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Original Article

Transformations of peripheral T cell subset distributions in oral cancer patients and the effect of P53 gene therapy in a short time

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Abstract: Objective: This study investigated whether T cells immune suppression exist in Oral Squamous Cell Carcinoma (OSCC) patients. We also investigated peripheral cellular immune responses to recombinant adenovirus vectors expressing wild type p53 (rAd-p53) in oral cancer patients. Materials and methods: We analyzed distributions of peripheral blood T cell subsets in OSCC patients group (OG, n=80) and healthy people group (HG, n=79) by flow cytometry. A cohort of 42 oral cancer patients was divided into the experiment group (EG, n=18) and the control group (CG, n=24), depending on whether patients received rAd-p53 gene therapy. Peripheral blood were collected before and after operation, then flow cytometric counts were performed to detect the transformations of the frequencies of peripheral blood T lymphocyte subsets. Results: OSCC patients show significantly lower percentages of CD3+CD4+, CD4+CD45RA+, CD8+CD28+, and CD3+HLA-DR- cells, and significantly higher percentages of CD4+CD45RO+, CD4+CD25+FoxP3+, CD8+CD28-, and CD3+HLA-DR+ cells. And advanced stage OSCC patient show a significantly higher proportion of CD8+CD28+ cells than early stage patients. After treatment, the oral cancer patients received surgery alone had a decreasing tendency in CD3+CD8+ cells. But the rAd-p53-treated patients had no significant fluctuations in the proportion of any T cell subsets. Conclusion: OSCC patients show a suppressive T cells immune state in general. Surgery alone may weaken anti-tumor immunity within a short time period, whereas regional injection of rAd-p53 as adjuvant therapy for cancer surgery could enhance anti-tumor immunity.

Keywords: Oral cancer, oral squamous cell carcinoma, T cells, cellular immunity, recombinant adenoviral human p53, flow cytometry

Introduction

Studies have presented that directly injection of a replication-defective type-V adenovirus–mediated transfer and expression wild-type p53 promotes the regression of large, established tumors and prevents the growth of new tumors in animal models and patients. A phase II clinical trial in China showed that rAd-p53 gene therapy combined with radiotherapy significantly improved 5-year overall survival and progression-free survival in the treatment of patients with advanced head and neck cancer, comparing with radiotherapy alone [1]. Our group has also evaluated the safety and effectiveness of direct injection into the tumor bed of rAd-p53 after surgery in oral cancer patients [2]. Various T cell subsets play a dominant role in the cell-mediated, anti-tumor immune response, and the frequency of peripheral blood lymphocyte subsets reflect the nature of antitumor capacity. However, the immune response to rAd-p53 in humans is not well known, and an anti-tumor immune response is essential for cancer patients. Therefore, we want to explore the peripheral T cellular immune status in oral squamous cell carcinoma (OSCC) patients and responses after regional administration of rAd-p53 in oral cancer patients.

Flow cytometric analysis of peripheral blood lymphocyte phenotypes has been used in a wide range of medical conditions, especially widely used in malignancy [3]. In our study, we

Table 1. Percentage of T cells and T cell subsets between healthy people group and OSCC patients group

Lymphocyte marker	Cell type	HG (n=79)	OG (n=80)	*P* value
CD3+	T Cells	68.0±10.0	66.3±9.9	0.272
CD3+CD4+	Helper T (Th) cells	39.0±7.1	35.4±9.6	0.009**
CD3+CD8+	Cytotoxic T (Tc) cells	25.1±7.4	26.3±10.3	0.396
CD4+CD45RA+	Naive Th cells	16.6±7.8	10.4±6.4	P<0.001***
CD4+CD45RO+	Memory Th cells	25.1±6.3	27.4±7.5	0.036*
CD4+CD25+Foxp3+	Treg Cells	7.6±2.8	9.1±3.8	0.007**
CD8+CD28+	Cytotoxic Tc cells	15.3±5.0	11.7±5.4	P<0.001***
CD8+CD28-	Suppressor Tc cells	15.1±6.9	18.3±8.6	0.010*
CD3+HLA-DR+	Activated T cells	1.7±1.3	2.8±2.3	P<0.001***
CD3+HLA-DR-	Resting T cells	66.5±10.2	62.8±11.6	0.036*

*P<0.05, **P<0.01, ***P<0.001, unpaired Student's t-test.

determined the percentage of peripheral blood T cells and T cell subsets–based on cluster of differentiation (CD) phenotypes by flow cytometry, then we analyzed the rAd-p53 treatment-related transformations in OSCC patients to determine whether rAd-p53 treatment affect T cellular immune.

Materials and methods

This study was approved by the ethics committee of Chinese P.L.A General Hospital, but was not registered in any international clinical trial registry platform. All enrolled patients signed a consent form.

Patients and healthy control

The inclusion criteria were as follows. From Feb 2012 to Oct 2015, Patients who were hospitalized in oral and maxillofacial ward, histologically diagnosed with primary oral squamous cell carcinoma (OSCC) after surgical resection and had not never been hospitalized for other therapies such as radiotherapy and chemotherapy before were adopt in this study. Patients with smoking, alcohol abuse, hematological, hepatic and renal disease, heart failure, chronic infection, autoimmune disease, splenectomy, and other cancers and drugs administration which could affect immune were excluded in the study. Healthy volunteers who were finally proved healthy were from physical examination center of PLA general hospital. The criteria for the health control group were: no smoking or alcohol abuse, no history of cancer; no immune system disease; no immunoactive or immunosuppressive drugs administration history; no evident infection during the last three months; and no other severe systemic disease.

Treatment protocol

Patients in CG underwent routine operation alone. Patients in EG underwent routine operation plus p53 gene therapy. The rAd-p53 (Gendicine, 1×10^{12} viral particles/ampoule) was from Shenzhen SiBiono Gene Tech. Patients in EG received a direct injection of rAd-p53 at a dose of 5×10^{12} viral particles into tumor bed after tumor resection during operation. And 1×10^{12} viral particles were injected at the third day, fifth day, and seventh day after operation, respectively. When after operation injection, the rAd-p53 was diluted with 4 ml saline and 1ml 2% lidocaine. Peripheral blood samples were drawn 1 day before surgery and 10 days after operation for flow cytometry. In this study, we analyzed peripheral T cell immune responses by detecting transformations of T cell subset distributions after regional administration of rAd-p53 in the short follow-up period.

Clinicopathologic feature

After histological examination of primary lesion and neck dissection tissues, we record relevant data, such as origin, tumor size, differentiation degree and regional lymph nodes metastasis. The TNM classification of the tumors was confirmed according to pathology reports, medical records and radiological findings.

Flow cytometry

The conjugated monoclonal antibodies (Beckman Coulter, Switzerland) used in this study include CD3-PC7, CD4-PC5, CD8-FITC, HLA-DR-PE, CD28-PE, CD45RA-FITC, CD45RO-PE, CD25-FITC, FoxP3-PC7. CD markers for T cell subsets shown in **Table 1**. Peripheral venous blood was collected into a 5-ml vacutainer tube containing liquid EDTA-Na_2 as an anticoagulant and processed within 4 h of collection. Anticoagulated venous blood was separated

Table 2. Basic information of case population

	HG (n=79)	OG (n=80)	*P* value	CG (n=24)	EG (n=18)	*P* value
Year (mean)	55	58	0.196	61	58	0.355
Sex (n)						
Male	53	51	0.739	15	13	0.742
Female	26	29		9	5	
Tumor differentiation (n)						
Well		37		10	5	0.642
Moderately		25		9	8	
Poorly		18		5	5	
TNM stage (n)						
Stage I		20		5	3	0.960
Stage II		18		4	4	
Stage III		18		6	4	
Stage IV		24		9	7	
Primary location (n)						0.599
Tongue		34		6	8	
Buccal mucosa		11		4	3	
Mouth Floor		14		5	4	
Gingiva		12		6	1	
Palate		3		2	1	
Bone		3		1	1	
Lip		3				

Unpaired student's t-test or person chi-square test.

into 100 μl aliquots and incubated with the appropriate fluorochrome-conjugated monoclonal antibodies at the manufacturer's recommended concentration for 20 min at room temperature in the dark. Blood samples were subjected to red blood cell lysis using a Q-Prep Workstation (Beckman Coulter) and an ImmunoPrep Reagent System (Beckman Coulter). Prepared samples were detected immediately using a Cytometer FC 500 MPL (Beckman Coulter) flow cytometer. Lymphocyte subsets were analyzed by quadrant statistics, data analysis was done with CXP software. FlowCheck™ fluorospheres (Beckman Coulter) were used for calibrating the instrument before analysis to ensure fluorescence coefficient of variation (CV) values of less than 2%. For each analysis, 10,000 events were counted.

Statistical analysis

Statistical analyses were conducted using the statistical software SPSS 19.0 (IBM, Corp). Unpaired t-test analysis or Chi-square test was performed for comparisons between two independent groups such as EG and CG and one-way ANOVA test for multi-groups. Paired-sample t-test analysis or Mann-Whitney U test was performed for comparisons between two paired groups, like the pre-treatment and post-treatment data in EG or CG. Data with Gaussian distribution was show with mean ± standard deviation, while data with non-Gaussian distribution was show with median. The level of significance was set at $P<0.05$.

Results

Case population

Finally, we accepted 80 persons in OSCC group (OG) and 79 persons for the healthy group (HG). From OSCC group, 18 persons who want a rAd-p53 gene therapy were divided into experimental group (EG) and 24 persons agreed to be divided to control group (CG). Basic information of each groups are summarized in **Table 2**. There were no statistical differences in the age and gender distribution, clinical stage and tumor differentiation by statistics analysis.

Distributions of peripheral blood T cell subsets in OSCC patients and healthy people

There was no significant difference in the percentage of total T cells between OSCC patients and healthy people. In the T cell subsets, OSCC patients had a significantly lower percentage of Th cells than the healthy people. There was no significant difference in the proportion of Tc cells between the OSCC patients and healthy people. In the subsets of Th cells, OSCC patients had a significantly lower proportion of naive Th cells and significantly higher proportion of memory Th cells. The proportion of Treg cells in the OSCC patients was significantly higher than that in healthy people. In the subsets of Tc cells, OSCC patients showed a significantly lower proportion of cytotoxic Tc cells and a significantly higher proportion of suppressor

Table 3. Percentage of T cells and T cell subsets with different tumor differentiation degree

Cell type	Well differentiation (n=37)	Middle differentiation (n=25)	Poorly differentiation (n=18)	*P* value
T cells	65.0±9.3	67.5±11.5	67.3±8.6	0.571
Helper T cells	35.3±9.8	35.8±10.5	35.2±8.4	0.973
Cytotoxic T cells	24.1±9.4	27.9±11.8	28.6±9.2	0.217
Naive Th cells	11.2±6.8	10.1±5.7	9.3±6.7	0.585
Memory Th cells	26.8±7.4	27.7±8.9	28.3±6.0	0.751
Treg cells	9.0±3.9	8.4±2.2	10.0±5.2	0.356
Cytotoxic Tc cells	11.0±6.1	12.1±4.2	12.7±5.4	0.504
Suppressor Tc cells	17.7±7.8	17.7±10.3	20.4±7.8	0.520
Activated T cells	2.8±2.7	2.2±1.5	3.6±2.2	0.150
Resting T cells	61.3±11.7	64.4±12.4	63.7±10.2	0.547

One-way ANOVA test.

Table 4. Percentage of T cells and T cell subsets with different TNM stage

Cell type	Early stage (n=39)	Advanced stage (n=41)	*P* value
T cells	64.5±10.4	68.0±9.1	0.111
Helper T cells	36.1±10.9	34.8±8.3	0.556
Cytotoxic T cells	23.6±8.3	28.9±11.3	0.019*
Naive Th cells	10.2±6.6	10.7±6.3	0.727
Memory Th cells	27.6±8.9	27.2±6.1	0.819
Treg cells	9.5±4.6	8.6±2.9	0.286
Cytotoxic Tc cells	11.4±5.8	12.0±5.1	0.620
Suppressor Tc cells	17.0±7.1	19.6±9.8	0.185
Activated T cells	2.9±2.2	2.7±2.5	0.718
Resting T cells	60.4±12.2	65.1±10.5	0.070

*$P<0.05$, unpaired Student's t-test.

Tc cells than those healthy people. We found that the proportion of activated T cells was significantly higher and the proportion of resting T cells was significantly lower in OSCC patients than in those in healthy people (**Table 1**).

Analysis of T cell subset distributions with clinicopathologic features

In accordance with the OSCC differentiation degree, we divided OSCC patients into well differentiation, middle differentiation and poor differentiation groups. According to the TNM stage method, we divided the TNM stage of OSCC patients with stage I, stage II, stage III and stage IV groups. Then we divide stage I and stage II patients to early stage group, stage III and stage IV patients into advanced stage group. We analyzed the abnormal T cell subsets distributions in the different groups. There were no significant differences in other T cell subsets among the different tumor differentiation groups (**Table 3**). Advanced stage OSCC patients show a significantly higher proportion of cytotoxic T cells than early stage OSCC patients. There were no significant differences in other T cell subsets among different TNM stage groups (**Table 4**).

The rAd-p53 injection associated changes of T cell subset distributions

To evaluate the potential impact on T cell subsets by rAd-p53, we first compared the percentage of T subsets between EG and CG. There was no significant difference in the percentage of each lymphocyte subset of pre-operation check between EG and CG. Then we compared after-operation T cell subset distributions with pre-operation T cell subset distributions to investigate whether rAd-p53 affect T cell subset distributions. We found that, after treatment, CG only showed a significantly lower proportion of Cytotoxic Tc cells. And in EG, there was no significant change of each T cell subset (**Table 5**).

Discussion

Cancer is an uncontrolled growth of self-cells. Immune cells must distinguish cancer cells from normal cells to launch an anti-tumor immune response. Immune modulation in cancer patients refers to a range of treatments that are aimed at harnessing the immune system to achieve tumor control and potential eradication of disease [4]. Immunotherapy has long been expected to become a powerful anticancer treatment that can be tumor-specific and less toxic [5]. To design an effective immune therapy for OSCC patients, knowledge of the immune status in cancer development is of great importance. In addition, earlier work has shown cellular changes may affect the prognosis of cancer patients. For example, a reduced percentage of Tc cells in the peripheral blood of cancer patients appeared to be asso-

Table 5. Proportions of T cells and T cell subsets in control group and experimental group

Cell type	CG	EG	*P* value	CG			EG		
				Pre-operation	After operation	*P* value	Pre-operation	After operation	*P* value
T Cells	72.2	64.7	0.303	72.2	69.4	0.330	64.7	66.3	0.231
Helper T cells	39.9	36.9	0.629	39.9	39.5	0.391	36.9	37.3	0.896
Cytotoxic T cells	24.3	23.0	0.638	24.3	23.3	0.157	23.0	22.7	0.913
Naive Th cells	8.2	10.4	0.334	8.2	9.4	0.587	10.4	9.9	0.556
Memory Th cells	28.0	29.7	0.939	28.0	27.9	0.764	29.7	29.0	0.170
Treg Cells	8.4	7.6	0.509	8.4	7.5	0.808	7.6	9.2	0.231
Cytotoxic Tc cells	12.4	11.5	0.291	12.4	10.6	0.024*	11.5	11.2	0.384
Suppressor Tc cells	16.7	12.8	0.106	16.7	15.9	0.493	12.8	16.1	0.094
Activated T cells	2.1	2.2	0.321	2.1	2.1	0.867	2.2	3.3	0.266
Resting T cells	69.0	63.2	0.493	69.0	68.2	0.346	63.2	62.1	0.528

*$P<0.05$, unpaired Student's t-test or Mann-Whitney U test.

ciated with a poorer prognosis [6]. Therefore, detecting autologous tumor-specific T-cell responses and the tumor-specific T-cell response induced by anti-tumor therapy or immunotherapy is necessary in oral cancer patients.

T cells are major effectors cells of cellular immune responses. Previous studies have shown that HNSCC patients had a significantly lower number of circulating CD3+ T cells than did those in the healthy control [7]. Uematsu et al found that oral cancer patients have a lower proportion of peripheral blood T cells than healthy subjects [8]. We found no significant difference in the proportion of CD3+ T cells between OSCC patients and the healthy control group. However, this does not mean that OSCC patients have a normal T cell immune function, for different T cell subsets showed different functions.

T cells are categorized into two major subsets-Th cells and Tc cells. Th cells play a pivotal role in generating and maintaining anti-tumor immune responses [9, 10]. In particular, cytokines produced by Th cells, such as IL-12 and IFN-γ, have a stimulating effect on the cellular immune response [11, 12]. Th cells also can secrete critical inflammatory cytokines, including IFN-γ and TNF-α, for proper resolution of infectious and neoplastic diseases indirectly [13]. Miyazaki established Th cell lines that could show selective cytotoxic activity against autologous tongue cancer cells in vitro [14]. This may provide important evidence of Th cells in the antitumor function to OSCC. Th cells decreased significantly in oral cancer patients when compared to normal controls, and the cytokine response in patients seems to be skewed from protective Th1 to immunosuppressive Th2 type [15, 16]. We also found in our study, a significantly lower percent of Th cells in the OSCC patients than in the healthy controls. A lack of their "help" may succeed in weakening other immune effectors cells to exert normal functions.

Tc cells could mediate target cell apoptosis by secreting lytic granules. This important role in immune-mediated tumor elimination has been proven in experimental murine tumor models [17]. During induction oral cancer in rats, the frequency of Tc cells increased significantly in the treated group compared to the control mice [18]. We also found a higher proportion of Tc cells in advanced OSCC patients. Yasumura et al proved in vitro that Tc cells are capable of killing autologous tongue squamous cell carcinoma cells [19]. This suggests that Tc cells play an important role in monitoring and fighting with OSCC. Laad et al found that Tc peripheral blood cells decreased in oral cancer patients compared to healthy individuals [20]. However, we found no significant difference in Tc cell distributions between OSCC patients and healthy control subjects. We continue to study Tc cell subsets to assess Tc cells function. CD28 is a major costimulatory molecule required for functional T-cell activation and provides requisite secondary signaling for the initiation of an immune response [21]. CD8+CD28+ T cells and CD8+CD28- T cells are named cytotoxic Tc cells and suppressor Tc cells, respectively.

Cytotoxic Tc cells recognize and kill tumor cells expressing peptides that are presented by MHC class I molecules, while suppressor Tc cells suppress B and T cell function due to the lack of a costimulatory signal [22]. In our study, we found a significantly lower percentage of cytotoxic Tc cells and a significantly higher percent of suppressor Tc cells in OSCC patients. It suggested that more suppressor Tc cells may have a suppressive function in OSCC patients.

Treg cells are commonly thought to negatively regulate cells of the human immune system. They can alter dendritic cell maturation and antigen-presenting ability, as well as directly inhibit T cell proliferation and function [23]. Gasparoto et al found that purified CD4+CD25+ T cells in OSCC patients exhibit stronger suppressive activity than those in healthy individuals [24]. Gaur P found that OSCC patients had a significantly higher proportion of peripheral blood Treg cells [25]. In our study, OSCC patients also had a significantly higher proportion of Treg cells. Their suppressive function to effector T cells may lead to immune anergy and facilitate OSCC cells to escape from immunologic surveillance. Al-Qahtani et al found that well-differentiated OSCC had significantly fewer FoxP3+ Tregs than moderate and poor differentiation in paraffin-embedded tissue sections [26]. We didn't find this tendency of Treg cells in peripheral blood.

To perform functions, T cells must be activated. Immunogenic activation of Th and Tc cells leads to rapid clonal expansion and differentiation of effector T cells. HLA-DR is often used as a late activation marker for T cells. More aggressive and deeply infiltrating laryngeal squamous cell carcinoma was most often characterized by significantly higher values of HLA-DR+ Th cells [27]. We found a significantly higher percentage of activated T cells and a significantly lower percentage of resting T cells in OSCC patients than in healthy people. We think that OSCC maybe immunogenic, although many antigens that are expressed by tumor cells are, in fact, self-antigens that are slightly altered or aberrantly expressed. However, due to the dysfunction of effectors T cells, the immune system cannot make efficient cascade reactions to kill OSCC cells.

The different expression of CD45 isoforms is cell type specific and depends on the stage of cell differentiation and activation status. In humans, CD45RA+ and CD45RO+ T cells are thought to be naive and memory T cells, respectively [28]. Memory T cells are critical for surveying the entire organism for signs of cancer recurrence and protection from disease development [29]. Previous study indicated that HNSCC patients had a significantly lower percentage of naive T cells and a higher percentage of memory T cells than the healthy control group. In our study, we found a significantly lower percent of naive Th cells and a significantly higher proportion of memory Th cells in OSCC patients. This may add evidence to the immunogenic feature of OSCC.

Surgery with or without chemotherapy or/and radiotherapy has been the primary method of cancer therapy. The myelosuppressive side effects of chemotherapy and radiotherapy are well known, while previous studies have indicated that the immunity of cancer patients after operation could recover to varying degrees via various mechanisms [30]. One the one hand, the tumor burden is removed, and the immunosuppressive agent was suppressed or eliminated. Alternatively, operation may induce a traumatic stress response and thus enhance antitumor immunity. However, some studies have revealed that the immunosuppression of patients with cancer lasts for a long time after therapy due to residual small lesions, operation trauma and the side effects of chemoradiotherapy. Bottcher et al have demonstrated that the distribution of peripheral blood T cells correlated with the systemic invasiveness of therapy that patients underwent and was affected most clearly by surgery with adjuvant radiochemotherapy [31]. In this study, we found CG showed a significantly lower proportion of cytotoxic Tc cells in the post-surgery check, but rAd-p53 group didn't show this tendency. We think surgery may weaken the function of cytotoxic Tc cells in a short time, but rAd-p53 may reverse this change.

Many studies have reported that the regional administration of adenovirus vectors expressing wt-p53 in orthotopic tumor models and patients with various types of tumors prevented tumor growth and mediated regression of large, established tumors. Our previous study also demonstrated that rAd-p53 combined with surgery controlled minimal residual disease,

reduced the recurrence of cancer and prolonged the survival of oral cancer patients. The effects of rAd-p53 are thought to be associated with the regulation of the cell cycle and the induction of apoptosis of exogenous wt-p53. Nevertheless, some studies have suggested that the close relationship between p53 and immunity is also pivotal for the efficient suppression and eradication of tumor cells [32]. For example, p53 stimulates the immune response as a tumor associated antigen. Alternatively, p53, as a transcription factor, directly activates the expression of some genes in immune cells and thus plays a regulatory role in the proliferation and function of these cells. A lack of wt-p53 function influences the innate immune response by interfering with the expression of inflammatory mediators [33]. Exogenous wt p53 transduced into tumor cells actives T cells as a tumor specific antigen [34]. Wt-p53 is commonly over-expressed in tumor cells that transfected by rAd-p53, causing the concentration of p53 in the cytoplasm to increase. A small number of necrotic tumor cells then release p53, which is then taken up by antigen presenting cells, processed and presented to T cells. Then p53 specific T cells are activated and induce the anti-tumor immune response [32]. Several studies have reported that wild-type and mutant p53 protein-specific peptides can induce in vivo MHC-II restricted T cell activation in normal or tumor bearing mice [35, 36]. We think this may enhance functions of Th cells, succeed to “help” increasing immune effector cells such as cytotoxic Tc cells. This may explain the diversity of cytotoxic Tc cells in EG and CG after operation in a short time.

Compared to healthy people, the distributions of T cell subsets in OSCC patients tend to show an immunosuppressive T cell immune status. Although OSCC trigger reactions in T cellular immune, the “fighting” ability is not sufficient and the negative regulating T cells are stronger. Surgery would exacerbate T cell immune suppression in the short term, whereas regional injection of rAd-p53 as adjuvant therapy during cancer surgery may reverse this change. The OSCC patients may draw more advantage from the regional injection of rAd-p53 as adjuvant therapy after cancer surgery than surgery alone.

Disclosure of conflicts of interest

None.

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