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Significance of the TGF-β1/IL-6 axis in oral cancer

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ABSTRACT

The aim of the present study was to explore specific molecular markers that could lead to new insights into the identification of innovative treatments in oral cancer. The role of TGF-β1 (transforming growth factor-β1) and its predictive power in the prognosis of oral cancer has been identified. Human oral cancer cell lines, including SCC4 and SCC25, were selected for cellular experiments. Changes in tumour aggressiveness, responses to treatment and the signalling pathway responsible were investigated *in vitro*. Furthermore, 125 oral cancer tissue specimens were constructed into tissue microarray blocks for immunohistochemical analysis to correlate the expression of TGF-β1 with clinical outcome. Using *in vitro* experiments, our results revealed that activated TGF-β1 signalling resulted in more aggressive tumour growth, augmented the epithelial–mesenchymal transition and more resistance to treatment. Activated IL-6 (interleukin-6) signalling could be the mechanism underlying the effects of TGF-β1 on oral cancer. Regarding clinical data, the incidence of TGF-β1 immunoreactivity in oral cancer specimens was significantly higher than in non-malignant epithelium and positively linked to IL-6 staining. Furthermore, expression of TGF-β1 was significantly correlated with the risk of lymph node involvement, disease recurrence and shorter survival in patients with pathological stage III–IV oral cancer. In conclusion, the TGF-β1/IL-6 axis had predictive power in the prognosis of oral cancer, and targeting TGF-β1 could represent a promising treatment strategy.

INTRODUCTION

Radical surgery remains the treatment of choice for oral cavity cancer. Post-operative CCRT (computer controlled radiation therapy) results in better loco-regional control and survival for locally advanced head and neck cancer patients with high risk factors [1]. Despite considerable advances in treatment, 40–50 % of patients with locally advanced disease relapse with local or distant disease progression [2]. Investigating specific molecular markers related to the imbalance of cell proliferation, the capacity for tissue invasion and treatment sensitivity

Key words: epithelial–mesenchymal transition, head and neck squamous cell carcinoma, interleukin-6 (IL-6), oral cancer, transforming growth factor-β1 (TGF-β1).

Abbreviations: ATM, ataxia telangiectasia mutated kinase; BrdU, bromodeoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; EMT, epithelial–mesenchymal transition; GFP, green fluorescent protein; HIF-1α, hypoxia-inducible factor-1α; HNSCC, head and neck squamous cell carcinoma; IL-6, interleukin-6; IRS, immunoreactive score; MMP-9, matrix metalloproteinase-9; OSCC, oral squamous cell carcinoma; 8-oxoG, 8-oxoguanine; PI3K, phosphoinositide 3-kinase; RFP, red fluorescent protein; RT, reverse transcription; STAT3, signal transducer and activator of transcription 3; TGF, transforming growth factor; TGF-βR, TGF-β receptor; TMA, tissue microarray; VEGF, vascular endothelial growth factor.

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could provide new insights into the identification of innovative treatments.

TGF (transforming growth factor)-β1 is a regulator of epithelial homoeostasis predominantly through canonical Smad-dependent mechanisms [3]. Although canonical TGF-β1 signalling inhibits epithelial proliferation, TGF-β1 overexpression has been reported to promote tumorigenesis via a paracrine effect on the tumour stroma [4]. In a different cellular context, TGF-β1 can promote tumour growth because it is able to induce changes in transcriptional activities through a Smad-independent pathway that the re-programmes epithelial cells. TGF-β1 is overexpressed in many types of cancer and correlates with tumour invasion [4,5]. Regarding HNSCC (head and neck squamous cell carcinoma), studies in mouse models and human samples illustrate that disruptions in TGF-β signalling promote epithelial carcinogenesis [4,6–8]. However, there are a number of contradictory reports concerning TGF-β1 expression levels in HNSCC [6,9]. Its role in oral cancer and tumour progression also remain to be elucidated. Therefore the aim of the present study was to determine the role of TGF-β1 in OSCC (oral squamous cell carcinoma).

Understanding the mechanisms underlying the role of TGF-β1 in oral cancer is pivotal in identifying novel targets for pharmacological intervention. It has been reported that TGF-β could activate several non-Smad pathways, including PI3K (phosphoinositide 3-kinase)/Akt and IL-6 (interleukin-6) signalling, which may be associated with treatment resistance [5,10,11]. According to a previous study [12], activated IL-6 signalling was associated with aggressive tumour behaviour in pharyngeal cancer. Activation of IL-6R (IL-6 receptor)/gp130 stimulated multiple pathways implicated in the regulation of tumour growth and metastatic spread [13]. Therefore it is hypothesized that overexpression of TGF-β1 and activated IL-6 signalling could mediate the aggressive tumour growth and treatment resistance in oral cancer. In the present study, we have investigated the prognostic value of TGF-β1, the underlying mechanisms responsible and the correlation with IL-6 signalling.

MATERIALS AND METHODS

Tissue specimens and characteristics of the patients

The Institutional Review Board of our hospital approved the present study. Patients who were at pathological stage I–II OSCC or had interrupted curative treatment for various reasons, and who had distant metastasis at diagnosis were excluded from the present study. Between 2005 and 2009, 125 OSCC cases with pathological stage III–IV OSCC (buccal, $n = 48$; gingival, $n = 24$; lip, $n = 10$; tongue, $n = 35$; palate, $n = 8$) who completed curative treatment at our hospital were enrolled in the study. Informed consent was obtained from the enrolled patients. The curative treatment for locally advanced oral cancer comprises surgery combined with adjuvant radiotherapy and/or chemotherapy, under guidelines proposed by the head and neck oncology team. Specimens retrospectively collected from these 125 patients were constructed into TMA (tissue microarray) blocks for immunohistochemical analysis by AutoTiss 1000 (Ever BioTechnology). When the blocks were available, H&E (haematoxylin and eosin)-stained slides were re-evaluated by a pathologist to assess the quality of TMA slides. Data concerning the initial diagnosis, staging, pathological factors, recurrence and survival were collected and are shown in Table 1.

Immunohistochemistry

Tissue sections from TMA blocks were mounted on to slides, deparaffinized with xylene, and dehydrated in a graded ethanol series. Blocks were incubated overnight with an anti-TGF-β1 antibody and anti-IL-6 antibody (1:20 dilution). A breast cancer specimen was used as a positive control for TGF-β1, and oral cancer specimens were stained for IL-6 as reported previously [12]. The results from the immunohistochemical staining were examined using the Good Speed scan slide scanning platform and were analysed by Image Pro Plus 6.3. This analysis demonstrated that the antibodies targeted proteins that were highly expressed in tumour epithelial cells (see Figure 1) compared with adjacent non-cancerous tissue. For histological evaluation of TGF-β1 and IL-6 staining, in addition to Image Pro Plus 6.3 analysis, slides were re-checked by a single pathologist. The specimens were assessed using a semi-quantitative IRS (immunoreactive score). The IRS was calculated by multiplying the staining intensity (graded as: 0 = no, 1 = weak, 2 = moderate and 3 = strong staining) and the percentage of positively stained cells (0 = <10 % of stained cells, 1 = 11–50 % of stained cells, 2 = 51–80 % of stained cells and 3 = >81 % of stained cells). The criterion for positive staining was a specimen with an IRS scoring grade $\geqslant$2.

Cell culture and reagents

The human oral cancer cell lines SCC4 and SCC25 were obtained from the Bioresource Collection and Research Center (BCRC). SCC4 is a squamous carcinoma cell line and SCC25 cells originated from a cancer progression model. The origins of SCC4 and SCC25 are both from squamous cell carcinoma of the mouth floor [14]. Human recombinant TGF-β1, the anti-TGF-β1 antibody and the anti-IL-6 antibody were obtained from R&D Systems. The TGF-β1–GFP (green fluorescent protein)-expression vector and TGF-β1–RFP (red fluorescent protein)-silencing vector were purchased from OriGene Technologies.

Table 1 Clinical and pathological characteristics of patients with locally advanced oral cancer according to negative or positive staining of TGF-β1 by immunohistochemistry

SPSS 14.0 was used to perform the log-rank test to determine the significant differences between the groups. All statistical tests were two-sided, with significance defined as $P < 0.05$*. W-D, well-differentiated; M-D, moderately differentiated; P-D, poorly differentiated.

Characteristic	Negative TGF-β1 staining	Positive TGF-β1 staining	*P* value
Patients (*n*)	47	78	
Age (years)	37–79	31–84	
$\geqslant$55 (*n*)	27	38	0.44
$<$55 (*n*)	20	40	
Differentiation			
W-D (*n*)	24	39	0.52
M-D (*n*)	17	25	
P-D (*n*)	3	10	
Unknown	3	4	
Clinical stage			
$\leqslant$III (*n*)	24	35	0.49
IV (*n*)	23	43	
Pathology stage			
III (*n*)	14	22	0.85
IV (*n*)	33	56	
Pathological lymph node metastasis			
N0 (*n*)	26	29	0.047*
N1–N3 (*n*)	21 (N1, $n = 9$; N2–N3, $n = 12$)	49 (N1, $n = 24$; N2–N3, $n = 25$)	
Risk factors†			
No (*n*)	13	20	0.81
Yes (*n*)	34	58	
Pathology T stage			
$\leqslant$T3 (*n*)	23	33	0.47
T4 (*n*)	24	45	
Adjuvant radiotherapy			
$<$4500 cGy (*n*)	13	15	0.37
$\geqslant$4500 cGy (*n*)	34	63	
Failure			
Local/regional failure and/or distant metastasis (*n*)	8	38	0.0004*
Disease-free (*n*)	39	40	

†Risk factors including extracapsular spread, and the presence of preineural, vascular or lymphatic invasion.

Cell growth and clonogenic assay

The effects of TGF-β1 signalling on the cell growth rate were assessed using cells transfected with a TGF-β1-expression vector/silencing vector or following pre-incubation in serum-free medium in the presence or absence of TGF-β1 (5 ng/ml) or anti-TGF-β1 antibody (10 μg/ml) for 48 h. A BrdU (bromodeoxyuridine)–FITC kit from BD Pharmingen was used to detect cell proliferation *in situ*. To examine the effects of TGF-β1 signalling on the treatment response, cells were pre-treated with TGF-β1 (5 ng/ml) or anti-TGF-β1 antibody (10 μg/ml) for 48 h before irradiation or cisplatin treatment. A cell proliferation assay, using an XTT kit from Biological Industries, was performed to examine the number of surviving cells. For the clonogenic assay, exponentially growing cells were irradiated with single doses of 0, 3, 6 and 9 Gy with a 6 MeV electron beam for radiation survival analysis. Immediately following irradiation, cells were counted, diluted and plated on to 60-mm-diameter culture dishes. After incubation at 37°C for 7–10 days, the plates were stained with Crystal Violet (Sigma) for colony counting. Colonies containing more than 50 cells were scored, and plating efficiency and surviving fractions were determined for each cell line. Furthermore, to determine the effects of IL-6 signalling on cell proliferation and the changes in related proteins *in vitro*, cells were incubated in serum-free medium in the presence or absence of IL-6 (60 ng/ml) or the anti-IL-6 antibody (5 μg/ml) for 24 h.

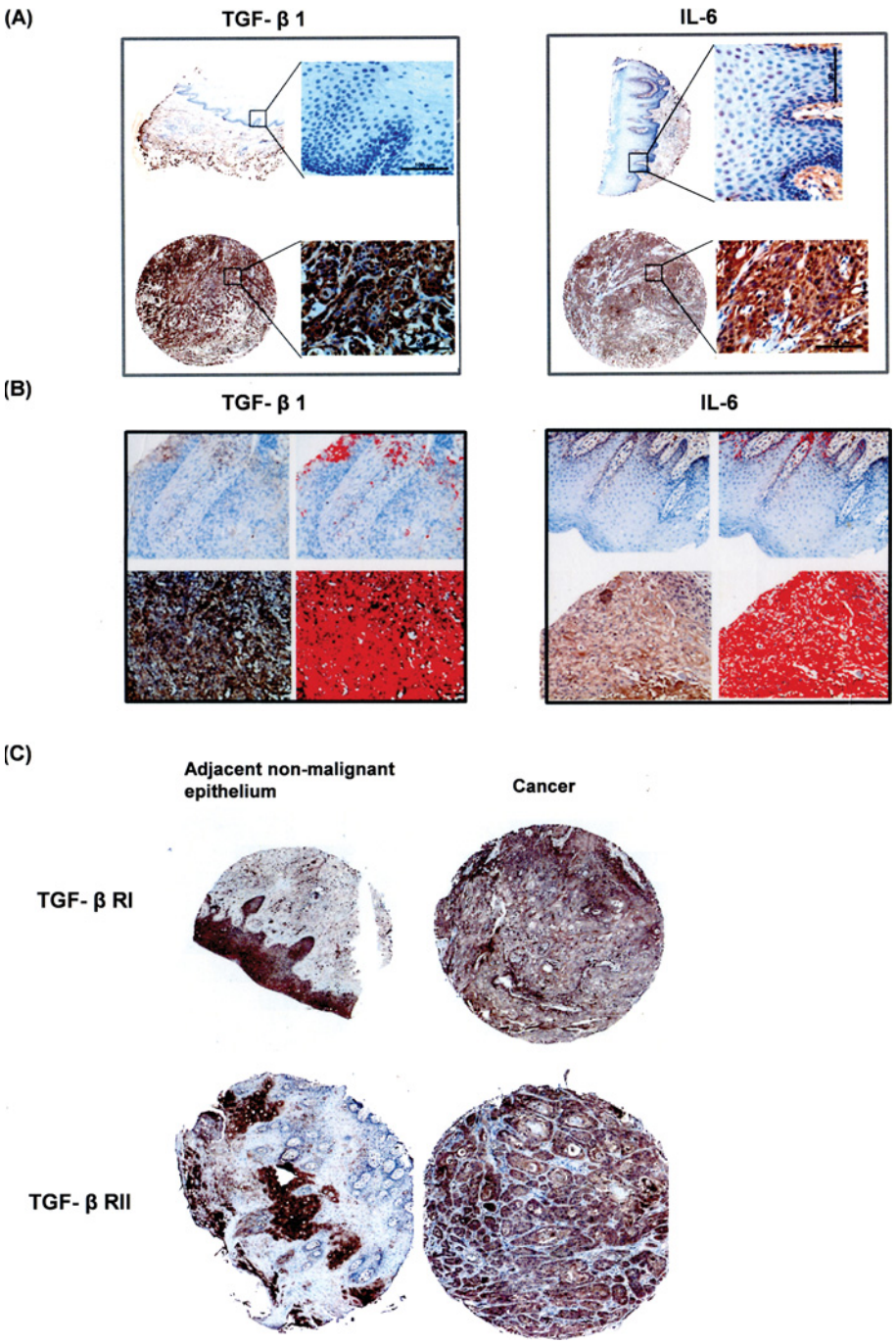

Figure 1 Immunohistochemical staining of human oral cancer TMA specimens

(A) Representative images of TGF-β1- and IL-6-positive staining by immunohistochemistry using the Good Speed scan slide scanning platform and Image Pro Plus 6.3. Upper row, adjacent non-malignant epithelium; lower panel, oral cancer tissue. Scale bars, 100 μm. (B) Representative data analysed by Image Pro Plus 6.3 are shown for each molecular marker. Left-hand panel, original immunohistochemical images; right-hand panel, Image Pro Plus 6.3 analysis with positive events marked in red. (C) Representative images of TGF-βRI- and TGF-βRII-positive staining by immunohistochemistry using the Good Speed slide scanning platform.

Immunoblotting

For Western blotting of whole cells, the cells were treated with lysis buffer (Calbiochem). An NE-PER kit (Pierce) was used to separate nuclear and cytoplasmic proteins. Equal amounts of protein were separated by SDS/PAGE and, after electrophoresis, proteins were transferred on to nitrocellulose membranes. Antibodies specific for TGF-β1, E-cadherin, vimentin, VEGF (vascular endothelial growth factor), MMP-9 (matrix metalloproteinase-9), HIF-1α (hypoxia-inducible

factor-1α) were obtained from Santa Cruz Biotechnology, antibodies specific for phospho-Akt, phospho-STAT3 (signal transducer and activator of transcription 3) (Tyr^{705}) and STAT3 were from Cell Signaling Technology and antibodies specific for IL-6 were from R&D Systems. After incubation with primary antibodies against the specific target protein (1:200–1:500 dilution), the membrane was incubated with an HRP (horseradish peroxidase)-conjugated secondary antibody and detected by ECL (enhanced chemiluminescence). To normalize protein loading, the membrane was re-probed with anti-(γ-tubulin) antibodies (1:1000 dilution).

To determine the effects of TGF-β1 signalling *in vitro*, proteins were extracted from cells transfected with a TGF-β1-expression vector/silencing vector or 48 h following pre-incubation in serum-free medium in the absence or presence of TGF-β1 (5 ng/ml) or an anti-TGF-β1 antibody (10 μg/ml).

Immunofluorescent staining

Cells demonstrating exponential growth were seeded on to coverslips for immunofluorescent staining with or without treatment. The slides were incubated for 1 h at room temperature (25 °C) with antibodies against TGF-β1 and phospho-ATM (ataxia telangiectasia mutated kinase), and with a Texas Red-conjugated secondary antibody. For TGF-β1, IL-6, phospho-H2AX, 8-oxoG (8-oxoguanine) and E-cadherin, slides were incubated with FITC-conjugated secondary antibody for 1 h and counterstained with DAPI (4′,6-diamidino-2-phenylindole) to visualize nuclei. After two further washes in PBST (PBS containing 0.05 % Tween 20), the specific target protein was visualized using a fluorescence microscope.

Real-time RT (reverse transcription)–PCR

Real-time RT–PCR was performed on RNA extracted from cells. RNA (2 μg) was reverse-transcribed with a random primer to obtain the first cDNA strand. The primer sequences were 5′-TACATCCTCGACGG-CATCTC-3′ and 5′-GCTACATTTGCCGAAGAGCC-3′ for IL-6; a β-actin primer was used as a loading control. The optimized PCR was performed on an iCycler iQ multicolour real-time PCR detection system. Significant fluorescent PCR signals from cells with different treatments were normalized to the mean value of the signals obtained from cells under control conditions.

ELISA for IL-6 levels in cellular supernatants

Cells were cultured in 1 ml of serum-free medium for 24 h in six-well plates. The medium was collected and clarified by centrifugation at 3000 ***g***. The levels of IL-6 in the supernatants were analysed by ELISA using an HS human IL-6 immunoassay kit (R&D Systems).

Cell migration and cell invasion assay

The capacity for cell invasion was determined using a cell invasion assay (Trevigen). After incubation for 24 h, the number of cells in the bottom chamber was determined by measuring the fluorescent anion calcein, released from intracellular calcein acetoxymethyl ester. To validate the cell migration experiments, scratch assays were carried out. A 2-mm-wide scratch was drawn across each cell layer using a pipette tip. The plates were photographed at the times indicated.

Statistical analysis

Survival probability analyses were performed using a Kaplan–Meier test. Significant differences between groups were assessed using a log-rank test. Multivariate analyses were performed using a Cox regression model for overall survival. All statistical tests were two sided.

Significant differences between samples *in vitro* were determined using a Student's *t* test. Results are presented as means $\pm$ S.D. Each experiment was independently carried out at least twice, with three repeats each. A probability level of $P < 0.05$ was adopted throughout to determine statistical significance, unless otherwise stated.

RESULTS

Levels of TGF-β1 in oral cancer tissues

Using immunohistochemical analysis, increased TGF-β1 expression was present in tumour tissues compared with adjacent non-malignant epithelial tissues (Figure 1). TGF-β1 may exhibit autocrine behaviour to stimulate tumour cells through TGF-βR (TGF-β receptor) I and TGF-βRII. Therefore we evaluated further whether TGF-βRI and TGF-βRII were overexpressed in oral cancer by immunohistochemical analysis of TMA slides. The results revealed that the levels of TGF-βRI and TGF-βRII were similar in tumour tissues and the adjacent non-malignant epithelial tissues (Figure 1C). Of the 125 OSCC tissues assayed for TGF-β1 with TMA blocks, 78 (62 %) gave positive immunoreactivity [59 % (33 out of 56) in $\leqslant$T3 compared with 65 % (45 out of 69) in T4; $P = 0.47$]. The presence of cervical lymph node metastasis is reported to be an important predictor of oral cancer [15]. From the clinical data, there was a positive correlation between TGF-β1-positive staining and the incidence of lymph node metastasis ($P = 0.047$) and disease recurrence ($P < 0.001$) (Table 2). A total of 70 % (49 out of 70) of patients with cervical lymph node metastasis demonstrated positive staining for TGF-β1. In contrast, 53 % (29 out of 55) of patients without lymph node metastasis expressed TGF-β1. Furthermore, 83 % (38 out of 46) of patients with disease recurrence (including local/regional failure and distant metastases) demonstrated positive staining for TGF-β1, and only

Table 2 Correlation between clinical predictors and molecular markers

Significant correlations are indicated with *.

	P value	
Clinical factor	TGF-β1-positive staining	IL-6-positive staining
Clinical stage ($\leqslant$III compared with IV)	0.49	0.79
Lymph node metastasis (negative compared with positive)	0.036*	0.007*
Risk factors (no compared with yes)†	0.81	0.001*
Pathology stage (III compared with IV)	0.85	0.118
Failure (local/regional failure and/or distant metastasis compared with disease-free)	0.0004*	0.003*

†Risk factors including extracapsular spread, and the presence of preineural, vascular or lymphatic invasion.

50 % (40 out of 79) of patients classed as having disease-free status expressed TGF-β1.

Role of TGF-β1 in tumour aggressiveness and EMT (epithelial–mesenchymal transition)

After stable transfection with the TGF-β1–GFP-expression vector or TGF-β1–RFP-silencing vector, the TGF-β1-expression/silencing vectors were demonstrated to significantly regulate TGF-β1 expression in SCC4 cells (Figure 2A). To determine further TGF-β1 signalling in oral cancer cell lines, we activated the pathway by TGF-β1 stimulation and suppressed it with an anti-TGF-β1 antibody. We found that regulating TGF-β1 had a significant effect on the proliferation rate of oral cancer cells determined using direct viable cell number counting (Figure 2B), and cell proliferation *in situ*, detected using a BrdU–FITC kit (Figure 2C). Furthermore, inhibited TGF-β1 signalling by the anti-TGF-β1 antibody or the TGF-β1-silencing vector attenuated the invasion and migration ability of oral cancer cells (Figure 3A). EMT is a key event during invasion and we have determined in the present study whether it was the underlying mechanism responsible for the effects of TGF-β1 on oral cancer. Treatment with the anti-TGF-β1 antibody induced oral cancer cells to increase their epithelial characteristics, as determined by changes in the expression of E-cadherin (Figures 3B and 3C) and vimentin (Figure 3C). Furthermore, EMT could have induced a number of invasion-related factors, including VEGF, HIF-1α and MMP-9. As shown in Figure 3(C), the TGF-β1-expression vector or TGF-β1 stimulation resulted in the increased expression of VEGF, HIF-1α and MMP-9. The molecular events underlying EMT are complex [16]. Previously, we have reported that activation of IL-6/STAT3 signalling induced aggressive tumour behaviour and EMT changes in pharyngeal cancer [12]. Therefore we determined further whether the alteration of IL-6 signalling involved the TGF-β1-induced EMT changes in oral cancer. Using Western blotting, real-time RT–PCR, ELISA and immunofluoresence analysis (Figure 4), TGF-β1 had a significant effect on IL-6 signalling associated with the changes in EMT-related proteins. Moreover, as shown in Figure 5, IL-6 signalling significantly regulated the cell proliferation rate and the EMT-related changes, but had no obvious impact on the expression of TGF-β1. On the basis of these results, we suggest that TGF-β1-induced aggressive tumour behaviour and EMT changes might be mediated by the activation of IL-6 signalling.

TGF-β1 correlates with the treatment resistance of oral cancer cells

The IC_{50} (half-maximal inhibitory concentration) of cisplatin in SCC25 cells was determined to be 5.9–6.2 μg/ml. As demonstrated in Figures 6(A) and 6(B), inhibition of TGF-β1 was associated with greater sensitivity to cisplatin and irradiation, as demonstrated using a cell proliferation assay. The radiation sensitivity of SCC4 transfectants was evaluated further using a clonogenic assay, which takes into account different kinds of radiation-induced cell death. The results in Figure 6(C) show that the TGF-β1-expression vector induced cells to be more resistant to irradiation. Moreover, as demonstrated in Figure 6(D), TGF-β1 inhibition augmented radiation-induced cell death. The capacity for DNA repair is the major determinant of radiosensitivity and the ATM protein is the most proximal signal transducer after radiation-induced DNA damage [17]. Defective or absent ATM causes extreme cellular sensitivity to irradiation. Using Western blotting and immunofluorescence analysis, inhibition of TGF-β1 significantly increased oxidative DNA damage, as shown by increased 8-oxoG and phospho-H2AX (histone H2AX), and attenuated irradiation-induced nuclear accumulation of phospho-ATM (Figure 7).

TGF-β1 and IL-6 are linked with the clinical outcome of OSCC

The roles of TGF-β1and IL-6 in the clinical outcome of stage III–IV human OSCC were estimated further using immunohistochemistry. To screen the expression

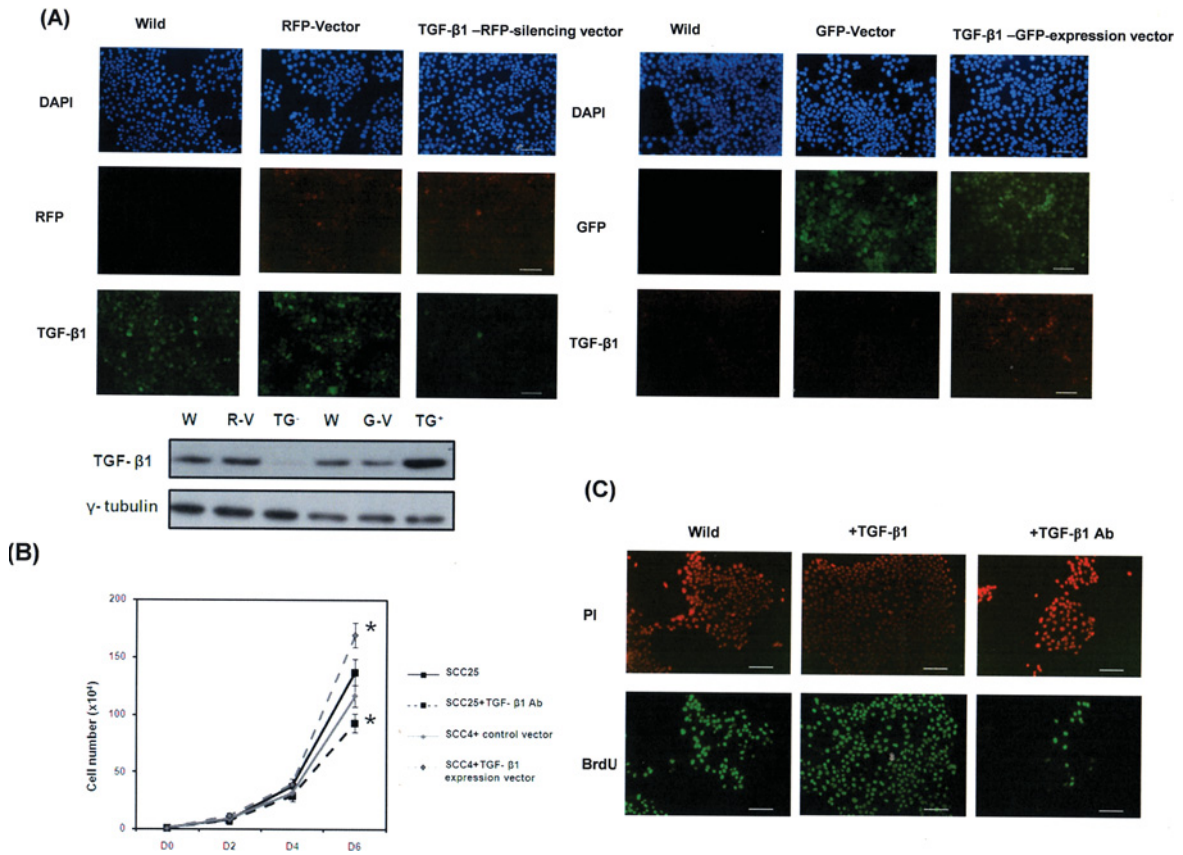

Figure 2 Effect of TGF-β1 in cancer cell proliferation

(A) Effects of the TGF-β1–RFP-silencing vector and TGF-β1–GFP-expression vector on the level of TGF-β1 in SCC4 cells as determined by immunofluorescence. Expression of TGF-β1 in SCC4 transfectants was also examined by Western blot analysis. W or Wild, wild-type; R-V, cells with control–RFP vector; TG-, cells with the TGF-β1–RFP-silencing vector; G-V, cells with control–GFP vector; TG^+, cells with the TGF-β1–GFP-expression vector. (B) Effect of TGF-β1 on the proliferation rate of cancer cells. The same number of cells (10^4) were plated on to each plate on day (D) 0 and were allowed to grow in their respective cultures. The number of viable cells after incubation for 2, 4 and 6 days were counted. $^*P < 0.05$. (C) Effect of TGF-β1 inhibition on SCC25 cancer cell proliferation *in situ* as detected by immunofluorescence. Upper panel, nuclei stained with propidium iodine (PI); lower panel, slides were stained with BrdU–FITC. Scale bars, 100 μm.

of TGF-β1 and IL-6 in human oral cancer samples, immunostaining was performed on TMAs consisting of 125 samples. Of the 125 tissue specimens assayed for TGF-β1 and IL-6, 78 (62%) and 76 (61%) were positive respectively. A significant positive correlation was observed in the cancer specimens that stained positively for TGF-β1 and IL-6 (Figure 8A). As shown in Table 2, positive staining for TGF-β1 and IL-6 was significantly correlated with the incidence of lymph node metastasis and disease recurrence. Furthermore, using univariate analysis, disease recurrence and positive staining for TGF-β1 and IL-6 were all significantly linked with shorter survival (Table 3 and Figure 8B; $P < 0.05$). Using multivariate analysis, the expression of TGF-β1 and disease recurrence were significant predictors for overall survival in the 125 oral cancer patients, but only disease recurrence was significantly linked with shorter disease-free survival (Table 4). These findings highlight the contribution of the TGF-β1/IL-6 axis to poor prognosis in oral cancer.

DISCUSSION

HNSCC results from an accumulation of genetic and epigenetic aberrations affecting numerous cellular processes. Studies in mouse models and human HNSCC samples have illustrated that disruption to TGF-β1 signalling promotes epithelial carcinogenesis. However, there is controversy about the expression levels of TGF-β1 in HNSCC and at which stage of HNSCC development TGF-β1 begins to be overexpressed [4,5,6,8]. Lu et al. [8] have suggested that TGF-β1 overexpression may be an early event during HNSCC development. In the present study, we have shown that TGF-β1 overexpression was observed in patients with

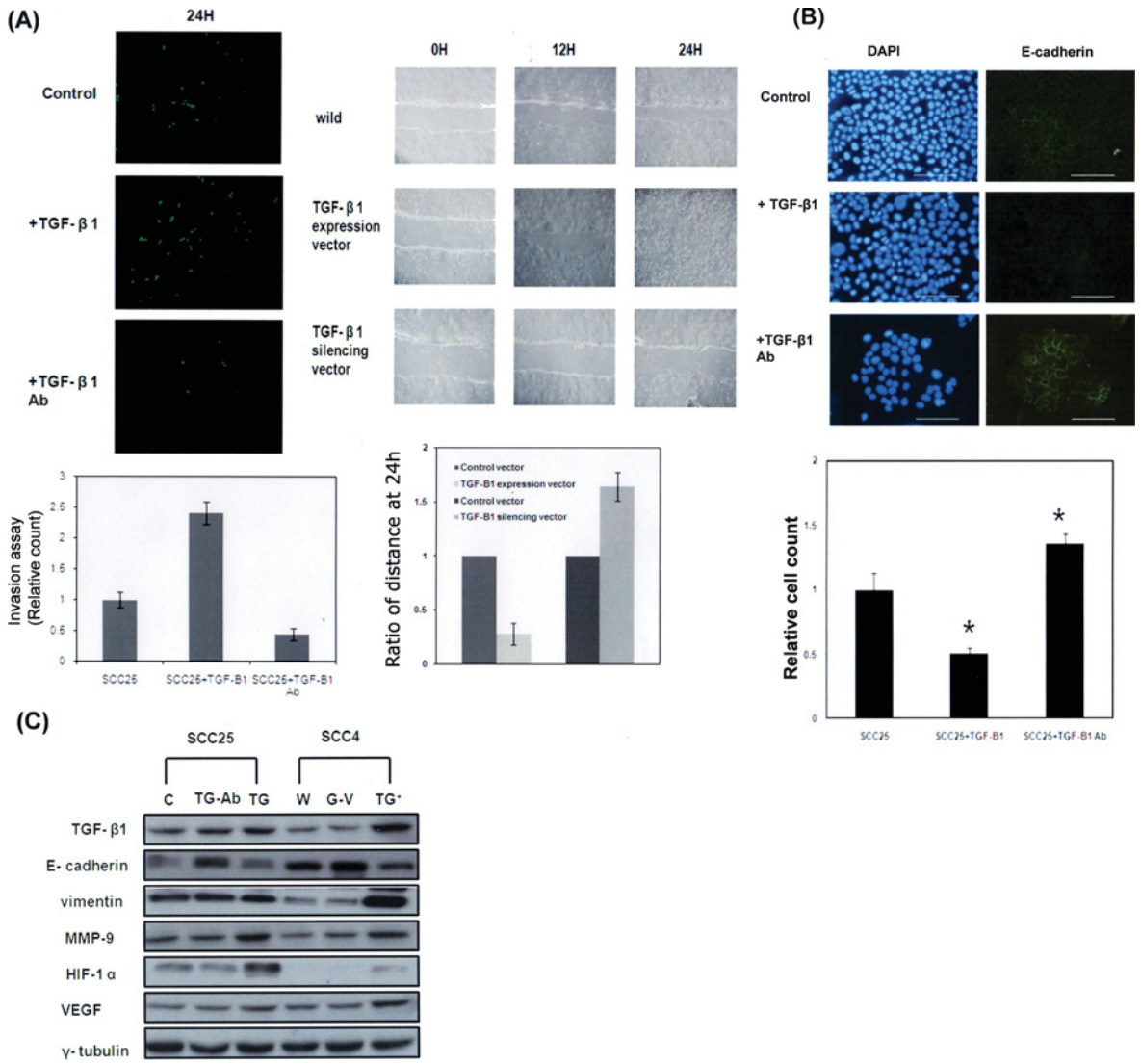

Figure 3 Effect of TGF-β1 on tumour aggressiveness and EMT changes

(A) The invasive capacities of oral cancer cells with or without TGF-β1 regulation were evaluated using an invasion assay in SCC25 cells (left-hand panels) and a migration assay in SCC4 cells (right-hand panels). Representative images and quantification are shown. For the invasion assay, the relative number of invading cells normalized to that under control conditions was calculated. For the migration assay, the relative ratio, normalized to the distance under control conditions at 24 h after scratching, was calculated. Results are means $\pm$ S.D. for three separate experiments; $^{*}P < 0.05$. Ab, antibody; Wild, wild-type. (B) E-cadherin levels in SCC25 cells was evaluated by immunofluorescence and quantified. Representative images are shown (left-hand panel, immunofluorescence image stained with DAPI for the nucleus; middle panel, immunofluorescence image stained with an anti-E-cadherin antibody). Scale bars, 100 μm. Right-hand panel, quantification of E-cadherin expression. Levels of E-cadherin were determined by dividing the number of cells positive for E-cadherin immunofluorescence by the total cell number for each condition. $^{*}P < 0.05$. (C) Change in EMT-associated proteins in cells with regulated TGF-β1 levels was evaluated by Western blot analysis. C, cells under control conditions; TG-Ab, cells treated with the anti-TGF-β1 antibody; TG, cells treated with TGF-β1; W, wild-type; G-V, cells transfected with control–GFP vector; TG^{+}, cells transfected with TGF-β1–GFP-expression vector.

locally advanced oral cancer. Our results revealed that TGF-β1 was overexpressed in 78 (62 %) cancer specimens compared with that in adjacent non-malignant oral epithelium (Figure 1 and Table 1). It has been reported that cancer cells that lose the tumour-suppressive arm of the TGF-β1 pathway accrue tumorigenic effects to directly enhance tumour growth. In some cancers, TGF-β1 can promote tumour cell proliferation by stimulating the production of autocrine mitogenic factors through Smad-independent transcriptional events such as PI3K hyperactivation [5]. To investigate whether TGF-β1 was responsible for the aggressive behaviour of oral cancer, tumour growth rate and invasion capacity were evaluated while regulating TGF-β1 signalling. Results obtained from experiments counting viable cells and from cell proliferation assays (Figure 2)

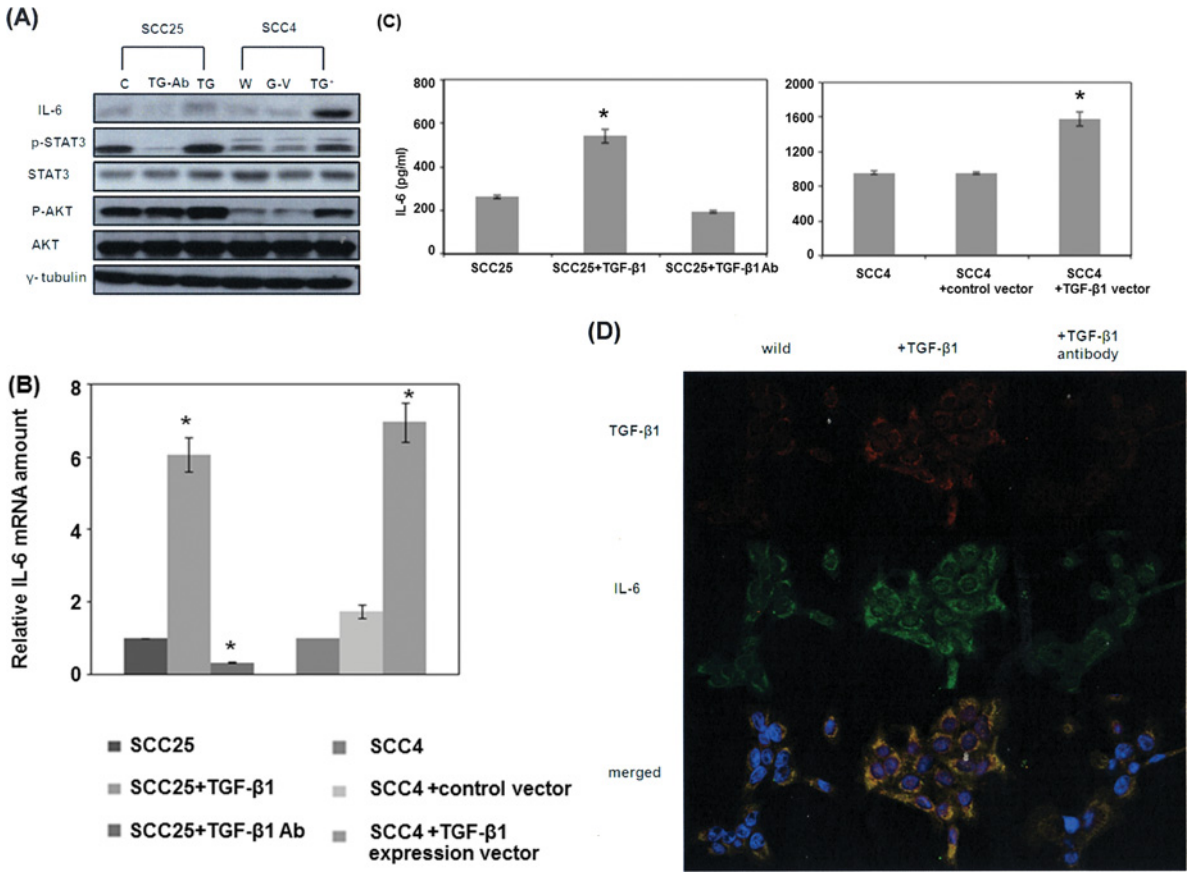

Figure 4 Effect of TGF-β1 expression on IL-6 signalling

(A) Effect of TGF-β1 on IL-6 signalling was evaluated by Western blotting in SCC25 and SCC4 cells. p-, phospho-. (B) Influence of TGF-β1 on the level of IL-6 in cells was examined by real-time RT–PCR. The IL-6/β-actin ratio in cancer cells normalized to cells without treatment was calculated. Values are means $\pm$ S.D. for three separate experiments; $^*P < 0.05$. (C) Influence of TGF-β1 on the level of IL-6 in the supernatants of cells was examined by ELISA. Values are means $\pm$ S.D. for three separate experiments; $^*P < 0.05$. (D) Effect of TGF-β1 on the expression of IL-6 in SCC25 cells was examined by immunofluorescence. Representative images are shown. Top row, cells stained with an anti-TGF-β1 antibody; middle row, cells stained with an anti-IL-6 antibody; bottom row, merged images with DAPI used to stain nuclei. Wild, wild-type.

revealed that inhibiting TGF-β1 resulted in decreased tumour cell proliferation. Moreover, the decrease in TGF-β1 significantly attenuated the invasive capacity detected in cellular invasion assays and migration assay.

A central issue in understanding the role of TGF-β1 in the progression of epithelial cancers is the elucidation of the mechanisms underlying the signalling cascades. The molecular and phenotypic changes involved in the transformation of an epithelial cell type to a mesenchymal cell type appear to be functionally relevant to the invasive characteristics of epithelial tumours [16]. As reported previously, TGF-β1 plays an important role in the induction of EMT in various cancers [18]. In the present study, we found that increased TGF-β1 expression resulted in a decrease in E-cadherin, a hallmark of EMT, and an increase in vimentin, VEGF and MMP-9 expression in oral cancer cells (Figures 3B and 3C). We have reported previously [12] that activated IL-6 signalling was associated with EMT and aggressive tumour behaviour in pharyngeal cancer. TGF-β1 has been reported to up-regulate IL-6 expression, and the TGF-β1/IL6 axis could mediate the aggressive tumour behaviour and treatment resistance observed in lung cancer [11]. In the present study, therefore, the association between TGF-β1 and IL-6 signalling in oral cancer was examined. Using Western blot analysis, ELISA and real-time RT–PCR analysis, we have demonstrated that TGF-β1 stimulated IL-6 expression and activated major downstream mediators, including phospho-Akt and phospho-STAT3 (Figure 4). Moreover, regulating IL-6 signalling resulted in significant changes in the cell proliferation rates and the levels of EMT-related proteins, but had no obvious impact on the expression of TGF-β1 (Figure 5). On the basis of these findings, it is suggested that activated IL-6 signalling induced by TGF-β1, and

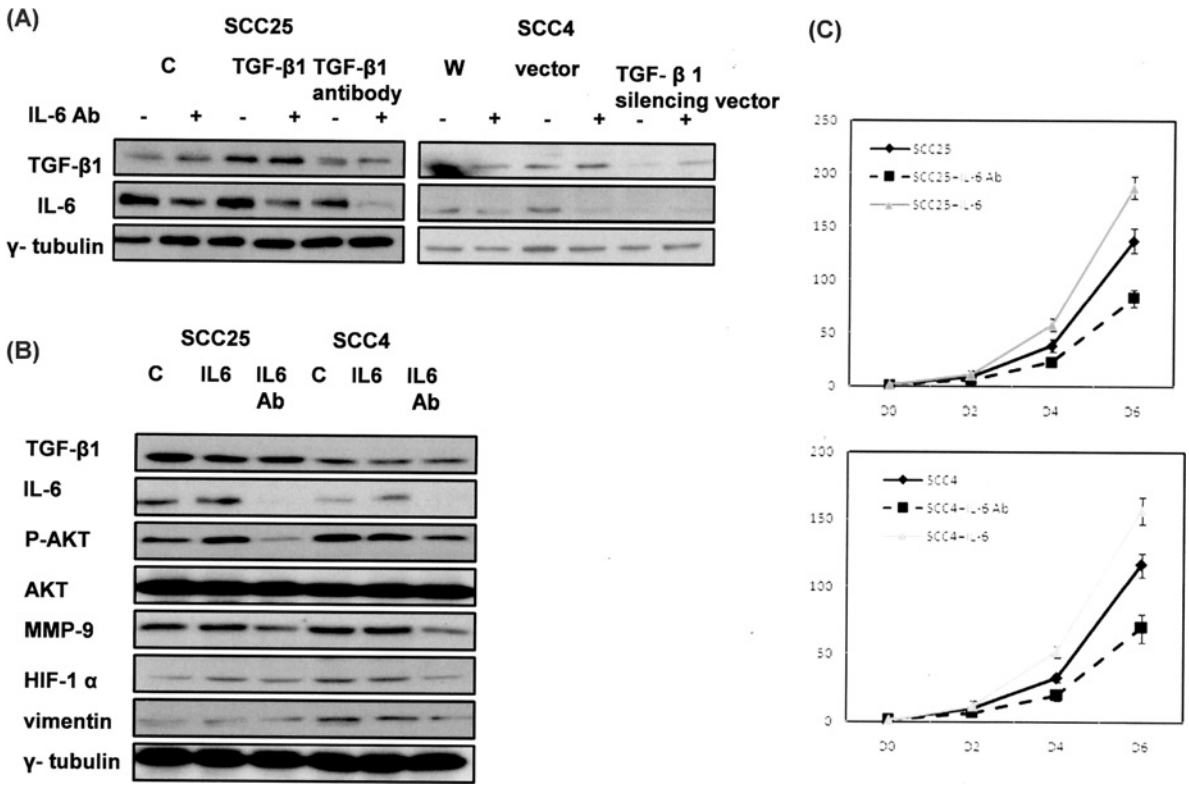

Figure 5 Effect of IL-6 on TGF-β1 expression and the proliferation rate in oral cancer cells

(A) Effect of IL-6 on the level of TGF-β1 was examined by Western blotting. Representative blots from experiments performed in triplicate are shown. (B) Effect of IL-6 signalling on the levels of HIF-1α, TGF-β1, vimentin and MMP-9 in oral cancer cells. Representative blots from experiments performed in triplicate are shown. Ab, antibody; W, wild-type. (C) Effects of IL-6 on the proliferation rate of oral cancer cells. The same number of cells (10^4) were plated on day 0 and were allowed to grow in their respective cultures. The number of viable cells after incubation for 2, 4 and 6 days was counted. The relative viable cell number normalized to that under control conditions was calculated. Values are means $\pm$ S.D. for three separate experiments; $^*P < 0.05$.

subsequent EMT changes, could be responsible for the more aggressive tumour behaviour observed in TGF-β1-positive oral cancers.

Radiotherapy and chemotherapy are crucial treatments for locally advanced oral cancer. It has been suggested that higher levels of TGF-β1 are associated with greater resistance to treatment [19–21]. In the present study, the cell killing effect induced by cisplatin or radiation was augmented by inhibiting TGF-β1 (Figure 6). Extensive DNA damage caused by radiation or anticancer agents can result in cell death or sensitivity to clinical treatment if left unrepaired [22]. Previous studies have reported that TGF-β signalling could be an important factor in the recruitment of ATM or for the phosphorylation of ATM [20,23]. To explore further the mechanisms potentially responsible for the radiosensitization effect induced by inhibition of TGF-β1 signalling, the recruitment of phospho-ATM, phospho-H2AX formation, an indicator of induced double-strand breaks [24], and oxidative DNA damage were examined after irradiation. The results revealed that inhibition of TGF-β1 attenuated nuclear accumulation of phospho-ATM and enhanced DNA damage induced by radiation (Figure 7).

Identification, development and selection of molecular targets are important in cancer therapy. According to the results from the cell lines used in the present study, the TGF-β1/IL-6 axis is likely to be important in the prognosis of oral cancer. The predictive powers of TGF-β1 and IL-6 were examined further in terms of the clinical outcome of locally advanced oral cancer. The immunohistochemical results demonstrated a positive correlation between TGF-β1-positive samples and those expressing IL-6 (Figure 8A). Moreover, using univariate analysis, enhanced expression of TGF-β1 and IL-6 were significantly associated with a higher incidence of lymph node metastasis, a higher recurrence rate after treatment and a shorter survival in stage III–IV oral cancer (Tables 2 and 3). Using multivariate analysis, disease failure and enhanced expression of TGF-β1 were significantly associated with shorter overall survival, but only disease failure retained predictive power on disease-free survival (Table 4). However, since our present study is a retrospective analysis of only stage III–IV oral cancers

Table 3 Univariate analysis to determine factors associated with prognosis

SPSS 14.0 was used to perform a Kaplan–Meier survival analysis, and a log-rank test was used to determine the significant differences between the groups. All statistical tests were two-sided, with significance defined as $P < 0.05$*.

Variable	*P* value for overall survival	*P* value for disease-free survival
Clinical stage ($\leqslant$III compared with IV)	0.66	0.45
Lymph node metastasis (negative compared with positive)	0.25	0.015*
Risk factors (no compared with yes)†	0.74	0.28
Pathology stage (III compared with IV)	0.74	0.47
Positive staining for TGF-β1 (negative compared with positive)	0.0002*	0.0004*
Positive staining for IL-6 (negative compared with positive)	0.001*	0.001*
Disease failure (negative compared with positive)‡	0.0001*	0.0001*

†Risk factors included extracapsular spread, and the presence of preineural, vascular or lymphatic invasion.
‡Disease failure refers to local/regional failure and/or distant metastasis.

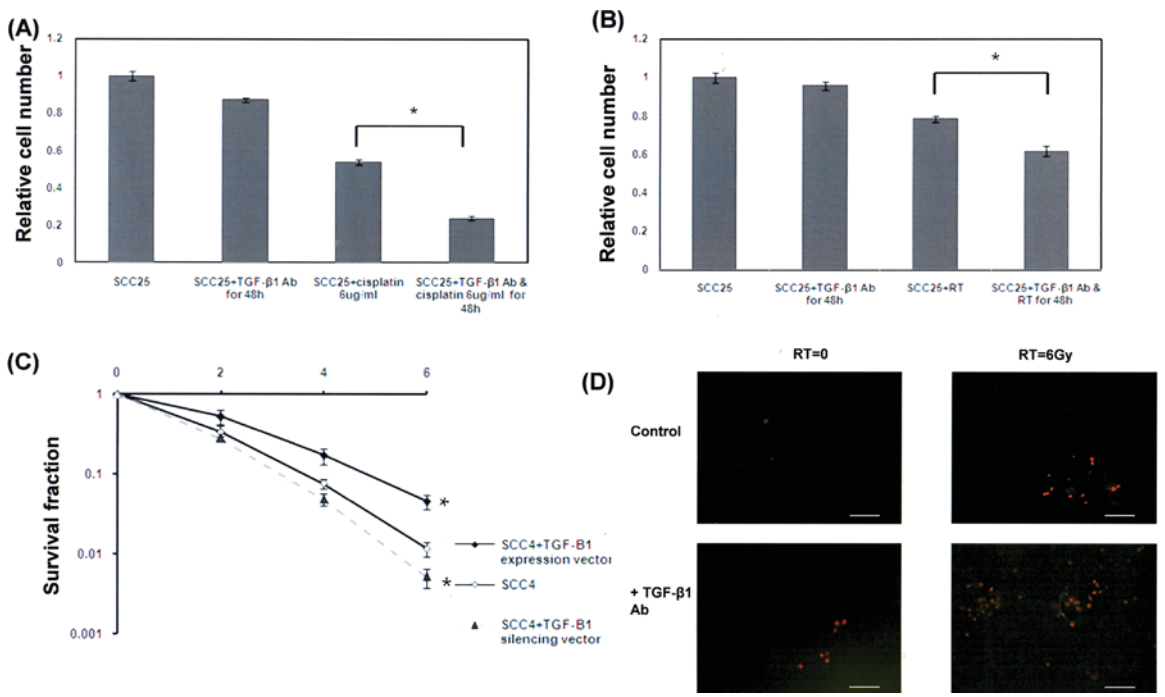

Figure 6 Effects of TGF-β1 on the treatment response

(A) Cells were treated with 0 or 6 μg/ml cisplatin in the presence or absence of the anti-TGF-β1 antibody, and the survival rate was determined at 48 h using an XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide] assay. The relative ratio normalized to that in SCC25 cells under control conditions for 48 h was calculated. (B) Cells were treated with 0 or 4 Gy in the presence or absence of the anti-TGF-β1 antibody, and the survival rate was determined at 48 h using the XTT assay. The relative ratio normalized to that in SCC25 cells under control conditions was calculated. (C) SCC4 transfectants were irradiated with 0, 2, 4 and 6 Gy, and the survival fractions were determined by measuring the colonies after irradiation and dividing by the plating efficiency. Values are means $\pm$ S.D.; $P < 0.05$. (D) Effect of TGF-β1 on radiation (RT)-induced cell death was demonstrated using annexin V–propidium iodide (PI) staining in cells 24 h after 6 Gy irradiation following pre-incubation with or without the anti-TGF-β1 antibody (Ab). Scale bars, 100 μm.

from a single institute, further investigations including more patients with different disease stages in a prospective trial are needed.

In conclusion, activated TGF-β1/IL-6 pathways could be responsible for more aggressive tumour growth and resistance to treatment in oral cancer. The results of the present study support the emerging hypothesis that the TGF-β1/IL-6 axis is a significant predictor, and targeting TGF-β1 signalling may represent a promising treatment strategy for HNSCC.

Table 4 Multivariate analysis to determine the association of molecular markers with the prognosis of patients (overall survival and disease-free survival)

SPSS 14.0 was used to perform a Kaplan–Meier survival analysis, and a log-rank test was used to determine the significant differences between the groups. All statistical tests were two-sided, with significance defined as $P < 0.05$*. HR, hazard ratio; CI, confidence interval.

	Overall survival		Disease-free survival	
Variable	HR (95 % CI)	*P* value	HR (95 % CI)	*P* value
TGF-β1 staining	3.5183 (1.1310–10.9442)	0.0298*	2.0445 (0.9104–4.5915)	0.0832
IL-6 staining	2.7056 (0.9833–7.4445)	0.0539	2.1034 (0.9574–4.6211)	0.0641
Disease failure†	2.3612 (1.0834–5.1461)	0.0307*	5.6018 (2.7845–11.2699)	0.0001*
Pathological stage	1.2015 (0.4757–3.0348)	0.6978	1.2354 (0.5844–2.6115)	0.5799
Lymph node metastasis	1.0687 (0.4937–2.3131)	0.8661	1.3330 (0.7280–2.4409)	0.3517
Risk factor	0.8349 (0.3305–2.1093)	0.7027	1.0858 (0.5051–2.3341)	0.8331

†Disease failure refers to local/regional failure and/or distant metastasis.

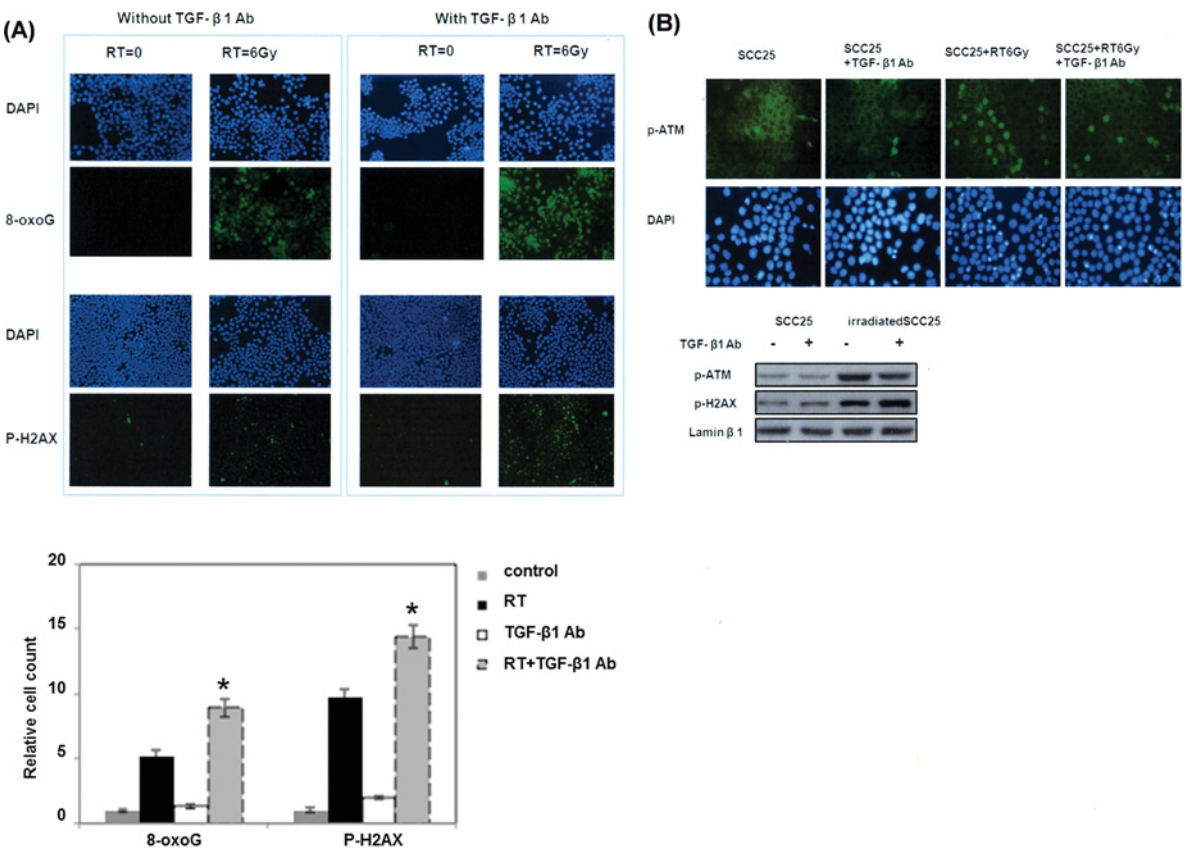

Figure 7 Effect of TGF-β1 on radiation-induced 8-oxoG, phospho-ATM and phosho-H2AX levels

(A) Influence of TGF-β1 on radiation (RT)-induced 8-oxoG and phospho-H2AX was evaluated by immunofluorescence in cells 30 min after irradiation following pre-incubation with or without the anti-TGF-β1 antibody (Ab). Representative images and quantitative results are shown. The quantification of 8-oxoG and phospho-H2AX expression was calculated by dividing the number of cells positive for the target protein by the total cell number for each condition. Values are means $\pm$ S.D. for three separate experiments. (B) The influence of TGF-β1 on radiation-induced nuclear accumulation of phospho-ATM was evaluated by immunofluorescence and Western blot analysis in cells 30 min after irradiation. p-, phospho-.

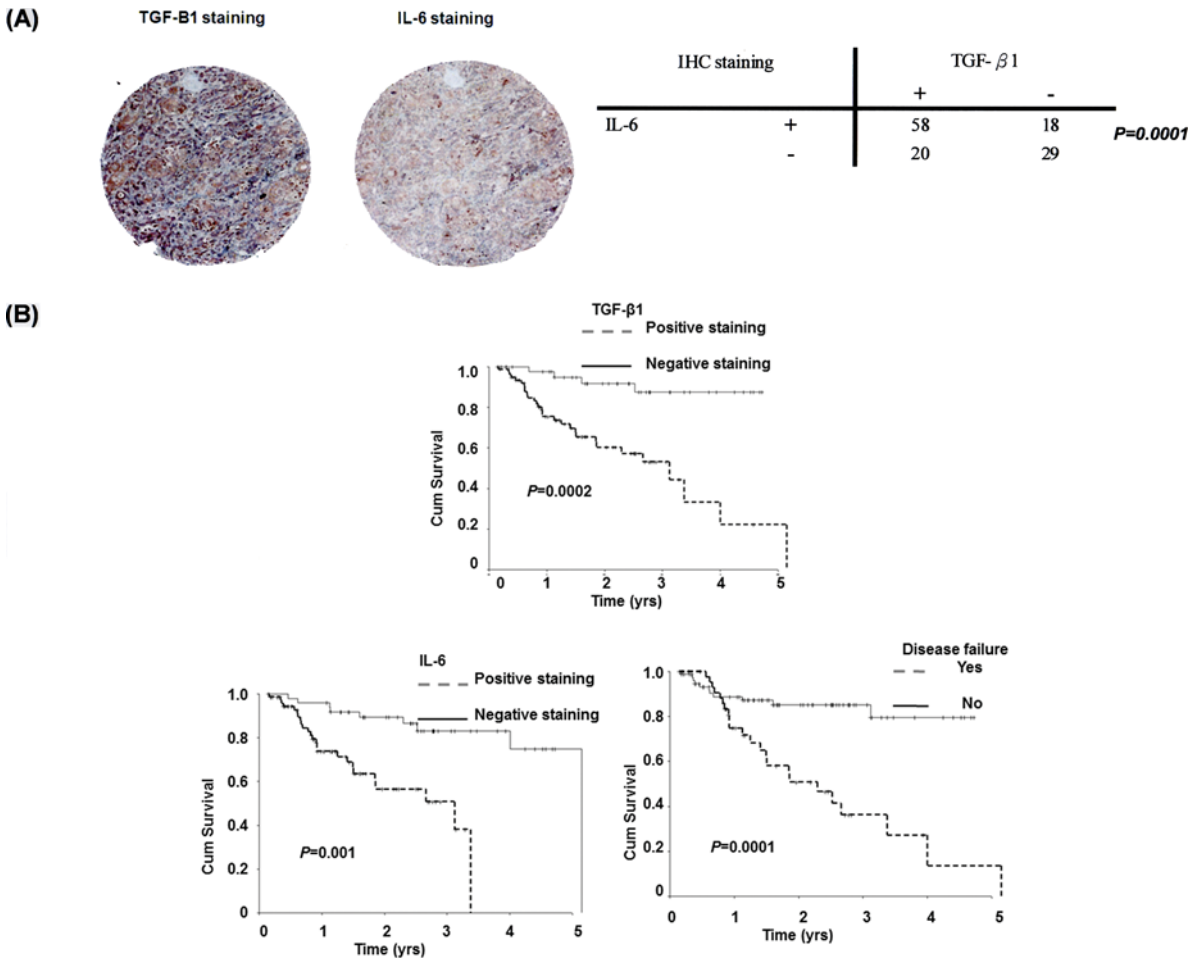

Figure 8 **Effect of the levels of TGF-β1 and IL-6 in the clinical outcome of oral cancer**

(A) Positive immunohistochemical (IHC) staining for IL-6 was significantly linked with TGF-β1 expression in human oral cancer specimens. Representative images of a selected tumour specimen positive for TGF-β1 and IL-6 staining are shown. (B) Overall survival differences according to the positive staining of TGF-β1 and IL-6, and disease failure.

AUTHOR CONTRIBUTION

Miao-Fin Chen performed the study and drafted the paper. Wen-Hung Wang conceived part of the study and performed the statistical analysis. Paul-Yang Lin helped in the histology and immunohistochemical staining. Kuan-Der Lee conceived the study and participated in its design and co-ordination. Wen-Cheng Chen conceived the study, participated in its design and co-ordination, and assisted in editing the paper prior to submission. All authors read and approved the final paper.

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