For pharmacologic inhibitor studies, SACC-83 was supplemented 30 min before scratched with vehicle (DMSO), 10 μM U0126 (Promega), or 10 μM LY294002 (Calbiochem). Wounded monolayers were then incubated for 24 h either in serum-free medium alone or with the addition of human recombinant epiregulin (R&D System). Wounded monolayers were photographed (40×) at 0 and 24 h after scratched. In vitro wound closure was represented by the decreased distance remaining uncovered by cells. Average rate of wound closure was calculated as the equation of decreased distance/incubated time. Data were presented as mean ± standard error from three independent experiments. To inhibit cell proliferation, parallel experiments were conducted with the addition of 0.5 μg/ml of mitomycin C. Migratory activity was assessed by the rate of wound closure in the presence of mitomycin C, while proliferative activity was determined as the rate of wound closure in the absence of mitomycin C subtracts the rate of wound closure in the presence of mitomycin C.

Transwell invasion and migration assays

Cell invasion assays were performed using Transwell chambers (Corning Costar) with a polycarbonate membrane (6.5 mm diameter, 8 μm pore size). The membrane was coated with 20 μg extracellular matrix (ECM) gel (Sigma—Aldrich) before use. The lower chamber contained 10% FBS medium alone or with addition of 2 nM human recombinant epiregulin. The overnight serum starved cells were harvested and seeded in the upper chambers with 1×10^5 cells/well in serum-free medium containing 0.2% BSA. For pharmacologic inhibitor studies, the cells were supplemented with corresponding inhibitors 20 min before seeded. After incubation for 12 h at 37 °C in 5% CO_2, cells on the top surface of the membrane were wiped off with a cotton swab, and the membrane were fixed and stained with crystal violet. Cells on the bottom surface of the membrane were examined with a light microscope at magnification of 200×. Cells from 6 to 10 random fields across three replicate wells were captured for counting and the average number of invaded cells per field represented activities of Transwell invasion assay. For cell migration assays, the same procedure was performed, except that Transwell chambers were not coated with ECM gel.

Western blot analysis

Western Blot analysis was performed as previously described.[22] Briefly, overnight serum starved SACC cells were incubated in serum-free medium with addition of 2 nM epiregulin for 0—240 min. At selected time points, cells were washed with PBS and lysed with lysis buffer, containing 1% Nonidet P-40, 5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 100 mM sodium orthovanadate and 1:100 protease inhibitors mixture (Sigma—Aldrich). The protein concentrations were determined using the BCA assay (Pierce). Forty microgram aliquots of the protein extracts were subjected to 12% SDS polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membrane (Millipore). The membrane was blocked in the blocking buffer (5% nonfat dry milk in TBS-T) for 1 h, probed with primary antibodies (1:1000 for phosphor-ERK1/2 and phosphor-Akt, 1:500 for COX-2) in the same buffer, and incubated for at least 16 h at 4 °C. Then the membrane was washed extensively with TBS-T, and incubated with 1/5000 dilution of horseradish peroxidase-conjugated secondary antibodies (Zymed) for 1 h. After extensive washes, detection of the active bands was performed using the ECL kit (Applygen Technologies Inc., China). In order to normalize the protein loading and transfer, membranes were stripped and reprobed in a similar process with antibodies against ERK1/2 (1:1000), Akt (1:1000), or α-tubulin (1:1000), as appropriate. Except for α-tubulin (Santa Cruz), all other primary antibodies including phosphor-ERK1/2, phosphor-Akt (Thr308), ERK1/2, Akt and COX-2 were purchased from Cell Signaling.

Statistical analysis

The data are expressed as mean ± standard error and were subjected to one-way ANOVA. Multiple means were compared using Tukey's post hoc test. $P < 0.05$ was considered as statistically significant.

Results

Genes up-regulated in SACC-LM

To examine expression profile of genes associated with lung metastatic behavior, a transcriptomic microarray analysis between SACC-83 and SACC-LM was performed. The genes obtained from the genechips for SACC-83 and SACC-LM populations were filtered by a threshold of threefolds change in expression level between the two cell lines. 1340 genes met the criteria: 397 were up-regulated and 943 down-regulated in SACC-LM. Among the up-regulated genes, eight genes, which may be associated with lung metastasis, were picked for validation by quantitative real-time PCR, as shown in Table 1. Among them, the expression of epiregulin was 350-folds higher in SACC-LM than in SACC-83.

Epiregulin promoted migration of SACC-83 through ERK1/2 cascade and PI3K/Akt pathway

To examine whether epiregulin could promote migration of SACC-83, in vitro wound closure assay and Transwell migration assay were applied. In a preliminary experiment, epiregulin treated SACC-83 cells showed a promoted wound closure with maximum effect at 2 nM (data not shown). Therefore, 2 nM epiregulin was used in the following experiments.

The average rate of wound closure in the absence of mitomycin C for SACC-LM during 24 h was 1.59-fold higher than that for SACC-83 ($P = 0.0001$). When stimulated with 2 nM epiregulin for 24 h, the average rate of wound closure for SACC-83 accelerated from 4.67 ± 0.32 μm/h to

Table 1 The expression profile of genes up-regulated in SACC-LM compared with SACC-83

Accession no.	Gene name	Abbreviation	Fold difference[a]	Fold difference[b]
NM_001432.1	Homo sapiens epiregulin	EREG	4.55	350.92
AY151286.1	Homo sapiens cyclooxygenase 2b mRNA, complete cds; alternatively spliced	COX-2	4.51	7.66
NM_005980.1	Homo sapiens S100 calcium-binding protein P	S100p	43.21	23.61
NM_014432.1	Interleukin 20 receptor, alpha	IL-20Rα	46.50	7.16
NM_001544.2	Intercellular adhesion molecule 4, isoform 1 precursor	ICAM-4	5.67	5.79
NM_002851.1	Protein tyrosine phosphatase, receptor-type, Zpolypeptide 1	PTPRZ	6.64	22.16
NM_004925.2	Aquaporin 3	AQP3	5.29	6.46
NM_005923.2	Homo sapiens mRNA for ASK1, complete cds	ASK1	3.04	4.92

All fold change comparing SACC-LM to SACC-83 (SACC-LM/SACC-83).
[a] Fold difference indicated by the microarray analysis.
[b] Fold difference validated by quantitative real-time PCR.

6.46 ± 0.36 μm/h ($P = 0.0025$) (Fig. 1A). Addition of mitomycin C in SACC-83 exhibited a slight reduction of wound closure promoted by epiregulin (Fig. 1A), suggesting that epiregulin accelerated wound closure would be primarily due to promotion of cell migration rather than cell proliferation. Treatment of cells with 10 μM U0126, a specific inhibitor of MEK, not only totally abolished epiregulin promoted wound closure ($P = 0.0001$), but also further inhibited wound closure as compared to untreated cells ($P = 0.0004$) (Fig. 1B). Treatment with 10 μM LY294002, a specific inhibitor of PI3 K, exhibited little effect on epiregulin promoted wound closure ($P = 0.1542$) (Fig. 1B).

For Transwell migration assay, the migrated cells for SACC-LM on the bottom surface of the membrane were 1.74-fold higher than those for SACC-83 ($P = 0.004$). When stimulated with epiregulin for 12 h, the average number of migrated SACC-83 cells per field increased from 150.2 ± 10.4 to 286.9 ± 19.0 ($P = 0.016$) (Fig. 2A and B). Treatment of SACC-83 with 10 μM U0126 or 10 μM LY294002 could inhibit epiregulin induced migration by 43.5% ($P = 0.0006$) and 39.5% ($P = 0.0023$), respectively (Fig. 2C).

Epiregulin facilitated invasion of SACC-83 through ERK1/2 and PI3K/Akt pathway

For Transwell invasion assay, the invaded cells for SACC-LM on the bottom surface of the membrane were 1.60-fold higher than those for SACC-83 ($P = 0.0001$). When stimulated with epiregulin for 12 h, the average number of invaded SACC-83 cells per field on the bottom surface of the membrane increased from 42.8 ± 2.7 to 64.1 ± 4.8 ($P = 0.0001$) (Fig. 2A and B). For pharmacologic inhibitor studies, treatment of SACC-83 with 10 μM U0126 or 10 μM LY294002 could inhibit epiregulin induced invasion by 86.7% ($P = 0.0191$) and 94.7% ($P = 0.0109$), respectively (Fig. 2C), suggesting both ERK/MAPK and PI3K/Akt pathway involved in epiregulin promoted cell invasion.

Activation of ERK1/2 and Akt by epiregulin in SACC-83

Phosphorylation level of ERK1/2 and Akt in SACC-83 was dramatically increased after the cells were treated with epireg-

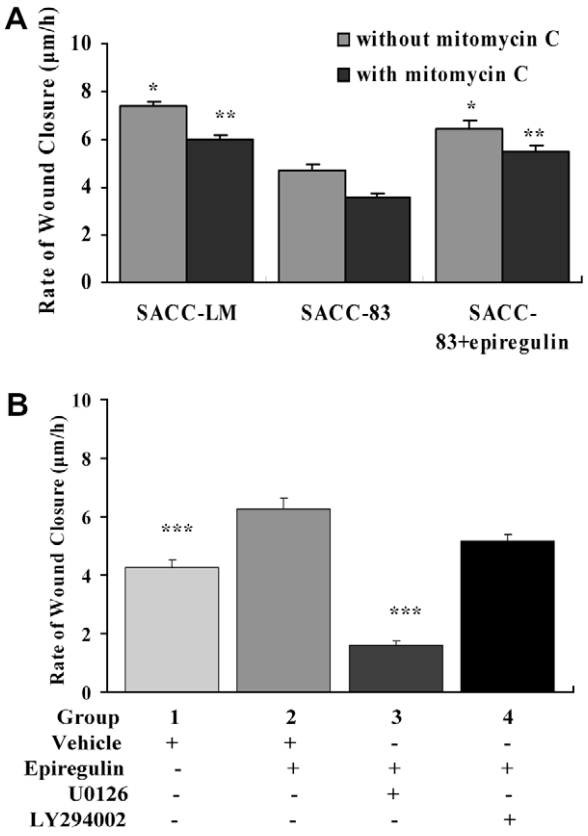

Figure 1 The effects of epiregulin on in vitro wound closure of SACC cells. (A) The rates of wound closure for SACC cells in the absence (gray bar) or presence (black bar) of mitomycin C represented the migratory or proliferative activity, as described in ''Materials and methods''. (B) In the inhibitor assay, the rates of wound closure in the absence mitomycin C were compared in differentially treated SACC-83 cells. Results in A and B represented mean ± standard error of three experiments carried out at least three times (* significantly promoted wound closure without mitomycin C vs. SACC-83, $P < 0.005$; ** significantly promoted wound closure with mitomycin C vs. SACC-83, $P < 0.001$; *** significantly delayed wound closure vs. Group 2, $P < 0.0005$).

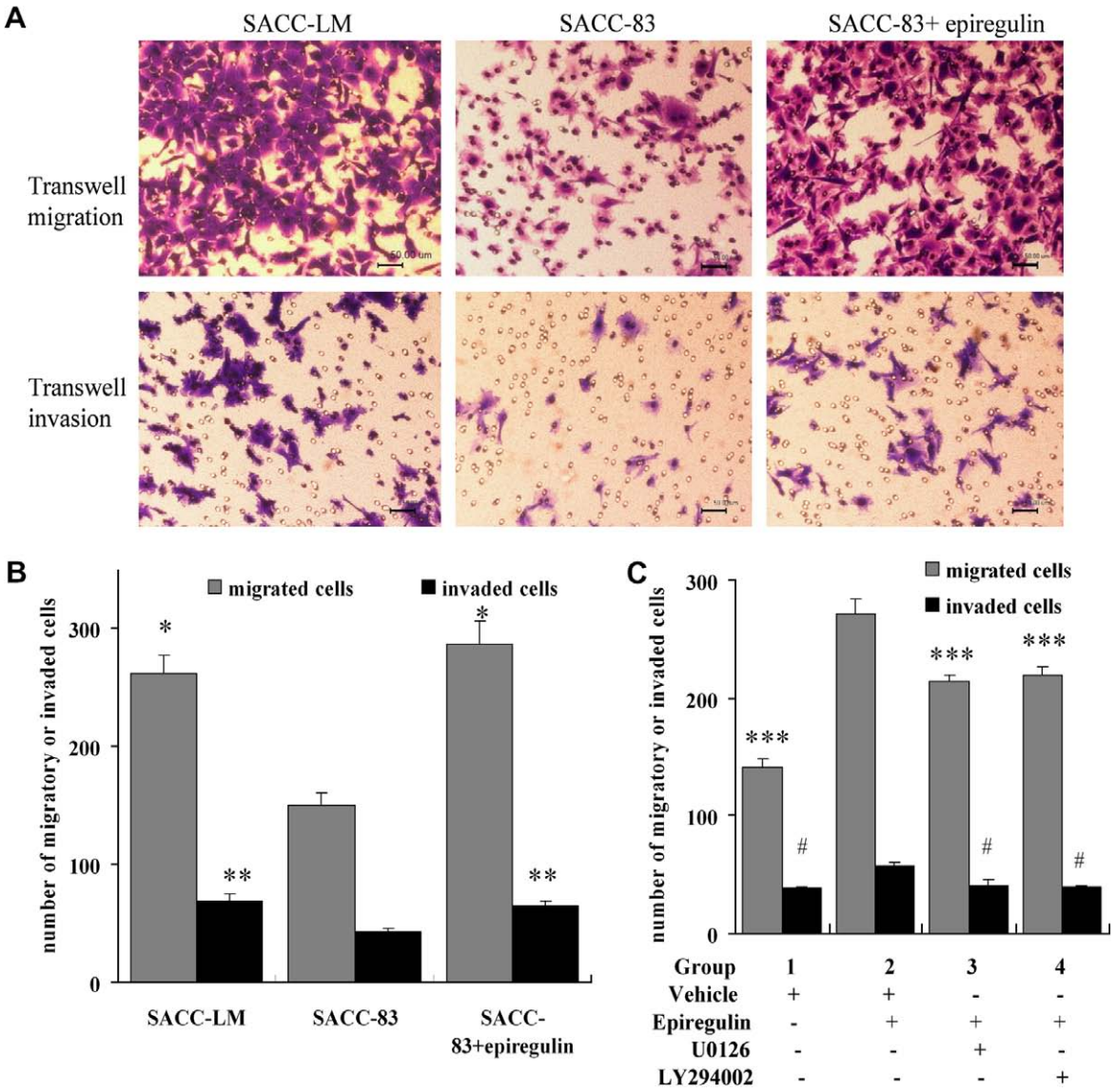

Figure 2 The effects of epiregulin on cell migration and invasion examined by Transwell assays. (A) Light microscope showed the migrated or invaded SACC cells on the bottom surface of the membrane. (B) The average number of cells per field represented the migratory (gray bar) or invasive (black bar) activity during 12 h in Transwell assays. (C) For the inhibitor assay, the average number of the migrated (gray bar) or invaded (black bar) cells was compared in differentially treated SACC-83 cells. Results in B and C represented mean ± standard error of three experiments carried out at least three times (* significantly increased migration vs. SACC-83, $P < 0.001$; ** significantly increased invasion vs. SACC-83, $P < 0.05$; *** significantly decreased migration vs. Group 2, $P < 0.005$; # significantly decreased invasion vs. Group 2, $P < 0.05$).

ulin for 10 min. The increase continued at least until 1 h and dropped back to the basal level by 4 h (Fig. 3A and B). These results indicated epiregulin could strongly activate ERK1/2 and Akt in SACC-83.

Up-regulation of COX-2 by epiregulin in SACC-83 through ERK1/2 and PI3K/Akt pathways

The mRNA expression of COX-2 in SACC-83 was significantly increased at 30 min after the cells were treated with epiregulin, quickly reached the maximum level at 1 h and then exponentially dropped back to the basal line by 24 h (Fig. 4A). The protein expression of COX-2 was also up-regulated by epiregulin (Fig. 4C). We could not observe any significant change of S100p, AQP3, ICAM-4, IL-20Rα and ASK1 in SACC-83 cells after epiregulin treatment for 24 h (data not shown). For pharmacologic inhibitor studies, pretreatment of SACC-83 with 10 μM U0126 or 10 μM LY294002 for 30 min, partially inhibited epiregulin induced COX-2 mRNA expression (Fig. 4B). In addition, the inhibitors also partially blocked epiregulin induced COX-2 protein expression (Fig. 4C), suggesting that both ERK1/2 and PI3K/Akt pathways participated in epiregulin induced COX-2 expression.

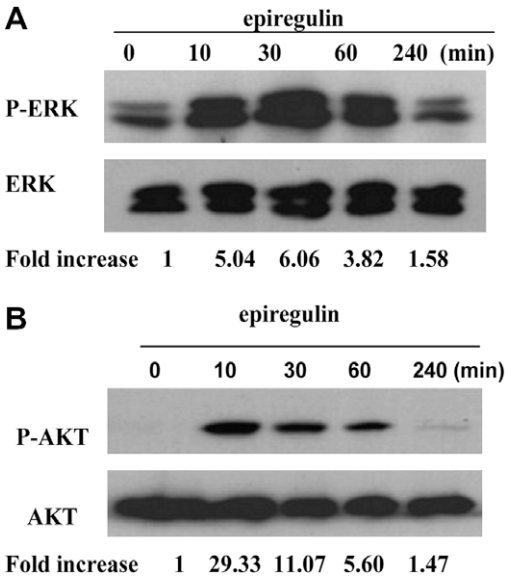

Figure 3 The effects of epiregulin on activation of ERK1/2 and Akt in SACC-83 cells examined by Western Blot analysis. Phosphorylated ERK1/2 (P-ERK1/2) or Akt (P-Akt) in SACC-83 cells treated with 2 nM epiregulin for various time was detected and visualized by immunoblotting with phosphospecific antibodies to either ERK1/2 (A) or Akt (B). Immunoblot images were analyzed with Bandscan Image Analysis software. The signal intensity of phosphorylated ERK1/2 or Akt was normalized the intensity of corresponding total ERK1/2 or Akt. The data showed fold increase over untreated SACC-83 cells (time 0). Results represented one of three independent sets of experiments.

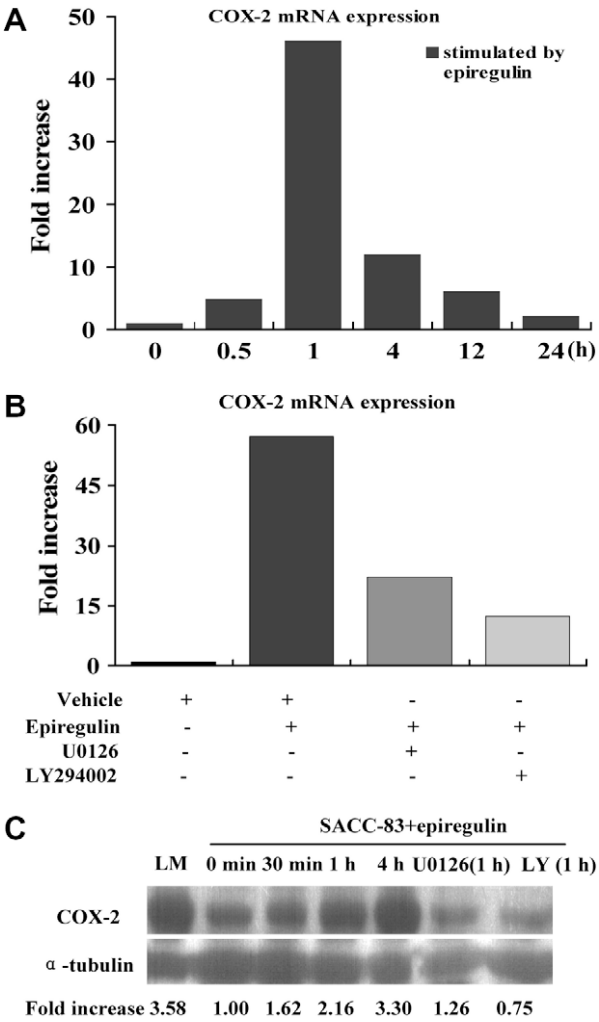

Figure 4 The roles of epiregulin in upregulating COX-2 mRNA and protein expression in SACC-83 cells. (A) Quantitative real-time PCR showed that the relative expressions of COX-2 mRNA in SACC-83 cells treated with 2 nM epiregulin for various time. (B) For the inhibitor assay, the relative expressions of COX-2 mRNA at 1 h were compared in differentially treated SACC-83 cells. (C) The protein expressions of COX-2 in untreated SACC-LM cells and differentially treated SACC-83 cells were compared by Western Blot analysis. Immunoblot images were analyzed with Bandscan Image Analysis software. The signal intensity of COX-2 was normalized the intensity of corresponding α-tubulin. The data showed fold increase over untreated SACC-83 cells (time 0). Results represented one of three independent sets of experiments.

Discussion

Previously, two cell lines, SACC-83 with lower lung-metastatic rate and SACC-LM with higher lung-metastatic rate, were established in our laboratory to explore metastasis mechanism of salivary adenoid cystic carcinoma. With comparative microarray analysis, 8 genes, which were up-regulated in SACC-LM and considered to be related in distinct aspects of tumor metastasis, were selected for quantitative real-time PCR validation. Recently, epiregulin were reported to participate in pulmonary metastases in some invasive cancers.[23,24] The present study showed that epiregulin was 350-folds higher in SACC-LM cells than in SACC-83, implying that overexpression of epiregulin was either a causative factor for metastasis or just the outcome during the SACC-LM cells acquired augmented metastatic abilities. If it is the former, it means that epiregulin may be related to lung metastasis of SACC. Therefore, we examined its effects on migration and invasion of SACC-83 cells.

Epiregulin did significantly promote migration and invasion of SACC-83 cells and its effects could be completely blocked by MEK inhibitor. ERK1/2 and PI3K/Akt are main downstream molecules in EGFR mediated signal cascades.[25] As a pan-EGFR ligand, epiregulin may elicit a more potent mitogenic signal than epidermal growth factor (EGF). Bradley et al reported that compared with EGF or TGF-α, epiregulin was more effective to promote ERK/MAPK activation.[26] ERK pathway plays key roles in angiogenesis, cell proliferation, migration, invasion and metastasis.[27,28] In some malignant tumors, such as malignant mesothelioma or breast cancer, ERK activities detected in metastatic cells and tissues were shown to be higher comparing with the responding non-metastatic tumors.[29,30] In SACC-83 cells, epiregulin stimulated the increase of phospho-ERK1/2 by six folds, and epiregulin promoted cell migration and invasion could be blocked by MEK inhibitor, suggesting increased activation of ERK1/2 participated in epiregulin promoted cell migration and invasion.

The role of Akt in metastasis was disputed recently. Most researchers considered that Akt may promote progression of

primary tumors into metastases.[31,32] Nonetheless, some publications recently challenged the traditional views towards Akt. Expression of activated Akt1, potently blocked in vitro migration and invasion of breast cancer cell lines, and it is suggested that Akt1 may be a possible suppressor of cancer invasion and metastasis.[33,34] In the present study, epiregulin could induce Akt activation rapidly in SACC-83 and PI3K inhibitor partially blocked epiregulin advanced migration and invasion of SACC-83 cells, suggesting that activation of Akt was involved in epiregulin promoted migration and invasion in SACC-83 cells. However, the Akt expression, especially ratio of Akt1 to Akt2 in general untreated cells may reflect the real effect of Akt on metastases. Further studies will be needed to elucidate the specific roles of Akt in lung metastasis of SACC.

Except for ERK1/2 and PI3K, ErbB-mediated signal pathways also include Signal Transducers and Activator of Transcription (STAT), and the phospholipase Cγ (PLCγ).[25] PLCγ pathway is commonly associated with cell motility by numerous growth factors including EGF.[35] Besides, it is reported that ErbB-mediated recruitment of JAK1/2 to phosphorylate STAT1 and STAT3 mainly contributes to cell migration in primary esophageal keratinocytes.[36] As a pan-ErbB ligand, epiregulin is potential to activate ErbB complexes.[10] This may be partially explained why the effect of epiregulin could just partially be blocked by ERK or Akt specific inhibitors. The roles of STAT and PLCγ signaling in response to epiregulin need to be illuminated in the future study.

Recently, COX-2 has been linked to various aspects of cancer progression.[37,38] COX-2 expression in human tumors could be induced by various growth factors, cytokines, oncogenes, and other factors.[39] Overexpressed COX-2 could induce cell migration and invasion in certain cancer cells.[40,41] In the present study, we also found that the mRNA and protein expressions of COX-2 in SACC-LM were much higher than those in SACC-83 and epiregulin could up-regulated COX-2 expression in SACC-83 via ERK1/2 and Akt signaling pathways. Those data suggest that the increased expression of COX-2 in SACC-LM could be partially due to higher expression of epiregulin, and COX-2 may also participate in epiregulin promoted cell migration and invasion in SACC-83 cells.

In conclusion, epiregulin, which was dramatically up-regulated in SACC-LM with higher lung-metastatic rate, could promote cell migration and invasion of SACC-83 cells with lower lung-metastatic rate through activation of ERK1/2 and Akt. The results indicate that epiregulin could be a causative factor for SACC-LM to acquire augmented metastatic abilities derived from SACC-83 cells.

Conflict of interest statement

None declared.

Acknowledgements

We gratefully acknowledge Prof. Kui-hua Zhang from Peking University School and Hospital of Stomatology for critical comments on this manuscript.

This work was supported by the National Natural Science Foundation of China (Grant No. 30572050) and the Natural Science Foundation of Beijing, China (Grant No. 7062069).

References

1. Szanto PA, Luna MA, Tortoledo E, White RA. Histologic grading of adenoid cystic carcinoma of the salivary glands. *Cancer* 1984;**54**(6):1062—9.
2. Zhang JL, Peng B, Chen XM. Expressions of nuclear factor kappa B, inducible nitric oxide synthase, and vascular endothelial growth factor in adenoid cystic carcinoma of salivary glands: correlations with the angiogenesis and clinical outcome. *Clin Cancer Res* 2005;**11**(20):7334—43.
3. Sung MW, Kim KH, Kim JW, Min YG, Scong WJ, Roh JL, et al. Clinicopathologic predictors and impact of distant metastasis from adenoid cystic carcinoma of the head and neck. *Arch Otolaryngol Head Neck Surg* 2003;**129**(11):1193—7.
4. Li SL, Liu XP, Zhang KH. Establishment of a human cancer cell line from adenoid cystic carcinoma of the minor salivary gland. *Zhonghua Kou Qiang Yi Xue Za Zhi* 1990;**25**(1):29—31, 62.
5. Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer* 2003;**3**(6):453—8.
6. Yokota J. Tumor progression and metastasis. *Carcinogenesis* 2000;**21**(3):497—503.
7. Toyoda H, Komurasaki T, Uchida D, Takayama Y, Isobe T, Okuyama T, et al. Epiregulin: a novel epidermal growth factor with mitogenic activity for rat primary hepatocytes. *J Biol Chem* 1995;**270**(13):7495—500.
8. Toyoda H, Komurasaki T, Uchida D, Morimoto S. Distribution of mRNA for human epiregulin, a differentially expressed member of the epidermal growth factor family. *Biochem J* 1997;**326**(1):69—75.
9. Riese 2nd DJ, Komurasaki T, Plowman GD, Stern DF. Activation of ErbB4 by the bifunctional epidermal growth factor family hormone epiregulin is regulated by ErbB2. *J Biol Chem* 1998;**273**(18):11288—94.
10. Shelly M, Pinkas-Kramarski R, Guarino BC, Waterman H, Wang L, Lyass L, et al. Epiregulin is a potent pan-ErbB ligand that preferentially activates heterodimeric receptor complexes. *J Biol Chem* 1998;**273**(17):10496—505.
11. Citri A, Yarden Y. EGF-ERBB signalling: towards the systems level. *Nat Rev Mol Cell Biol* 2006;**7**(7):505—16.
12. Yoon S, Seger Y. The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. *Growth Factors* 2006;**24**(1):21—44.
13. Osaki M, Oshimura M, Ito H. PI3K-Akt pathway: its functions and alterations in human cancer. *Apoptosis* 2004;**9**(6):667—76.
14. Luo J, Manning BD, Cantley LC. Targeting the P13K-Akt pathway in human cancer: rationale and promise. *Cancer Cell* 2003;**4**(4):257—62.
15. Zeng Q, McCauley LK, Wang CY. Hepatocyte growth factor inhibits anoikis by induction of activator protein 1-dependent cyclooxygenase-2— implication in head and neck squamous cell carcinoma progression. *J Biol Chem* 2002;**277**(51):50137—42.
16. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{\Delta\Delta CT}$ method. *Methods* 2001;**25**(4):402—8.
17. Yoshikawa M, Kojima H, Wada K, Tsukidate T, Okada N, Saito H, et al. Identification of specific gene expression profiles in fibroblasts derived from middle ear cholesteatoma. *Arch Otolaryngol Head Neck Surg* 2006;**132**(7):734—42.
18. Zhang Z, Reenstra W, Weiner DJ, Louboutin JP, Wilson JM. The p38 mitogen-activated protein kinase signaling pathway is coupled to toll-like receptor 5 to mediate gene regulation in response to *Pseudomonas aeruginosa* infection in human airway epithelial cells. *Infect Immun* 2007;**75**(12):5985—92.

19. Wang V, Davis DA, Haque M, Huang LE, Yarchoan R. Differential gene up-regulation by hypoxia-inducible factor-1 alpha and hypoxia-inducible factor-2 alpha in HEK293T cells. *Cancer Res* 2005;**65**(8):3299—306.
20. Dowen SE, Crnogorac-Jurcevic T, Gangeswaran R, Hansen M, Eloranta JJ, Bhakta V, et al. Expression of S100P and its novel binding partner S100PBPR in early pancreatic cancer. *Am J Pathol* 2005;**166**(1):81—92.
21. Dveksler GS, Basile AA, Dieffenbach CW. Analysis of gene expression: use of oligonucleotide primers for glyceraldehyde-3-phosphate dehydrogenase. *PCR Methods Appl* 1992;**1**(4): 283—5.
22. Zeng QH, Chen SQ, You ZB, Yang F, Carey TE, Saims D, et al. Hepatocyte growth factor inhibits anoikis in head and neck squamous cell carcinoma cells by activation of ERK and Akt signaling independent of NF kappa B. *J Biol Chem* 2002;**277**(28):25203—8.
23. Nicholson BE, Frierson HF, Conaway MR, Seraj JM, Harding MA, Hampton GM, et al. Profiling the evolution of human metastatic bladder cancer. *Cancer Res* 2004;**64**(21):7813—21.
24. Gupta GP, Nguyen DX, Chiang AC, Bos PD, Kim JY, Nadal C, et al. Mediators of vascular remodelling co-opted for sequential steps in lung metastasis. *Nature* 2007;**446**(7137): 765—70.
25. Katz M, Amit I, Yarden Y. Regulation of MAPKs by growth factors and receptor tyrosine kinases. *Biochim Biophys Acta-Mol Cell Res* 2007;**1773**(8):1161—76.
26. Draper BK, Komurasaki T, Davidson MK, Nanney LB. Epiregulin is more potent than EGF or TGFalpha in promoting in vitro wound closure due to enhanced ERK/MAPK activation. *J Cell Biochem* 2003;**89**(6):1126—37.
27. Giehl K. Oncogenic Ras in tumour progression and metastasis. *Biol Chem* 2005;**386**(3):193—205.
28. Reddy KB, Nabha SM, Atanaskova N. Role of MAP kinase in tumor progression and invasion. *Cancer Metastasis Rev* 2003;**22**(4):395—403.
29. de Melo M, Gerbase MW, Curran J, Pache JC. Phosphorylated extracellular signal-regulated kinases are significantly increased in malignant mesothelioma. *J Histochem Cytochem* 2006;**54**(8):855—61.
30. Adeyinka A, Nui Y, Cherlet T, Snell L, Watson PH, Murphy LC. Activated mitogen-activated protein kinase expression during human breast tumorigenesis and breast cancer progression. *Clin Cancer Res* 2002;**8**(6):1747—53.
31. Shaw LM, Rabinovitz I, Wang HH, Toker A, Mercurio AM. Activation of phosphoinositide 3-OH kinase by the alpha 6 beta 4 integrin promotes carcinoma invasion. *Cell* 1997;**91**(7): 949—60.
32. Park BK, Zeng X, Glazer RI. Akt1 induces extracellular matrix invasion and matrix metalloproteinase-2 activity in mouse mammary epithelial cells. *Cancer Res* 2001;**61**(20): 7647—53.
33. Yoeli-Lerner M, Yiu GK, Rabinovitz I, Erhardt P, Jauliac S, Toker A. Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. *Mol Cell* 2005;**20**(4):539—50.
34. Irie HY, Pearline RV, Grueneberg D, Hsia M, Ravichandran P, Kothari N, et al. Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial—mesenchymal transition. *J Cell Biol* 2005;**171**(6):1023—34.
35. Chen P, Xie H, Sekar MC, Gupta K, Wells A. Epidermal growth factor receptor-mediated cell motility: phospholipase C activity is required, but mitogen activated protein kinase activity is not sufficient for induced cell movement. *J Cell Biol* 1994;**127**(3):847—57.
36. Andl CD, Mizushima T, Oyama K, Bowser M, Nakagawa H, Rustgi AK. EGFR-induced cell migration is mediated predominantly by the JAK-STAT pathway in primary esophageal keratinocytes. *Am J Physiol Gastroint Liver Physiol* 2004;**287**(6): G1227—37.
37. Tsujii M, Kawano S, DuBois RN. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *Proc Natl Acad Sci USA* 1997;**94**(7):3336—40.
38. Basu GD, Pathangey LB, Tinder TL, Lagioia M, Gendler SJ, Mukherjee P. Cyclooxygenase-2 inhibitor induces apoptosis in breast cancer cells in an in vivo model of spontaneous metastatic breast cancer. *Mol Cancer Res* 2004;**2**(11): 632—42.
39. Singh B, Berry JA, Vincent LE, Lucci A. Involvement of IL-8 in COX-2 mediated bone metastases from breast cancer. *J Surg Res* 2006;**134**(1):44—51.
40. Takaoka K, Kishimoto H, Segawa E, Hashitani S, Zushi Y, Noguchi K, et al. Elevated cell migration, invasion and tumorigenicity in human KB carcinoma cells transfected with COX-2 cDNA. *Int J Oncol* 2006;**29**(5):1095—101.
41. Singh B, Berry JA, Shoher A, Ramakrishnan V, Lucci A. COX-2 overexpression increases motility and invasion of breast cancer cells. *Int J Oncol* 2005;**26**(5):1393—9.