

Prognostic Significance of Intratumoral Natural Killer Cells in Primary Resected Esophageal Squamous Cell Carcinoma

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Background: Natural killer (NK) cells are important effector cells in the defense against tumors. The present study retrospectively examines the intratumoral NK cells in primary resected esophageal squamous cell carcinoma (ESCC) and the correlation between the patient's outcome and the intratumoral NK cells infiltration.

Methods: Immunohistochemistry was used to analyze the intratumoral NK cell infiltration in 38 archival specimens from patients with primary resected ESCC.

Results: According to the cut-off point of the staining for intratumoral NK cell infiltration, 14 (37%) cases had high level infiltration and 24 (63%) low. The 5-year survival of patients with high level NK infiltration was significantly better than that of patients with a low level of NK infiltration ($p < 0.01$). Multivariate analysis did not show NK cell infiltration to be a significant prognostic factor.

Conclusions: Intratumoral NK cell infiltration is associated with a favorable outcome in ESCC. Intratumoral NK cell infiltration might be used as a variable with prognostic value in primary resected ESCC.
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Key words: natural killer cell, esophageal squamous cell carcinoma.

Squamous cell carcinoma of the esophagus accounts for almost all esophageal malignancy in Taiwan. The prognosis for patients with ESCCs has only slightly improved during recent years. Even at the resectable stage, the results of surgical excision have been unsatisfactory, with 5-year survival rates of about 20%.⁽¹⁾ This situation makes the availability of prognostic factors for outcome highly desirable in order to guide the use of (neo) adjuvant treatments.

Natural killer (NK) cells constitute 3-5% of peripheral blood lymphocytes in humans, and their activity often reflects immunocompetence and surgi-

cal resectability in patients with malignancy.⁽²⁻³⁾ These cells have a particular role in immunosurveillance of neoplasms and represent a small proportion of human gastrointestinal tumor infiltrating lymphocytes.⁽⁴⁾ Activation of NK cells is an early event and does not involve the expansion of antigen-specific cells, as is characteristic of the adaptive immune response. NK cells are therefore part of the innate, immediate-acting arm of the immune system. NK cells prefer to attack cells that have deregulated classical MHC class I molecules. The "missing self hypothesis"⁽⁵⁾ proposes that this function of NK cells

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serves to protect the host against transformed cells, which often deregulate expressions of MHC class I proteins to escape recognition by cytotoxic T cells.

Although the number of NK cells coming into contact with neoplastic cells constitutes a small percentage of the infiltrating lymphocytes, they can play an important role in the prognosis of gastrointestinal tumors.^(4,6) Some immunologic markers have been reported to be significant prognostic factors in esophageal squamous cell carcinoma⁽⁷⁻⁸⁾ but the clinical importance of intratumoral NK cell infiltration has been unclear. The purpose of this study was to clarify the correlation between intratumoral NK cell infiltration and the prognosis of patients with primary resected esophageal squamous cell carcinomas.

METHODS

Patients

Tumor samples were obtained from 38 consecutive patients who underwent surgical intervention for ESCC at the center for Thoracic Surgery at Taichung Veterans General Hospital between June 1994 and July 1996. All the patients were male. No pre-operative radiotherapy or chemotherapy had been performed. All patients have been followed up for overall survival.

Immunohistochemistry

Paraffin-embedded tumor specimens that had been fixed in neutral-buffered formalin were sectioned (4 μ m) and placed on poly-L-lysine-coated glass slides. After microwave pretreatment in 10mM citric acid monohydrate (pH 6.0) three times for 5 min at 750 W, the slides were incubated overnight at 4C with monoclonal antibodies against CD57 (dilution 1:50; Dakopatts, Denmark). After a second incubation with biotin-conjugated anti-mouse antibody, slides were incubated with avidin-biotin-peroxidase reagent (LSAB Kit, Dakopatts, Denmark). Reaction products were visualized by immersing the slides in diaminobenzidine tetrachloride and finally counterstained with hematoxylin. A normal prostate gland was used for positive control of the CD57 antibody.

Using light microscopy, the immunohistochemical staining of CD57 was examined by two observers without knowledge of the clinical outcome. Twenty-five areas containing cancerous tissue were selected and the number of NK cells was counted at a magni-

fication of x200 according to Coca et al.⁽⁹⁾ The degree of intratumoral NK cell infiltration was classified into two groups: patients with a low level of NK infiltration (<25 NK cells found in 25 intratumoral fields) and patients with a high level of NK infiltration (>25 NK cells found in 25 intratumoral fields).

Statistical analysis

Survival rates were calculated by the Kaplan-Meier method for analysis of censored data. The significance of difference in survival was analyzed by means of the log-rank test. In multivariate analysis, independent prognostic factors were determined by the Cox proportional hazards model. The level of significance of all tests was set at 0.05.

RESULTS

A total of 38 samples of primary resected ESCCs were processed for intratumoral NK cell infiltration by immunohistochemistry. Table 1 lists the clinicopathological features and NK cell infiltration.

Intratumoral NK cell infiltration in ESCCs

According to the cut-off point of the immunohistochemical staining for NK cell infiltration, 14 (37%) cases had high level infiltration (Fig. 1) and

Table 1. Clinicopathological Features and NK Cell Infiltration in ESCCs

Variable	Total	NK Cell Infiltration	
		High	Low
Gender			
Male	38	14	24
Female	0		
Age			
< 60	18	7	11
> 60	20	7	13
Stage (UICC,1987)			
I & II	16	10	6
III & IV	22	4	18
Lymph Node Metastasis			
Positive	21	5	16
Negative	17	9	8
Surgical Intervention Thoracotomy			
with Esophagectomy	32		
Trans-hiatal Esophagectomy	6		

Abbreviations: NK: natural killer; ESCCs: esophageal squamous cell carcinomas.

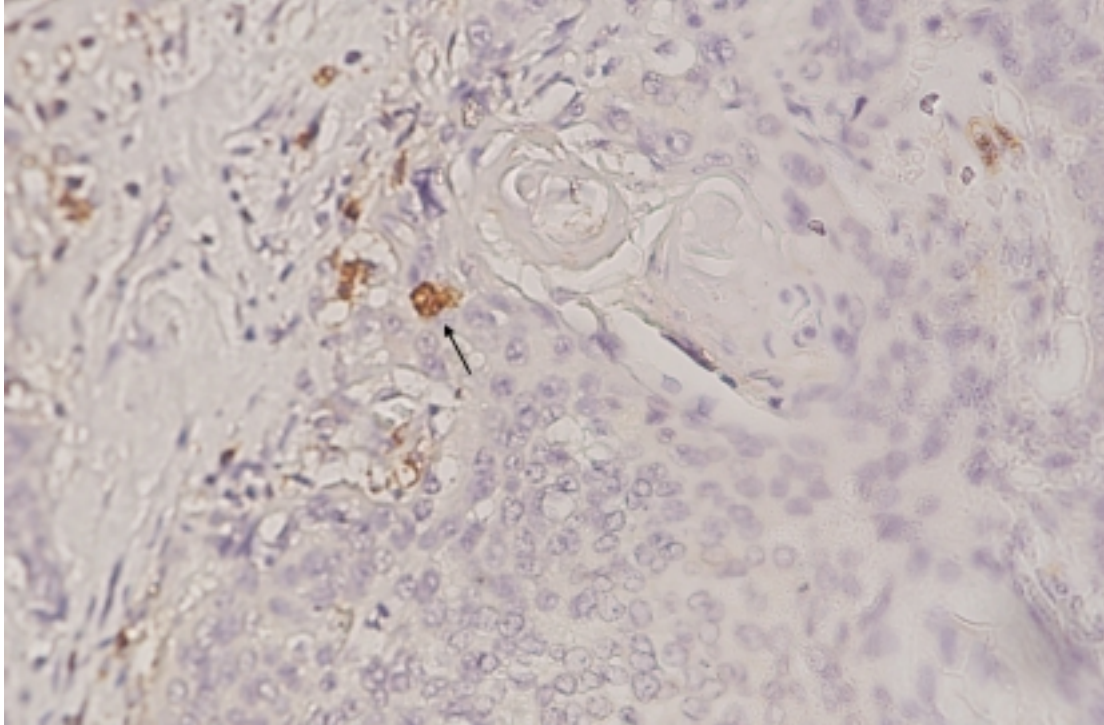


Fig. 1 Immunohistochemical staining of esophageal squamous cell carcinoma cells with CD57 antibody. Positive cells are identified in the stroma of the tumor (arrow) (original magnification x400).

24 (63%) had low level. No significant relationship between NK cell infiltration and the clinical parameters of age, stage or lymph node metastasis was found.

Correlation between NK cell infiltration in ESCCs and survival

The survival of all 38 patients had been noted every 3 months for the first 2 years after the end of treatment; afterward every 6 months. At the end of the follow-up period (July 31, 2002) 6 of the 38 patients (16%) were still alive. Analysis of the immunohistochemical staining of NK cell infiltration with respect to survival is shown in Figure 2. Analysis based on the log-rank test revealed that patients with a high level of intratumoral NK cell infiltration had a significantly more favorable overall survival than patients with low level NK cell infiltration ($p = 0.0068$).

A multivariate analysis of the clinical and immunohistochemical data indicated that only the T4

tumor had a p value < 0.05 (Table 2).

DISCUSSION

Clinical immune responses to autologous cancer cells may be mediated mainly by nonspecific TILs such as NK cells⁽¹⁰⁻¹¹⁾ or by tumor specific lympho-

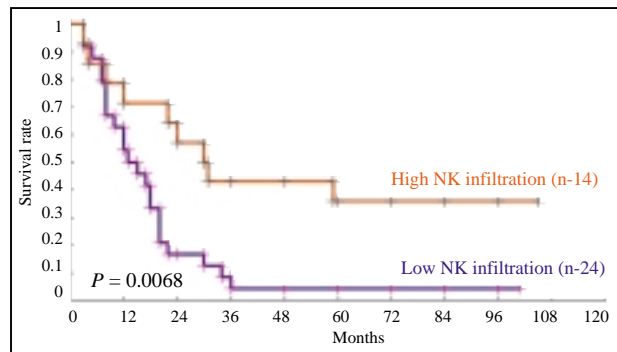


Fig. 2 Overall survival rates of 38 patients with primary resected ESCC in relation to NK cell infiltration.

Table 2. Multivariate Analysis of Prognostic Factors of Survival using Cox's Proportional Hazard Model

Parameters	Coefficient	<i>p</i>	Risk Ratio	95% CI*
Age	0.03	0.073	1.03	1.00-1.07
Depth of invasion				
T1			1	
T2	1.31	0.407	3.72	0.17-82.81
T3	1.23	0.331	3.44	0.29-41.35
T4	3.05	0.042†	21.05	1.11-399.52
Stage				
I	1			
II	-0.70	0.445	0.49	0.008-3.01
III	-1.24	0.142	0.29	0.06-1.51
IV				
Lymph Node Metastasis				
N0	1			
N1	1.08	0.263	2.94	0.45-19.34
NK Infiltration				
Low	1			
High	0.49	0.378	1.63	0.55-4.85

* 95% CI = 95% confidence interval.

† *p* < 0.05

cytes.⁽¹²⁻¹³⁾ Recently, in patients with colorectal,⁽¹³⁾ gastric⁽¹⁴⁾ and lung cancer,⁽¹⁵⁾ an immunohistochemical analysis of NK cells was performed and it was reported that a high level of intratumoral infiltration of NK cells was found to be associated with a favorable outcome. Contrary to previous reports, Cho et al⁽¹⁶⁾ found the numbers of NK cells did not improve prognosis in esophageal squamous cell carcinoma. The cut-off point of the immunohistochemical staining for NK cells infiltration in their study was different than previous reports, and they divided the patients into four groups (most abundant, abundant, moderate, scanty) to compare the prognosis. They also evaluated the NK cells in the mesenchymal stroma and the prognosis did not correlate with the number of NK cells in the stroma. As previously reported, we did not evaluate the NK cells in the stroma because of the variations over the stroma around the tumor nest. In the present study, we found a positive correlation between intratumoral NK cell infiltration and surgical outcome in patients with esophageal squamous cell carcinoma. By univariate analysis, depth of tumor invasion, lymph node metastasis, disease stage and NK cell infiltration all correlated with surgical outcome. The Cox multivariate proportional analysis was performed using the covariates patient

age, depth of tumor invasion, lymph node metastasis, disease stage and NK cell infiltration. Although, in univariate analysis, the prognostic significance of NK cell infiltration was *p* = 0.007, NK cell infiltration was not picked up as an independent prognostic factor. Perhaps this is because NK cell infiltration correlates strongly with the depth of tumor invasion and the sample size was still too small to show the significance. Analysis of more cases may be necessary.

Human MHC HLA class I deregulation is a widespread phenomenon in tumor biology.^(7,17) Between 39% and 88% of tumors derived from HLA+ epithelia are HLA deficient.^(7,16,18) This probably reflects tumor escape mechanisms selected owing to the role of HLA molecules in presenting immunogenic peptides to T cells.⁽¹⁹⁾ However, such alteration in MHC expression might expose the tumor variants to NK cell attack, since the NK cells can lyse HLA class I deficient targets.

Great progress has been made in understanding how NK cells detect loss of MHC class I molecules on tumor cells; NK cells express inhibitory MHC class-I-specific receptors.⁽²⁰⁾ But what stimulates NK cells to attack tumor cells? A new candidate that triggers NK cells to attack tumor cells is the non-classical MHC class I molecule termed MICA/B (for MHC I chain-related-A/B); the NKG2D ligands.⁽⁸⁾ The activating NK receptor NKG2D plays a major role in NK-mediated cytotoxicity against tumors.⁽²¹⁻²²⁾ Jinushi et al demonstrated MICA/B might play an important role in susceptibility to NK cells in hepatocellular carcinomas.⁽²³⁾ We are continuing the research on the MICA/B and NK cells in esophageal squamous cell carcinomas based on the result of the present study.

In conclusion, NK cells are important effector cells for the defense against tumors. Our investigation demonstrates that intratumoral NK cell infiltration is associated with a favorable prognosis in primary resected esophageal squamous cell carcinomas. To date, pathological staging remains the most reliable determinant of prognosis in ESCC, and the main factor in choice of curative treatment. However, it is of great interest to verify new biological markers to define the risk of relapse or to decide on the use of adjuvant treatment. Intratumoral NK cell infiltration might be developed as a good prognostic factor in primary resected esophageal squamous cell carcinoma.

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腫瘤中自然殺手細胞在手術切除之食道鱗狀上皮癌的預後重要性

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背景：自然殺手細胞是對抗腫瘤的重要作用細胞。本篇研究即在探討以手術切除之食道鱗狀上皮癌中，自然殺手細胞浸潤的情形，以及這個情形與病患預後的關係。

方法：38個連續接受手術切除之食道鱗狀上皮癌的檢體，利用免疫化學染色的方法來分析腫瘤中自然殺手細胞浸潤的情形。

結果：依據腫瘤中自然殺手細胞浸潤情形的界定，14個病例 (37%) 被認定為高度自然殺手細胞浸潤，24個病例(63%)被認定為低度自然殺手細胞浸潤。高度自然殺手細胞浸潤之病患的五年存活率顯著優於低度自然殺手細胞浸潤之病患 (p 值小於0.01)。多變異分析並無法顯示自然殺手細胞浸潤情形為一顯著之預後因子。

結論：在手術切除之食道鱗狀上皮癌中，腫瘤中有高度自然殺手細胞浸潤的病患較佳的預後。

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關鍵字：自然殺手細胞，食道鱗狀上皮癌。