Journal of Cellular Physiology

IL-1β Promotes Malignant Transformation and Tumor Aggressiveness in Oral Cancer

CHIA-HUEI LEE,[1]* JEFFREY SHU-MING CHANG,[1] SHIH-HAN SYU,[1] THIAN-SZE WONG,[2] JIMMY YU-WAI CHAN,[2] YA-CHU TANG,[1] ZHI-PING YANG,[1] WEN-CHAN YANG,[1] CHIUNG-TONG CHEN,[3] SHAO-CHUN LU,[4] PEI-HUA TANG,[3] TZU-CHING YANG,[4] PEI-YI CHU,[5] JENN-REN HSIAO,[6] AND KO-JIUNN LIU[1,7,8]**

[1]*National Institute of Cancer Research, National Health Research Institutes, Zhunan, Taiwan*

[2]*Department of Surgery, University of Hong Kong, Hong Kong, Special Administrative Region, China*

[3]*Biotechnology and Pharmaceutical Research, National Health Research Institutes, Zhunan, Taiwan*

[4]*Department of Biochemistry and Molecular Biology, College of Medicine, National Taiwan University, Taiwan*

[5]*Department of Pathology, St. Martin De Porres Hospital, Chiayi, Taiwan*

[6]*Department of Otolaryngology, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan*

[7]*School of Medical, Department of Laboratory Science and Biotechnology, Taipei Medical University, Taipei, Taiwan*

[8]*Institute of Clinical Pharmacy and Pharmaceutical Sciences, National Cheng Kung University, Tainan, Taiwan*

Chronic inflammation, coupled with alcohol, betel quid, and cigarette consumption, is associated with oral squamous cell carcinoma (OSCC). Interleukin-1 beta (IL-1β) is a critical mediator of chronic inflammation and implicated in many cancers. In this study, we showed that increased pro-IL-1β expression was associated with the severity of oral malignant transformation in a mouse OSCC model induced by 4-Nitroquinolin-1-oxide (4-NQO) and arecoline, two carcinogens related to tobacco and betel quid, respectively. Using microarray and quantitative PCR assay, we showed that pro-IL-1β was upregulated in human OSCC tumors associated with tobacco and betel quid consumption. In a human OSCC cell line TW2.6, we demonstrated nicotine-derived nitrosamine ketone (NNK) and arecoline stimulated IL-1β secretion in an inflammasome-dependent manner. IL-1β treatment significantly increased the proliferation and dysregulated the Akt signaling pathways of dysplastic oral keratinocytes (DOKs). Using cytokine antibodies and inflammation cytometric bead arrays, we found that DOK and OSCC cells secreted high levels of IL-6, IL-8, and growth-regulated oncogene-α following IL-1β stimulation. The conditioned medium of IL-1β-treated OSCC cells exerted significant proangiogenic effects. Crucially, IL-1β increased the invasiveness of OSCC cells through the epithelial-mesenchymal transition (EMT), characterized by downregulation of E-cadherin, upregulation of Snail, Slug, and Vimentin, and alterations in morphology. These findings provide novel insights into the mechanism underlying OSCC tumorigenesis. Our study suggested that IL-1β can be induced by tobacco and betel quid-related carcinogens, and participates in the early and late stages of oral carcinogenesis by increasing the proliferation of dysplasia oral cells, stimulating oncogenic cytokines, and promoting aggressiveness of OSCC.

J. Cell. Physiol. 230: 875–884, 2015.

Abbreviations: EMT, epithelial-mesenchymal transition; GRO-α, growth-regulated oncogene-α; IL-1β, Interleukin-1 beta; IL-6, Interleukin-6; IL-8, Interleukin-8; OSCC, oral squamous cell carcinoma.

Conflict of interest: The authors declare there's no conflict of interest.

The microarray data used in this study have been deposited in NCBIs Gene Expression Omnibus (GEO) http://www.ncbi.nlm.nih.gov/geo/ and can be accessed through GEO Series accession number GSE37991 (Lee et al.), GSE13601 (Estilo et al.), and GSE25104 (Peng et al.).

Contract grant sponsor: National Science Council, Taiwan;
Contract grant number: NSC100–3112-B-400–014.
Contract grant sponsor: National Health Research Institutes, Taiwan;
Contract grant number: CA-101-PP-01 and CA-101-PP-06.
Contract grant sponsor: Ministry of Health and Welfare, Executive Yuan, Taiwan
Contract grant numbers: DOH101-TD-C-111–003, DOH101-TD-C-111–005, DOH100-TMP002–3.

*Correspondence to: Chia-Huei Lee, National Institute of Cancer Research, National Health Research Institutes, R2, R1211, Keyan Road, Zhunan Town, Miaoli County 350, Taiwan.
E-mail: chlee124@nhri.org.tw
**Correspondence to: Ko-Jiunn Liu, National Institute of Cancer Research, National Health Research Institutes, 2F, No.367, Sheng Li Road, Tainan 704, Taiwan.
E-mail: kojiunn@nhri.org.tw

Manuscript Received: 17 February 2014
Manuscript Accepted: 5 September 2014

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 10 September 2014.
DOI: 10.1002/jcp.24816

Oral squamous cell carcinoma (OSCC) represents a major health problem worldwide, and its patients are associated with a particularly poor 5-year survival rate. The principal obstacles in the treatment of OSCC and causes of poor prognosis are lymph node metastasis and local recurrence. Previous studies have shown that carcinogen exposure and chronic inflammation are two conditions that contribute substantially to the initiation, promotion, and progression of OSCC (Lee et al., 2003; Mignogna et al., 2004; Rao et al., 2010). Numerous studies have extensively investigated the association between alcohol consumption, betel quid chewing, and cigarette smoking and OSCC (Ko et al., 1995; Lee et al., 2003; Lin et al., 2011). These three risk factors are proposed to act synergistically to initiate or promote OSCC carcinogenesis (Ko et al., 1995; Lee et al., 2003; Wen et al., 2005). Results from an epidemiological study indicated that among regular users of alcohol, betel quid and cigarette, the risk of developing OSCC is increased by 123 folds (Ko et al., 1995).

Oral lichen planus (OLP) is a chronic inflammatory autoimmune disease that affects the oral mucosa in approximately 1–2% of the general adult population (Carrozzo et al., 2004; Lodi et al., 2005). Patients with OLP are typically associated with a clinically relevant increased risk of OSCC (Gandolfo et al., 2004; Mignogna et al., 2004; Rodstrom et al., 2004). The cytokine network and activated innate immunity during the inflammation response can account for malignant transformation in OLP (Mignogna et al., 2004). The constitutive activation of the nuclear factor kappa B (NF-κB), a hallmark of inflammatory responses, and the presence of several cytokines, including IL-1β, IL-6, and IL-8, are frequently associated with OSCC (Rao et al., 2010). These cytokines are also involved in lymph node metastasis and indicate poor prognosis in patients with OSCC (Takamune et al., 2008; Furuta et al., 2012). Increasing evidence has shown that IL-1β is one of the critical proinflammatory cytokines involved in tumour pathogenesis (Voronov et al., 2003; Apte et al., 2006). Two distinct signals are essential for the activation and secretion of IL-1β. The first signal upregulates the expression of pro-IL-1β by activating NF-κB, whereas the second signal stimulates the assembly of inflammasomes and activation of caspase-1 or -5, which promotes the cleavage of pro-IL-1β to produce active IL-1β for secretion. In most cases, pro-IL-1β is not activated without the presence of an inflammasome in the same cell. An inflammasome is a multi-protein complex consisting of the pattern recognition receptors (PRRs) NLRP1, NLRP3, NLRC4, NLRP6, or AIM2 (Schroder and Tschopp, 2010), an adaptor protein PYCARD, and caspase-1 or -5. In response to pathogen- and danger-associated molecular pattern signals, the PRRs interact with PYCARD to form an inflammasome and activate procaspase-1 (Franchi et al., 2009). Both IL-1β and inflammasomes are upregulated in several tumour types. However, IL-1β and inflammasomes might have contrasting effects on antitumour immunity and the induction of oncogenic factors (Apte and Voronov, 2008). In some cancer types, IL-1β eliminates malignant cells by stimulating antitumor immunity and increasing the effects of chemotherapy. A study by Allen et al. (2010) has shown the correlations between attenuated IL-1β and IL-18 levels and increased tumour burden, indicating that NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitis and colitis-associated cancer. A study by Chen et al. (2012) has further shown that tumour inflammasome-derived IL-1β recruits neutrophils and improves local recurrence-free survival in EBV-induced nasopharyngeal carcinoma. However, IL-1β may favour tumour development and progression by creating a microenvironment that confers survival advantages to tumour cells or through autocrine signalling. A study by Zienolddiny et al. (2004) indicated that the polymorphisms of IL-1β gene are associated with increased risk of non-small cell lung cancer. Arlt et al. (2002) identified that IL-1β plays a key role in the induction of constitutive NF-κB activity and chemoresistance in pancreatic carcinoma cells. IL-1β also promotes the stemness and invasiveness of colon cancer cells through Zeb1 activation (Li et al., 2012). A study by Li et al. (2004) have identified that IL-1β is a salivary biomarker for OSCC detection. However, the role of IL-1β in oral carcinogenesis has yet to be characterised.

The purpose of this study is to investigate the mechanisms by which carcinogen exposure may contribute to OSCC and to evaluate the potential tumour-promoting role of IL-1β in this human malignancy. We found that upregulated pro-IL-1β expression is proportional to the severity of oral malignancy in 4-Nitroquinolin-1-oxide (4-NQO) and arecoline co-induced mouse OSCC. We showed that the activation of inflammasomes and exposure to NNK and arecoline contributed, at least partly, to the secretion of IL-1β in OSCC cells. We also demonstrated that the pro-tumorigenic effects of IL-1β in OSCC are likely through stimulating an oncogenic cytokine network in the tumour microenvironment whereby increasing angiogenesis and invasiveness.

Materials and Methods

Chemicals and reagents

Caspase inhibitors, Z-YVAD-FMK and Z-WEHD-FMK, were obtained from R&D System (Minneapolis, MN) and BioVision (San Francisco, CA). Recombinant human IL-1β was purchased from R&D System. NNK, 4-NQO and arecoline were purchased from Sigma–Aldrich (St Louis, MO). Western blot was performed using antibodies against human IL-1β, IL-1 receptor (IL-1R) (Merk Millipore, Billerica, MA), NLRP3 (Abcam, Cambridge, UK), caspase-1, caspase-5, NLRP1, AIM2, and PYCARD (Cell Signaling, Danvers, MA).

Clinical samples

Paired OSCC specimens and their matched, non-tumour epithelial parts for microarray study were obtained from 40 previously untreated OSCC patients who received curative surgery during 2002–2009 at the National Cheng Kung University Hospital (Lee et al., 2013). Tumour and non-tumour pair-wised OSCC specimens for qPCR study were obtained from 32 previously untreated OSCC patients who received curative surgery during 2007–2009 at the Queen Mary Hospital. Fresh-frozen tissues were preserved in liquid nitrogen until ready to use. Clinical parameters, including age, social history, pathological features and TMN stage, were collected by chart review. This study was approved by the Institutional Human Experiment and Ethic Committee (HR-97–100) and the Institutional Review Board of the University of Hong Kong (UW12–123). Informed consent was obtained from each patient. For each clinical sample, a 5-μM thick pathological section was obtained, stained with hematoxyline and eosin, and observed under light microscope. Only specimens that contained target tissue (OSCC or non-tumour epithelium) in more than 80% area of their corresponding pathological sections were used for subsequent experiments. Total RNA from each sample were extracted using an RNeasy mini kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. The extracted total RNA were quantified and confirmed for OD 260/280 values between 1.8 and 2.2 and OD 260/230 values greater than 1.

Cell culture

Cell lines established from human dysplasia oral mucosa (DOK) (Chang et al., 1992) and human OSCC, including OE-CM1, OC3, CAL27, SCC-15, and TW2.6, were routinely cultured as previously described (Rheinwald and Beckett, 1981; Lin et al., 2004; Kok et al., 2007). Authentication of DOK and OSCC cells

was performed by DNA short tandem repeat analysis using GenePrint® 10 System (Promega, Madison, WI) and GeneMapper software version 3.7 (Life Technologies, Grand Island, NY).

Combination of 4-Nitroquinolin-1-oxide (4-NQO) and arecoline induced mice oral transformation

The mouse oral cancer was induced with 4-NQO and arecoline as described before (Chang et al., 2010). The animal experimental protocol was approved by Institutional Animal Care and Use Committee. C57BL/6JNarl mice (8-week of age) were purchased from the National Laboratory Animal Center (Taipei, Taiwan). The mice were sacrificed at different time points to determine the occurrence of pre-neoplasm and neoplasm in the tongue. The tongue was carefully separated into two representative parts. One part was processed for histopathological examination after being fixed in 10% buffered formalin and stained with H&E. The other part of tongue was homogenized with the Precellys homogenizer (Bertin Technologies, Artigues-Près-Bordeaux, France) two times for 20 sec at 6500 rpm with a 10-sec pause between the homogenization steps. Total RNA was isolated with RNeasy kit (Qiagen) according to the manufacturer's protocol, and subjected to PCR analysis. Since the tongue analyzed contains tissue with heterogeneous histological types, the RNA level of a certain gene determined reflects an overall expression level in the entire tumour microenvironment.

Real-time quantitative PCR (qPCR) and PCR

Synthesis of cDNA from total RNA and qPCR was performed as described previously (Lee et al., 2013). The primer sequences for qPCR and PCR are as follows: 5'-TACCTGTCCTGCGTGTT-GAA-3' (forward) and 5'-TCTTTGGGTAATTTTTGGGATCT-3'for human pro-IL-1β; 5'-TCGTGCTGTCGGACCCATAT-3' (forward) and 5'-GTCGTTGCTTGGTTCTCCTTGT-3'for mouse pro-IL-1β; and 5'-CCAACCGCGAGAAGATGA-3' (forward) and 5'-CCAGAGGCGTACAGGGATAG-3' for β-actin (ACTB). PCR for mouse pro-IL-1β was carried out using 0.5 μg cDNA, 1 X Taq polymerase buffer, 1 μM each of the forward and reverse primers, 0.2 mM dNTPs, 2 mM MgCl, and 0.5 U GoTaq® Flexi DNA polymerase (Promega) in a final reaction volume of 20 μl. PCR was performed with 30 cycles of a denaturation step at 95 °C for 30 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 1 min.

IL-1β ELISA

Cells were seeding in a 24-well culture plate (2×10^5cells/well) and grown in 2 ml of serum free medium for 16 h. The supernatants were collected and the secreted IL-1β was quantified using ELISA for human IL-1β (R&D System) following the manufacturer's protocol. Concentrations of measured IL-1β were normalized to the cell number determined in parallel.

Cell proliferation assays

Cell proliferation assay was carried out with CellTrace™ Cell Proliferation kits (Life Technologies) following the manufacturer's protocol.

Cytokine antibody array analysis

Human cytokines including IL-1Bα, IL-2, IL-3, IL-5, IL-6, IL-7, IL-8, IL-1B0, IL-1B3, IL-1B5, G-CSF, GRO, GRO-α, IFN-γ, MCP-1, MCP-3, MIG, RANTES, TGF-β1, TNF-α, and TNF-β were measured by antibody array (RayBio Human Cytokine Antibody Array 1, RayBiotech, GA) following the manufacturer's protocol. Conditioned medium subjected to cytokine assay were prepared from 10^5 cells seeded onto 6-cm dish and cultured in 2 ml of serum-free medium with or without IL-1β (1 ng/ml) treatment for 16 h.

Inflammation cytometric bead array (CBA) analysis

The production of cytokine in the culture supernatant was determined by a Human Inflammation CBA Kit (BD PharMingen). Conditioned medium subjected to CBA assay was prepared as those used for cytokine antibody array analysis.

Tube formation assay

The HUVECs were suspended in a 2:1 mixture of a serum-free M200 medium containing LSGS and an OSCC cell conditioned medium (OCM), and then cultured in 48-well plates coated with a polymerized growth factor-reduced Matrigel (BD Biosciences, Bedford, MA). The numbers of tube-like structures with closed networks of vessel-like tubes were then counted in four random fields.

Morphology assay

Morphology assay was carried out described previously (Sheu et al., 2013). Briefly, tissue culture dishes (BD Biosciences) were coated with 5 μg/ml of collagen Type I (BD Biosciences) in 0.02 N glacial acetic acid at 4 °C overnight, then aspirated out the coating solution. The coated plate was washed with PBS once before use. TW2.6 cells were seeded at a density of 2×10^6 cells in 10-cm plates. After adhesion, cells were cultured in the presence or absence of IL-1β (1 ng/ml). Phase-contrast pictures were taken 48 h after IL-1β treatment.

Cell migration assays

Cell migration assay was carried out using Oris Cell Migration system (Platypus Technologies, Madison, WI) and transwell system as described previously (Park and Han, 2009; Sheu et al., 2013). For Oris Cell Migration system, TW2.6 cells (10^5) were seeded at 150 μl per well and incubated for 16 h to permit cell adhesion. After removal of the inserts, the cells were cultured in serum-free medium with or without IL-1β (1 ng/ml). Migrations were observed microscopically after removal of the inserts for 48 h. Cell population in end-point assays were fixed with 4% paraformaldehyde in PBS and stained with 10% Giemsa for 1 h.

Statistical analysis

Comparisons between groups were statistically evaluated using Student's T-test for non-paired data and paired T-test for paired data, when the assumption of normality was fulfilled. When the assumption of normality was violated (Shapiro-Wilk Test P-value <0.05), comparisons between groups were performed using Wilcoxon rank sum test for non-paired data and Wilcoxon signed rank test for paired data.

Results

Upregulated IL-1β expression is associated with betel quid and/or tobacco-related OSCC

In a mouse oral cancer model established through 4-NQO (a carcinogen of tobacco smoke-related heterocyclic amine) and arecoline (an alkaloid found in the areca nut) co-induction, we observed that the increases in the overall levels of pro-IL-1β mRNA in the tongue correlated with the presence of malignant pathological changes of the oral tissues (from mild to severe dysplasia and OSCC) (Fig. 1A). We then evaluated the upregulation and secretion of the IL-1β protein in the pre-

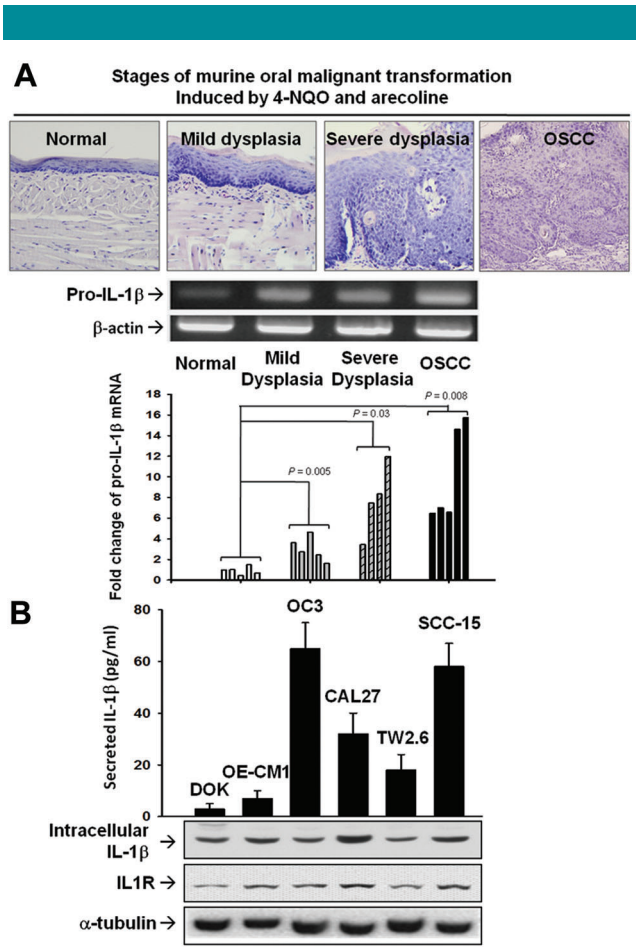

Fig. 1. IL-1β expression and secretion in 4-NQO and arecoline co-induced mouse OSCC model and in oral cancer cell lines. (A) The levels of pro-IL-1β mRNA progressively increased during different oral cancer development stages in the 4-NQO and arecoline co-induced mouse OSCC model. Top panel: representative histopathological sections of tongue tissues with different stages of malignant transformation (magnification, 200×). Quantitative RT-PCR experiments were conducted using the total RNA harvested from tongue tissue with various pathological statuses in mice exposed to 4-NQO and arecoline. Middle panel: representative gel views of the pro-IL-1β PCR fragments. Bottom panel: quantified result of the RT-PCR experiments. Thirty PCR thermal cycles were performed in each group. Each group contained five mice, except the severe dysplasia group (n = 4). (B) Top panel: the levels of secreted IL-1β by DOK and OSCC cell lines were evaluated using the ELISA. Data are presented as mean ± SD from four separate experiments. Bottom panel: Western blot analysis of intracellular IL-1β (17 kD) and IL-1R in the DOK and OSCC cell lines.

neoplastic DOK and OSCC cell lines derived from smokers and/or betel quid chewers. As shown in Figure 1B, we detected intracellular IL-1β and IL-1R expression in all tested cells ($P < 0.001$). Consistent with our mouse model data, the secreted IL-1β was more abundant in the OSCC cells than in the DOK.

Our previous cDNA microarray study (n = 28) (Lee et al., 2013) showed the significant overexpression of pro-IL-1β in 75% of the tumours compared with their matched non-tumour tissues obtained from OSCC patients with betel quid chewing and smoking habits ($P < 0.001$; Fig. 2A). Using real-time PCR analysis of a second group of paired samples from smokers (n = 32), we also found that 66% of the OSCC tumours showed significantly upregulated pro-IL-1β mRNA expression (≥2-fold change; $P = 0.017$) compared with the matched normal tissues (Fig. 2B). To confirm these findings, we surveyed the publicly available microarray studies in the Oncomine database (www.oncomine.org) and found similar correlations in several independent studies on oral cancer (n = 6). As shown in Figures 2C and D, two previous studies (Estilo et al., 2009; Peng et al., 2011) showed increased levels of the pro-IL-1β transcripts in tumour samples compared with normal tongue or normal oral cavity samples ($P < 0.001$ for both). Because the presence of inflammasome is necessary for IL-1β secretion, the above results suggested that inflammasomes might be constitutively activated in OSCC cells.

The inflammasome pathway is involved in the IL-1β production stimulated by NNK and Arecoline

To determine whether the production of IL-1β in OSCC is triggered by inflammasomes, we analysed the transcription levels of various inflammasome components with clinical array database obtained from OSCC patients with smoking and betel quid chewing habits (Lee et al., 2013). As shown in Figure 3A, transcripts of AIM2, NLRP1, PYCARD, CASP-1, and CASP-5 (>2-fold change) were significantly increased in OSCC tumours compared with the adjacent non-tumour epithelia. Results of Western blot analysis showed that the AIM2 expressions were abundant in all tested OSCC cells. Most OSCC cells expressed a substantial level of NLRP1 and NLPR3. We also detected active caspase-1 (p20) and caspase-5 (p20) in all analysed cells (Fig. 3B). To evaluate the requirement of active caspase-1 and caspase-5 for IL-1β secretion in OSCC cells, we added various caspase inhibitors to the culture media of the two cell lines known to secrete high levels of IL-1β: the OC3 and SCC-15 cells. As shown in Figure 3C, the caspase-1, 4 inhibitor Z-YVAD-FMK and the caspase-1, 4, 5 inhibitor Z-WEHD-FMK significantly reduced IL-1β secretion to 35–66% of the levels in the OC3 and SCC-15 cells, indicating that inflammasome-activated caspase-1 and caspase-5 are involved in IL-1β production in OSCC cells. No significant toxicity in these two cell lines was observed with the addition of caspase inhibitors (data not shown). We then assessed the effect of arecoline and NNK, two carcinogens related to betel quid and cigarette, respectively, on IL-1β secretion by OSCC cells. We selected the TW2.6 cell line because its basal level of secreted IL-1β is lower than that of other OSCC cell lines (Fig. 1B). TNF-α treatment was used as a positive control of IL-1β secretion. We have observed that arecoline and NNK treatment significantly increased IL-1β production within 1 h and 4 h of administration (Fig. 3D). However, Z-WEHD-FMK pre-treatment attenuated these effects, suggesting that arecoline and NNK, at least partially, increase IL-1β production through the inflammasome pathway. These data indicated that inflammasome components are constitutively expressed and respond to the stimulations generated by arecoline and NNK to promote IL-1β secretion in OSCC cells.

IL-1β promotes proliferation and dysregulates oncogenic signalling in DOK and OSCC Cells

As shown in Figures 1A and B, we observed increased levels of IL-1β during progression from an oral lesion to OSCC, and confirmed that DOK and OSCC cells expressed IL-R. We therefore evaluated the potential survival advantages gained by the DOK and OSCC cells from the presence of IL-1β in an inflammatory oral lesion. As shown in Figure 4A, the proliferation rates of the DOK increased significantly (1.89-fold; $P < 0.001$) relative to the control cells after culture with IL-1β (1 ng/mL) for 144 h. The proliferation rates of the TW2.6 cells were slightly increased (approximately 1.3-fold; $P < 0.01$) under the same conditions (Fig. 4B). To identify the cancer-related pathways activated by IL-1β treatment, we conducted

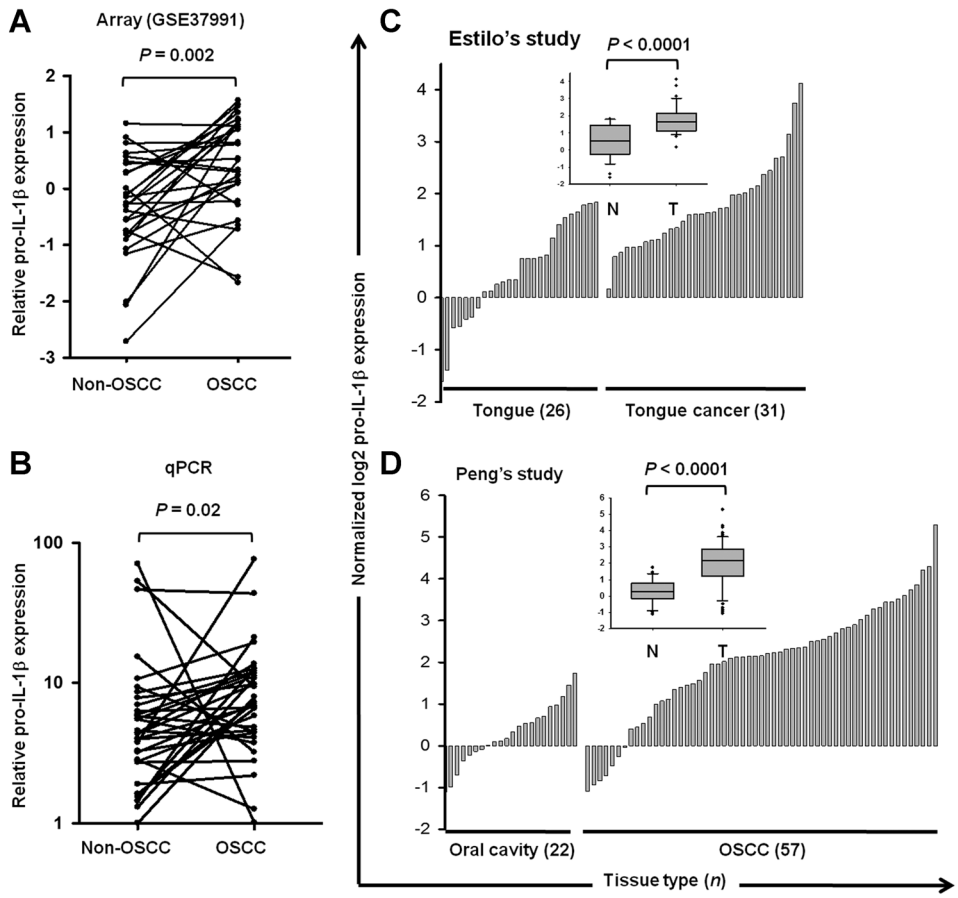

Fig. 2. The clinical association between pro-IL-1β expression and human oral cancer associated with smoking or/and betel quid chewing. (A) Array data (n = 28) and (B) qPCR analysis (n = 32) demonstrated the significant overexpression of pro-IL-1β in OSCC tumour tissue versus the matched non-tumour tissue obtained from smokers and betel quid chewers. The pro-IL-1β expression profiles of (C) normal tongue and tongue cancer, and (D) normal oral cavity and OSCC, were obtained from publicly available microarray data sets (Estilo et al., 2009; Peng et al., 2011) in Oncomine (https://www.oncomine.com). Each of the normal and tumour samples is plotted in order of increasing levels of pro-IL-1β. Inset: box plots display the median values of the array data and the 25th and 75th percentiles. The minimum and maximum values are indicated as whiskers. Points indicate outliers.

time-course Western blot analyses using IL-1β (1 ng/mL)-treated DOK and TW2.6 cells. As shown in Figure 4C, in the DOK, we identified that phosphorylated Akt (pAkt) and ERK1/2 (pERK1/2) significantly increased after IL-1β stimulation for 10 min, and then reduced gradually from 20 min to 60 min. The amount of pAkt remained augmented 160 h after IL-1β treatment, whereas pERK1/2 declined. In the TW2.6 cells, the ERK-MEK pathway was highly activated 160 h after IL-1β treatment, whereas the pAkt pathway was slightly upregulated and then downregulated within 160 h. These results indicated that IL-1βincreases DOK cell proliferation and dysregulates signalling in DOK and TW2.6 cells after long-term stimulation, suggesting that IL-1β is an influential cytokine for oral lesion and tumour cells.

IL-1β stimulates a pro-tumorigenic cytokine network in DOK and OSCC cells

To identify the downstream mediators induced by IL-1β in the tumour microenvironment, we conducted cytokine antibody arrays using DCM/IL-1β (+), the conditioned medium of IL-1β−treated DOK, and OCM/IL-1β (+), the conditioned medium of IL-1β-treated TW2.6 OSCC cells. As shown in Figure 5A, IL-1β treatment induced similar cytokine patterns in the conditioned media from the two cell lines. In response to IL-1β treatment, the DCM/IL-1β (+) contained significantly increased levels of GM-CSF, GRO-α, IL-6, and IL-8, and marginally increased levels of RANTES, IL-1α, and MCP-1. The OCM/IL-1β (+) contained further upregulated levels of all cytokines induced by IL-1β in DOK cells, with the exception of RANTES. We observed the marked upregulation of IL-6 production in response to IL-1β stimulation. Using inflammation CBA with a broad detection range, we confirmed the increased production of IL-6 in the IL-1β-treated DOK and TW2.6 cells (Fig. 5B). In addition, we observed substantially increased IL-8 secretion, beyond the detection limit (5 ng/mL), in the IL-1β-treated TW2.6 cells (Fig. 5B). Previous studies have shown that IL-6, IL-8, and GRO-α are associated with aggressive clinical manifestations of head and neck cancers (Shintani et al., 2004; Liao et al., 2010; Shinriki et al., 2011; Yadav et al., 2011). Therefore, we further investigated the effects of OCM/IL-1β (+) and IL-1β on OSCC malignancies.

IL-1β contributes to aggressiveness of OSCC by inducing angiogenesis and EMT

We used HUVECs to evaluate the effects of the conditioned medium of TW2.6 cells on angiogenesis in vitro. We observed

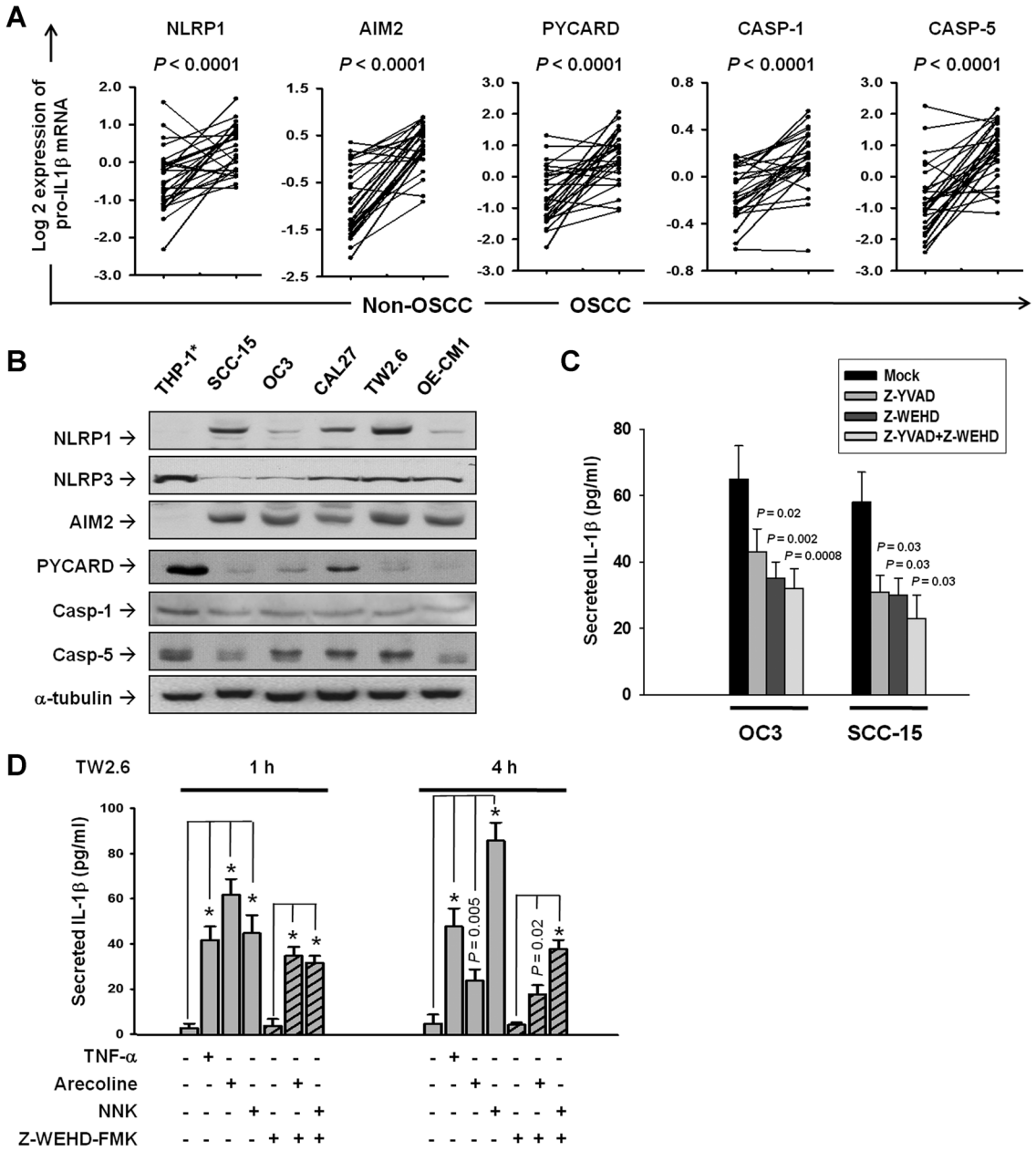

Fig. 3. The activation of inflammasomes, and exposure to arecoline and NNK, contribute to IL-1β production in OSCC. (A) Microarray data indicated that CASP-1, CASP-5, and the inflammasome components (AIM2, PYCARD, and NLRP1) were significantly upregulated in tumours compared with the adjacent non-tumour epithelia obtained from OSCC patients with betel quid and cigarette consumption. (B) Western blot analysis of CASP-1, CASP-5, NALP1, NALP3, AIM2, and PYCARD in OSCC cells. (C) Treatment with Z-YVAD-FMK (2 μM, a CASP-1 inhibitor) or/and Z-WEHD-FMK (2 μM, a CASP-5 inhibitor) for 8 h reduced the levels of secreted IL-1β in the OC3 and SCC-15 cells (mean ± SD from at least three separate experiments). There was a significant decrease in IL-1β production in caspase inhibitor(s)-treated cells as compared to the untreated controls. (D) Exposure to arecoline and NNK stimulated the secretion of IL-1β by the OSCC cells. The TW2.6 cells were treated with TNF-α (1000 unit/mL), arecoline (10 mM), or NNK (10 mM) for 1 h and 4 h, with or without a pretreatment of Z-WEHD-FMK (5 mM) for 4 h (mean ± SD from at least 3 separate experiments-). There was a significant increase or decrease in IL-1β production in TNF-α, arecoline, NNK or Z-WEHD-FMK treated cells as compared to the untreated controls.

that the OCM/IL-1β (+) derived from the TW2.6 cells induced a 2.7-fold higher proportion of tubular structures in the HUVECs compared with the medium alone (Fig. 6A). To evaluate the effects of IL-1β on OSCC cell invasiveness, we compared the expression of epithelial-mesenchymal transition (EMT)-related molecules in non-metastatic TW2.6 cells before and after IL-1β treatment. As shown in Figure 6B, the expression of Snail and Slug was upregulated in IL-1β-treated TW2.6 cells. Vimentin expression was also upregulated, whereas E-cadherin expression was downregulated, suggesting that IL-1β induces the EMT in the TW2.6 cells. The possibility that IL-1β induces EMT in OSCC cells was supported by morphology changes. We observed that TW2.6 have a squamous-cell like shape in control medium while display

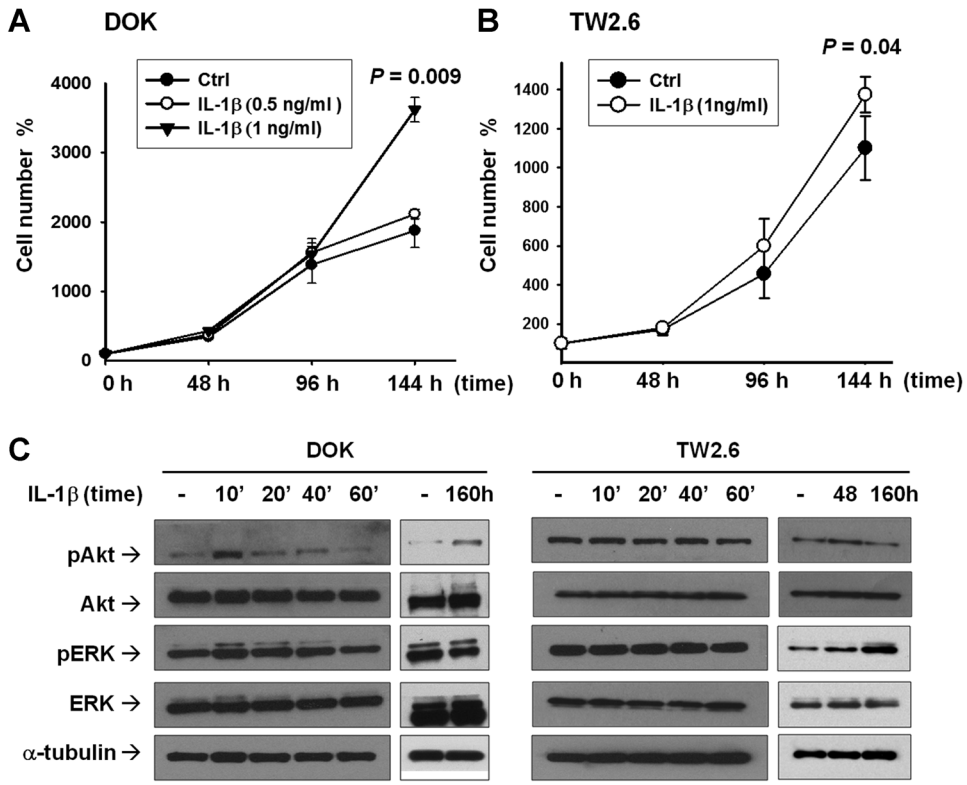

Fig. 4. IL-1β treatment accelerates proliferation and induces signalling aberrations in DOK and OSCC cells. The growth rates of the (A) DOK and (B) TW2.6 cells in the absence or presence of IL-1β (0.5 and 1 ng/mL) (mean ± SD from at least three independent experiments). (C) Altered Akt and MEK-ERK signalling in the IL-1β (1 ng/mL)-treated DOK and TW2.6 cells as determined by Western blot analyses. ', minutes.

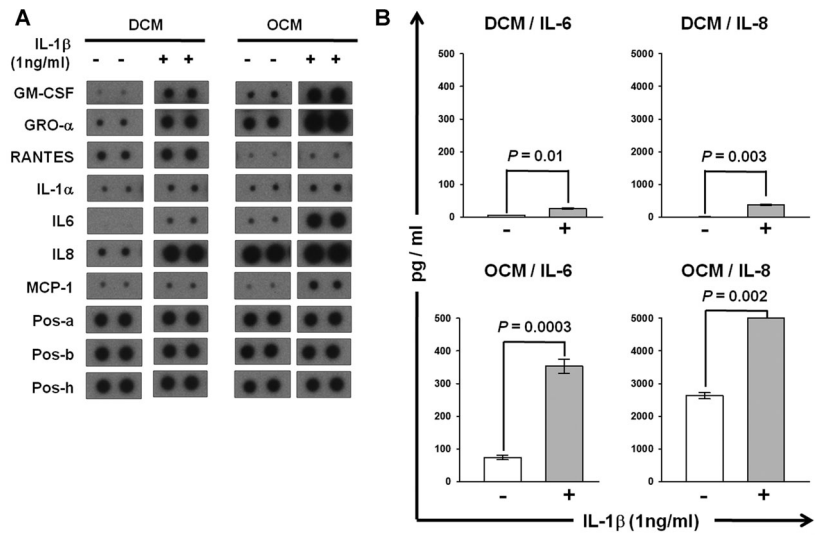

Fig. 5. IL-1β stimulates DOK and OSCC cells to release oncogenic cytokines. (A) Human cytokine antibody arrays and (B) human inflammation CBA were used to detect secreted cytokines in the conditioned media of the DOK and TW2.6 cells (DCM and OCM, respectively) after stimulation with IL-1β for 16 h. Pos-a, Pos-b, and Pos-h indicate the positive control spots on columns a, b, and h of the cytokine array, respectively (the original images of the hybridized cytokine array are shown in Supplementary Fig. S1). The media collected from cells without IL-1β treatment served as the control samples. (B) Two independent experiments were performed for IL-6 and IL-8.

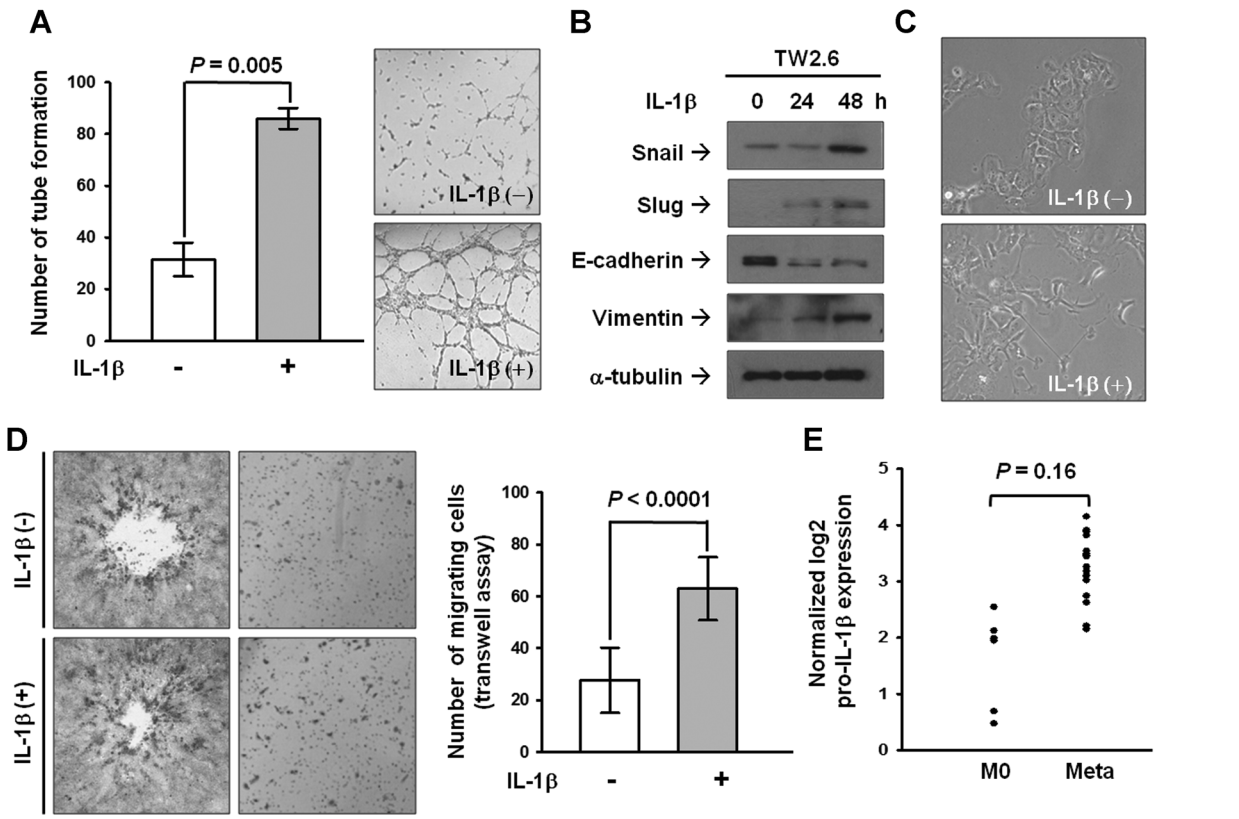

Fig. 6. IL-1β promotes angiogenesis, EMT and migration in OSCC. (A) Angiogenesis was evaluated using the tube forming assay. Tube formation by HUVECs incubated with OCM derived from the TW2.6 cells in the presence or absence of IL-1β. Tube formation was visualized under a microscope and counted in four random fields. Data are presented as mean ± SD from three independent experiments, each with four replicates. Representative images are shown. (B) Western blot analysis of Sail, Slug, E-cadherin, and Vimentin expression using the whole-cell lysates harvested from the TW2.6 cells with or without IL-1β treatment for 48 h. α-Tubulin provided the loading control. (C) TW2.6 cells display distinct morphologies in the presence and absence of IL-1β (1 ng/ml) for 48 h (phase-contrast images at 100 × magnification). Cells were grown on collagen-coated culture dishes. (D) The migratory capacity of the IL-1β-treated TW2.6 cells was evaluated using Oris migration system (left panels) and transwell migration assays (right panels). Representative images are shown. Quantified results of the transwell migration assay were shown on the right. (E) Upregulation of IL-1β expression in human OSCC with lymph node metastasis. M0: no metastasis. Meta: metastasis.

spindle-like shape in the presence of IL-1β (Fig. 6C). To further establish if the IL-1β-treated TW2.6 cells display enhanced migratory ability, as conferred by the EMT, we conducted in vitro migration assays. The migration capacity of the TW2.6 cells markedly increased after 48 h culture in the presence of IL-1β, as compared to that of the control cells (Fig. 6D). These data suggest that the OCM/IL-1β (+) derived from the TW2.6 cells promotes angiogenesis in a paracrine manner, and that TW2.6 cells display increased invasiveness as a consequence of IL-1β-induced EMT. The clinical array data from our previous investigations (Lee et al., 2013) provide supporting evidence that elevated IL-1β expression level is associated with lymph node metastasis of OSCC (Fig. 6E).

Discussion

The results of epidemiological studies support the notion that carcinogen exposure and a chronic inflammatory microenvironment are causative factors of OSCC and precede the malignant process (Jeng et al., 2003; Rao et al., 2010). In this study, we demonstrated that high level of IL-1β is closely associated with oral malignant transformation in a mouse OSCC model induced by 4-NQO and arecoline (Fig. 1). We further showed that IL-1β promotes cell proliferation and dysregulates signalling pathways in human DOK (Fig. 4A), and provided evidence to suggest that IL-1β may be induced by betel quid and tobacco-related carcinogens and involved in the early stage of OSCC oncogenesis.

A substantial portion of OSCC patients do not quit smoking or betel quid chewing despite warning of the risks (Poveda-Roda et al., 2010). Therefore, it is likely that IL-1β induced by carcinogen exposure also participates in the progression of OSCC. As shown in Figure 3D, arecoline and NNK induced IL-1β production in TW2.6 OSCC cells. IL-1β stimulated the secretion of oncogenic cytokines (Fig. 5) and induced the EMT, consistent with increased angiogenesis and migratory capacities as well as morphology changes (Fig. 6) in TW2.6 OSCC cells. Our findings strongly suggest that IL-1β not only plays a pro- tumorigenic role in the early stage of oral cell transformation but also enhances the aggressiveness of OSCC. Caspase-1 inhibitors did not completely suppress the IL-1β production induced by arecoline and NNK (Fig. 3D). These data suggested that in addition to the inflammasome pathway, other signalling pathways such as the NF-κB signalling pathway are also involved in the stimulatory effects of arecoline and NNK in IL-1β production. To the best of our knowledge, our study is the first to show that NNK and arecoline increase IL-1β production in oral cancer cells. This finding may explain the

close association between oral cancer and the consumption of cigarette and betel quid. IL-1β might play various roles in various pathologic states. IL-1β promotes the malignant transformation process in oral dysplasia cells by increasing their proliferation. After tumour onset, IL-1β might then enhance malignant progression by increasing metastasis and recurrence other than growth rates.

Metastasis is a major cause of poor outcome in advanced OSCC. Previous investigations have shown that increased levels of several cytokines, such as IL-6, IL-8, and GRO-α, correlated with lymph node metastasis and poor prognosis in patients with head and neck cancers, including OSCC. A study by Yadav et al. (2011) showed that recombinant IL-6 treatment induces EMT-related changes in the OSCC cell line CAL27 through the JAK/STAT3/Snail signalling pathway. As shown in Figure 1B, the two cell lines that secreted the highest levels of IL-1β, OC3 and SCC-15, were the two most aggressive cell lines among the OSCC cells tested, and showed the highest invasiveness in transwell assay (data not shown). We identified that the presence of recombinant human IL-1β in the growth media of the DOK and TW2.6 cells substantially increased the secretion of IL-6, IL-8, and GRO-α (Fig. 5). We then showed that IL-1β induced the EMT and enhanced migratory capacity in the TW2.6 cells. Our findings suggested that in tumour sites, IL-1β secreted by infiltrated immune cells and OSCC cells might provide an inflammatory microenvironment that promotes angiogenesis and EMT, and contributes to metastasis by inducing the release of oncogenic cytokines, including IL-6, IL-8, and GRO-α, from cancer cells. Indeed, we have observed that IL-1β was expressed mainly in epithelial cells of dysplastic tissue, while it was present in both malignant epithelial cells and infiltrating immune cells in neoplastic tissue (Supplemental Fig. 2). In line with our observations, EMT induced by inflammation (Dohadwala et al., 2010) and IL-1β (Li et al., 2012) has been noted in head and neck cancers as well as colon cancer. A study by Arlt et al. (2002) revealed that the autocrine production of IL-1β is associated with constitutively activated NF-κB in pancreatic cancer cells. In addition, Storci et al. (2010) showed that the activation of the NF-κB pathway upregulates Slug expression. In IL-1β-treated TW2.6 cells (Fig. 6B), the upregulation of Snail and Slug expression might result from NF-κB activation by IL-1β in the growth medium. Although our findings support the idea that IL-1β plays an oncogenic role and promotes malignant onset and aggressiveness in OSCC, we cannot eliminate the possibility that IL-1β recruits immune cells to the tumour environment and facilitates anti-tumour immunity during the early stages of tumour development. Therefore, further investigation of the effects of IL-1β during various stages of oral cancer in immune-competent mice is warranted.

In summary, in this study we have characterised the role of IL-1β in OSCC pathogenesis and showed the close association between carcinogen exposure, IL-1β and oral cancer progression, as well as the possible promoting effects of IL-1β during metastasis. Further detailed studies on the regulation of IL-1β production during various stages of oral cancer are required to facilitate the efficient design of cancer treatments that target IL-1β.

Acknowledgements

We were grateful for support of clinical samples from the National Cheng Kung University Hospital and Queen Mary Hospital.

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