

Significance of Expression of Complement C4d in Esophageal Squamous Cell

Carcinoma

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Key Words: Esophageal cancer, complement C4d, immune response evasion,
immune tolerance.

Running Title: C4d Expression in Esophageal Squamous Cell Carcinoma

experimental sturdy

Submission date: July 23, 2016

Abstract. Background/Aim: Esophageal squamous cell carcinoma (ESCC) is one of the most difficult malignancies to cure. C4d is a degradation product of the classical complement pathway and is suggested as an early diagnostic marker for other SCCs. The purpose of this study was to clarify the association of complement C4d with ESCC. Patients and Methods: Immunohistochemical staining for C4d was performed on surgical specimens obtained from 114 patients with ESCC. Results: Positive C4d expression was observed in 70 (61.4%) cases and negative expression in 44 (38.6%) cases. There was a significant inverse correlation between C4d expression and depth of tumor invasion ($p=0.0001$), lymph node metastasis ($p=0.011$), lymphatic invasion ($p=0.033$), and TNM stage ($p=0.0021$). Kaplan–Meier analysis showed that negative C4d expression tended to lead to shorter overall survival ($p=0.232$). Conclusion: C4d expression in ESCC might be useful in developing treatment strategies or suppression of ESCC.

Esophageal squamous cell carcinoma (ESCC) is the major histological type of esophageal cancer in East Asian countries (1) and the eighth most common form of cancer worldwide (2). It is one of the most difficult malignancies to cure. It has a poor prognosis compared with other types of digestive cancer because of its high frequency of lymph node metastasis and recurrence (3). The overall 5-year survival rate of patients with ESCC is <20% (4), therefore early diagnosis is one of the most important factors for patient survival.

Two hypotheses are proposed for the role of the immune system in cancer initiation and progression. Firstly, the traditional hypothesis is that long-term inflammatory reactions promote cancer progression. Secondly, the cancer immunoediting theory postulates that the immune system protects the host against cancer development (5-11). Complement is a central component of innate immunity and plays an essential role in immune surveillance and homeostasis (12). The

complement system is an essential part of the inflammatory response and innate immunity to infection (13).

In various tumor types, complement activation, particularly of the classical pathway, is reported to be related to carcinogenesis (14-16). C4d is a degradation (activation) product of the classical complement pathway. It is suggested that C4d is a useful early diagnostic marker in lung cancer or oropharyngeal SCC and related to their prognosis (17, 18).

The purposes of the present study were to analyze the clinical significance of C4d in ESCC by immunohistochemistry, and to examine the relationship between C4d expression and clinicopathological parameters and patient survival.

Patients and Methods

Patient characteristics. Between May 2009 and March 2015, 199 patients with ESCC underwent transthoracic esophagectomy by the McKeown technique at the Dokkyo

Medical University Hospital, Japan. Surgical tissue samples were obtained from 114 patients, which excluded 69 patients who had received neoadjuvant therapy and 16 who underwent salvage surgery. There were 97 male (85.1%) and 17 female (14.9%) patients, with a mean age of 69 years (range=40-86 years). Tumor stage and disease grade were classified according to the seventh edition of the TNM classification of the International Union Against Cancer (UICC) (19). All patients signed informed consent forms according to our institutional guidelines.

Immunohistochemistry. Resected specimens were fixed with 10% neutral-buffered formalin and embedded in paraffin blocks. Four-micrometer-thick sections were deparaffinized with xylene, hydrated through a series of graded alcohol solutions, and immersed in 3% hydrogen peroxide in absolute methanol for 30 min. The sections were washed with distilled water and phosphate-buffered saline (PBS; pH 6.0). Antigens were retrieved by immersion in preheated 0.01 M citrate buffer (pH

6.0) and heating in an autoclave at 120°C for 20 min. The sections were incubated with rabbit anti-C4d polyclonal antibody (1: 50 dilution; Biomedica, Vienna, Austria) at room temperature for 1 h in a high-humidity chamber. After washing in PBS, the sections were incubated with biotinylated anti-rabbit IgG for 30 min at room temperature, followed by incubation with an avidin–biotin–peroxidase complex solution (VECTASTAIN® ABC Kit; Vector Laboratories, Burlingame, CA, USA). Peroxidase activity was visualized by incubation in 0.02% 3,3'-diaminobenzidine tetrahydrochloride with hematoxylin, dehydrated, and mounted in aqueous medium. C4d staining was evaluated by two pathologists blinded to the clinicopathological background of the patients. Normal epithelial tissue, present on each slide, was used as internal control. Negative C4d expression was defined as identical or weaker staining than in the internal control, and positive C4d expression was defined as stronger staining than in the internal control.

Statistical analysis. Statistical analysis was performed by χ^2 and *t*-tests. Survival parameters were determined using the Kaplan–Meier method and compared using the log-rank test. Univariate and multivariate survival analyses were performed using the Cox proportional hazards regression model. A value of $p < 0.05$ was considered statistically significant in all analyses.

Results

Immunohistochemistry for C4d. Representative results of immunohistochemistry for C4d are shown in Figure 1. In normal squamous epithelium of the esophagus, C4d staining was positive in the cytoplasm of the basal layer (Figure 1a). In ESCC, C4d staining in the cytoplasm varied among the tumor tissues. Several staining patterns were observed for C4d expression in the same tumor tissues. We evaluated C4d staining at the invasive front of the tumor tissue. We defined identical or weaker staining in ESCC than in normal squamous epithelium as negative C4d expression,

and stronger staining in ESCC than in normal squamous epithelium as positive C4d expression.

Relationship between C4d expression and clinicopathological findings. The

relationship between C4d expression and clinicopathological characteristics is shown

in Table I. Positive C4d expression was observed in 70 (61.4%) cases and negative

expression in 44 (38.6%). There was a significant inverse correlation between C4d

expression and depth of tumor invasion ($p=0.0001$), lymph node metastasis

($p=0.011$), lymphatic invasion ($p=0.033$), and TNM stage ($p=0.0021$). There was a

significant correlation between C4d expression and tumor location ($p=0.001$).

However, there was no significant association with patient age, gender, or blood

vessel invasion.

Relationship between C4d and survival. Kaplan–Meier analysis showed that

negative C4d expression tended to lead to shorter overall survival ($p=0.232$) (Figure

2a). No association was found between C4d staining and disease-free survival

(Figure 2b). Multivariate analysis also showed no significant association.

Discussion

There are three complement activation pathways: classical, mannose-binding lectin, and alternative. The three complement pathways converge in the activation of C3, finally forming the membrane attack complex (MAC), which causes cell death (20).

C4d is produced by the classical pathway, which is activated by C1qrs complex and

derives from C4b, which has an internal thioester in the molecule. When C4d is

cleaved from C4b, the covalent bond between C4d and the tissue membrane

remains intact. Covalent-bound C4d has a longer half-life, and therefore remains at

the site (21). According to this theory, the tissue membrane should be stained for

C4d. However, in our study, C4d staining was mainly in the cytoplasm and membrane.

Covalent-bound C4d is used for immunological evaluation. For example, C4d is used as a marker for antibody-mediated rejection (AMR) of renal allografts.

Diagnostic criteria for AMR of renal allografts have been established, and quantitative cutoff values for C4d added to the Banff criteria (22). C4d expression in peritubular capillaries is one of the diagnostic criteria of AMR. C4d expression reflects the function of the immune system that removes non-self tissue. Therefore, it is possible that the negative C4d expression in the current study reflected an aberrant immune response as a result of immune evasion.

Our study showed that negative C4d expression was associated with progression of ESCC, advanced depth of tumor invasion, lymph node metastasis, lymphatic invasion, and TNM stage. C4d expression and tumor location were

significantly correlated. We hypothesize that advanced cancer may inactivate the immune system and suppress expression of C4d.

Corrales *et al.* have suggested that complement-deficient mice have decreased tumor growth compared with wild-type mice (5, 23, 24). Ajona *et al.* reported positive C4d expression to be associated with progression of cancer, such as depth of tumor invasion, lymph node metastasis, and TNM stage, or prognosis of the disease in oropharyngeal or lung cancer (17, 18). From these reports, we suggest that complement activation promotes carcinogenesis. Hence we believe complement activation is related to the traditional hypothesis.

In contrast, our study showed the relationship between negative C4d expression and advanced cancer. This suggests that negative C4d expression induces immune response evasion, that is, immune tolerance. Immune tolerance might inhibit cell death induced by MAC. If cancer cell death is not induced, cancer cells continue to grow and carcinogenesis is promoted. MAC might contribute to the

increase in cancer cells in advanced ESCC, while MAC removes cancer cell in other tumor types. This might apply to the immunoediting theory in ESCC.

Ajona *et al.* showed by computed tomography that plasma C4d levels in asymptomatic patients with lung cancer (histologically, 53.6% of patients had adenocarcinoma and 46.4% had SCC) were higher than in controls (18). The plasma C4d level may be a sensitive screening marker. Patients diagnosed with stage I lung cancer had lower plasma C4d levels than those diagnosed with stage II disease. Higher C4d expression evaluated by immunohistochemistry and a higher plasma C4d level resulted in significantly worse overall survival. These results suggest that plasma C4d levels may predict the risk of lung cancer in asymptomatic individuals and be of value in early diagnosis. In our study, patients with early-stage ESCC had higher levels of C4d expression than those with advanced-stage disease.

Consequently, evaluation of C4d expression may be useful as a screening tool in ESCC. However, in advanced ESCC, the evaluation of C4d expression would not be

useful for screening because of decreased C4d expression. Ajona *et al.* examined immunohistochemistry as well as plasma C4d levels in patients with lung cancer (18). Therefore, the role of C4d in ESCC should be examined by other procedures.

The expression of complement protein and induction of cancer initiation and progression by complement may differ in each type of tumor.

Patients with upper thoracic ESCC have more superficial tumor invasion than patients with middle or lower thoracic ESCC. As a result, this suggests that there might be a significant relationship between C4d expression and tumor location. But, we considered that there are no relationship between C4d expression and tumor location. Our statistical analysis showed that there was no significant relationship between C4d expression and patient prognosis. Most of our patients had early-stage ESCC, which might have been a factor in this result.

Finally, we indicated that C4d might be useful for early diagnosis in ESCC. If complement activation can be stopped at the stage where C4d is cleaved from C4b,

such as C1qrs complex, it might be possible to suppress the development of ESCC.

A study on the relation between immune response and carcinogenesis of C4d may be useful in the early diagnosis, treatment planning, and suppression of ESCC.

In conclusion, the expression of C4d was lower in advanced than in early ESCC and normal squamous epithelium of the esophagus. Moreover, a lower expression of C4d tended to lead to shorter overall survival. Further research is needed to establish the role of C4d in ESCC, and for its use in early diagnosis and treatment of ESCC.

Acknowledgements

This study was supported by the Uehara Memorial Foundation. The authors would like to thank H. Ozeki, Y. Ohashi and N. Yuzawa for their secretarial assistance and N. Suzuki, a member of the Research Support Center Division of Clinical Science, for her technical assistance.

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Table I. *Relationship between C4d expression and clinicopathological characteristics in patients with esophageal squamous cell carcinoma.*

Figure 1. *Representative results of immunohistochemistry for C4d. a: C4d expression in normal squamous epithelium of the esophagus. C4d staining was positive in the cytoplasm of the basal layer. b: Positive C4d expression was stronger in esophageal squamous cell carcinoma (ESCC) than normal squamous epithelium. c: Negative C4d expression was defined as identical or weaker staining in ESCC than in normal squamous epithelium.*

Figure 2. *Relationship between C4d expression and postoperative overall (a) and disease-free (b) survival . The 5-year overall survival rate in patients with negative C4d expression was 61.0% and 68.6% in those with positive C4d expression. Negative C4d expression tended to lead to shorter overall survival than positive C4d*

expression did. The 5-year disease-free survival rate in patients with negative C4d expression was 64.3% and 69.4% in those with positive C4d expression. No association was found between C4d staining intensity and disease-free survival.

