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Original Research

CD8$^+$ T cell response to human papillomavirus 16 E7 is able to predict survival outcome in oropharyngeal cancer

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KEYWORDS
Human papillomavirus;
Oropharyngeal carcinoma;
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Abstract ***Introduction:*** Immunological response to human papillomavirus (HPV) in the development and progression of HPV16+ oropharyngeal squamous cell carcinoma (OPSCC) (accounting for the majority of viral associated cases) is largely unknown and may provide important insights for new therapeutic strategies.

Methods: In this prospective clinical trial (UKCRN11945), we examined cell-mediated immune responses to HPV16 E2, E6 and E7 in peripheral blood using IFN-γ enzyme-linked immunosorbent spot assay. CD56$^+$, CD4$^+$, CD8$^+$ and regulatory T cell frequencies were also discerned by flow cytometry. Fifty-one study participants with oropharyngeal carcinoma were recruited. Control subjects were those undergoing tonsillectomy for benign disease. All patients were treated with curative intent by radiotherapy ± chemotherapy. Disease-specific survival was investigated by multivariate analysis.

Results: HPV16 DNA was detected in 41/51 of the OPSCC participants. T cell responses against HPV16 E6 or E7 peptides were detected in 33/51 evaluable patients, respectively and correlated with HPV status. Matched pre- and post-treatment T cell responses were available for 39/51 OPSCC cases. Within the whole cohort, elevated post-treatment CD8$^+$ response to HPV16 E7 correlated with longer disease free survival (multivariate DFS $p < 0.03$). Within the HPV + OPSCC cohort, a significant increase in regulatory T cells ($p < 0.02$) was noted after treatment.

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Conclusions: This is the first study to provide survival data in OPSCC stratified by cell-mediated immune response to HPV16 peptides. Within the HPV16+ OPSCC cohort, enhanced immunoreactivity to antigen E7 was linked to improved survival. An increase in regulatory T cell frequencies after treatment may suggest that immunosuppression can contribute to a reduced HPV-specific cell-mediated response.

1. Introduction

In head and neck squamous cell cancer (HNSCC), immunodeficiency has been shown to correlate with a poor prognosis [1]. However, few studies have addressed the specific role of cell-mediated immunity in human papillomavirus (HPV)–associated OPSCC [2,3]. A study by de Jong *et al.* [4] demonstrated that baseline HPV16 specific $CD4^+$ T cell response in cervical cancer patients is either absent or severely impaired (despite a relatively robust immune status). Spanos *et al.*[5] recently showed that functional immune response was critical to achieving adequate tumour clearance with chemoradiation in a murine model. A further study revealed that $CD8^+$ T cells reactive to wild-type p53 are significantly reduced after surgical excision of HPV16 tumours [6]. Hoffman *et al.*[7] investigated $CD8^+$ T cells reactive to HPV16 E7 in a small cohort of patients with OPSCC. As predicted, these were commonly detected in patients with a positive HPV16 status but the study lacked survival analysis as a clinical outcome measure.

Most of the studies above focus on immune response at the point of diagnosis and before curative therapy. At present, it is unclear whether conventional treatment protocols for OPSCC [1] inhibit or promote tumour immune response mechanisms [8]. Distel *et al.* [9] investigated intra-tumoural immune profiles before and after primary chemoradiation and concluded that post-therapy cytotoxic T-lymphocyte cells (CTL) were depleted to a lesser extent than immunosuppressive T regulatory cells. This is in contrast to the findings of Al-Taei *et al.* [10] who found decreased systemic HPV-specific T cell responses and accumulation of immunosuppressive influences in oropharyngeal cancer patients following radical therapy.

Natural regulatory T cells (T_{regs}) are important immunosuppressive cells and have been correlated with a poor prognosis in HNSCC [1,11]. They have an important role in homeostasis and can be characterised by high co-expression of $CD4^+$ and $CD25^+$ [12]. T_{regs} inhibit T cell activity by induction of ATP hydrolysis and apoptosis via the Fas/FasL pathway [13]. An increasing body of evidence would suggest that they are responsible for ameliorating tumour-specific immune responses [14] and as such are a potential barrier to immunotherapy [7,15].

In order to understand better the immune profile in patients with OPSCC, we report the first study to examine long-term survival outcomes in relation to T-lymphocyte subsets and specific cell-mediated immune response to HPV16 E2, E6 and E7.

2. Methods

2.1. Study population

This project received formal approval by the National Research Ethics Service Committee of East of England (12/EE/44). Clinical samples were obtained from Cambridge University Hospitals NHS Trust between June 2011 and July 2013.

The study comprised 51 patients with primary oropharyngeal squamous cell carcinoma and 11 control subjects with benign disease. Disease stage was classified using the tumour regional lymph node metastasis (TNM) classification of malignant tumours [16].

A baseline sample of the tumour was taken for histological analysis and the remainder processed for DNA ± RNA extraction. A consultant histopathologist with expertise in head and neck pathology reviewed each tissue block to ensure adequate tumour sampling.

The trial protocol can be downloaded from the United Kingdom Clinical Research Network (https://www.ukctg.nihr.ac.uk; study ID 11945). Clinical data for all subjects are shown in Table 1.

2.2. Lymphocyte cell preparation

A blood sample (40 ml) was drawn at baseline after diagnosis and 3 months post-treatment. In order to examine the specificity of the IFN-γ enzyme-linked immunosorbent spot assay (ELISPOT), healthy patients were recruited to the study, each of whom underwent tonsillectomy for a benign condition.

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll–Paque (GE Healthcare, Sweden) and washed with phosphate buffered saline (PBS). The extracted PBMCs were then mixed with foetal calf serum (FCS)

Table 1
Clinical and histopathological data. OPSCC patients had a mean age of 58 years and the male-female ratio was 4:1.

Stage	Sex	Subsite	Age	PS	Histol.	Smoker	p16 IHC	DNA seq	PCR HPV16 E6/E7 DNA	PCR (PGMY)	Nested PCR	PCR HPV16 E6/E7 mRNA	DNA ISH	Primary treatment	Neck dissection	Clinical f/u (months)	Clinical outcome
IVB	M	Tonsil	61	0	Poor	Current	+	16	+	+	+	−		CRT	+	43	
III	M	BoT	48	0	Poor	Never	+	Nil	+	−	−	+		CRT	+	47	
IVA	M	Tonsil	72	1	Mod	Former	+	16	−	+	+		+	RT	−	11	††
IVA	M	BoT	59	0	Well	Current	+	16	−	+	+	−		CRT	+	43	
IVB	F	BoT	68	3	n/a	Never	+	16	+	+	+	−		RT	−	14	†
IVA	F	BoT	49	0	Poor	Never	+	16	+	+	+		+	CRT	+	51	
IVA	M	BoT	33	0	Mod	Never	+	18	+	+	+		+	CRT	−	4	
III	F	Tonsil	67	1	Poor	Never	+	16	−	+	+		+	RT	+	40	
IVA	M	BoT	57	0	Poor	Current	+	16	+	+	+		+	RT	−	44	
IVA	M	BoT	65	0	Poor	Never	+	Nil	+	+	+	+		CRT	+	43	
IVA	F	Tonsil	61	0	Mod	Current	+	16	+	+	+	−		CRT	+	48	
I	M	BoT	77	1	n/a	Former	+	16	+	+	+		−	RT	−	7	†
IVA	M	Tonsil	51	0	Poor	Current	+	16	+	+	+		+	CRT	−	12	††
IVA	M	Tonsil	49	1	Poor	Never	+	16	+	−	+	−		RT	−	4	†
IVA	M	BoT	57	1	n/a	Never	+	16	+	+	+	−		CRT	−	40	
IVA	M	BoT	64	0	Mod	Former	+	16	+	+	+		+	CRT	+	47	
IVA	M	BoT	75	1	Mod	Former	+	16	+	+	+	+		RT	+	39	
IVA	M	Tonsil	47	0	Poor	Never	+	16	+	+	+	−		CRT	+	39	
III	F	Tonsil	60	0	Mod	Former	+	16	+	+	+	+		RT	−	39	
IVA	F	Tonsil	62	0	Poor	Never	+	16	+	+	+		+	RT	+	42	
IVA	M	Tonsil	82	1	Poor	Never	+	16	+	+	+		−	RT	−	4	†
IVA	M	BoT	52	0	Poor	Never	+	16	+	−	+	+		CRT	+	43	
IVA	M	Tonsil	58	0	Well	Current	+	16	+	+	+	+		CRT	+	30	
III	M	BoT	51	0	Mod	Never	+	16	+	+	+		+	CRT	−	53	
IVA	M	BoT	40	0	Mod	Never	+	16	−	−	+		+	CRT	−	13	††
IVA	M	Tonsil	56	2	Poor	Never	+	33	+	+	+	+		CRT	+	37	
IVA	M	Tonsil	46	0	Poor	Current	+	16	−	+	+		+	RT	+	38	
III	M	BoT	53	0	n/a	Never	−	16	+	+	+		−	CRT	−	37	
IVA	M	BoT	78	1	Poor	Former	+	16	+	−	+	−		RT	+	37	
IVA	M	Tonsil	58	0	Mod	Never	+	16	+	+	+		+	CRT	+	39	
IVA	M	Tonsil	55	0	Mod	Current	+	Nil	+	+	+	+		CRT	+	35	
IVA	M	BoT	81	1	Poor	Former	+	16	+	+	+	+		RT	−	12	††
IVA	M	Tonsil	59	0	n/a	Former	+	16	−	+	+	−		RT	+	36	
IVA	M	Tonsil	61	0	Poor	Current	+	16	−	−	+		+	RT	+	41	
IVA	F	BoT	51	0	Poor	n/a	+	16	+	+	+		+	CRT	+	44	
III	M	Tonsil	53	1	n/a	Former	+	16	+	+	+		−	CRT	−	41	# 10 months
III	F	Tonsil	40	0	Poor	Current	+	33	+	+	+		+	RT	−	40	
I	M	Tonsil	67	0	n/a	Former	+	16	−	−	+		−	RT	−	51	
IVA	M	Tonsil	52	0	Poor	Current	+	16	+	+	+		+	CRT	+	44	
IVA	F	Tonsil	63	1	Poor	Never	+	16	+	+	+		+	CRT	+	44	
IVA	M	BoT	47	0	Poor	Never	+	16	+	+	+		+	CRT	+	48	
IVA	M	Soft pal.	61	2	Poor	Current	−	Nil	−	−	−		−	RT	+	34	# 16 months
IVA	F	Tonsil	56	0	Mod	Current	−	Nil	−	−	−		−	CRT	+	48	
III	M	BoT	60	0	Poor	n/a	−	n/a	n/a	n/a	n/a		−	CRT	+	37	

(*continued on next page*)

Table 1 (*continued*)

Stage	Sex	Subsite	Age	PS	Histol.	Smoker	p16 IHC	DNA seq	PCR HPV16 E6/E7 DNA	PCR (PGMY)	Nested PCR	PCR HPV16 E6/E7 mRNA	DNA ISH	Primary treatment	Neck dissection	Clinical f/u (months)	Clinical outcome
III	M	BoT	71	0	Mod	Current	–	n/a	n/a	n/a	n/a		–	CRT	–	35	# 11 months
III	M	BoT	56	1	Mod	Current	–	n/a	–	–	–	–		CRT	–	15	# 15 months
IVA	M	Tonsil	53	0	Poor	Never	+	n/a	–	–	–	–		CRT	–	40	
IVA	M	Tonsil	42	0	n/a	Former	+	n/a	–	–	–	–		CRT	–	41	
III	M	Tonsil	43	1	Mod	Current	–	n/a	–	–	–	–		CRT	–	13	††
IVA	M	Tonsil	77	1	n/a	Former	–	n/a	–	–	–	–		RT	+	37	
IVB	M	BoT	77	1	Mod	Former	–	n/a	–	–	–	–		RT	+	38	# 22 months

The majority had local advanced disease (stage IVA ~70%). No patient had distant metastases (stage IVC). Treatment involved chemotherapy (cisplatin 100 mg/m^2, days 1, 22, 43) given concurrently with radiation therapy (65 Gy in 35 fractions to both primary and nodal sites). In two subjects with significant renal or cardiac disease, cetuximab 400 mg/m^2 IV loading dose 1 week before the start of radiation therapy, then 250 mg/m^2 weekly. For patients with limited nodal disease (usually >3 cm), a selective or modified radical neck dissection was performed before the start of chemoradiation. The multi-disciplinary team assessed clinical response based primarily on surface measurements at direct laryngoscopy, supplemented by radiographic imaging (^{18}F-fluoro-2-deoxyglucose positron emission tomography/computed tomography [FDG PET/CT]). Disease free survival and overall survival were determined after a median period of 43 months.
BoT, base of tongue; Well, well differentiated SCC; Mod, moderately differentiated SCC; Poor, poorly differentiated SCC; PS, Eastern Co-Operative Group (ECOG) physiological performance status; CRT, chemoradiotherapy; RT, radiotherapy; HPV, human papillomavirus; n/a, not available.
Clinical outcome:#, locoregional recurrence; †, non-malignant cause of death; ††, malignant cause of death.

supplemented with 10% dimethyl sulfoxide (Sigma) before storage in liquid nitrogen.

Pre- and post-therapy samples for each patient were tested in the same experiment. After thawing and washing in cold sterile P10 (PBS + 10% FCS), PBMCs underwent depletion of CD56$^+$, CD8$^+$ and CD25$^+$ cells using magnetic microbeads (Miltenyi Biotec, Surrey, UK) according to the manufacturer's instructions. Purity of the monocyte selection (CD4$^+$/CD8$^+$/CD56$^+$) was verified by fluorescence-activated cell sorter analysis (see below). 0.5–1.5 × 10^6 CD4$^+$, CD8$^+$ or CD56$^+$ cells were each added to a 15-ml conical tube and washed with culture medium (RPMI 1640 supplemented with 10% FCS, 2-mM glutamine, 100-IU/ml penicillin and 100-μg/ml streptomycin; Gibco, Paisley, UK).

2.3. ELISPOT assays for IFN-γ

To measure IFN-γ release, flat-bottomed 96-well polyvinylidene difluoride membrane–backed plates (Millipore, Bedford, UK) were first washed with sterile PBS before coating with 50 μl of mouse IgG1 anti-human IFN-γ monoclonal antibody (1-D1K, 15 μg/ml in 0.1M NaHCO3 buffer; pH 9.5, Mabtech, Sweden) and incubation at 4 °C overnight.

The next day, coated wells were washed ×6 with PBS before blocking with 50 μl of culture medium (CM) for 3 h at 37 °C. Aliquots of CD4$^+$/CD8$^+$/CD56$^+$ cells in CM (100 μl–1 × 10^5) were added to each well.

Stimulatory mAbs to CD28 and CD49d (Pharmingen, Oxford, UK) both at 0.5 μg/ml were then added for CD4$^+$ T cell assays [17]. Only stimulatory mAbs to CD28 were added for CD8$^+$ T cell assays [18].

To induce non-specific cytokine production (required for CD4$^+$ or CD8$^+$ cells), 2 μl of phorbol myristate acetate (5 μg/ml; Sigma–Aldrich, UK) and 10 μl of ionomycin (100 μM concentration; Sigma–Aldrich, UK) were added to each positive control well.

Peptide libraries spanning the entire length of HPV16 E2, E6 and E7 proteins (Mimotopes, Victoria, Australia) were grouped in pools and added to the CD4$^+$/CD8$^+$ cell culture medium to achieve a final concentration of 1 μM for each peptide, prior to incubation for 18 h at 37 °C in 5% CO_2.

The cells were then discarded and the plate washed ×6 with PBS/0.05% Tween-20. Each well was then coated with 50 μl (4 μg/ml) of biotinylated anti-human IFN- antibody (7-B6-1-biotin, Mabtech, Sweden) and the plates incubated for 2 h at 37 °C. A further ×6 wash with PBS/0.05% Tween-20 preceded the addition of a streptavidin–alkaline phosphatase conjugate for 1 h (prepared according to the manufacturer's instructions and added at a volume of 100 μl/well; Biorad, UK). The reaction was terminated by washing with tap water and allowed to air-dry. The number of spots in each well was subsequently counted with digital image software (AutoImmun Diagnostik, Germany).

2.4. Flow cytometry

Harvested cells (~1 × 10^5) were washed in sterile P10 (PBS + 10% FCS) and incubated with the relevant mAbs (each at 25 μg/ml) to surface markers (anti-human CD4/fluorescein isothiocyanate; anti-human CD8/R-phycoerythrin-Cy5; anti-human CD56/phycoerythrin; DAKO, Denmark) for 15 min at 4 °C. A further aliquot of $CD4^+$ cells was separated into $CD25^{neg}$ [negative], $CD25^{int}$ [intermediate] and $CD25^{high}$ T cells (the latter two groups representing the T_{reg} population) after staining with anti-human CD25/phycoerythrin (BD Biosciences, USA). Purity of the T_{reg} population was further assessed in a subgroup of patients after staining for FoxP3. All stained cells were washed again with sterile P10 before fixation in 1% paraformaldehyde (Sigma, UK).

2.5. HPV stratification

HPV stratification methods included consensus $p16^{INK4A}$ IHC, PGMY (L1) PCR; type-specific HPV16 DNA PCR and DNA sequencing in all patients. In some patients, sufficient quality fresh frozen samples allowed HPV16 E6/E7 mRNA PCR. In all others HPV DNA *in situ* hybridization was performed (Fig. S1). HPV positive status defined as evidence of HPV16 L1/E6/E7 DNA or HPV16 E6/E7 mRNA ± episomal or integrative pattern on HPV DNA ISH ± p16INK4A expression (>70% tumour cell staining).

2.6. Statistical analysis

Statistical calculations were performed using SPSS version 21 (Chicago, IL, USA). Rates of disease free survival (DFS) were estimated by means of the Kaplan–Meier method and were compared by the log-rank test. A two-tailed p value of <0.05 was defined as significant.

3. Results

3.1. HPV-specific T cell responses correlate with tumour HPV status

In total, 41/51 of tumour samples were classified as HPV positive - defined by evidence of HPV16 L1/E6/E7 DNA or HPV16 E6/E7 mRNA ± HPV DNA ISH episomal/integrative staining pattern. Immunohistochemical analysis for expression of $p16^{INK4a}$ was demonstrated for all HPV+ OPSCC samples but also present for 2 out of 10 OPSCC samples categorised as HPV negative.

When linking HPV16 status with the ELISPOT data, we found that 80% (33/41) of the patients who had detectable HPV16 DNA in tumours also had $CD4^+$ or $CD8^+$ T cell response to HPV16 E6/E7. Only one patient with a HPV negative tumour displayed such a response, suggesting a correlation is likely between HPV positive status of the tumour and T cell response to HPV antigens. Data summarising HPV status and T cell response are shown in Fig. S1.

3.2. $CD4^+$ and $CD8^+$ T cell response to HPV16 E7 reduce post-treatment for the majority of patients

Specific IFN-γ responses to HPV16 E2, E6 and E7 by both $CD4^+$/$CD8^+$ T cells were investigated at initial diagnosis of malignancy and three months after completion of chemoradiation for 39/51 patients. Pre-treatment $CD4^+$ response to E2 was detected for eight patients (8/30); this frequency decreased slightly after treatment (8/39). One patient demonstrated a $CD8^+$ response to E2 prior to treatment only.

Overall, $CD8^+$ T cell responses to HPV16 E6 and E7 peptides were detected pre-treatment in 60% (18/30) and 70% (21/30) of the patients, respectively. Post-treatment evaluation revealed that there was no significant change in $CD8^+$ response to E6 (39.5%; 15/38; $p < 0.16$) and a significant decreased response to E7 peptide (44.7%; 17/38; $p < 0.02$; Fig. 1).

Pre-treatment $CD4^+$ T cell responses to HPV16 E6 and E7 peptides were detected in 60% (18/30) and 70% (21/30) of the patients, respectively. Post-treatment, there was a significant decrease in ELISPOT responses to both E6 (35.9%; 14/39; $p < 0.02$) and E7 peptides (43.6%; 17/39; $p < 0.01$; Fig. 1).

To ascertain sensitivity and specificity of the IFN-γ assay, patients undergoing tonsillectomy for benign

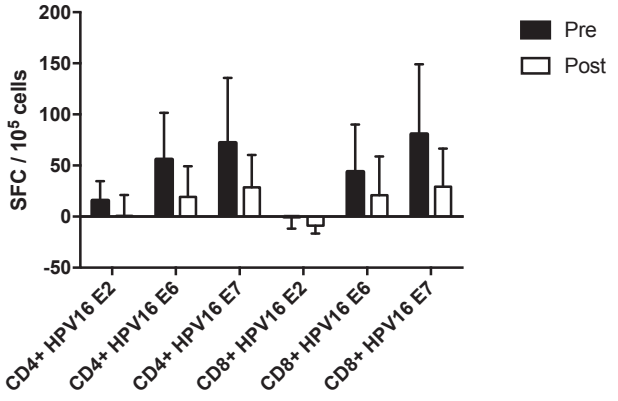

Fig. 1. HPV16 E2, E6 and E7 response detection by IFN-γ ELISPOT pre- and post-radical therapy. A response was considered positive if the average number of spot forming cells (SFCs) in HPV antigen well was 2 standard deviations (SDs) above the average of negative control. The frequency of cytokine secreting $CD4^+$/$CD8^+$/$CD56^+$ cells were then derived by the formula: number of spots/number of cells per well. For this study, we assume that increased staining of $CD4^+$ equates to $CD4^+$ T cells; we assume that increased staining of $CD8^+$ equates to $CD8^+$ T cells; we assume that increased staining of $CD56^+$ equates to NK cells. HPV, human papillomavirus

disease were also subjected to the same analysis at baseline. All displayed no positive IFN-γ response for either $CD4^+$ or $CD8^+$ T cells except for one subject (1/11). The same subject displayed evidence for HPV16 L1 DNA on initial HPV stratification.

IFN-γ production by CD56 cells (NK cell population) was utilised to discern non-specific (innate) immune response within the clinical subgroups. Fig. S2 shows no significant difference was detected for all patients pre- or post-treatment.

3.3. Decreased CD4:CD8 ratio following radical therapy

The CD4:CD8 ratio pre-treatment was similar to that seen in healthy controls (3.03 versus 2.87). However, $CD4^+$ (0.74 versus 0.42 × 10^9/L) and $CD8^+$ (0.37 versus 0.33 × 10^9/L) cell numbers were reduced post-therapy with a larger proportionate decrease in $CD4^+$ T cells ($p < 0.04$; Fig. 2). Haemoglobin level (13.1 versus 12.1 g/dl), total lymphocytes (1.4 versus 1.1 × 10^9/L), monocytes (1.6 versus 1.3 × 10^9/L) and platelet count (298 versus 307 × 10^9/L), all remained stable before and after treatment.

3.4. $CD4^+/CD25^+$ T cell frequencies increase in HPV+ OPSCC patients after treatment

Frequency of $CD4^+$ cells with high co-expression of $CD25^+$ were examined for the majority of patients [34/51] using gate-settings as previously described (Fig. 3) [19,20]. Although the proportion of $CD4^+/CD25^+$ T_{regs} did not differ at baseline between the patient groups,

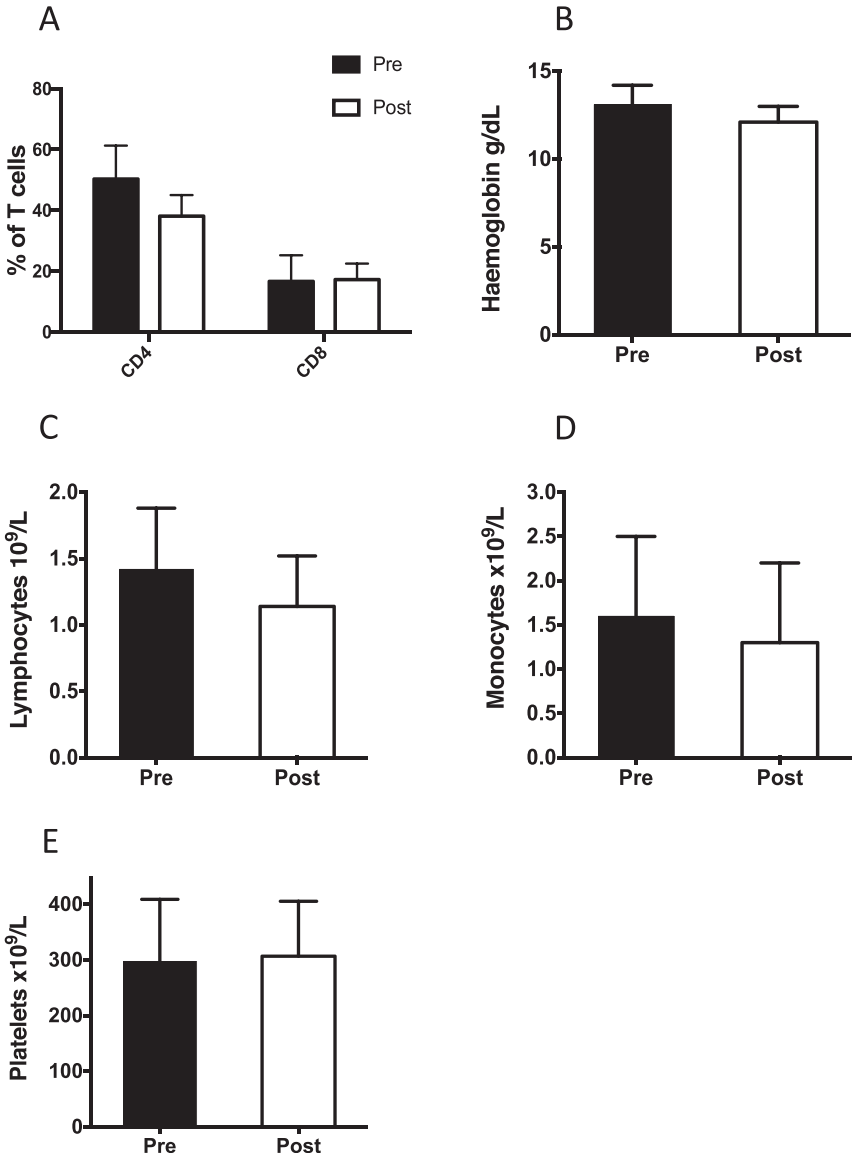

Fig. 2. Mean haematological parameters (±SD) in OPSCC patients before and after radical therapy. The graph shows changes in (A) CD4:CD8 ratio (B) haemoglobin (C) lymphocytes (D) monocytes and (E) platelets. The CD4:CD8 ratios were calculated using the frequencies of CD4+ and CD8+ population. All other subgroups were taken from the routine full blood count (FBC) in the clinic.

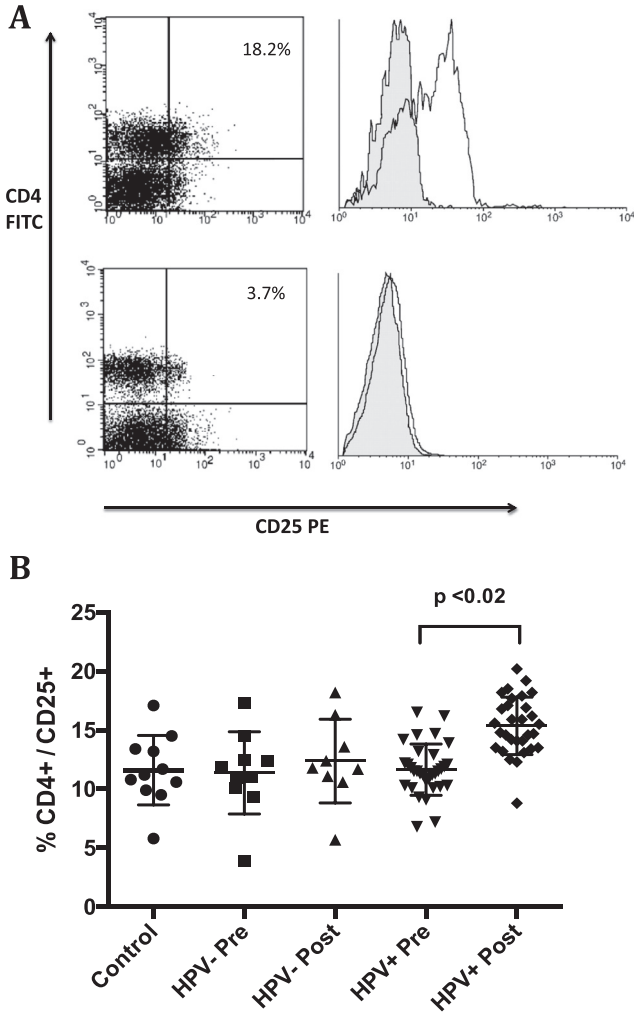

Fig. 3. CD25 expression on $CD4^+$ T cells using flow cytometry. Flow activated cell sorting (FACS) was undertaken using the FacsCalibur flow cytometer (BD Biosciences) and analysed with Flowjo software (Treestar, USA). A) The left panel dotplots illustrate the expression of CD25 on $CD4^+$ T cells. The right panels depict histograms used to quantitate CD25 expression: clear-coloured histograms represent staining with a specific antibody to CD25. Grey histograms represent control staining. Representative patients with high (18.2%) and low (4.2%) CD25+ expression are illustrated on the top and bottom rows respectively. B) Frequency distribution (%) of CD4+/CD25+ for control, HPV− and HPV+ patients before and after treatment. HPV, human papillomavirus.

there was significant elevation in HPV+ OPSCC patients after treatment (11.6% versus 15.4%; $p < 0.02$).

FoxP3 expression in the CD4+/CD25 T cell subsets revealed the majority of CD4+/$CD25^{high}$ T cells expressed FoxP3, whereas CD4+/$CD25^{neg}$ T cells and CD4+/$CD25^{int}$ T cells expressed FoxP3 at very low and moderate levels, respectively (Fig. S3).

Within a subset of the HPV cohort, a further functional analysis looked at the effect of CD25+ cell depletion on antigen specific IFN-γ responses against E6/E7 by both the CD4 and CD8 T cell population (Fig. S4). Cytokine response was enhanced significantly for the post-treatment CD8+ group only (*HPV16 E6 $p < 0.03$; **HPV16 E7 $p < 0.02$).

3.5. Improved disease free survival with elevated CD8+ T cell response to HPV16 E7

The average DFS for all patients was 43.7 months (±SE 2.5). For the HPV16 + cohort, the DFS period increased to 47.3 months (±SE 2.3). In those with an increased $CD8^+$ T cell response to E7, the average DFS period was 49.6 months (± SE 2.3 Fig. 4 and Fig. S5). A multivariate proportional hazards model using Cox regression analysis revealed HPV16 ($p < 0.02$), retained or enhanced $CD8^+$ T cell response to E7 ($p < 0.03$) and smoking ($p < 0.04$) to have significant influence on DFS.

4. Discussion

Oropharyngeal squamous cell carcinoma is the possible outcome of infection with high-risk HPV and is preceded by a phase of persistent HPV infection during which the host immune system fails to eliminate the virus. Fortunately, the majority of oral cavity HPV infections are cleared before oncogenic conversion [21].

In this prospective observational study, we report a systematic analysis of IFN-γ response in primary oropharyngeal carcinoma. The significant finding of this data set would suggest an attenuated response by $CD4^+$/$CD8^+$ T cells to HPV16 peptides after completion of radical therapy. Specific factors found to influence DFS include HPV16 status, smoking and post-treatment $CD8^+$ response to HPV16 E7.

As yet, the majority of HNSCC studies investigating interaction between T cell response and the HPV16 E6 or E7 oncoproteins are small and confined to a non-clinical setting [2,3,5,6,10,22,23]. This is in contrast to the more extensive research evaluating specific immune response in cervical cancer [4,24–29].

The improved clinical outcome with retained $CD8^+$ response to HPV16 E7 may contradict data from previous studies that show a more prominent immunogenic role for E6 [27]. This situation may have arisen due to the more preserved status of E7 (compared to E6) in pre-malignant and malignant disease [28]. E7 has also been used as the target epitope for many therapeutic vaccination trials for CIN [25,29].

HPV E6 is transcribed off the same transcript as E7 and thus should be expressed in similar frequencies and quantity. It may be reasonable to assume a similar antiviral T cell response, particularly for E6*1, which is the splice isoform of E6 expressed at high levels (along with E7) in tumours. E7 is expressed at the expense of full length E6, therefore a spectrum of responses to E7 and E6*1 and not full length E6 in tumours is possible [26].

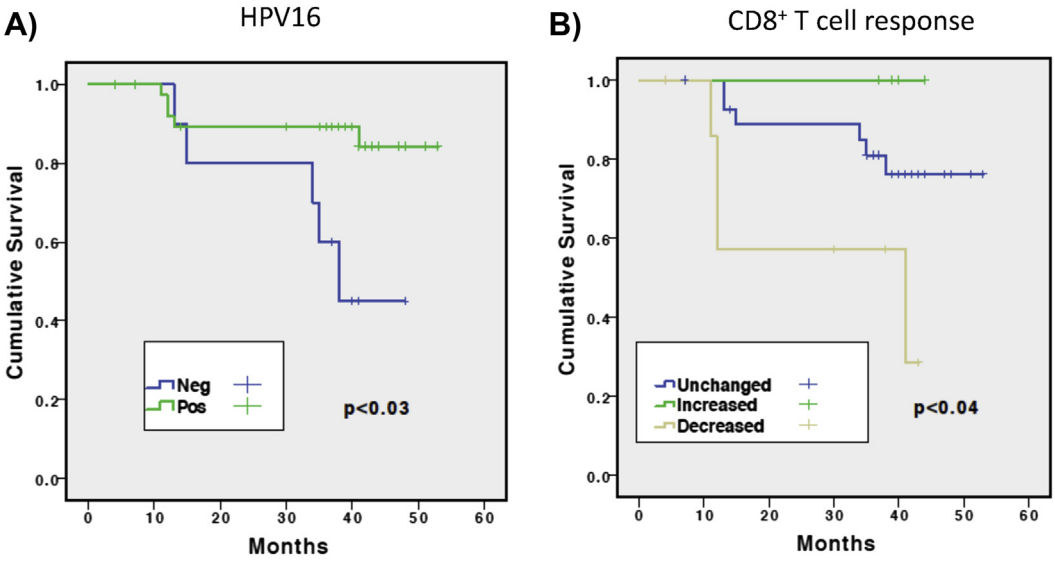

Fig. 4. Disease free survival (DFS) stratified by (A) HPV16 status and (B) Post-treatment CD8 response to HPV16 E7. A multivariate model was developed using Cox regression to investigate the effect of clinical factors on disease free survival (HPV16, $\Delta CD4^{+}$ T cell response to E6/E7 [pre-treatment versus post-treatment], $\Delta CD8^{+}$ T cell response to E6/E7, $p16^{INK4a}$, T_{reg} frequency (%), T stage, N stage, sex, physiological performance status, oropharyngeal subsite, histology grade, smoking, concurrent chemotherapy, age and $CD8^{+}$/Treg ratio [pre/post/Δ ratio]). HPV, human papillomavirus.

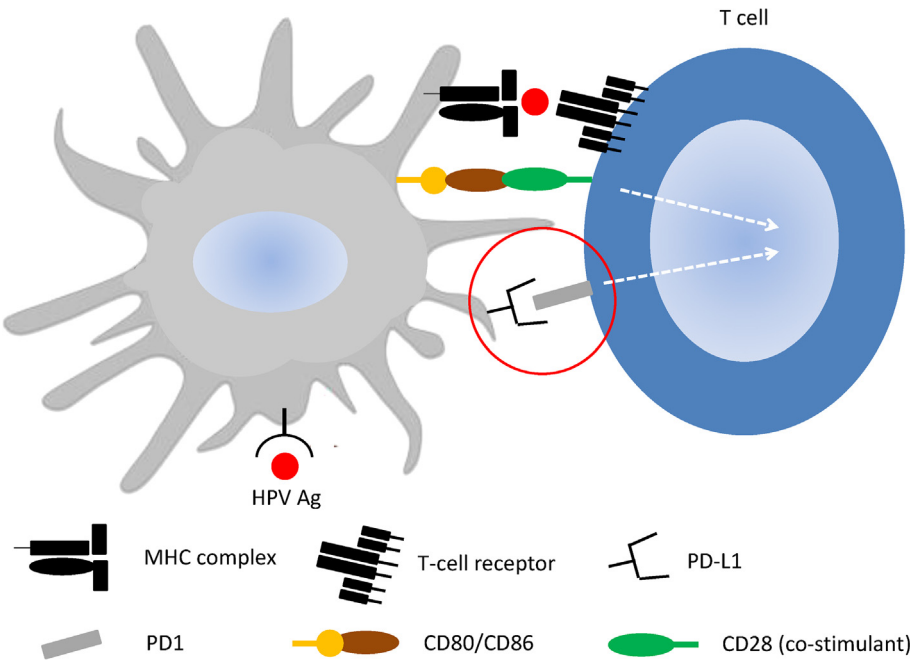

Fig. 5. Hypothetical role for PD-1 (programmed death protein 1) in HPV associated OPSCC. PD-1 is an inhibitory receptor expressed on various immune cells, including activated T cells and T_{reg} cells. By blocking the interaction between PD-1 and its primary ligand, PD-L1 (red circle), T cell effector functions are enhanced by increasing proliferation and cytokine activity (IFN-γ). Data from this study would suggest that further work is required to discern the balance between cytotoxic and regulatory T cells (before and after treatment). HPV, human papillomavirus.

CD8+ cytokine response to the HPV16 E7 epitope was more likely in our HPV16 RNA+/DNA+ group—as such it may simply act as a proxy marker for improved clinical outcome regardless of the immune phenotype. However, this specific response could have clinical relevance as CD8+ T cells are the dominant immune killer cells for virus-infected cells, intracellular bacteria and cancer cells. Previous studies [23,30] also indicate that patients with HPV16+ HNSCC exhibit an increased number of T cells specific for peptides derived

from the HPV E7 oncoprotein. Also, HPV-specific T cell responses [as a marker of HPV infection] would presumably lead to *generalised* correlations with improved survival for most or all CD4+ and CD8+ T cell responses (E2, E6 and E7).

The reduced T cell responses after treatment noted in this study cannot be explained by a concurrent decrease in lymphocytes or memory T cells; absolute numbers for both groups remained unaffected after radical therapy (Fig. 3). Instead, there was a significant increase in regulatory T cells noted within the HPV16+ cohort before and after the treatment (11.6% versus 15.4%; $p < 0.02$). These data suggest that immunosuppression can influence HPV-specific immune response post-treatment; in addition they would support animal models where an immunodeficient host failed to clear HPV16 positive tumour cells after exposure to cisplatin [5].

Whiteside *et al.* demonstrate a higher Treg frequency and a lower CD8+/Treg ratio post chemoradiotherapy with no stratification by HPV status [31]. The study authors suggest that the Treg fraction in PBMC is relatively resistant to chemoradiotherapy, unlike the CD4+ and CD8+ T cells. This hypothesis was tested *in vitro* and demonstrated supportive data. Our study indicates that increased frequency of Tregs [post-treatment] is restricted to HPV + cohort (Fig. 3b). If confirmed to be the case, important drivers of this resistance could include pathways related to PDZ-domain proteins (E6), pRB, p107, p130, wild-type p53 or E7.

Although DFS was not significantly influenced by T_{reg} frequency before and after treatment, we suggest further functional studies may be of benefit. In particular, investigation of PD1 or PDL-1 expression from tumour infiltrating lymphocytes] pre- or post-treatment may be of clinical value [32]. A previous study has demonstrated increased PD1 expression from tumour infiltrating lymphocytes is linked to improved survival in HPV+ OPSCC [33]. It may be of interest to determine whether changes in (or selection for) a PD1-high tumour cell population drives the balance between cytotoxic and regulatory T cells (Fig. 5).

Our data reveal two patients assigned to the HPV− OPSCC cohort tested positive for $p16^{INK4A}$. Potential reasons for this observation include intra-observer/inter-observer variation of the immunohistochemical background stain. In addition, a small but significant cohort of the false positive HPV− samples has $p16^{INK4A}$ mutations that may account for accumulation of inactive $p16^{INK4A}$ [34]. These data reinforce our previous assertion that ***p16+ status alone*** is ***not a safe or justifiable*** method of assigning HPV status to a tumour [1].

Limitations of this research are the small sample size and the possible insensitivity of the assay due to the localised nature of the infection. The reduced cohort size may result in less power to detect associations with E6 or E7. Problems with detection of CMI response in peripheral blood are not surprising since HPV16 is a specific oropharyngeal subsite infection and consequently the number of circulating memory cells would be hypothesized to be small. The E2 antigen may also have a more important role in viral control at an earlier stage of infection [35]. It would also be of interest to obtain information regarding sustainability of the IFN-γ ELISPOT response at a more distant time-point after primary treatment (>5−10 years). We postulate that this may continue to decrease as antigen exposure becomes diminished (with clearance of infection or the cancer). Conversely, a rising $CD4^+/CD8^+$ T cell response may indicate recurrence of disease.

In summary, we have found that the frequency of IFN-γ secreting $CD4^+/CD8^+$ T cells specific for HPV16 E6 and E7 peptides were decreased in most patients 3 months after radical therapy. Further to this, we observe a significantly improved DFS outcome in the small subset of patients who retain or enhance their $CD8^+$ E7 response. HPV16 and smoking status were also found to correlate with survival. Increased immunosuppressive influences after treatment (for the HPV16 cohort) are supported by a concomitant rise in T_{reg} frequencies. Although the E6 and E7 epitopes are apparently immunogenic in patients with HPV-associated oropharyngeal carcinoma, it is unclear why specific T cells are unable to eliminate or prevent oncogenic change at an earlier stage. Further studies are required to explain this resistance of tumour targets to cytotoxic T cells and to find potential strategies that will increase the chances of developing HPV-based therapeutic vaccine in the future.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejca.2016.08.012.

Conflict of interest statement

None declared.

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