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Tunicamycin-induced endoplasmic reticulum stress up-regulates tumour-promoting cytokines in oral squamous cell carcinoma☆

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ABSTRACT

Aims: Signal transducer and activator of transcription (STAT)-3 lies at the convergence point of key pathways involved in many malignancies including oral squamous cell carcinoma (OSCC). Endoplasmic reticulum stress (ERS) and the unfolded protein response have been shown to be involved in the pathogenesis and progression of different cancers by influencing key cellular processes such as apoptosis. We investigated the differential expression of STAT3 pathway-related genes and proteins under ERS in OSCC.

Methods: Three normal oral keratinocyte (NOK) and three OSCC cell lines were subjected to tunicamycin to induce ERS for 24 h or to the vehicle medium as control. A pathway-focussed array was used to analyse the modulation of STAT3 pathway gene expression under ERS using qPCR. The expression of key regulated proteins was investigated in the cell lines using immunocytochemistry and in 76 OSCC and 9 normal oral mucosa (NOM) tissue samples using tissue microarray technology and immunohistochemistry.

Results: ERS resulted in up-regulation of interleukin-6 receptor (IL6R) gene in NOK cell lines ($p = 0.001$) and IL5 ($p = 0.005$) and IL22 ($p = 0.024$) in OSCC cell lines. Greater STAT3 ($p = 0.019$) and leukaemia inhibitory factor receptor ($p = 0.042$) protein expression was observed in treated than untreated NOK cell lines.

Conclusions: The gene and protein regulation patterns show that ERS plays a role in modifying the tumour microenvironment in OSCC by up-regulating tumour-promoting cytokines.

1. Introduction

Oral cancer generally refers to malignancies of the lips (excluding skin of lip) and oral cavity, 90% of which are oral squamous cell carcinomas (OSCC). Oral cancer had an estimated global annual incidence of 300,400 new cases and accounted for 145,400 deaths worldwide in 2012 [1]. Despite advances in treatment options survival rates have not improved significantly [2]. Mortality rates have been rising, and with late diagnosis the overall 5-year survival rate is approximately 50% [3].

Signal transducer and activator of transcription (STAT)-3 is one of seven proteins in the STAT family. It has multiple context-dependent functions in development, physiology and pathology, and plays a central role in oncogenic signalling pathways [4,5]. STAT3 is activated by a variety of growth factors and cytokines such as interleukin (IL)-6, IL-22, leukaemia inhibitory factor (LIF), epidermal growth factor, and vascular endothelial growth factor (VEGF), that bind to their corresponding receptors [6]. STAT3 up-regulates genes and proteins that activate pathways such as the Ras and mitogen activated protein kinase (MAPK) pathways as well as the STAT3 pathway itself. The activation of these pathways ultimately promotes cell proliferation, metastasis, angiogenesis, cell survival, and anti-tumour immune suppression [2,6–8].

The endoplasmic reticulum (ER) is the organelle responsible for the secretion and post-translational modification of transmembrane and secretory proteins. Normally, proteins are folded in the ER and modified before being exported. Much of this process is energy-dependent and sensitive to changes in glucose supply, calcium levels, and oxygen balance. When a cell is subjected to metabolic stress as a result of infection, inflammation, and other noxious stimuli, or when the calcium, oxygen, and/or glucose balance in the ER is disturbed, the protein folding process is deranged resulting in ER stress (ERS), where mis-

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Table 1
Antibody dilutions and controls used for immunohistochemistry.

Antibody	Type	Dilution	Positive Controls	Catalogue no.
IL-6R	Rabbit polyclonal	1:200	Spleen	ab128008
TLR-4	Mouse monoclonal [76B357.1]	1:100	Tonsil	ab22408
LIF-R	Rabbit polyclonal	1:500	Colon	ab101228
STAT3	Mouse monoclonal [9D8]	1:1600	Breast cancer	ab119352
IL-5	Rabbit polyclonal	1:100	Breast cancer	NOVNBP119749
IL-22	Mouse monoclonal [IMG8F1EE2]	1:200	Tonsil	ab134035

folded or unfolded proteins accumulate in the ER [9]. To remedy ERS, the unfolded-protein response (UPR) is activated. UPR comprises three pathways: inositol-requiring enzyme 1-alpha (IRE1α), protein kinase-like ER kinase (PERK), and activating transcription factor 6-alpha (ATF6α). Collectively, they act to overcome ERS and re-establish the normal function of the ER [10].

Upon ERS, UPR-dependent apoptotic pathways may be activated when the stress is irrevocable. In contrast, cancer cells are believed to have evolved mechanisms to evade apoptotic pathways, and thrive under stressful conditions. The mechanisms by which cancerous cells evade these pathways are not well understood [11].

The relationship between STAT3 and UPR pathways has been investigated in inflammatory diseases, infections and, more recently, in cancer. It is known that IRE1α and PERK activate STAT3, which in turn promotes survival through up-regulation of anti-apoptotic proteins [12]. The aim of the current study was to identify and analyse differential STAT3-related gene expression profiles in cells derived from OSCC and normal oral keratinocytes (NOK) and to correlate the findings with relevant protein expression patterns.

2. Methods

Ethical approval to conduct this study was obtained from the University of Otago Human Ethics Committee (Health: H16/016) on 23 February 2016.

2.1. Cell Culture

Six cell lines including immortalised NOK cell lines (OKF4/TERT-1, OKF6/TERT-1, OKP7/bmi/TERT; Harvard Skin Disease Research Center, Boston, MA, USA) and OSCC cell lines (SCC4, SCC15, SCC25; ATCC, Manassas, VA, USA) were used in this study. Culture media for OSCC and NOK cells were used as described in previous publications [13,14]. When the cells reached 40% confluence, they were maintained in consensus medium consisting of Dulbecco Modified Eagle Medium (DMEM), Keratinocyte Serum-Free Medium (KSFM), and antimicrobials. At 80% confluence, they were detached by incubation with 2 ml of 0.25% trypsin-EDTA (Gibco, Thermofisher Scientific) then seeded into 6-well microtitre plates at a concentration of 16,000 cells/cm^2.

2.2. Induction of ERS

After 12 h, the media were discarded and fresh consensus medium containing 2.5 μg/ml tunicamycin (Sigma-Aldrich) was added to one 6-well plate for 24 h to induce ERS. As a control, reagents comprising

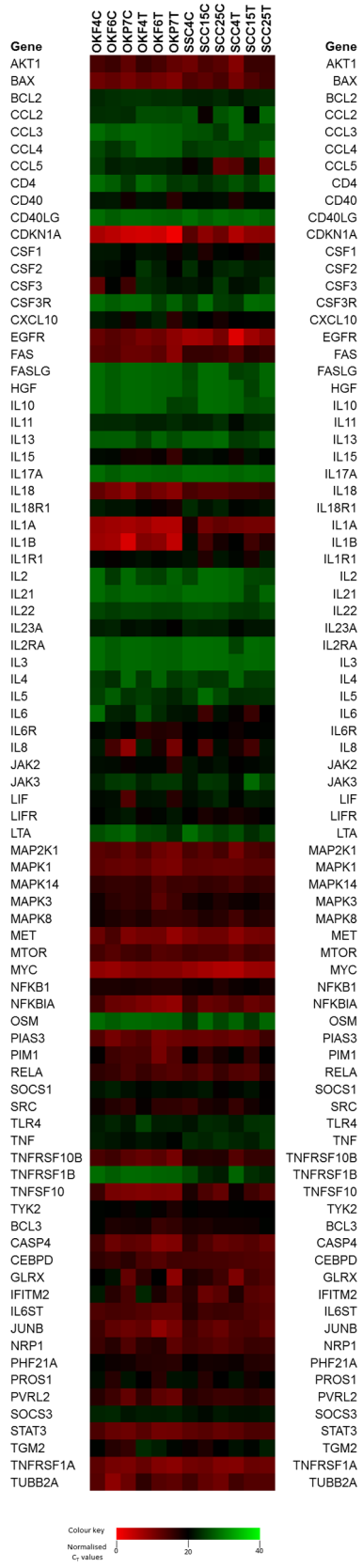

Fig. 1. Heat map of the normalised C_T values of the genes.

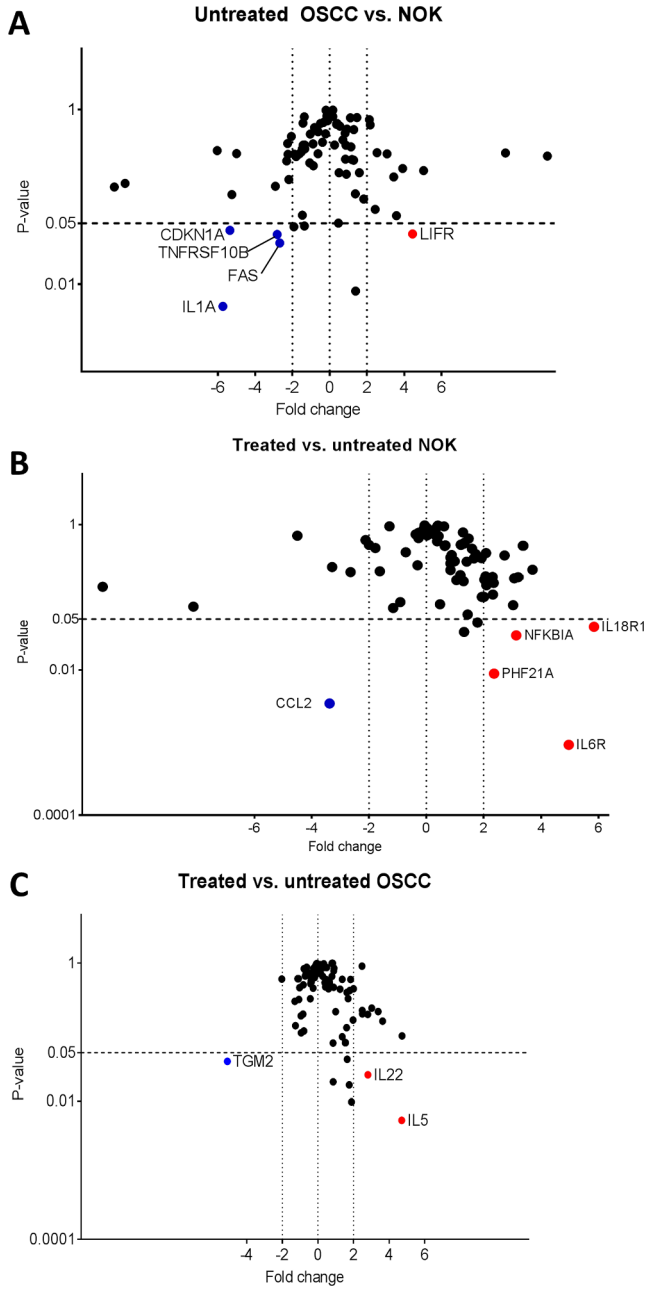

Fig. 2. Volcano plots showing significantly regulated genes in (A) untreated (*i.e.* without ERS) OSCC compared to untreated NOK cell lines, (B) treated compared to control NOK cell lines, and (C) treated compared to control OSCC cell lines. The significantly regulated genes with two- or more fold-regulation are to the right (red dots, up-regulated) and left (blue dots, down-regulated) of the respective dotted vertical lines. Genes lying below the dashed horizontal line are those with statistically significant fold-regulation.

volume-to-volume equivalent 25 mM sodium hydroxide (NaOH) vehicle in the consensus medium without tunicamycin were added to another 6-well plate. The treatment was terminated after 24 h by discarding the medium then incubating the cells with sterile phosphate buffered saline (PBS) for 5 min at room temperature.

2.3. Polymerase chain reaction analysis

Purelink™ RNA Mini Kit (Ambion) was used in conjunction with TRIzol® reagent (Invitrogen, CA, USA) to isolate RNA from the cell lysates. Reverse transcription (RT) was carried out with a RT^2 First Strand Kit (SA Biosciences). The RT^{2}SYBR Green Master-mix, cDNA synthesis reaction, and RNase-free water were mixed in a PCR loading reservoir. Using an 8-channel multi-pipettor, 25 µl was added into each of the 96 wells of the Human IL6/STAT3 Signalling Pathway Plus PCR Array (SA Biosciences; PAHS-160Y) which included control wells for genomic DNA contamination, positive PCR, and RT. Real-time RT^2-PCR was executed using an Applied Biosystems QuantStudio™ 6 K Flex Real Time PCR Machine.

The results were analysed using GeneGlobe Data Analysis Centre (Qiagen, Germany) software and Prism Graph Pad (Prism version 7.01, California, USA). Reference genes were chosen using qBase + software (Biogazelle – Zwijnaarde, Belgium). Accordingly, test genes were normalized using the three most stable genes. The $2^{(-\Delta\Delta CT)}$ were calculated to give a fold change. Pairwise analysis of control and treatments for each cell line was also undertaken. A cycle threshold (C_T) value of 35.00 was chosen as the cut-off value. Genes with more than 2-fold regulation were considered highly regulated, while statistical significance was set at $p < 0.05$.

2.4. Immunocytochemistry

The cells were cultured in 1-well glass chamber slides (NUNC Lab-Tek II; ThermoFisher Scientific), plated at 16,000 cells/cm^2 and then treated with test or control media as described above for 24 h. The slides were fixed in ice-cold 100% acetone for 10 min, and then washed with PBS and stored at −4 °C.

Immunocytochemistry (ICC) was carried out on the slides using the Ventana BenchMark XT automated IHC/ISH staining instrument (Ventana Medical Systems, Tucson, USA) then counter-stained with haematoxylin. Primary antibodies were diluted in PBS at a pre-determined concentration (Table 1). IgG matched isotypes (Ventana, Tucson, USA and Dako-Global Science, Auckland, New Zealand) were used for negative control slides. Three representative images at 100× magnification from each ICC slide were captured for analysis using a mounted Olympus DP21 camera on a BX53 light microscope (Olympus New Zealand, Auckland, New Zealand). Fiji (Image J) software [15] was used to quantify the percentage of the area of the positive staining to the entire area of positive and negative (counter-) staining cells using the command (Analyse > Measure). The command (Image > Adjust Threshold) was used to adjust the staining threshold using visual comparison with corresponding colour photomicrographs.

2.5. Immunohistochemistry

Seventy-six consented formalin-fixed paraffin-embedded OSCC tissues and nine consented normal oral mucosa (NOM) tissues were chosen from the archive of the Oral Pathology Centre at the University of Otago. The NOM tissue comprised accessions diagnosed as NOM or those that contained NOM distant from the diagnosed oral lesions, where the diagnosis was unrelated to any malignant or potentially malignant process. The OSCC samples were categorised into three prognostic groups. The first group comprised superficially-invasive OSCC (SI-OSCC), defined as T1 OSCC [16,17] with less than 0.5 mm

Table 2
Fold change and fold regulation in significantly regulated genes in tunicamycin-treated (test) and tunicamycin-untreated (control) NOM and OSCC cell lines.

Significantly regulated STAT3 pathway-related genes in untreated OSCC compared to untreated NOK cell lines							
	Average ΔC_t		$2^{-\Delta Ct}$		Fold Change	p value	Fold Regulation
Gene	OSCC	NOK	OSCC	NOK	OSCC/NOK	*t*-test	OSCC/NOK
CDKN1A	5.18	2.50	0.02754	0.17645	0.16	0.04	−6.41
FAS	7.25	5.91	0.00657	0.01664	0.39	0.03	−2.53
IL1A	6.40	3.53	0.01183	0.08639	0.14	0.01	−7.30
LIFR	8.77	10.99	0.00230	0.00049	4.66	0.04	4.66
TNFRSF10B	7.97	6.56	0.00399	0.01059	0.38	0.04	−2.65
Significantly regulated STAT3 pathway-related genes in tunicaymin-treated compared to untreated NOK cell lines							
	Average ΔC_t		$2^{-\Delta Ct}$		Fold Change	p value	Fold Regulation
Gene	Test	Control	Test	Control	Test/Control	*t*-test	Test/Control
CCL2	16.41	14.74	0.00001	0.00004	0.31	0.003	−3.19
IL18R1	9.10	12.02	0.00183	0.00024	7.57	0.039	7.57
IL6R	7.86	10.34	0.00430	0.00077	5.58	0.001	5.58
NFKBIA	4.34	5.91	0.04927	0.01662	2.97	0.030	2.97
PHF21A	7.90	9.08	0.00419	0.00185	2.27	0.009	2.27
Significantly regulated STAT3 pathway-related genes in tunicamycin-treated compared to untreated OSCC cell lines							
	Average ΔC_t		$2^{-\Delta Ct}$		Fold change	p value	Fold regulation
Gene	Test	Control	Test	Control	Test/Control	*t*-test	Test/Control
IL22	16.11	17.52	0.00001	0.00001	2.65	0.024	2.65
IL5	14.71	17.06	0.00004	0.00001	5.12	0.005	5.12

Table 3
Independent-sample *t*-test of protein expression in tunicamycin-treated compared to untreated (control) NOK cell lines. Significantly greater extents of STAT3 (p = 0.019) and LIF-R (p = 0.042) expression were seen in treated compared to untreated NOK cell lines. No significant differences were found when comparing the expression of IL-6R, TLR-4, IL-5, and IL-22 in tunicamycin-treated compared to untreated NOK cell lines. *Denotes statistical significance at the 0.05 level. ** Where variances were unequal, Independent Mann-Whitney *U* Test was done. The results for both IL-6R and IL-5 were not statistically significant (p = 0.1 and p = 0.4, respectively).

Treated *vs* untreated (Control) NOK cell lines		Levene's test for equality of variances		*t*-test for equality of means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean difference	Std. Error difference	95% Confidence interval of the difference	
									Lower	Upper
STAT3	Equal variances assumed	1.901	0.240	−3.777	4	0.019*	−0.201	0.053	−0.348	−0.053
TLR-4	Equal variances assumed	5.182	0.085	−1.751	4	0.155	−0.269	0.154	−0.696	0.157
IL-6R	Equal variances assumed	9.789	0.035**	−2.307	4	0.082	−0.127	0.055	−0.279	0.026
	Equal variances not assumed			−2.307	2.009	0.147	−0.127	0.055	−0.362	0.107
LIF-R	Equal variances assumed	0.002	0.964	−2.961	4	0.042*	−0.007	0.002	−0.013	−0.000
IL-5	Equal variances assumed	8.208	0.046**	−1.239	4	0.283	−0.159	0.128	−0.514	0.197
	Equal variances not assumed			−1.239	2.175	0.332	−0.159	0.128	−0.667	0.352
IL-22	Equal variances assumed	2.225	0.210	−0.976	4	0.384	−0.063	0.065	−0.244	0.117

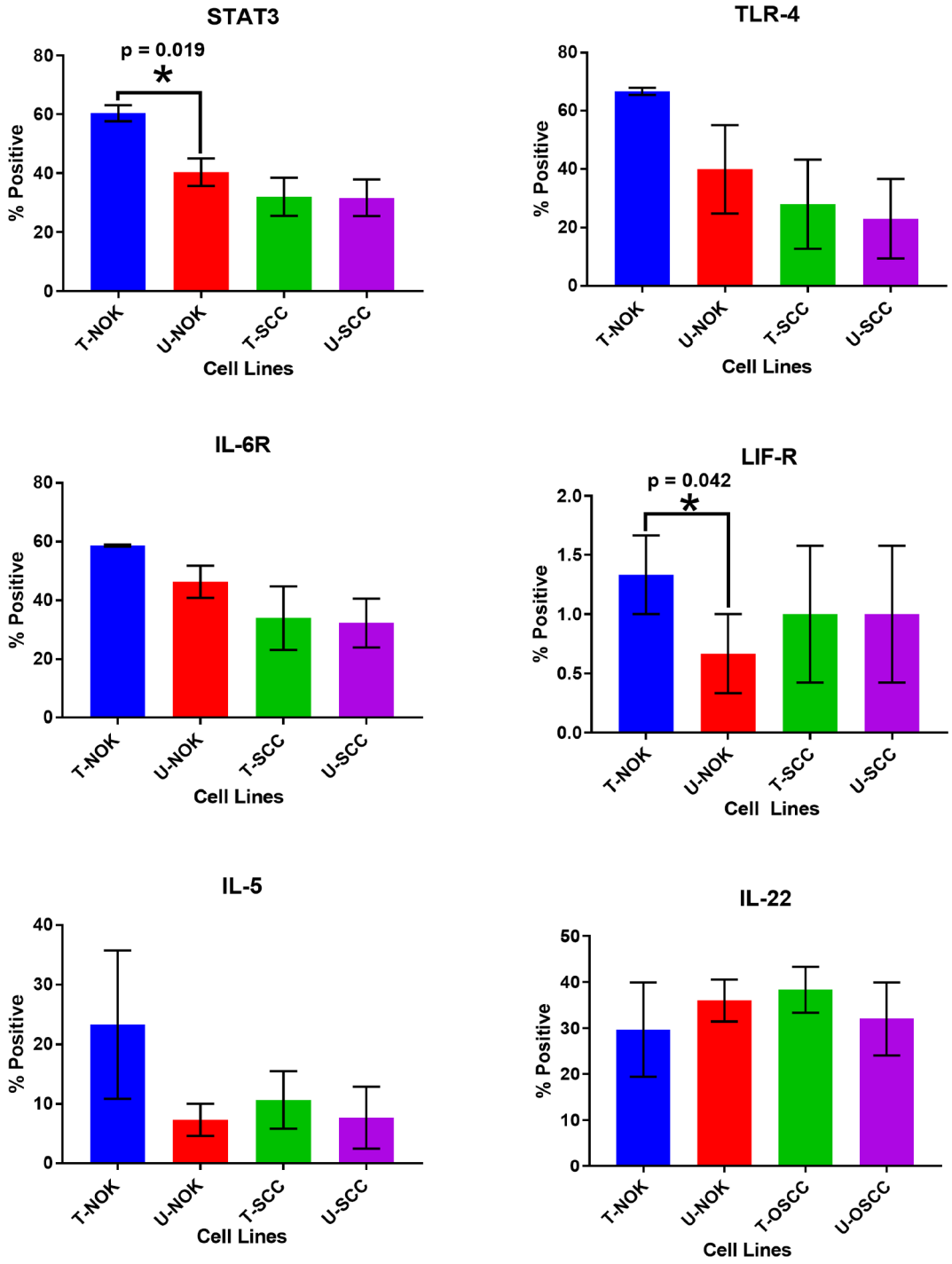

Fig. 3. Bar graphs showing significantly greater extents of immunocytochemical STAT3 (p = 0.019) and LIF-R (p = 0.042) protein expression in tunicamycin-treated compared to untreated NOK cell lines. No significant differences were found between the expression of IL-6R, TLR-4, IL-5, and IL-22 in tunicamycin-treated compared to untreated NOK cell lines, nor in any of STAT3, IL-6R, LIF-R, TLR-4, IL-5, and IL-22 in tunicamycin-treated compared to untreated OSCC cell lines. T-NOK: Treated NOK cell lines. U-NOK: Untreated NOK cell lines. T-SCC: Treated SCC cell lines. U-SCC: Untreated SCC cell lines. Error bars represent standard error of the mean.

invasion measured from the basement membrane (n = 13). Lesions with an invasion depth of > 1 mm were classified into well-differentiated (WD-OSCC) and moderately-differentiated OSCC (MD-OSCC) according to the current WHO classification in conjunction with the Bryne's classification which has been validated to correlate well with prognosis [18–20] and thus there were 43 WD-OSCCs, and 20 MD-OSCCs. Tissue microarray (TMA) blocks were constructed using a manual tissue arrayer-1 (MTA-01, Beecher Instruments, Estigen,

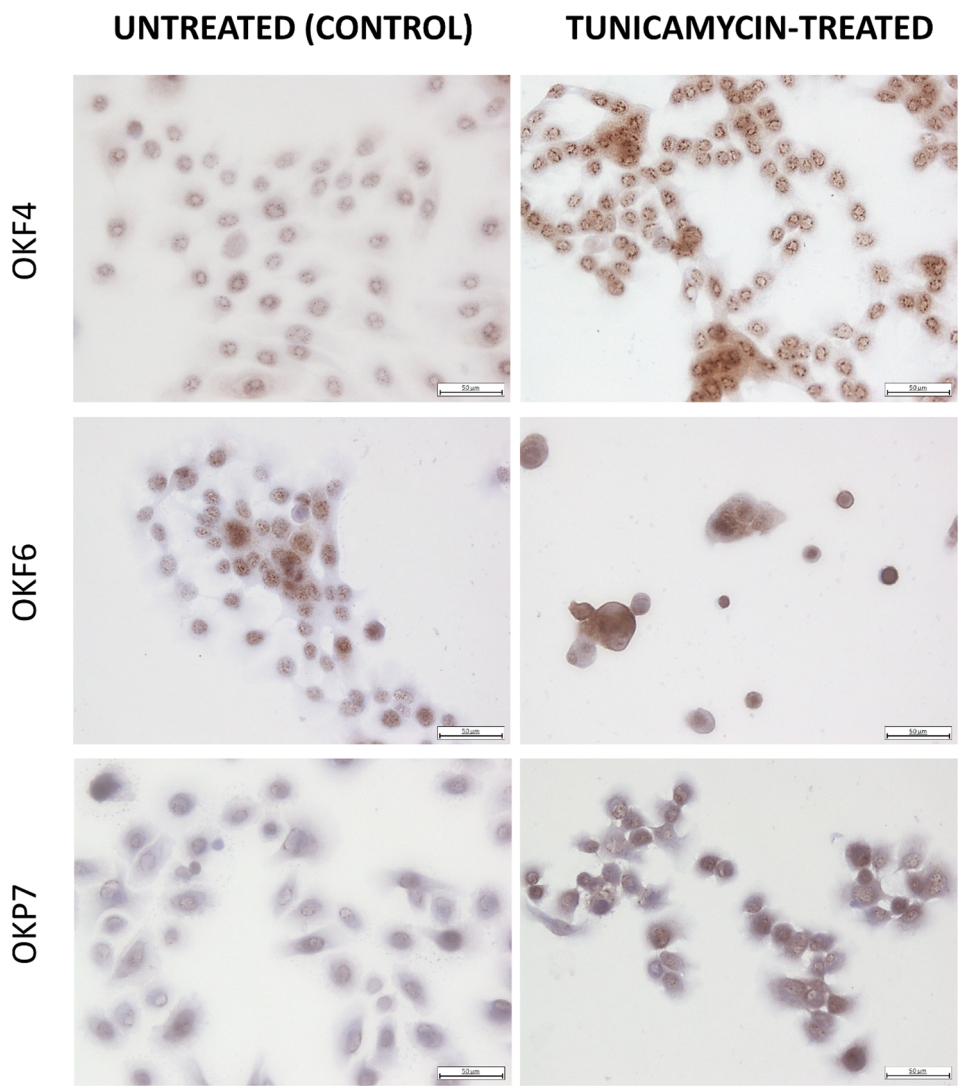

Fig. 4. Photomicrographs showing immunocytochemical expression of STAT3 protein in NOK cell lines (400x magnification. Scale bar: 50 μm).

Estonia) from the samples selected. Control tissue cores from the spleen, tonsil, colon, and breast cancer were also incorporated. Sections of 4 μm in thickness were cut from the TMA blocks and placed on adhesive slides. IHC and automated semi-quantitative analysis of the digitised images were performed as described for ICC.

2.6. Statistical analysis

A mean score was calculated from the triplicate values for each marker or tissue sample for the assessment of protein expression for ICC and IHC. Independent sample t-tests were carried out to compare the extent of expression of each protein between the groups. One-way ANOVA test with post-hoc Tukey's was performed for pair-wise comparison between OSCCs from different sites and different prognostic groups. Pair-wise Spearman's correlation test was performed between the markers tested. The significance level was set at $p < 0.05$. Statistical tests were performed and graphs were plotted using SPSS (IBM, Version 22.0, NY, USA), Prism Graph Pad (Prism version 7.01, California, USA), and Microsoft Excel (Version 14.0.7177.5000) software.

3. Results

3.1. Gene expression

CDKN1A, TNFRSF10B, FAS, and IL1A were significantly down-regulated whereas LIFR was significantly up-regulated in untreated OSCC compared to untreated NOK cell lines (Fig. 1, Fig. 2A, and Table 2). In response to tunicamycin treatment, CCL2 was significantly down-regulated whereas IL18R1, NFKBIA, PHF21A, and IL6R were

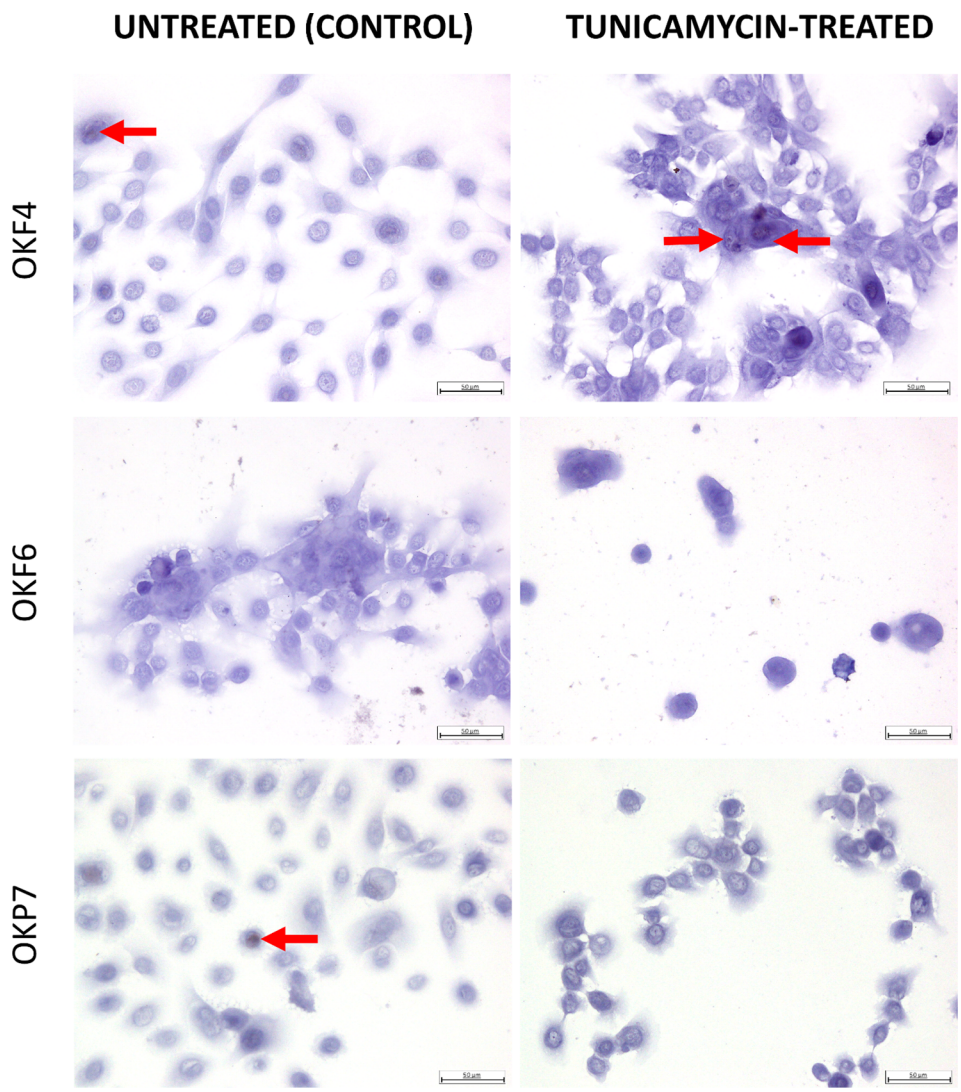

Fig. 5. Photomicrographs showing immunocytochemical LIF-R protein expression in NOK cell lines. Red arrows indicate some of the occasional cells that show weak nuclear staining for LIF-R (400× magnification. Scale bar: 50 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significantly up-regulated in NOK cell lines (Fig. 1, Fig. 2B, and Table 2). In OSCC cell lines, tunicamycin treatment resulted in significant up-regulation of IL22 and IL5 but significant down-regulation of TGM2 (Fig. 1, Fig. 2C, and Table 2).

3.2. Immunocytochemistry

In NOK cell lines, a significantly higher extent of STAT3 ($p = 0.019$; Table 3; Figs. 3 and 4) and LIF-R ($p = 0.042$; Table 3; Figs. 3 and 5) expression was seen in tunicamycin-treated compared to untreated cells (Table 3, Fig. 3). The expression of IL-6R, IL-5, and TLR-4 was higher in treated NOK cell lines than untreated ones, but not to the point of statistical significance (Table 3; Fig. 3).

Qualitative changes observed suggested an increase in intensity of STAT3 staining in treated NOK cell lines, particularly in OKP7 cell line (Fig. 4). LIF-R was weakly expressed in the nuclei of occasional cells in both treatment and control groups of NOK cell lines (Fig. 5). Among OSCC cell lines, the expression of the all the proteins was higher in treated cell lines. None of these differences were statistically significant (Table 4; Fig. 3).

When untreated OSCC cell lines were compared with untreated NOK cell lines, no statistically significant differences were detected in any of the parameters assessed (Table 5; Fig. 3).

3.3. Immunohistochemistry

STAT3 ($p = 0.046$) and IL-6R ($p = 0.027$) were expressed to significantly higher extents in OSCC compared to NOM tissue whereas LIF-R expression was significantly higher in NOM than OSCC tissue ($p = 0.033$; Table 6, Fig. 6). The expression of TLR-4, IL-6R, and IL-5

Table 4
Independent-sample t-tests showing no significant differences in protein expression in tunicamycin-treated compared to untreated (control) OSCC cell lines.

Treated *vs* untreated (control) OSCC cell lines		Levene's test for equality of variances		*t*-test for equality of means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean difference	Std. error difference	95% Confidence interval of the difference	
									Lower	Upper
STAT3	Equal variances assumed	0.003	0.957	−0.036	4	0.973	−0.003	0.0877	−0.246	0.239
TLR-4	Equal variances assumed	0.029	0.873	−0.236	4	0.825	−0.049	0.207	−0.623	0.525
IL-6R	Equal variances assumed	0.362	0.580	−0.113	4	0.916	−0.015	0.137	−0.395	0.364
LIF-R	Equal variances assumed	0.101	0.767	−0.176	4	0.869	−0.001	0.007	−0.020	0.0174
IL-5	Equal variances assumed	0.019	0.898	−0.408	4	0.704	−0.029	0.070	−0.224	0.166
IL-22	Equal variances assumed	1.166	0.341	1.167	4	0.308	0.063	0.054	−0.087	0.214

Table 5
Independent-sample t-tests showing no significant differences in protein expression in untreated OSCC compared to untreated NOK cell lines.

Untreated OSCC *vs* NOK cell lines		Levene's test for equality of variances		*t*-test for equality of means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean difference	Std. error difference	95% Confidence interval of the difference	
									Lower	Upper
STAT3	Equal variances assumed	0.229	0.657	1.108	4	0.330	0.085	0.076	−0.127	0.296
TLR-4	Equal variances assumed	0.006	0.943	0.812	4	0.462	0.167	0.206	−0.404	0.738
IL-6R	Equal variances assumed	0.961	0.382	1.389	4	0.237	0.138	0.099	−0.137	0.412
LIF-R	Equal variances assumed	4.826	0.093	−0.711	4	0.517	−0.004	0.005	−0.018	0.011
IL-5	Equal variances assumed	2.784	0.171	−0.088	4	0.934	−0.005	0.057	−0.163	0.153
IL-22	Equal variances assumed	1.600	0.275	−0.756	4	0.492	−0.040	0.053	−0.187	0.107

Table 6
Independent sample t-tests between OSCC and NOM tissue samples. Significantly greater extents of STAT3 (p = 0.046) and IL-6R (p = 0.027) proteins were seen in OSCC than NOM tissue, and of LIF-R (p = 0.033) in NOM than OSCC tissue. * Denotes statistical significance at the 0.05 level.

OSCC versus NOM tissue		Levene's test for equality of variances		*t*-test for equality of means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean difference	Std. error difference	95% confidence interval of the difference	
									Lower	Upper
STAT3	Equal variances assumed	0.016	0.898	2.033	75	0.046*	0.096	0.047	0.002	0.190
TLR-4	Equal variances assumed	0.023	0.881	1.044	75	0.300	0.062	0.059	−0.056	0.179
IL-6R	Equal variances assumed	1.899	0.172	2.259	74	0.027*	0.104	0.046	0.012	0.195
LIF-R	Equal variances assumed	0.026	0.873	−2.167	74	0.033*	−0.102	0.047	−0.196	−0.008
IL-5	Equal variances assumed	2.706	0.104	0.346	75	0.730	0.016	0.045	−0.074	0.105
IL22	Equal variances assumed	1.792	0.185	1.077	76	0.285	0.036	0.034	−0.031	0.103

proteins was higher in OSCC than NOK but did not reach statistical significance (Table 6).

Except for LIF-R, the proteins were expressed to a higher extent in intra-oral than lip vermilion SCC but these differences were not statistically significant (Table 7). Apart from IL to 22, there were no significant differences in the expression of the proteins investigated in OSCC tissue from different sites (p = 0.001, Table 8). Pair-wise analysis using the post-hoc Tukey's test showed significantly greater extent of IL-22 expression in OSCC tissue from the lip vermilion than buccal mucosa (p = 0.002) and the tongue (p = 0.008, Table 9). ANOVA tests showed that STAT3 expression was significantly different among different prognostic groups of OSCCs (p = 0.035, Table 10). Pair-wise analysis using the post-hoc Tukey's test showed significantly greater extent of STAT3 expression in moderately-differentiated OSCC than superficially-invasive OSCC (p = 0.031; Table 11, Fig. 6D). No other statistically significant differences were found when comparing the expression of the proteins in different prognostic groups of OSCC.

Pair-wise Spearman's tests revealed statistically significant correlations between the expression of STAT3 and TLR-4 (p = 0.036; Table 12), STAT3 AND IL-5 (p = 0.003, Table 12), and IL-6R and LIF-R (p = 0.029, Table 12) in OSCC.

4. Discussion

NOK response to tunicamycin-induced ERS (TI-ERS) involved significant up-regulation of STAT3 and LIF-R proteins, and IL6R and IL18R1 genes, both of which are STAT3 and NFκB signalling activators. ERS is known to induce STAT3 and NFκB signalling and up-regulate IL-6 [21]. Both STAT3 and IL-6R proteins were expressed to significantly higher extents in OSCC tissue. In addition, STAT3 protein was also expressed to significantly greater extents in MD-OSCC compared with SI-OSCC. This suggests that overexpression of STAT3 may be involved in mechanisms that influence the depth of invasion and degree of differentiation. Whilst there is contrasting evidence for the prognostic value of STAT3 expression [8,22], over-expression of both IL-6 and IL-6R observed in many cancers, including OSCC, has generally been correlated with poor clinical outcome [23].

Up-regulation of the aforementioned receptors in response to ERS in NOK, unlike OSCC, was accompanied by the significant up-regulation of a potent inhibitor of NFκB (*i.e.* NFKBIA) and high up-regulation of STAT3 inhibitor (*i.e.* SOCS1) at the mRNA level, which may account for the down-regulation of the majority of the STAT3- and NFκB-dependent tumour-promoting genes in NOK. These findings suggest that inhibition of NFκB and STAT3 activation may have been compromised in the examined OSCC cell lines whereas they remained intact in the NOK cell lines. Indeed, a study conducted on a Taiwanese population suggested that NFKBIA gene mutations may increase the risk of OSCC [24].

On the other hand, OSCC cells responded to TI-ERS by up-regulation of IL5 and IL22. IL5 is both a pro- and anti-inflammatory cytokine [25,26]. In mice, over-expression of IL5 has been shown to inhibit wound healing *via* an enhanced inflammatory response [27]. Its over-expression in cancers has shown variable effects [28,29]. In one study IL-5 inhibition led to decreased OSCC tumour size through inhibition of eosinophils [30] and in another study IL-5 was shown to promote tumour cell growth through up-regulation of T cell leukaemia/lymphoma 1A (TCL1A) [31].

IL22 is an anti-inflammatory cytokine which is important in immune-to-epithelial cell signalling and is secreted by natural killer and T cells [25,32]. While IL22 promotes healing under physiological conditions [33], it has shown both tumour-promoting and tumour-suppressing roles [34,35]. In OSCC, up-regulation of IL22 through STAT3 signalling has been reported [36]. In the context of ERS, IL22 was shown to suppress palmitate- and TI-ERS in a STAT3-dependent manner through negatively affecting the IRE1α/XBP1 arm of the UPR [32,37]. To this end, it seems that IL22 was up-regulated in direct response to TI-ERS in the current study.

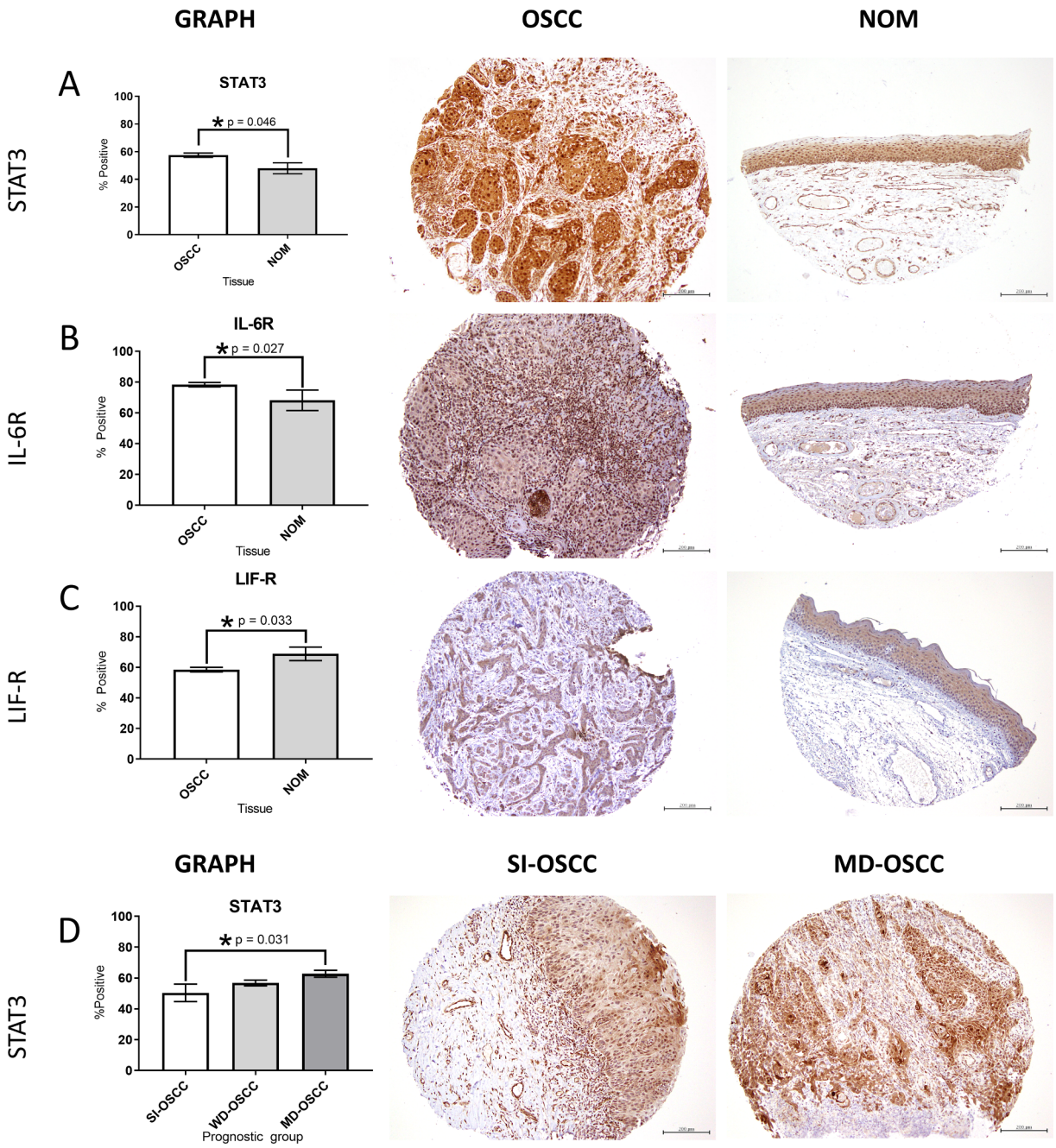

Fig. 6. The bar graphs show the extents of STAT3 (A), IL-6R (B), and LIF-R (C) protein expression in OSCC and NOM tissue and the extents of STAT3 protein expression in OSCC in different prognostic groups (D). (A) STAT3 protein was expressed to a significantly greater extent in OSCC than NOM tissue ($p = 0.046$). (B) IL-6R protein was expressed to a significantly greater extent in OSCC than NOM tissue ($p = 0.027$). (C) LIF-R protein was expressed to a significantly greater extend in NOM than OSCC tissue ($p = 0.033$). (D) STAT3 protein was expressed to significantly greater extents in superficially-invasive than moderately differentiated OSCC tissue. Error bars represent standard error of the mean.

Importantly, both IL5 and IL22 have been shown to activate STAT3 and/or NFκB resulting in a feed-forward loop that involves activation and re-activation of STAT3 signalling [34,36,38]. While the effects of these responses are quenched under physiological conditions, sustained signalling may condition the tumour microenvironment (TME) to favour disease progression [34,36,38]. It has also been shown that IL-22 suppresses ERS by stimulating XBP1 splicing [37], and that XBP1 is required for the differentiation of eosinophils, which are a major source of IL-5 [39]. Further work is required to examine the underlying mechanisms and effects of IL-5 and IL-22 up-regulation in the TME.

One of the novel findings was the expression of LIFR in OSCC. LIFR is a cytokine receptor for LIF, and has multiple functions in

Table 7
Independent Samples t-tests showing no significant differences in the expression of the investigated proteins between intra-oral and lip vermilion OSCC. * Where variances were unequal, an Independent Mann-Whitney U Test was done. There was no significant difference between expression of STAT3 in IO-SCC and L-OSCC (p = 0.35).

Intra-oral versus lip vermillion SCC		Levene's test for equality of variances		*t*-test for equality of means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean difference	Std. error difference	95% Confidence interval of the difference	
									Lower	Upper
STAT3	Equal variances assumed	5.753	0.019*	−1.174	67	0.244	−0.040	0.035	−0.109	0.028
	Equal variances not assumed			−0.970	23.927	0.342	−0.040	0.042	−0.126	0.046
TLR-4	Equal variances assumed	1.253	0.267	−0.093	67	0.926	−0.004	0.042	−0.089	0.081
IL-6R	Equal variances assumed	2.690	0.106	−1.696	66	0.095	−0.051	0.030	−0.111	0.009
LIF-R	Equal variances assumed	2.209	0.142	0.558	66	0.579	0.019	0.034	−0.049	0.086
IL-5	Equal variances assumed	0.653	0.422	−0.432	68	0.667	−0.013	0.029	−0.070	0.045
IL22	Equal variances assumed	3.737	0.057	−4.164	67	0.000	−0.094	0.023	−0.139	−0.049

Table 8
ANOVA tests of protein expression extents in OSCC based on site. A significant difference in IL-22 protein expression was seen in OSCC tissue from different sites (p = 0.001). *Denotes statistical significance.

		Sum of squares	df	Mean square	F	Sig.
STAT3	Between groups	0.073	4	0.018	1.118	0.356
	Within groups	1.040	64	0.016		
	Total	1.113	68			
TLR-4	Between groups	0.063	4	0.016	0.635	0.639
	Within groups	1.593	64	0.025		
	Total	1.657	68			
IL-6R	Between groups	0.050	4	0.012	0.947	0.443
	Within groups	0.827	63	0.013		
	Total	0.876	67			
LIF-R	Between groups	0.032	4	0.008	0.486	0.746
	Within groups	1.033	63	0.016		
	Total	1.065	67			
IL-5	Between groups	0.008	4	0.002	0.169	0.953
	Within groups	0.779	65	0.012		
	Total	0.787	69			
IL-22	Between Groups	0.165	4	0.041	5.53	0.001*
	Within Groups	0.479	64	0.007		
	Total	0.644	68			

Table 9
ANOVA test with pair-wise comparison of IL-22 expression in OSCC based on site using Tukey's posthoc test. Significantly higher extents of IL-22 staining was seen in OSCC tissue from the lip vermilion (LMV) than buccal mucosa (BM; p = 0.002) and the tongue (T; p = 0.008). * Denotes statistical significance at the 0.05 level. G: gingiva; FOM: floor of mouth.

(I) SITE	(J) SITE	Mean difference (I-J)	Std. Error	Sig.	95% Confidence interval	
					Lower bound	Upper bound
BM	FOM	−0.096	0.063	0.553	−0.273	0.081
	G	−0.087	0.045	0.307	−0.214	0.039
	T	−0.083	0.042	0.302	−0.202	0.036
	LMV	−0.170	0.043	0.002*	−0.290	−0.050
FOM	BM	0.096	0.063	0.553	−0.081	0.273
	G	0.009	0.055	1.000	−0.146	0.163
	T	0.013	0.053	0.999	−0.135	0.162
	LMV	−0.074	0.053	0.635	−0.221	0.075
G	BM	0.087	0.045	0.307	−0.039	0.214
	FOM	−0.009	0.055	1.000	−0.163	0.146
	T	0.005	0.029	1.000	−0.076	0.086
	LMV	−0.083	0.030	0.051	−0.166	0.000
T	BM	0.083	0.042	0.302	−0.036	0.202
	FOM	−0.013	0.053	0.999	−0.162	0.135
	G	−0.005	0.029	1.000	−0.086	0.076
	LMV	−0.088	0.025	0.008*	−0.158	−0.017
LMV	BM	0.170	0.043	0.002*	0.050	0.290
	FOM	0.074	0.053	0.635	−0.075	0.224
	G	0.083	0.030	0.051	−0.000	0.166
	T	0.088	0.025	0.008*	0.017	0.158

development, immunity and pathological processes [40]. Constitutive active LIF secretion has been detected in approximately 70% of 24 carcinoma cell lines of the breast, lung, pancreas, liver, stomach, colon, and melanocytes [41]. LIF and LIFR have shown contrasting roles in different contexts. LIF was reported to promote the growth of four cancer cell lines of the breast and pancreas, and suppress the growth of three others [42] and its over-expression in melanoma was shown to promote metastasis [43]. On the other hand, LIFR may also act as a

Table 10
ANOVA results of protein expression in OSCC based on prognostic groups. A significant difference in STAT3 protein expression in OSCC from different prognostic groups was seen (p = 0.035). * Denotes statistical significance at the 0.05 level.

		Sum of Squares	df	Mean Square	F	Sig.
STAT3	Between groups	0.107	2	0.054	3.514	**0.035***
	Within groups	1.006	66	0.015		
	Total	1.113	68			
TLR-4	Between groups	0.078	2	0.039	1.640	0.202
	Within groups	1.578	66	0.024		
	Total	1.657	68			
IL-6R	Between groups	0.037	2	0.019	1.449	0.242
	Within groups	0.839	65	0.013		
	Total	0.876	67			
LIF-R	Between groups	0.000	2	0.000	0.015	0.985
	Within groups	1.064	65	0.016		
	Total	1.065	67			
IL-5	Between groups	0.043	2	0.021	1.919	0.155
	Within groups	0.744	67	0.011		
	Total	0.787	69			
IL-22	Between Groups	0.031	2	0.016	1.680	0.194
	Within Groups	0.613	66	0.009		
	Total	0.644	68			

Table 11
ANOVA test with pair-wise comparison of STAT3 expression in OSCC based on prognostic groups using Tukey's posthoc test. Significantly higher extents of STAT3 expression were seen in moderately differentiated OSCC tissue (MD-OSCC) than superficially invasive OSCC (SI-OSCC; p = 0.31). * Denotes statistical significance at the 0.05 level. WD-OSCC: Well-differentiated OSCC.

(I) Prognostic group	(J) Prognostic group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence interval	
					Lower bound	Upper bound
SI-SCC	WD-SCC	−0.06439	0.04364	0.309	−0.169	0.040
	MD-SCC	−0.12479	0.04823	**0.031***	−0.240	−0.009
WD-SCC	SI-SCC	0.06439	0.04364	0.309	−0.040	0.169
	MD-SCC	−0.06040	0.03439	0.192	−0.143	0.0221
MD-SCC	SI-SCC	0.12479	0.04823	**0.031***	0.009	0.240
	WD-SCC	0.06040	0.03439	0.192	−0.022	0.143

tumour suppressor [44,45]. Significantly higher LIF-R expression was seen in NOK in the present study and a statistically significant correlation was found between IL and 6R and LIF-R expression in OSCC. The findings of the current study support the role of LIF-R in STAT3 activation in OSCC. Recently, a study of OSCC showed that LIF expressed by cancer-associated fibroblasts (CAF) in OSCC TME promoted invasion of OSCC, and that LIF was expressed to higher levels in CAF associated with OSCC than the malignant epithelial cells [46]. Furthermore, another study investigating LIF-R expression in the head and neck reported greater expression of LIF-R in nasopharyngeal carcinoma, suggesting that LIF-R expression is different in OSCC [47].

In conclusion, this is the first study to show that STAT3 pathway genes are differentially regulated under TI-ERS in OSCC. TI-ERS up-regulated tumour-promoting cytokines IL-5 and IL-22 in OSCC. These findings provide important potential targets for tailored immunotherapy. Further studies are required to determine the exact mechanisms by which TI-ERS up-regulates these cytokines in OSCC.

Table 12
Pair-wise Spearman's correlation test between the markers in OSCC tissue showing significant correlations between the immunohistochemical expression of STAT3 and IL-5 (p = 0.003), STAT3- and TLR-4 (p = 0.036), and LIF-R and IL-6R (p = 0.029). *Denotes significance at the 0.05 level. **Denotes significance at the 0.01 level.

Spearman's rho		STAT3	TLR-4	IL-6R	LIF-R	IL-5	IL-22
STAT3	Correlation coefficient	1.000	0.254*	0.043	0.033	0.354**	−0.034
	Sig. (2-tailed)	.	**0.036**	0.731	0.793	**0.003**	0.791
	N	69	68	66	67	68	62
TLR-4	Correlation coefficient	0.254*	1.000	0.045	−0.015	0.109	−0.220
	Sig. (2-tailed)	**0.036**	.	0.719	0.902	0.382	0.086
	N	68	69	66	67	67	62
IL-6R	Correlation coefficient	0.043	0.045	1.000	0.269*	0.150	−0.236
	Sig. (2-tailed)	0.731	0.719	.	**0.029**	0.230	0.067
	N	66	66	68	66	66	61
LIF-R	Correlation coefficient	0.033	−0.015	0.269*	1.000	−0.008	0.066
	Sig. (2-tailed)	0.793	0.902	**0.029**	.	0.950	0.615
	N	67	67	66	68	67	61
IL-5	Correlation coefficient	0.354**	0.109	0.150	−0.008	1.000	−0.212
	Sig. (2-tailed)	**0.003**	0.382	0.230	0.950	.	0.095
	N	68	67	66	67	70	63
IL-22	Correlation Coefficient	−0.034	−0.220	−0.236	0.066	−0.212	1.000
	Sig. (2-tailed)	0.791	0.086	0.067	0.615	0.095	.
	N	62	62	61	61	63	69

5. Conflict of interest statement

The authors declare no conflicts of interest.

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Appendix A. Supplementary material

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