erbB2 gene silencing and its effect on PTEN in SACC-83 salivary adenoid cystic carcinoma cells

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Abstract. erbB2 gene plays an important role in carcinoma formation. erbB2 overexpression was observed in many types of tumours, including salivary carcinoma. However, a putative erbB2 and PTEN interaction remains largely unknown in salivary adenoid cystic carcinoma cells. The purpose of this study was to silence erbB2 gene and investigate the functional relationship between erbB2 and PTEN in SACC-83 salivary adenoid cystic carcinoma cells. erbB2-specific siRNAs were transfected into SACC-83 cells using cationic liposome. RT-PCR, immunocytochemistry and Western blotting were employed to detect erbB2 and PTEN expression. Compared with the control groups, erbB2 mRNA expression was decreased in the erbB2-siRNA transfection group, and immunocytochemistry and Western blotting indicated a concordant erbB2 protein reduction. The average optical density values for erbB2 proteins in erbB2-siRNA transfected group were significantly lower than that in the control groups ($P<0.05$). On the other hand, PTEN expression at both mRNA and protein levels were not significantly affected by erbB2 silencing ($P>0.05$). In conclusion, the data indicate that siRNA could effectively silence erbB2 gene expression in SACC-83 cells, but PTEN expression appeared unaltered following erbB2 silencing. PTEN expression might not be strictly associated with erbB2 amplification in SACC-83 cells. Future studies will more closely examine the molecular and biological relationships of erbB2 and PTEN in salivary adenoid cystic carcinoma.

Introduction

Salivary adenoid cystic carcinoma (SACC) is a common oral tumour, known to be highly destructive and unpredictable (1-3). Previous research has demonstrated a possible role of oncogenic activation in SACC (4-6). erbB2, also called HER2, is a cell surface receptor tyrosine kinase (RTK) and becomes internalized upon ligand binding which can trigger a multitude of signaling pathways, such as MAPKs and PI3K. The erbB family consists of four structurally related tyrosine kinase receptors: erbB1, erbB2, erbB3, and erbB4. These four receptors contain multiple cytosolic tyrosines, which can interact with one or more adaptor proteins to activate downstream signaling pathways when phosphorylated. Furthermore, the erbB receptors display functional redundancy with overlapping signaling pathways. erbB2 is the preferred heterodimerization partner of all erbB proteins, and plays important roles in the lateral transmission of signals between other erbB receptors (7). Consequently, erbB2 has been shown to trigger signal transduction leading to cell growth and differentiation. Tumours showing erbB2 amplification/overexpression have been shown to demonstrate increased aggressiveness and metastatic potential and decreased overall survival (8-10). In previous studies, erbB2 gene expression was found to be elevated in many types of tumours, including salivary gland carcinoma (11-13).

PTEN is an important tumour suppressor gene located on chromosome 10q23.3. The encoded protein displays homology with the catalytic domain of tyrosine phosphatases, as well as the cytoskeletal proteins tensin and auzilin. PTEN proteins function to dephosphorylate phosphatidyl inositol 3, 4, 5-triphosphate produced by PI3K, thereby acting as a major negative regulator of the PI3K pathway via dual-specificity phosphorylation. PTEN can be deactivated by phosphorylation in various cell types. Upon phosphorylation, PTEN undergoes conformational changes accompanied by loss of activity (14). Impaired PTEN activities have been observed in many tumours (15,16), contributing to tumourigenesis via induction of apoptosis and cell cycle arrest. PTEN also regulates cell adhesion and migration through interactions with focal adhesion kinase. PTEN acts through inhibition of the PI3K/Akt pathway which promotes cell survival and proliferation (17). The biological function of PTEN is multifaceted, required for the control of cell cycle, cell growth, and apoptosis. PTEN can also be inactivated via gene mutation, loss of mRNA expression, hypermethylation, degradation or post-translational modification (18,19).

Extensive studies have investigated the functional interaction of PTEN with other genes. However, a putative PTEN

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and erbB2 interaction remains largely unknown. RNA interference (RNAi) is a key mechanism in host defense and gene regulation. Moreover, gene silencing using small interfering RNA (siRNA) has become a powerful approach to probe gene function in mammalian cells (20-22). In the present study, we employed siRNA to silence erbB2 gene expression in the SACC-83 cell line, and to evaluate the effect of erbB2 silencing on PTEN expression.

Materials and methods

Cell culture. The adenoid cystic carcinoma cell line SACC-83 was obtained from Peking University School of Stomatology (Beijing, China). SACC-83 cells were cultured in RPMI-1640 medium supplemented with 10% calf-serum and grown in humidified atmosphere at 37˚C with 5% CO_2. Experiments were performed with cells undergoing logarithmic growth.

siRNA transfection. siRNA target sequences were designed as previously described (23,24). The sequences of 19-21 nt were selected as RNAi target sites based on erbB2 conserved sequences. The BLAST homology search was performed to ensure no significant sequence homology with other human genome sequences. A nonspecific siRNA and a fluorescent siRNA were designed as control. These siRNAs were named erbB2-siRNA, control-siRNA, and FAM control-siRNA, respectively. The sequences were: erbB2-siRNA, sense 5'-GCCUCACAGAGAUCUUGAATT-3', antisense 5'-UUC AAGAUCUCUGUGAGGCTT-3'; control-siRNA, sense 5'-UUCUCCGAACGUGUCACGUTT-3', antisense 5'-ACG UGACACGUUCGGAGAATT-3'; FAM control-siRNA, sense 5'-UUCUCCGAACGUGUCACGUTT-3', antisense 5'-ACGUGACACGUUCGGAGAATT-3'. siRNAs were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China).

Four experimental groups were designed: blank control group, liposome control group, negative control-siRNA group, and erbB2-siRNA group. SACC-83 cells were grown in six-well plates in 2 ml growth medium without antibiotics for 24 h before transfection. siRNA-Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) complexes were prepared according to the manufacturer's instructions, and used in transfection when cells reached 50-70% confluence. Lipofectamine 2000 (5 μl) was added to 200 μl of Opti-MEMI reduced serum medium without serum, mixed gently and kept at room temperature for 5 min. Then 5 μl of 20 μM siRNA was added to 200 μl of Opti-MEMI reduced serum medium and mixed gently. The final concentration of siRNA when added to the cells was 50 nM. After incubation at room temperature for 5 min, the diluted siRNA and the diluted Lipofectamine 2000 were combined, mixed gently and kept at room temperature for 20 min. The siRNA-Lipofectamine 2000 complexes were added to each well containing cells and medium to a final volume of 2 ml and mixed gently by rocking the plate back and forth. Cells were incubated at 37˚C in a CO_2 incubator. The medium was replaced by complete medium after 6 h. Transfection efficiency was assayed by fluorescence microscopy of cells transfected with FAM control-siRNA for 6 h. Cells were harvested 48 h post-transfection and erbB2 and PTEN expression were assayed.

RT-PCR. Total RNAs were extracted from the transfected SACC-83 cells using TRIzol Reagent (Invitrogen). RNA quality and concentration were analyzed by measuring absorbance at 260 nm and 280 nm; the A_{260}/A_{280} ratios were in the range of 1.8-2.0. For single-stranded cDNA synthesis, the reverse transcription reaction was performed using the Revertaid First Strand cDNA synthesis kit (Fermentas, Burlington, Canada). The random hexamer primers and RevertAid™ M-MuLV reverse transcriptase were used with 2.0 μg of total RNAs from each sample. Subsequently, PCR was performed by standard methods and GAPDH was amplified as the internal control. The PCR reactions contained cDNA template 2 μl, forward primer 1 μl, reverse primer 1 μl, 2X *Taq* PCR MasterMix 25 μl, and dH_2O with a final volume of 50 μl. PCR was carried out under the following conditions: the template cDNA was first denatured at 94˚C for 5 min, followed by 30 cycles of amplification each of 94˚C for 30 sec, 59˚C for 30 sec, and 72˚C for 30 sec. Finally, the reaction was terminated at 72˚C for 7 min. GAPDH PCR was carried out for 20 cycles. PCR primers were designed according to published work and GenBank (23). The primer sequences were: erbB2, sense 5'-CTGTGCCCGAGTGTGCTA-3', antisense 5'-GTCCCCATCAAAGCTCTCC-3'; PTEN, sense 5'-TAGAGCGTGCAGATAATGACAAGGA-3', antisense 5'-TGAACTGCTAGCCTCTGGATTTGA-3'; The house-keeping gene GAPDH, sense 5'-GCACCGTCAAGGCTGA GAAC-3', antisense 5'-TGGTGAAGACGCCAGTGGA-3'. The primers were synthesized commercially (Sangon Biotech Co., Ltd. Shanghai, China). All results were confirmed in five independent PCRs. After amplification, PCR products were electrophoresed on a 2% (w/v) agarose gel and visualized by ethidium bromide staining to identify the amplified product of the expected size. For semi-quantification, an image of the gel was captured, and the intensity of the bands was quantified using the GeneTools from Syngene gel analysis system.

Immunocytochemical analysis. Anti-erbB2 and anti-PTEN antibodies were rabbit polyclonal antibodies, and the secondary antibody was goat anti-rabbit antibody (Beijing Boisynthesis Biotechnology Co., Ltd., Beijing, China). SACC-83 cells were seeded on glass coverslips. Cells were fixed with 4% (v/v) formaldehyde for 30 min at 4˚C and rinsed 3 times with PBS. Immunocytochemistry assay was performed according to the manufacturer's protocol. The antibodies were diluted to 1:200 in PBS. The seeded slides were incubated at 4˚C with primary antibody overnight. On the following day, the slides were rinsed in PBS and incubated at room temperature with secondary antibody for 10 min. The cells were stained with 3,3'-diaminobenzidine (DAB). The immunoreactivity was detected with a light microscope.

Western blot analysis. The infected SACC-83 cells were harvested. Total cellular proteins were extracted and separated by 8% SDS-polyacrylamide gel electrophoresis. Subsequently, the proteins were transferred onto nitrocellulose membranes. The membranes were blocked by TBST with non-fat milk for 1 h at room temperature. The membranes were washed and incubated with the primary antibodies for anti-erbB2, anti-PTEN, and anti-GAPDH (Beijing Boisynthesis Biotechnology Co.) for 2 h, followed by incubation with secondary antibodies

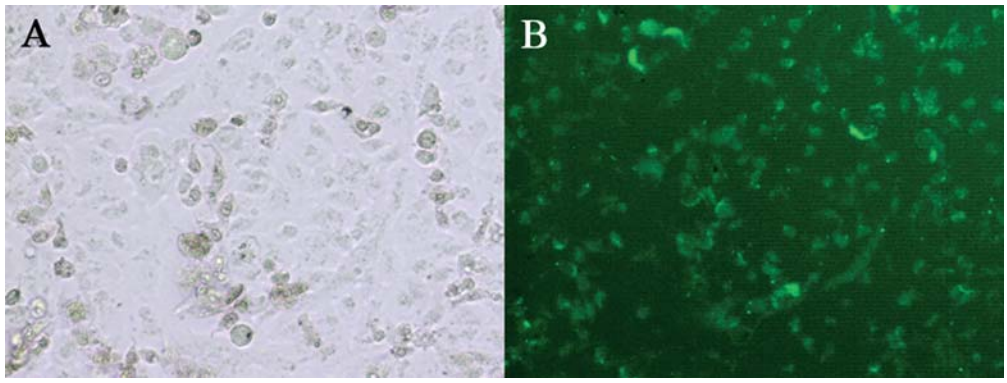

Figure 1. Transfection efficiency of siRNA. Following FAM control siRNAs were transfected, SACC-83 cells were observed via light microscope field (A) or fluorescent microscope field (B). The siRNA was transfected successfully.

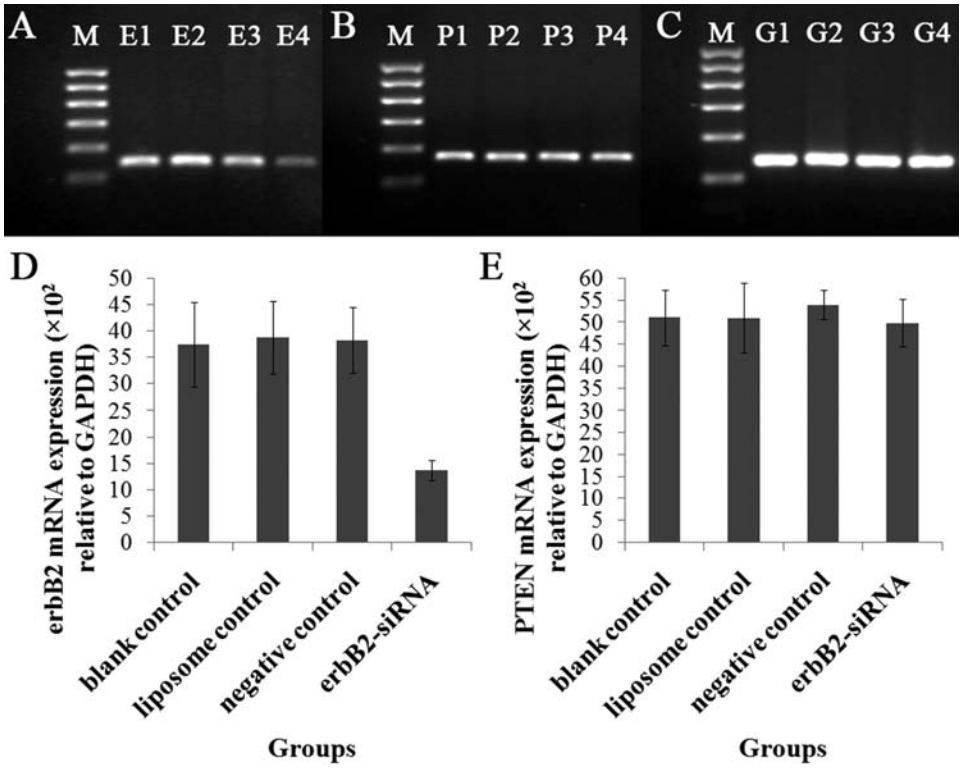

Figure 2. RT-PCR analysis of mRNA expression. The erbB2, PTEN, and GAPDH mRNA expression in the blank control group (E1, P1, G1), the liposome control group (E2, P2, G2), the negative control-siRNA group (E3, P3, G3), and the erbB2-siRNA group (E4, P4, G4), respectively. The results shown the mRNA of erbB2 was decreased in erbB2-siRNA tansfection group (A). The mRNA level of PTEN was not different among groups (B). The mRNA levels of erbB2 (D) and PTEN (E) were relative to GAPDH.

for 1 h. GAPDH was used as an internal control. Gel bands were detected with ECL.

Statistical analysis. For all measurements as needed, the statistical significance between groups was assessed by One-Way ANOVA or Kruskal-Wallis H-test based on homogeneity of variance test (SPSS 13.0, USA). $P<0.05$ was considered as statistically significant.

Results

siRNA transfection efficiency. The transfection efficiency was assayed by fluorescence microscopy after cells were transfected by FAM control-siRNA for 6 h. A robust transfection efficiency of 87% was observed (Fig. 1).

mRNA expression of erbB2 and PTEN. Following siRNA transfection of SACC-83 cells, the mRNA expression of erbB2 and PTEN was examined by RT-PCR. The PCR products were analyzed by electrophoresis on a 2% agarose gel, and the expected size was observed. erbB2 expression amplification was detected in SACC-83 cells. Importantly, erbB2-siRNA transfected cells exhibited significantly lower erbB2 mRNA expression compared with that of the blank control group, liposome control group, and negative control-siRNA group (Fig. 2). There was significant difference

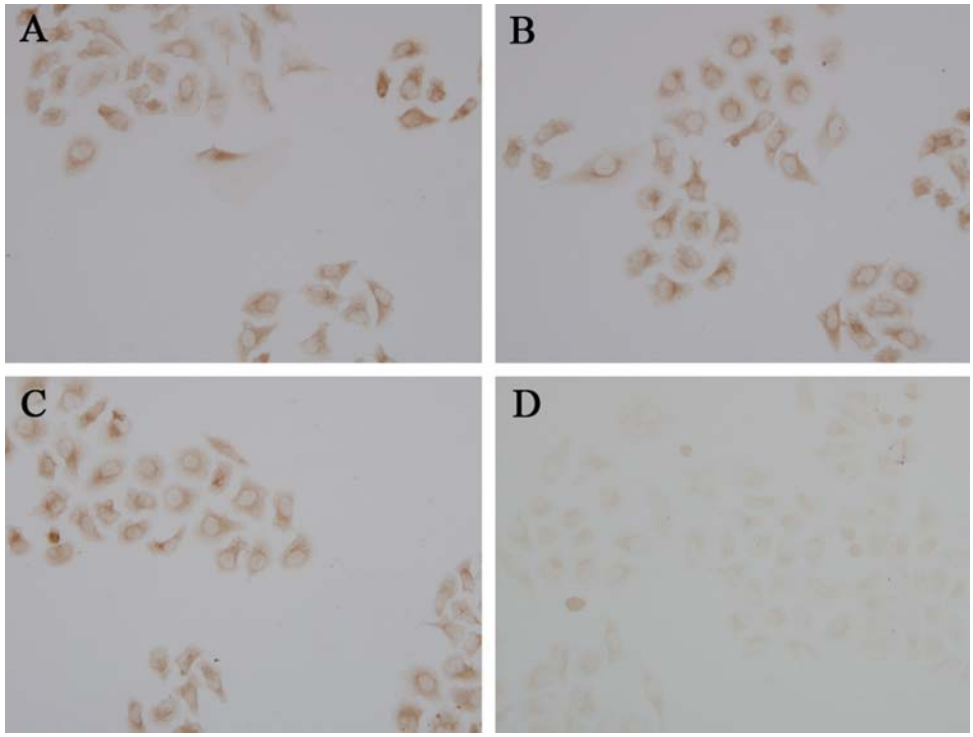

Figure 3. Immunocytochemistry of erbB2 in the blank control group (A), the liposome control group (B), the negative control-siRNA group (C), and the erbB2-siRNA group (D). The SACC-83 cells showed lower level of brown immunostaining in siRNA transfected cells than that in controls.

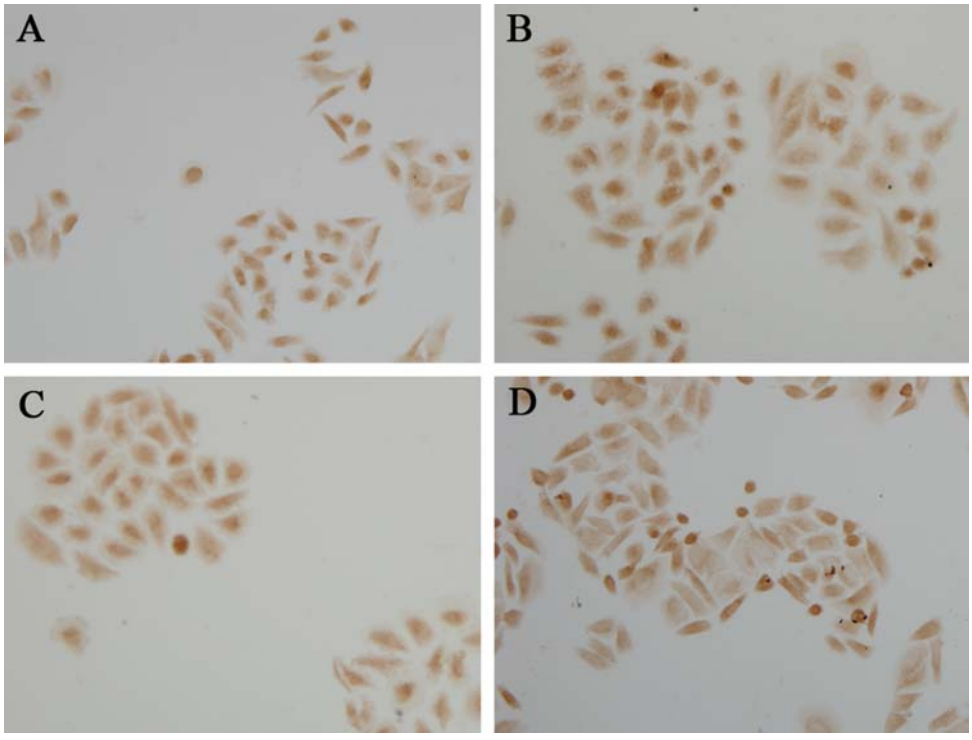

Figure 4. Immunocytochemistry of PTEN in the blank control group (A), the liposome control group (B), the negative control-siRNA group (C), and the erbB2-siRNA group (D). The SACC-83 cells showed brown immunostaining that was not different among groups.

between the silenced cells and control cells ($P<0.05$), estimated to be reduction in expression. Therefore, the expression of erbB2 mRNA in SACC-83 cells was knocked down successfully (Fig. 2A). On the other hand, the expression of erbB2 mRNA showed no significant difference among control groups ($P>0.05$). As shown in Fig. 2, the expression of PTEN mRNA was not affected following erbB2 knock-down, with equivalent expression of PTEN mRNA in erbB2

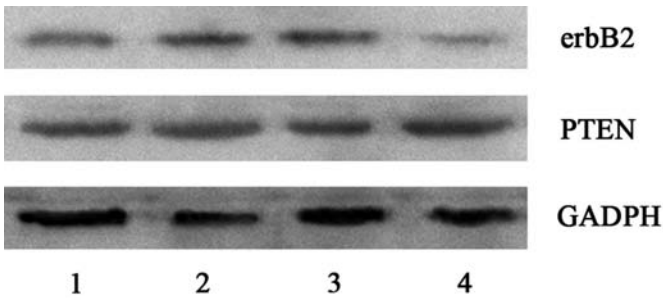

Figure 5. The protein expression of erbB2 and PTEN in the blank control group (1), the liposome control group (2), the negative control-siRNA group (3), and the erbB2-siRNA group (4). The protein level of erbB2 was decreased in erbB2-siRNA transfected cells, and the protein levels of PTEN were not different among groups. It was in accord with the mRNA levels.

silenced cells and control cells (Fig. 2B). No significant differences between groups were observed (P>0.05).

erbB2 and PTEN protein expression. Immunocytochemical staining of protein was analysed by Image-ProPlus software (Media Cybernetics, USA). The gray scale of the stained area was measured under identical conditions. The average optical density for erbB2 protein expression in the erbB2-siRNA transfection group was lower compared with the value in the blank control group, the liposome control group, and the negative control-siRNA group, respectively. It indicated marked reduction in erbB2 protein following erbB2-siRNA transfection. The observed differences were significant (P<0.05) (Fig. 3). Consistent with the above mRNA result, the average optical density for PTEN protein expression in the blank control group, the liposome control group, the negative control-siRNA group, and the erbB2-siRNA group showed no significant differences between groups (P>0.05) (Fig. 4). The protein expression of erbB2 and PTEN were evaluated by Western blotting. The results were in accord with the mRNA levels (Fig. 5).

Discussion

This study confirmed the power of *in vitro* RNAi knockdown of erbB2 in SACC-83 cells. erbB receptors, especially of erbB2, play an important role in carcinoma formation, and its dysfunction promotes tumourigenesis. Overexpression of the erbB2 gene was frequently observed in human tumours, including those of breast, lung, stomach, and oral cavity (25-27). Capable of stable and highly specific silencing of gene expression, siRNAs have been extensively applied to silence abnormal gene expression in the treatment of cancer (28). Given that erbB2 was found to be overexpressed in SACC (11,12), we wished to examine the functional consequence of erbB2 silencing in SACC-83 cells. In the present study, after erbB2-siRNA was transfected into the SACC-83 adenoid cystic carcinoma cells, erbB2 expression were significantly reduced at both mRNA and protein levels. Since erbB family mediated signaling played a critical role in cell growth, survival, adhesion, and motility (9), our data suggest that erbB2 gene is a feasible RNAi target for gene silencing therapy against salivary adenoid cystic carcinoma.

Few studies have examined the functional relationship of PTEN and erbB2 in SACC. In this study, we investigated the relationship between erbB2 and PTEN, specifically in SACC-83 cells. PTEN expression was examined following siRNA-mediated silencing of erbB2 gene in SACC-83 cells. We found that silencing of erbB2 did not induce changes in PTEN expression. Although PTEN and erbB2 both contribute to the progression of certain tumours, our data suggest that PTEN expression might not be strictly associated with erbB2 amplification in SACC.

In addition to expression regulation, PTEN might be regulated at multiple levels, such as mutation, loss of heterozygosity, promoter methylation, or activation of alternative signaling pathway. Several studies have shown synergistic effects of PTEN with other genes during tumourigenesis (29,30). On the other hand, there was also evidence that PTEN was not involved in tumour progression (31-33). Chen *et al* suggested that homozygous deletion of PTEN gene was unlikely to be a feature of oral squamous cell carcinoma, and speculated that cancerous growths from different tissues may have multiple means by PTEN inactivated (34). Another study has investigated PTEN mutation in ovarian cancers, and their results shown that PTEN mutation was not observed in primary ovarian cancers or ovarian cancer cell lines (35). Furthermore, siRNA knockdown of PTEN in erbB2 overexpressing breast cancer cells demonstrated robust responses of breast cancer patients to lapatinib monotherapy regardless of PTEN status (36). Overall, PTEN, a dual phosphatase which appears to play an important role in PI3K/Akt mediated anti-tumour signaling. In SACC-83 cells, however, PTEN and erbB2 expression appears independently regulated.

In summary, we demonstrated that erbB2 was effectively silenced in SACC cells via siRNA knockdown, providing proof of principle for using RNAi in trial protocol to investigate erbB2 biological function in SACC. Furthermore, the expression of PTEN was not altered following erbB2 silencing. Future studies will be required to examine the molecular and biological relationship of erbB2 and PTEN in SACC in greater detail.

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