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Quantification of tumor infiltrating Foxp3+ regulatory T cells enables the identification of high-risk patients for developing synchronous cancers over upper aerodigestive tract

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SUMMARY

Objectives: Patients with squamous cell carcinomas (SCC) of upper aerodigestive tract, either over head and neck (HNSCC) or esophagus (ESCC), frequently developed synchronous multiple cancers, leading to worse prognosis. This study validated whether suppression of host cancer immunosurveillance mediated by regulatory T cells (Treg) may predispose to the development of synchronous cancers.
Methods: Tumor tissues of 200 patients (100 ESCC only, 50 HNSCC only, and 50 synchronous SCCs) were quantitatively accessed for the tumor infiltrating Treg by immunohistochemistry. The density of Treg was also correlated to the level of Treg-associated inhibitory cytokines (IL-10, IL-35 and TGF-β1), and chemokine (CCL22).
Results: The density of tumor infiltrating Treg in the index tumor (*i.e.* the first malignancy diagnosed) of synchronous SCC group was higher than those of HNSCC or ESCC only ($p < 0.05$). Selecting the optimal cut-off value of Treg density as 34.6 cells/mm^2 by ROC curve, an increased Treg density of the index tumor can be an independent factor for developing synchronous SCCs (OR: 6.13; 95% CI: 2.84–13.26). The Treg density was positively correlated with serum IL-10 level and the degree of CCL22-positive cells infiltration in tumor. Furthermore, the serum inhibitory cytokine IL-10 level was higher in synchronous SCC than in non-synchronous ones ($p < 0.001$), that indicated the cellular immunosuppression in patients with synchronous cancers.
Conclusions: A more severe defect in cellular immunity may predispose to multifocal tumor. The Treg cell number in SCC may serve as a novel predictive biomarker for the risk of synchronous cancer development to initiate a proper surveillance program.

Abbreviations: SCC, squamous cell carcinoma; HNSCC, head and neck squamous cell carcinoma; ESCC, esophageal squamous cell carcinoma; UADT, upper aerodigestive tract; Treg, regulatory T cell; Foxp3, Forkhead box protein 3.

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Introduction

Squamous cell carcinomas (SCC) of the upper aerodigestive tract (UADT), including head and neck squamous cell carcinomas (HNSCC) and esophageal squamous cell carcinomas (ESCC), are common malignancies worldwide [1,2]. The incidence continues to increase in geographic areas where the uses of alcohol, betel nut and cigarette are highly prevalent. Despite of the multidisciplinary treatments to include surgery, radiotherapy, and chemotherapy, the prognoses remain poor [2,3].

Based on the well-established concept of field cancerization [4], the patients with ESCC or HNSCC may develop synchronous multifocal squamous cancers over the UADT [5–10]. The repetitive exposures to the common carcinogens are proposed to lead into genetic alterations in multi-focal locations of the UADT fields and ultimately develop with synchronous SCC with worse survival [5–7]. Alcohol drinking and the numerous Lugol-voiding lesions in esophageal background mucosa correlate with the synchronous SCC [8,11,12], but the predictive effectiveness of such parameters remains unsatisfactory. For example, the patients without alcohol exposure still develop the synchronous SCC. It is thus in need of a more accurate biomarker within the host to predict the development of synchronous SCC in UADT.

In addition to the traditional carcinogens exposures, the host tumor immune surveillance should be important for the carcinogenesis [13]. Whenever the transformed cells occur, the host immunosurveillance shall be initiated to protect against the tumor development. On the contrary, tumor itself can produce certain factors to inhibit or to escape from the host immunity to maintain tumor development [13]. Emerging evidence suggests regulatory T cells (Treg) are crucial in such immune escape [14,15]. Forkhead box protein 3 (Foxp3) is a key regulator for the development and function of Treg [16,17], and serves as the most specific marker to label the CD4+CD25+ Treg in human cancers [14,18]. Treg can be locally induced or selectively recruited into the tumor microenvironment mediated by IL-10, TGF-β or CCL22 [19–21]. The accumulation of Tregs can subsequently inhibit the host antitumor immune response, via cell–cell contact or secreting immunosuppressive cytokines, such as IL-10, IL-35 or TGF-β [15,19,22,23]. However, their clinical significances in SCC of the UADT are still inconclusive [24–26]. Accordingly, this study validated whether the regulatory T cells involved in the cancer-related immunosuppression can participate in the field cancerization phenomenon in SCC of UADT. In order to elucidate the mechanisms behind Treg accumulation within tumors, we evaluated the relationship between Treg-associated cytokines (IL-10, IL-35 and TGF-β1), chemokine (CCL22) expressions and the Treg density in tumor. Moreover, the study result shall be highly original to illustrate the Treg cell number in SCC predict the risk of the synchronous cancer development. It implicates a clinical outlook to initiate a proper surveillance program for certain high-risky SCC in UADT.

Materials and methods

Patients

This study consecutively recruited 300 patients who underwent meticulous endoscopic screening of the upper aero-digestive tract with image-enhanced endoscopy, including narrow-band imaging (NBI) and Lugol chromoendoscopy [8–10], from January 2008 to January 2011. Of these patients, 100 had ESCC only, 150 had HNSCC only, and 50 had synchronous SCC simultaneously in both esophagus and the head neck regions. The 100 patients with ESCC only and 50 patients with synchronous SCC were all consecutively enrolled. This study also randomly selected 50 patients with HNSCC only by matched tumor location to the HNSCC part of the synchronous group. The institutes' review board and ethics committees of both E-Da Hospital (EMRP-097-110) and National Cheng Kung University Hospital (BR-100-087) have approved the study.

Clinicopathologic features and survival follow-up of SCC patients

Among the enrolled patients with histology-confirmed SCC, demographic characteristics, substance use, and medical histories were collected via an interview with participants using a standardized questionnaire. The tumor was grouped by location: head and neck (oral cavity, oropharynx, hypopharynx) and esophagus. The index primary tumor was defined as the first malignancy diagnosed, and accordingly the patients were referred for endoscopic screening of upper aerodigestive tract. After meticulous screening for UADT, endoscopic biopsy was done for all suspected lesions detected by white-light, NBI, or Lugol chromoendoscopy. The number and multiform pattern of Lugol-voiding lesions in the esophageal background mucosa were also recorded (Supplementary Fig. 1) [11]. If a second primary SCC was detected during screening within 6 months after the diagnosis of index primary tumor, it was defined as synchronous SCC. The clinical staging for head and neck or esophageal cancer was determined following the TNM classification system [27]. Each one was then treated after a multi-disciplinary evaluation following the National Comprehensive Cancer Network (NCCN) guideline. All of the patients received regular follow-up and survival was determined from medical records or by telephone contact when appropriate.

Quantification of tumor infiltrating regulatory T cells and CCL22-positive cells

The tumor tissues of ESCC or HNSCC, and the index (first diagnosed SCC) and the latter or concurrent tumors of the synchronous SCC were all stained for the Foxp3+ lymphocytes by immunohistochemistry (Fig. 1). The tissue was treated with primary antibody against Foxp3 (clone 246A/E7; Abcam, Cambridge, UK) at a dilution of 1:40. The pathologist blinded to patients' clinical information quantified the staining of Foxp3+ Treg by analyzing five different fields (x200). Each observed field represent an area of 0.785 mm^2. The density (cells/mm^2) of intra-tumor infiltrating Treg was defined as the mean Foxp3+ lymphocyte numbers in five fields divided by the observed area.

The immunohistochemical staining for CCL22-postive infiltrating cells was done with primary antibody against CCL22 (ab9847; Abcam, Cambridge, UK) at a dilution of 1:50. The numbers of CCL22-positive infiltrating cells in tumor were then quantified by analyzing five different fields (x200). High CCL22-postive cell infiltration was defined as there are more than 5 cells in the observed area. In addition, the expressions of CCL22 in tumor cells were also evaluated. Cases were scored negative if no staining of the cytoplasm of tumor cells was found, and positive if cytoplasmic staining of tumor cells was presented. The positive cases were subdivided into two groups based on the intensity of staining (1 = weak; 2 = strong).

Serum levels of IL-35, IL-10 and TGF-β by enzyme-linked immunosorbent assay

Pre-treatment blood samples for serum levels of three Treg-associated cytokines (IL-10, IL-35 and TGF-β1) were taken from patients with SCC. Specific ELISA kits were used to measure serum IL-10, TGF-β1 (R&D Systems, Minneapolis, MN, USA), and IL-35 (BioLegend, San Diego, CA) levels, respectively, following