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Short communication

DETECTION OF THE STATUS OF TUMOUR-INFILTRATING CD4$^+$ T-CELL SUBPOPULATIONS IN SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK (HNSCC) USING REAL-TIME RT-PCR DETECTION OF CD4 mRNA

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Abstract: Several studies have reported tumour infiltrating CD4$^+$ T cells as a favourable prognostic factor in some types of cancer. We investigated 37 head and neck squamous cell carcinomas (HNSCC) at different stages, using immunohistochemical staining for CD4$^+$ infiltrates and real-time reverse transcription polymerase chain reaction (RT-PCR) detection of CD4 mRNA. The CD4$^+$ infiltrates were evaluated and expressed as a percentage according to the ratio of CD4$^+$ T cells to epithelial cells in the cancer cell nests and to the overall inflammatory cell infiltrate in the tumor stroma. The CD4 mRNA expression level strongly correlated with the CD4$^+$ infiltration score in the cancer epithelium (r_s = 0.858, P < 0.001) and in the cancer stroma (r_s = 0.797, P < 0.001). These results indicate that the real-time RT-PCR assay is a sensitive and reliable method for the detection of CD4 mRNA, and that it could be used to reassess CD4$^+$ infiltration status in resected specimens from patients with HNSCC.

Key words: Tumour infiltrating CD4$^+$ T cells, RT-PCR CD4 mRNA detection, HNSCC

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Abbreviations used: ECL – enhanced chemiluminiscence; EDTA – ethylendiaminetetraacetic acid; F – female; HNSCC – head and neck squamous cell carcinoma; Hpx- hypopharynx; IHC – immunohistochemistry; IL – interleukin; Lrx – larynx; M – male; MHC – major histocompatibility complex; NFDM – non-fat dry milk; PBS – phosphate buffer saline; PCR – polymerase chain reaction; PMSF – phenylmethylsulfonyl fluoride; pTNM – pathological Tumour Node Metastasis classification of cancer; RT – room temperature; RT-PCR – reverse transcriptase polymerase chain reaction; SD – standard deviation; SDS – sodium dodecyl sulfate; Th – T-helper immune cells; TRI reagent – TRIZOL reagent; SPSS – Statistical Package for the Social Sciences; UICC – International Union Against Cancer (Union Internationale Contre le Cancer)

INTRODUCTION

CD4 proteins are receptors that are localized on the surface of helper T cells, and are mostly responsible for recognizing peptide fragments associated with class II major histocompatibility complex (MHC) molecules [1]. MHC class I-restricted $CD4^+$ T cells, however, have been shown to be reactive to the melanoma antigen [2]. For signal transduction, CD4 and CD8 T-cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56*lck* [3]. Both subpopulations of helper T cells, Th1 and Th2, play a role in the elimination of tumours, either by the recruitment of tumouricidal myeloid cells [4] into the tumours or by the action of anti-angiogenic cytokines [5]. $CD4^+$ Th2 cells, a subpopulation of helper T cells, predominantly secrete IL-4, IL-5 and IL-10, and can, for example, eradicate melanoma metastases [6]. Some recent studies have shown that tumour-infiltrating $CD4^+$ T-cell subpopulations are associated with a good prognosis in HNSCC [7] and non-small-cell lung carcinoma [8]. On the other hand, in early breast cancer, infiltration of $CD4^+$ T lymphocytes reflected lymph node involvement [9]. The purpose of this study was to develop a real-time RT-PCR assay for the detection of CD4 mRNA in order to establish $CD4^+$ infiltration status in resected specimens from patients with HNSCC. Precise $CD4^+$ infiltration status may be a useful index for adjuvant therapies for patients with a poor prognosis.

MATERIALS AND METHODS

Patients and tissue samples

From 1998 to 2005, surgical specimens from patients with histologically confirmed HNSCC were obtained from the Department of Otorhinolaryngology and Cervicofacial Surgery, Clinical Centre Ljubljana, Slovenia. None of these patients had received preoperative radiation, chemotherapy or other medical interventions before the study. Altogether, 37 surgical specimens were included in our study. All the specimens were snap-frozen in liquid nitrogen and stored at -70ºC until used. The patients were 27 men and 10 women, ranging from 36 to 76 years of age (mean ± SD: 56.8 ± 11.4 years). The tumours were located in the larynx (24 patients) or in the hypopharynx (13 patients). Additional samples of all the tumours were fixed in 10% formalin and embedded in paraffin wax. Unstained 4-mm sections were then cut for immunohistochemical analysis. The histological classification of tumours was based on the World Health Organization criteria. All the tumours were staged according to the pTNM pathological classification of the UICC (International Union Against Cancer).

RNA extraction and cDNA synthesis

RNA was extracted from the frozen tissues using guanidium thiocyanate and phenol (TRI Reagent, Sigma) as recommended by the manufacturer. The mean total RNA yield was 2.0 μg (range 1.2-3.1 μg). First strand cDNA was synthesised using MultiScribeTM reverse transcriptase (Applied BioSystems,

Foster City, California, USA), with random hexamers, according to the manufacturer's instructions, in a total volume of 50 µl; samples were incubated for 10 minutes at 25ºC, 30 minutes at 48ºC and, finally, for five minutes at 95ºC.

Immunohistochemistry

Immunohistochemical staining for CD4 was performed on formalin-fixed, paraffin-embedded tissue sections of cancer specimens. After pretreatment with microwave POLAR PATENT PP-780 at 96ºC for 25 minutes in EDTA buffer at pH 8.0, the sections were incubated with 1:10 diluted monoclonal antibody anti CD4 (NCL-CD4-1F6; Novocastra, England). They were then stained as per the protocol of Streptavidin Biotin labelling with diaminobenzidine chromogen using a Tech Mate™ 500/1000 (DAKO, Glostrup, Denmark), and Chem Mate reagents and buffers (DAKO, Glostrup, Denmark), and counterstained with Mayer hematoxylin. All the sections were stained simultaneously, using appropriate specimens as positive and negative controls.

Evaluation of $CD4^+$ T cells

Tumour-infiltrating $CD4^+$ T cells were classified using at x200 magnification (Olympus Optical Co., Ltd, Tokyo, Japan) into two groups according to their location: $CD4^+$ T cells within cancer stroma adjacent to cancer cell nests, and $CD4^+$ T cells within the cancer cell nests themselves. The evaluation of infiltration was scored as a percentage semiquantitatively according to the ratio of $CD4^+$ T cells to epithelial cells in the cancer cell nests and to the overall inflammatory cell infiltrate in the tumor stroma, as follows: a score of - indicated 0%; +: 1-10%; ++: 10-50%; and +++: more than 50% $CD4^+$ cells.

Protein extraction

Frozen tissue was lysed in a buffer consisting of 20 mM Tris HCl, pH 8 (Merck), 150 mM NaCl (Merck), 1% (v/v) Triton X-100 (Sigma), 1 mM EDTA, pH 8 (Calbiochem), and 0.1 mM PMSF (Fluka). Tissue homogenization was performed by Polytron PT3000 (Kinematica AG), incubated for 60 minutes on ice and stored at -70ºC. The concentration of proteins in the cell lysates was determined immediately prior to use by the Bradford method (BioPhotometer, Eppendorff) using Roti-Nanoquant reagent (Roth). A protein extract from blood lymphocytes was used as a control sample. The method of extraction was the same as for the frozen tissue, but without homogenization.

Western blotting

30-100 µg of proteins of the cell lysate were electrophoresed at 200 V (at RT) for 50 min through an SDS-polyacrilamide gel (NuPAGE 10% Bis-Tris Gel, Invitrogen). After electrophoresis was completed and the gel equilibrated, the proteins were electroblotted onto a 0.45-µm nitocellulose membrane (Invitrogen) for 1 h at 35 V (at RT). The membrane was blocked overnight with 10% (w/v) non-fat dry milk (NFDM) in rinse buffer (1x PBS, Calbiochem and 0.05% (v/v) Tween-20, Sigma). The membrane was washed briefly in rinse buffer after

blocking, and incubated with mouse anti-CD4 (Novocastra) for 2 h at RT, diluted 1:200 in 10% NFDM in rinse buffer. Three washes, each for 5 min, were performed in rinse buffer, and the membrane was incubated with horseradish peroxidase-conjugated anti-mouse antibodies (Santa Cruz Biotehnology) for 2 h at RT, and diluted 1:2000 in 10% NFDM in rinse buffer. After three more washes, ECL detection was performed with Pierce ECL Western blotting Substrate and Hyperfilm ECL (Amersham).

Real-time PCR. Gene expression levels

Expression of the CD4 gene in tumour samples relative to their normal adjacent tissues was investigated using real-time quantitative RT-PCR based on the TaqMan® fluorescence methodology. A ready-made mixture of probes and primers specific for *CD4* gene expression was used (Assay-on-Demand™, Hs00181217_m1, Applied Biosystems, Foster City, USA). Human 18S rRNA (TaqMan predeveloped assay reagents for gene expression, Applied BioSystems) was used as the endogenous control gene. Reactions were performed with a TaqMan Universal PCR Master Mix (Applied BioSystems) in a 25 µl reaction volume. All the reactions were performed in triplicate and included a negative control. Quantification was done using the ABI Prism 7900 sequence detection system (Applied Biosystems). Cycling conditions were: two minutes at 50ºC, 10 minutes at 95ºC, and 40 cycles of 15 seconds at 95ºC, and one minute at 60ºC. The relative quantification of the mRNA levels of the target genes (quantity of transcripts of the target in the tumour samples relative to normal tissues) was determined using the $\Delta\Delta C_T$ method.

Briefly, the Ct of the target gene (CD4) was normalised to the Ct of the endogenous reference gene (18S rRNA) ($\Delta Ct = Ct\ (CD4) - Ct\ (18S)$ for the tumour and normal adjacent tissue. The difference in the CD4 expression level between the tumour and non-cancerous adjacent tissue was calculated as $R = 2^{-[\Delta Ct(tumour)\ -\ \Delta Ct\ (normal)]}$. The final results are expressed as the N-fold difference in tumour expression relative to the non-cancerous adjacent tissue.

Statistical analysis

Correlations between the CD4 mRNA expression level, $CD4^+$ T-cell infiltration score and patients' clinicopathological variables were analysed by calculating the Spearman's rank correlation coefficient (r_s) and the respective 2-tailed P values using SPSS software (SPSS Inc., Chicago, IL). $CD4^+$ expression and age were treated as continuous variables; the other variables were nominal (gender, tumour location) or ordinal (tumor staging, IHC evaluation of the $CD4^+$ T-cell infiltration). We separated the pTNM stages into pT and pN individual variables and ranked all the ordinal variables according to the pTNM stages and the IHC evaluation of the $CD4^+$ T-cell infiltration, with the higher result ranked as superior.

RESULTS

Immunohistochemistry and classification

The specimens of tumour tissue from HNSCC patients showed heterogenous patterns of infiltration by $CD4^+$ cells (Fig. 1). Immunohistochemical staining for CD4 revealed infiltration in the epithelial component of the cancerous tissue, within the cancer stroma or in both types (Tab. 1).

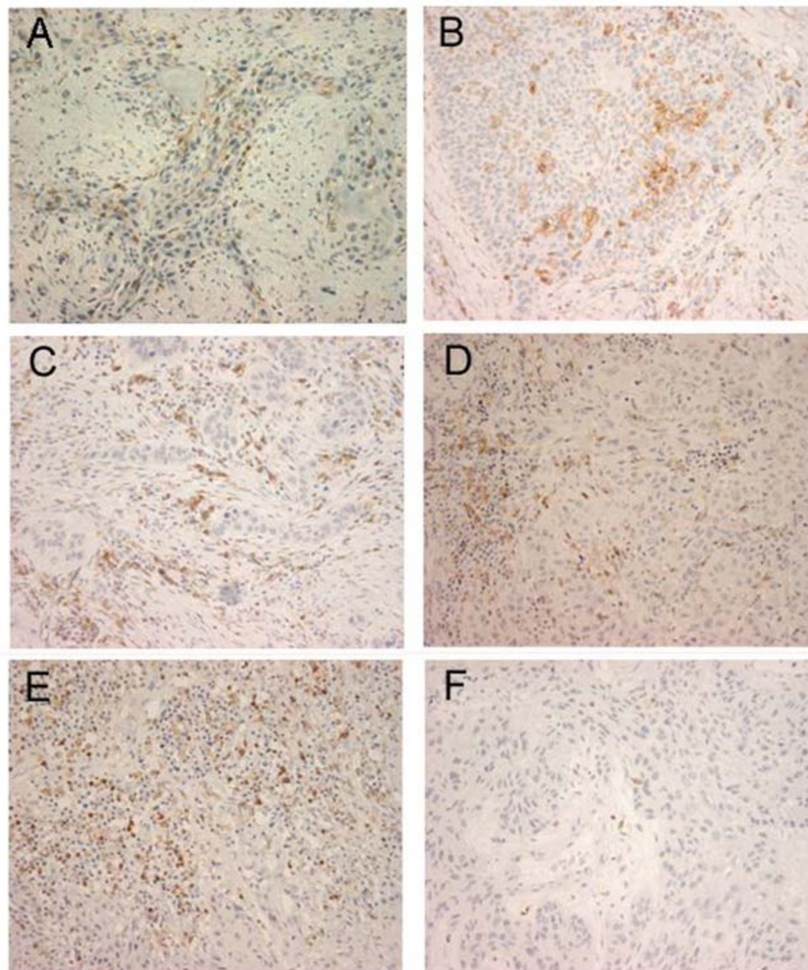

Fig. 1. Specimens of tumour tissue in HNSCC (head and neck squamous cell carcinoma) patients show heterogenous patterns of infiltration by $CD4^+$ T cells. In the first two cases (A and B), the infiltration of immunohistochemically labelled $CD4^+$ cells is seen predominantly in the epithelial component of the cancerous tissue. In the next two cases (C and D), the cells are more abundant in the stroma. In case E, the immunocompetent cells diffusely infiltrated both tumour components. The last case (F) is an example of HNSCC in which only a few $CD4^+$ cells were found to have infiltrated the tumour tissue. Orig. magnif. x 200 in all cases.

Tab. 1. Clinical and pathological characteristics of patients with HNSCC in relation to $CD4^+$ infiltration and *CD4* expression. Lrx – larynx. Hpx – hypopharynx. pTNM – pathological classification of the UICC (International Union Against Cancer). IHC/$CD4^+$ – immunohistochemistry $CD4^+$ infiltration scoring in S (cancer stroma) and E (cancer epithelium nests). CD4 exp. – expression in tumour samples calculated relative to normal adjacent sample.

Case	Age	Gender	Loc.	Tum.-pTNM	IHC/$CD4^+$		CD4 exp.
					S	E	
1	42	M	Lrx	T2N0M0	++	+	9
2	67	F	Lrx	T1N0M0	++	+	8
3	56	M	Lrx	T2N1M0	+	-	3
4	48	M	Lrx	T2N0M0	+++	++	26
5	59	M	Hpx	T3N2aM0	++	++	8
6	72	F	Lrx	T2N0M0	-	-	1
7	38	M	Lrx	T2N1M0	+++	++	32
8	47	M	Lrx	T3N2bM0	-	-	1
9	52	M	Lrx	T1N0M0	++	+++	42
10	71	M	Lrx	T2N0M0	+	++	9
11	39	M	Lrx	T3N2aM0	+	+	5
12	46	F	Lrx	T2N1M0	++	++	11
13	49	M	Lrx	T2N0M0	++	+++	36
14	52	M	Hpx	T3N2bM0	++	+++	14
15	58	F	Hpx	T2N1M0	++	+	6
16	62	M	Lrx	T1N0M0	++	+++	12
17	64	M	Lrx	T2N0M0	++	-	5
18	36	M	Lrx	T3N2aM0	+	-	2
19	68	M	Lrx	T2N0M0	++	++	31
20	72	F	Hpx	T4N2bM0	++	-	9
21	59	M	Hpx	T3N2aM0	++	-	8
22	76	M	Hpx	T2N0M0	++	++	12
23	71	F	Lrx	T1N0M0	++	+++	28
24	68	M	Lrx	T2N0M0	+	++	8
25	63	M	Lrx	T1N0M0	++	++	11
26	57	M	Lrx	T2N1M0	-	+	3
27	48	M	Lrx	T2N1M0	+	-	2
28	43	M	Lrx	T2N0M0	++	++	6
29	69	M	Hpx	T1N0M0	-	-	1
30	68	M	Hpx	T3N1M0	++	++	12
31	38	F	Hpx	T2N1M0	++	++	8
32	39	M	Hpx	T4N2bM0	-	-	1
33	56	F	Hpx	T2N1M0	++	+++	28
34	65	F	Hpx	T3N2bM0	+	-	2
35	54	M	Lrx	T1N0M0	-	-	1
36	56	F	Hpx	T3N1bM0	+++	+	23
37	62	M	Lrx	T2N1M0	++	+++	38

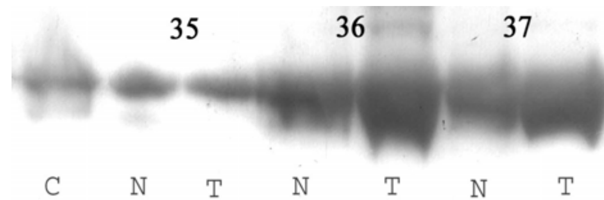

Fig. 2. CD4 Western blot of samples from three patients with HNSCC, using blood lymphocytes as a control (N – normal adjacent tissue T – tumour, C – control protein extract from blood lymphocytes). CD4 (56 kDa) expression was significantly higher in tumour samples 36 and 37, which had high infiltration in the epithelial component of the cancerous tissue and/or within the cancer stroma.

Gene expression levels

As shown in Fig. 3, CD4 mRNA expression level was strongly correlated with the $CD4^+$ infiltration score in the cancer epithelium ($r_s = 0.858$, $P < 0.001$) and in the cancer stroma ($r_s = 0.797$, $P < 0.001$). The CD4 mRNA expression level was also weakly inversely correlated with pT ($r_s = -0.190$, $P = 0.260$) and pN ($r_s = -0.211$, $P = 0.210$) but was not significantly related to the tumor location or patients' age or gender. The $CD4^+$ infiltration score in the cancer epithelium was inversely correlated with pT ($r_s = -0.296$, $P = 0.075$) and pN ($r_s = -0.272$, $P = 0.104$) but not with other clinicopathological variables. Whereas the $CD4^+$ infiltration score in the cancer stroma significantly correlated with the infiltration score in cancer epithelium ($r_s = 0.596$, $P < 0.001$), it did not show a significant association with other variables. The age of the patients was inversely correlated with pN ($r_s = -0.328$, $P = 0.048$), and the tumour location correlated with the pT ($r_s = 0{,}485$, $P = 0.002$) and pN stages ($r_s = 0{,}523$, $P = 0.001$). We also detected a strong correlation between pT and pN ($r_s = -0.831$, $P < 0.001$).

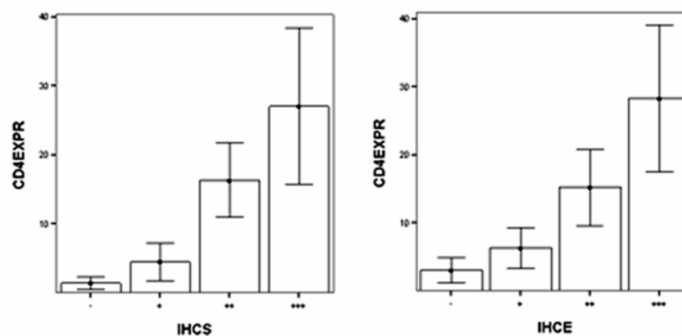

Fig. 3. The correlation between immunohistochemistry $CD4^+$ infiltration scoring in cancer stroma (IHCS) and in cancer epithelium nests (IHCE) and CD4 mRNA expression. Bars represent the mean values and error barrs 95% CI of the mean value for each score.

DISCUSSION

$CD4^+$ T cells play a central role in the anti-tumour immune response. Essentially, $CD4^+$ T cells recognise peptides presented on MHC class II molecules expressed primarily on antigen-presenting cells [4]. Although most tumour cells do not express MHC class II molecules, $CD4^+$ T cells can effect the anti-tumour response in the absence of $CD8^+$ T cells by secreting cytokines, such as interleukin-2, or by activating and recruiting effector cells such as macrophages and eosinophils [10]. However, the main role of $CD4^+$ T cells in the immune response to cancer is to prime $CD8^+$ cells and maintain their proliferation. The depletion of $CD4^+$ T cells by anti-CD4 antibodies or the analysis of CD4-knockout animals demonstrated that $CD4^+$ T cells are essential for the induction of antigen-specific $CD8^+$ T cells [11]. $CD4^+$ status may be a valuable prognostic marker and index of post-surgical host immune reactivity. However, patient prognosis may be difficult to predict before surgical resection, because biopsy specimens obtained by endoscopy may not be useful for determining CD4/CD8 status [12]. In this study, we evaluated the infiltration of $CD4^+$ T cells by immunohistochemistry and developed a sensitive RT-PCR assay for the detection of CD4 mRNA which could be used in order to precisely reassess the $CD4^+$ infiltration status. $CD4^+$ infiltrates were evaluated semiquantitatively according to the percentage of $CD4^+$ T cells localized within cancer stroma and cancer cell nests. The CD4 mRNA expression level was strongly correlated with the $CD4^+$ infiltration score in the cancer epithelium ($r_s = 0.858$, $P < 0.001$) and in the cancer stroma ($r_s = 0.797$, $P < 0.001$). Although the $CD4^+$ infiltration score in the cancer stroma also correlated with the infiltration score in the cancer epithelium ($r_s = 0.596$, $P < 0.001$), it did not show significant association with any other variables. Tumor-infiltrating activated $CD4^+CD69^+$ T cells were already associated with a good prognosis in head and neck squamous cell carcinoma [7], while the presence of high levels of both $CD8^+$ T cells and $CD4^+$ T cells was a significant indicator of a better prognosis for patients with non-small-cell lung carcinoma [8]. Therefore, instead of using the semiquantitative immunohistochemistry methodology, which can have standardization issues related to different antibodies and staining and scoring protocols, real-time RT-PCR assay is suggested as a much more convenient, reliable and sensitive method for the detection of CD4 mRNA.

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