ORAL DISEASES

Oral Diseases (2011) 17, 53–59. doi:10.1111/j.1601-0825.2010.01702.x

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ORIGINAL ARTICLE

Increase of *ZASC1* gene copy number in recurrent oral carcinoma

W-F Chiang[1,2,*], P-S Hung[2,*], S-Y Liu[3], T-C Yuan[2], K-W Chang[2], Y-P Chen[2], Y-C Liu[4], S-C Lin[2]

[1]*Department of Oral and Maxillofacial Surgery, Chi-Mei Medical Center, Liouying;* [2]*Institute of Oral Biology, School of Dentistry, National Yang-Ming University, Taipei;* [3]*Department of Oral and Maxillofacial Surgery, Chi-Mei Hospital, Yongkang;* [4]*Department of Ophthalmology, Taipei Veterans General Hospital, Taipei, Taiwan*

BACKGROUNDS: The chromosome 3q26 locus is a hotspot region carrying oncogenes that frequently altered in neoplasms. ZASC1 is a zinc finger protein transcription factor localized on 3q26. Our previous study showed the frequent amplification of 3q26, including the *ZASC1* gene, in oral squamous cell carcinoma (OSCC). This study investigated the copy number changes of *ZASC1* gene from primary to recurrent OSCC and the functions of ZASC1 in OSCC cells.

MATERIALS AND METHODS: A total of 27 OSCC patients with primary and recurrent tumors were examined for *ZASC1* and *TERC* copy number changes using Quantitative PCR analysis. Exogenous expression and knockdown of ZASC1 were carried out to specify the oncogenic potential of ZASC1 in OSCC cells.

RESULTS: A *ZASC1* copy number that has increased from primary to recurrent tumor counterparts in tissue pairs suggested the importance of ZASC1 in tumor progression. The increase of *ZASC1* gene copy number in recurrent tumors was associated with the consumption of betel quid in patients. OSCC cells expressing ZASC1-FLAG fusion protein showed increased proliferation. After the knockdown of endogenous *ZASC1* expression using small interference RNA, the growth and colony formation of SAS OSCC cells decreased.

CONCLUSIONS: The findings support the hypothesis that *ZASC1* localized on 3q26 contributes to the recurrence of OSCC.

Oral Diseases (2011) **17**, 53–59

Keywords: amplification; carcinoma; oral cavity; recurrence; *ZASC1*

Correspondence: Shu-Chun Lin, PhD, Institute of Oral Biology, School of Dentistry, National Yang-Ming University, No. 155, Li-Nong St., Sec.2, Taipei 112, Taiwan. Tel: +886 2 28267272, Fax: +886 2 2826 4053, E-mail: sclin@ym.edu.tw
*Contributed equally to this work
The authors declare no conflict of interest.
Received 7 December 2009; revised 5 March 2010; accepted 7 March 2010

Introduction

Oral squamous cell carcinoma (OSCC) is a common malignancy and causes death worldwide (Shieh *et al*, 2007; Chiang *et al*, 2008; Hung *et al*, 2008a,b; Liu *et al*, 2010). Its pathogenesis was associated with the oncogenic stimuli including addictive oral habits, viral infection and others (Ko *et al*, 1995; Shieh *et al*, 2007; Chiang *et al*, 2008; Scully and Bagan, 2009). Betel quid chewing was known as the most potent risk for the OSCC in Taiwanese and some other Southeastern Asians (Ko *et al*, 1995; Liu *et al*, 2010). Our previous study showed the occurrence of hypertetraploid chromosomal alterations in oral keratinocytes following long-term treatment with betel nut extract (Lu *et al*, 2006). The chromosome 3q25–28 region carries several well-known genes frequently altered in cancers, including *TERC* (human telomerase RNA template gene), *PIK3CA*, and *TP63*. Our previous studies also showed frequent amplification of 3q26–27 loci in OSCC associated with betel chewing (Lin *et al*, 2002, 2005; Chen *et al*, 2004).

Telomerase plays important roles in cellular immortalization and malignant transformation and is a hallmark of cancer (Lin *et al*, 2006). *TERC*, localized on 3q26.3 (the core region of 3q25–28 amplicon), encodes the RNA component essential for telomerase activity (Yokoi *et al*, 2003). A progressive increase in the *TERC* copy number after an increase in the severity of neoplasia has been reported in cervical carcinogenesis (Tu *et al*, 2009). We identified *TERC* amplification in brushed samples from 25% of betel nut chewers and patients with oral leukoplakia (32%), which suggested that *TERC* amplification could be an early event of oral carcinogenesis (Lin *et al*, 2005).

Krüpple-like factor (KLF) zinc finger protein family members share common Cys2His2 (C2H2) zinc finger DNA binding domains, and are involved in a diversity of biological or pathological situations including tumorigenesis (Nonet *et al*, 2001; Bureau *et al*, 2009). The *ZASC1* gene, localized on 3q26.3, encodes a 56-kDa KLF C2H2 transcription factor (Imoto *et al*, 2003; Bogaerts *et al*, 2005). ZASC1 interacts with DNA and

may function as a transcriptional repressor (Bogaerts *et al*, 2005). It was identified as a target for amplification in esophageal SCC (ESCC) and functional studies showed that ZASC1 expression promoted the growth of ESCC cells (Imoto *et al*, 2003). *ZASC1* is activated through 3q26 amplification, and its consequential overexpression is involved in the pathogenesis of ESCC (Imoto *et al*, 2003). We noted the drastic increase of the prevalence of *ZASC1* amplification from oral leukoplakia (18%) to OSCC (70%), which suggested that *ZASC1* alterations may be involved in a relatively later stage of neoplastic process than *TERC* is (Lin *et al*, 2005).

Local recurrence is a major cause of OSCC mortality (Tabor *et al*, 2004; Sinha *et al*, 2009; Liu *et al*, 2010), while no any clinocopathological was found to affect local recurrence in our previous study (Liu *et al*, 2010). There were only few studies addressing the mechanism underlying OSCC recurrence. A retrospective study reported the genetic similarity of the primary and recurrent head and neck squamous cell carcinoma (HNSCC), which suggests that the residual tumor cells were the source of recurrence (Tabor *et al*, 2004). Signatures of gene expression predisposed for local recurrence have been addressed (Ginos *et al*, 2004; Sinha *et al*, 2009). Sinha *et al* (Sinha *et al*, 2009) reported a high incidence of *p16* gene methylation in margins of tongue carcinomas. Patients with positive *p16* gene methylation in margins exhibited increased risk of recurrence. This study investigated the gene copy number of *ZASC1* in paired primary and recurrent OSCC and indicated the relevance of *ZASC1* amplification with OSCC recurrence.

Materials and methods

Subjects

Twenty-seven primary tumors were resected with a margin >1 cm and based on the stage of the lesion. Histopathological evaluation indicated that all margins were free of tumor involvement. Local recurrence was defined as tumor recurrence at a site <2 cm from primary lesion within 3 years (Tabor *et al*, 2004). The clinical parameters of cases with available primary and recurrent tumor tissues were listed in Table 1. Tumor components were retrieved from resected tissues using glass needles under the microscope. All patients quitted the betel chewing after the resection of primary tumors, while around a half of the original drinkers and smokers contacted these substances occasionally after the resection of primary tumors. Another subset of 46 primary OSCC samples was used in this study to compare the discrepancies in gene copy number between primary and recurrent tumors (Lin *et al*, 2005). The protocols of this study were approved by both the National Yang-Ming University Institute Review Board and the Chi-Mei Hospital Review Board.

Cell culture

Cell samples analyzed were from an SAS OSCC cell line and normal human oral keratinocytes (NHOK) that had been tested in our previous studies were cultivated under conditions previously described (Lin *et al*, 2005; Hung *et al*, 2008a).

Table 1 Clinicopathological parameters of primary OSCC

Parameter	*Value*
Age (years)	52.5 ± 1.8
Median recurrence duration (months)	9
Median survival after recurrence (months)	36
Median survival after primary resection (months)	41
Alcohol drinking	
Non-drinker (0)	1
Social drinker (1)	12
Habitual drinker (2)	14
Betel quid chewing	
Non-chewer (0)	1
Chewer, <10 qpd (1)	5
Chewer, 10–20 qpd (2)	10
Chewer, >20 qpd (3)	11
Cigarette smoking	
Non-smoker (0)	6
Smoker, <1 ppd (1)	8
Smoker, 1–2 ppd (2)	10
Smoker, >2 ppd (3)	3
Primary site	
Buccal mucosa	13
Tongue	6
Others	8
Lymphovascular permeation	
Absence	26
Presence	1
Perineural invasion	
Absence	26
Presence	1
TNM stage	
T1, 2	18
T3, 4	9
N0	18
N+	9
Stages I, II	11
Stage III, IV	16
Survival	
Alive	13
Dead (All died of OSCC)	14

The scores within parenthesis were used for the analysis in Figure 2. qpd, quid per day; ppd, pack per day; TNM, Tumor, Node, Metastasis; OSCC, oral squamous cell carcinoma.

qPCR

DNA was isolated from tissues and cells using a DNA isolation kit (Qiagen, Valencia, CA, USA). Quantitative PCR (qPCR) analysis was performed with specific primers (Table 2) to determine the gene copy numbers for *TERC* and *ZASC1*. PCR reactions were performed using a kit (SYBR Green PCR Master Mix; Applied Biosystems, Foster City, CA, USA) and a sequencing detection system (7900HT Fast Real-Time quantitative PCR System; Applied Biosystems). EN, LN, ET, and LT were the number of cycle at which the fluorescence signal passed threshold (Ct) for the experimental primers of NHOK DNA, *LINE1* primer for NHOK DNA, experimental primers for tumor DNA, and *LINE1* primer for tumor DNA, respectively (Chiang *et al*, 2008). EN – LN and ET – LT were the ΔCt of NHOK DNA and tumor DNA, respectively. ΔΔCt was the

Table 2 Primers used for qPCR and qRT-PCR

	Forward primer	*Reverse primer*
qPCR		
TERC	CCTCGTCCACCAGTCCCTAT	AACAGTCCATTGGCAGTTGAGA
ZASC1	AAGCATTCAGCTGACTTGCCTC	GAATAATCAAGTTGTCTCATGGAC
LINE1	CCGCTCAACTACATGGAAACTG	GCGTCCCAGAGATTCTGGTATG
qRT-PCR		
ZASC1	GATGTACAGTTCTCCTCAAGC	TACCACATGGCTATGCAAGTC
GAPDH	AACCATGAGAAGTATGACAACA	GAGTCCTTCCACGATACCAAAG

qPCR, quantitative PCR; qRT-PCR, quantitative real-time PCR.

difference of ΔCt values between NHOK and tumor sample. The copy number was calculated as $2^{\Delta\Delta Ct}$ (Lin *et al*, 2005). The quantitative data shown are means and standard error (s.e.) from triplicate experiments. The specificity of the qPCR reactions was validated using a melting-curve analysis.

qRT-PCR
Total RNA was isolated using a reagent (Trizol; Molecular Research Center, Cincinnati, OH, USA) and reverse-transcribed to cDNA. PCR assays with specific primer pairs (Table 2) were used to analyze changes in *ZASC1* mRNA expression. Amplification of *GAPDH* served as an internal control. ZC, GC, ZT, and GT of SAS cells were defined as the Ct for *ZASC1* primer of control cDNA, *GAPDH* primer for control cDNA, *ZASC1* primer for treated cDNA and *GAPDH* primer for treated cDNA, respectively. The difference in *ZASC1* mRNA expression was calculated as $2^{(ZC-GC)-(ZT-GT)}$. The quantitative data shown are means and s.e. from triplicate experiments.

Western blot analysis
Whole cell lysates (50 μg) were electrophoretically separated on a 10% denaturing polyacrylamide gel. A mouse antibody (Anti-Flag; Sigma-Aldrich, St Louis, MO, USA) was used to detect the expression of ZASC1-Flag fusion in cell lysate (Hung *et al*, 2008b). GAPDH signals were used as internal controls.

ZASC1-Flag ectopic expression
cDNA of *ZASC1* was cloned into the pCMV-3Tag-3 vector with 3 × Flag tagging sequences in the C-terminal of the cloning site (Stratagene, La Jolla, CA, USA) (Hung *et al*, 2008b). The construct was confirmed by sequencing and designated pCMV-ZASC1-Flag. The plasmid and a vector alone (VA) control were transfected into SAS cells. The cells were treated with 300 μg ml^{-1} G418 (Invitrogen, Carlsbad, CA, USA) for 3 days to enrich the transfected cell population (Hung *et al*, 2008b).

ZASC1 knockdown
siRNA synthesized (5′-AACCGCCAGCTAAACTT-TGTA-3) against nt 953–973 in exon 4 of *ZASC1* was designated si-*ZASC1*. si-*ZASC1* and scrambled siRNA control (Ambion, Austin, TX, USA) were transfected into SAS cells using a transfection reagent (siPORT amine; Ambion) according to the manufacturer's protocols.

Cell growth
The viability of cells was measured using a trypan blue exclusion assay. The growth curves were plotted and statistically analyzed.

Anchorage-independent colony formation
Cells were suspended in 1.3% methylcellulose (Fluka, Seelze, Germany) in culture medium at a density of 1×10^5 per well, plated in 6-well culture plates on a layer of 0.9% agarose (Sigma-Aldrich) in culture medium containing 10% fetal bovine serum, and then cultured for 1 week at 37°C. Agar growth medium was replaced every other day. The number of colonies with diameters >50 μm in five fields per well – a total of 15 fields in triplicate experiments – were counted (Hung *et al*, 2008b).

Statistical analysis
Statistical significance, set at $P < 0.05$, was determined using *t*-tests, Mann–Whitney tests, and linear regression (Prism 5; GraphPad, San Diego, CA, USA).

Results

Recurrent OSCC had higher ZASC1 CN than primary counterparts
Twenty-seven pairs of tissue were analyzed. The mean time for local recurrence was 14 months. qPCR showed that the gene copy numbers of *TERC* were 4.4 ± 0.5 in primary and 6.7 ± 1.1 in recurrent OSCC ($P = 0.07$; Figure 1a). The gene copy numbers of *ZASC1* were 5.4 ± 0.6 in primary and 8.0 ± 1.1 in recurrent OSCC ($P = 0.04$; Figure 1b). The gene copy numbers for both *TERC* and *ZASC1* were 1.5 times higher in recurrent than in primary tumors. The fold change of gene copy numbers from primary to recurrent tumors was plotted in Figure 1c. A strong correlation between the fold change of *ZASC1* and the fold change of *TERC* was identified ($P < 0.0001$; Figure 1c).

We previously identified the gene copy numbers of *TERC* and *ZASC1* in 46 microdissected primary OSCC samples (Lin *et al*, 2005). During a mean follow-up period for 27 months, five (11%) patients recurred and three (6%) patients exhibited neck metastasis in this subset. The integration of the present

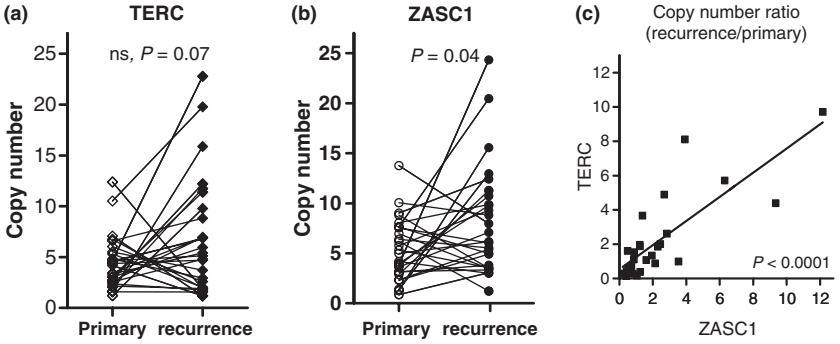

Figure 1 Gene copy number of *TERC* and *ZASC1* in OSCC tissue pairs. Before-and-after plot of *TERC* (**a**) and *ZASC1* (**b**) copy numbers in primary and recurrent tumors, respectively. ns, not significant. Paired *t*-test. (**c**) Linear regression dot plot and analysis of the gene copy number ratio of recurrent and primary tumors

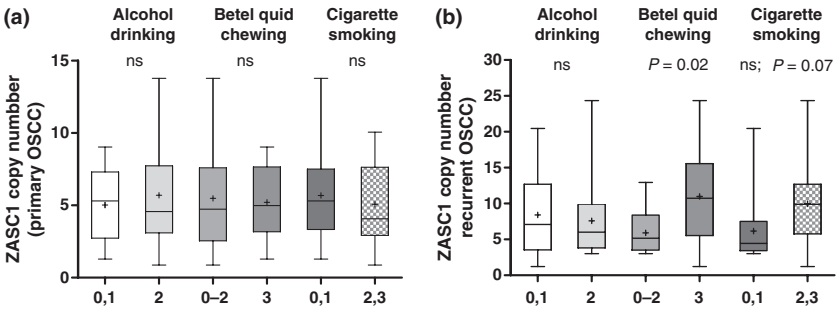

Figure 2 Gene copy number of *ZASC1* in relation to oral habits. Box and whisker plot of primary (**a**) and recurrent (**b**) tumors. Horizontal bars, median values, +, mean values, ns, not significant. Un-paired *t*-test. Please refer to Table 1 for the scores in *X*-axis

cohort with the previous subset gave 32 primaries with recurrence and 38 primaries without recurrence or metastasis. Cross analysis indicated the gene copy numbers of *TERC* in tumors with recurrence were 4.2 ± 0.6 and 2.6 ± 0.6 in tumors without recurrence or metastasis. The difference was not statistically significant ($P = 0.07$, un-paired *t*-test). The gene copy numbers of *ZASC1* in tumors with recurrence were 5.2 ± 0.7 and 3.4 ± 0.5 in tumors without recurrence or metastasis. The difference was statistically significant ($P = 0.04$, un-paired *t*-test). The gene copy numbers for both *TERC* and *ZASC1* were around 1.5 times higher in primary tumors with recurrent than primary tumors without recurrence.

Higher ZASC1 copy numbers in recurrent OSCC were associated with more betel quid consumption

To address the association between oral habits and the gene copy number changes of *ZASC1*, the consumption of alcohol, betel quid, and cigarette (Table 1) were analyzed with gene copy number of *ZASC1* in primary and recurrent tumors. The analysis indicated an absence of association between oral habits and *ZASC1* gene copy number in primary tumors (Figure 2a). However, the amount of betel quid consumption was statistically associated with the higher *ZASC1* gene copy number in recurrent tumors ($P = 0.02$; Figure 2b). Although the *ZASC1* gene copy number in recurrent tumors was also higher in patients consumed more tobaccos, the difference was not statistically significant ($P = 0.07$; Figure 2b). The *ZASC1* gene copy number was not associated with other clinocopathological parameters (detailed analysis not shown).

Ectopic ZASC1 expression increased SAS cell proliferation

Quantitative RT-PCR analysis showed that *ZASC1* mRNA expression was five times higher in SAS cells transfected with pCMV-ZASC1-Flag plasmid than in cells transfected with VA control (Figure 3a). Western blotting detected the expression of a ~60 kDa ZASC1-Flag fusion protein (Figure 3b). The proliferation of SAS cells transfected with pCMV-ZASC1-Flag was significantly higher than in VA-transfected cells (Figure 3c). The difference in anchorage-independent colony formation, however, was not statistically significant ($P = 0.08$; Figure 3d).

Blocking ZASC1 expression decreased the in vitro oncogenic potential of SAS cells

ZASC1 mRNA expression was significantly reduced to < 10% in si-ZASC1 treated cells relative to si-scramble-treated cells ($P < 0.01$, Figure 4a). The growth of SAS cells significantly decreased following the knockdown of *ZASC1* mRNA expression ($P < 0.05$, Figure 4b). Anchorage-independent colony formation was also significantly lower in si-*ZASC1*-treated cells ($P < 0.001$, Figure 4c).

Discussion

In these 27 OSCC with paired primary and recurrent specimens, we found that the *ZASC1* gene copy number increased significantly in the recurrent OSCC specimens compared with their primary OSCC. Comparison between different subsets also identified a significant increase in *ZASC1* gene copy number in primary OSCC with recurrence than without recurrence. Although the

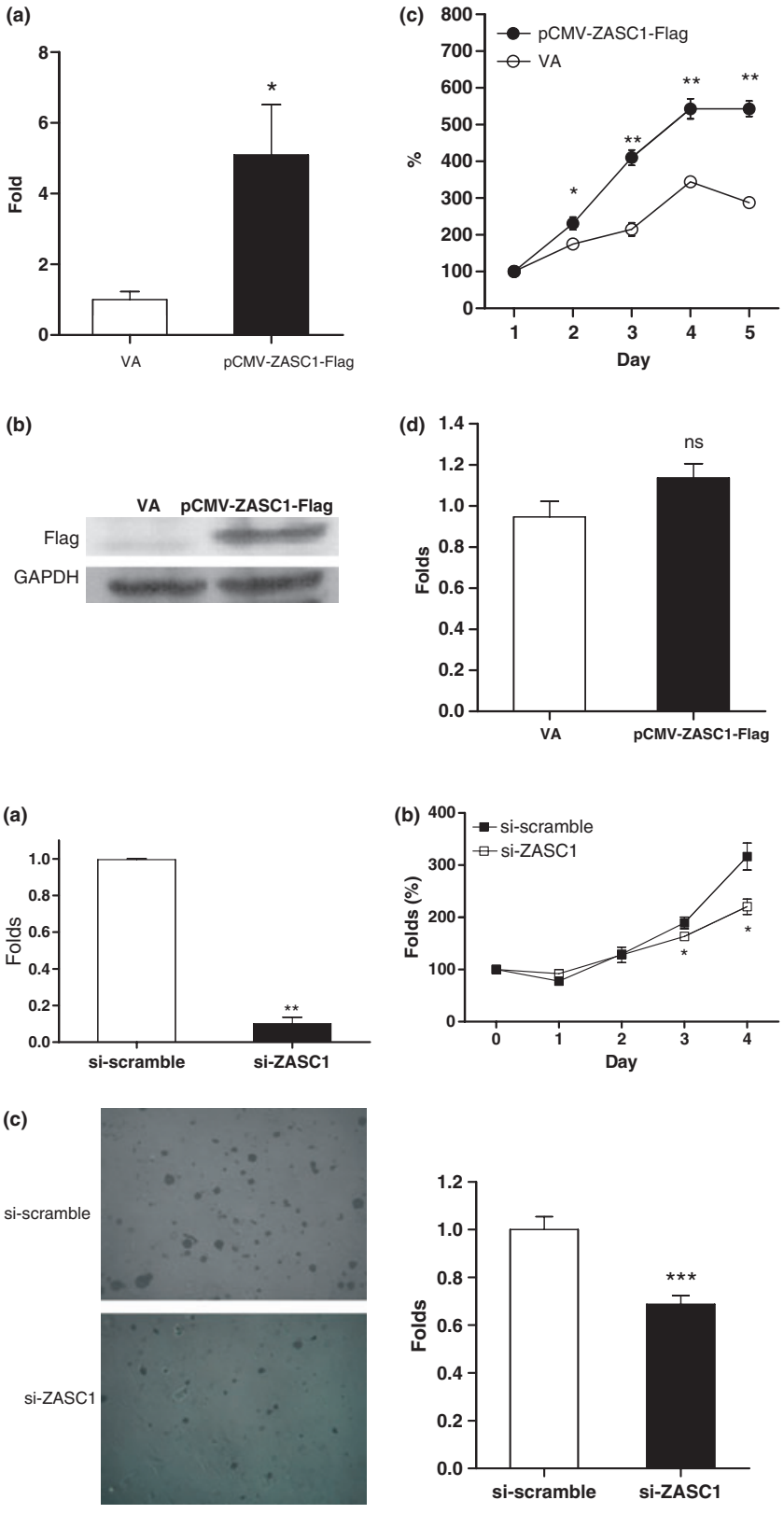

Figure 3 Phenotypic analysis of SAS cells transfected with vector alone (VA) and pCMV-ZASC1-Flag (**a**) qRT-PCR analysis. (**b**) Western blotting was used to detect the ZASC1-Flag fusion protein. (**c**) Growth curve. (**d**) Anchorage-independent colony formation. Data are means ± s.e. from at least a triplicate analysis. $*P < 0.05$, $**P < 0.01$; ns, not significant; Mann–Whitney analysis

Figure 4 Phenotypic analysis of SAS with the treatment of si-scramble and si- *ZASC1*. (**a**) qRT-PCR analysis. (**b**) Growth curve. (**c**) Anchorage-independent colony formation. Lt, representative fields; Rt, quantitation. Bars, 100 μm. Data are means ± s.e. from at least a triplicate analysis. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; Mann–Whitney analysis

TERC gene copy number also increased, the difference was only borderline statistically significant. It is likely that *TERC* copy number increases more in the earlier stage of the tumorigenic cascade (Lin *et al*, 2005). The increase of the gene copy numbers of *ZASC1* and *TERC* during recurrence suggests that 3q26.3 is an important locus for tumor progression in the 3q25–28 region. Although we were unable to stratify *ZASC1* and *TERC* amplification

as determinants of clinicopathological status, the importance of changes in *ZASC1* and *TERC*, as well as other 3q26.3 events such as *PIK3CA* amplification and overexpression, for disease progression should not be underestimated. The 3q26.3 oncogenic genes should be considered targets for OSCC diagnosis and therapy.

Our study showed the local recurrence as a major cause of treatment failure of OSCC (Liu *et al*, 2010). Residual cancer cells in the resection margins or cells with certain genetic alterations in the fields adjacent to the tumors may underlie the recurrence (Sinha *et al*, 2009). We identified high *ZASC1* and *TERC* gene copy numbers in paired primary and recurrent tissue, which confirmed that recurrent lesions have genetic alterations similar to those of primary lesions. However, because these genetic alterations become more conspicuous in recurrent lesions, we hypothesize that chronic stimuli from carcinogenic substances in the oral environment generate accumulated 3q26.3 genomic alterations. Our results seemed to partially substantiate this postulation as the *ZASC1* copy number was irrelevant to oral habits in primary tumors, while it was significantly higher in recurrent tumors with more conspicuous betel quid consumption. Alternatively, increased proliferative activity in precancerous fields arisen in the oral mucosa of areca chewers can allow the development of genetic instability and higher amplitude of gene copy number changes. Epidemiologic clues indicated that betel was a stronger risk factor than alcohol or tobacco for OSCC susceptibility (Ko *et al*, 1995). Betel nut extract treatment resulted in karyotypic alterations of oral keratinocyte (Lu *et al*, 2006). This study provided clinical evidences linking the betel quid consumption to *ZASC1* amplification. The methylation status of *p16* in resection margin was validated as a predictor of tongue cancer recurrence (Sinha *et al*, 2009). Impaired cells that carry the 3q26.3 alterations in the tissue surrounding resected tumors may selectively increase advantages for tumor recurrence. Although this study demonstrated a higher copy number of *ZASC1* in recurrent tumors relative to non-recurrent tumors, additional studies in a large number of cases is needed to insight the feasibility of using *ZASC1* amplification in surgical margin to evaluate tumor recurrence (Lin *et al*, 2005).

In view of the eminent *ZASC1* amplification in OSCC and its association with OSCC recurrence (Lin *et al*, 2005), the functional and phenotypic roles of *ZASC1* in OSCC are still obscure. Transfection of pCMV-ZASC1-Flag plasmid significantly increased the proliferation of SAS cells but induced only limited anchorage-independent growth. The increase of *ZASC1* mRNA expression and Flag confirmed the expression of ectopic ZASC1-Flag. It was unclear whether ZASC1-Flag fusion protein causes differential activity other than ZASC1, which may underlie the discrepancies in growth and colony formation. Thereby, the knockdown of endogenous *ZASC1* expression in SAS cells was followed to insight the phenotypic influences. *ZASC1* knockdown significantly decreased the *in vitro* oncogenic potential. The findings of ZASC1 in the regulation of OSCC growth were in agreement with the findings in ESCC (Imoto *et al*, 2003). ZASC1 was found to interact with α-N-catenin by its zinc finger domains for nuclear transportation of catenin molecules (Bogaerts *et al*, 2005). Although nuclear localization of catenin was an importnat event for malignancies, it did not appear eminent in HNSCC or OSCC (Gasparoni *et al*, 2002; Rodriguez-Pinilla *et al*, 2005). The oncogenic potential of ZASC1 in OSCC could be independnet from its interaction with catenin. Because ZASC1 is a transcription factor, its expression may activate a panel of genes regulating tumorigenesis. The study of ZASC1 expression and the expression of these targets in OSCC tissues may validate diagnostic or therapeutic uses.

In this study, we identified the high concordance in *ZASC1* and *TERC* gene amplification in OSCC recurrence. ZNF217 is a KLF protein that promotes the immortalization of mammary epithelial cells by activating telomerase activity (Nonet *et al*, 2001). Because TERC is also essential for regulating telomerase activity, and both the clinical and functional clues in this study confirm the role of ZASC1 in oral tumorigenesis, the synergism of *ZASC1* and *TERC* in OSCC development and progression requires further study.

Acknowledgements

We thank Dr C-J Liu, Ms Y-S Lo and Ms L-T Kuo for their help. This study was supported by Chi-Yang grant 95CY004, grant NSC96-2628-B-010-MY3 and grant NSC 97-2314-B-003-MY3 from the National Science Council, Taiwan.

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