RESEARCH COMMUNICATION

G1/S-specific Cyclin-D1 Might be a Prognostic Biomarker for Patients with Laryngeal Squamous Cell Carcinoma

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Abstract

Objective: To investigate the prognostic role of antigen KI-67 (Ki-67) and G1/S-specific cyclin-D1 (cyclin-D1) in patients with laryngeal squamous cell carcinoma (LSCC). Methods: Immunohistochemical staining (IHS) was used to determine the protein expression of Ki-67 and cyclin-D1 in LSCC tissues. Kaplan-Meier survival curves was calculated with reference to Ki-67 and cyclin-D1 levels. Results: Cyclin-D1 and Ki67 were expressed in the nuclei of cancer cells. Among the total of 92 cancer tissues examined by immunohistochemistry, 60 (65.22%) had cyclin-D1 overexpression and 56 (60.87%) had Ki67 overexpression. Cyclin-D1 overexpression is associated with the advanced stage of the cancer (P=0.029), but not with gender, age, stage of cancer, histological differentiation, anatomical site, smoking history and alcohol consumption history. Ki67 overexpression is not associated with the advanced stage, gender, age, histological differentiation, anatomical site, smoking history and alcohol consumption history. A statistically significant correlation was found between lymph node status and the expression of Ki67 (p = 0.025). Overexpression of cyclin-D1 was correlated to shorter relapse-free survival period (P<0.001). Conclusions: Overexpression of cyclin-D1 can be used as a marker to predict relapse in patients with LSCC after primary curative resection.

Keywords: Cyclin-D1 - Ki-67 - laryngeal squamous cell carcinoma - prognostic biomarker - survival time

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Introduction

Squamous cell carcinomas are the malignant tumors of epithelial cells in the skin and mucous membranes, and thus spread to the different part of human body, especially bronchi, skin, cervix, esophagus, oral cavity, pharynx and larynx. In addition to age, gender, and particularly, the immune status of individual genetic predisposition include the development of a neoplastic disease (Reichart, 1991). It is believed that a positive family history (at least one family member had a squamous cell carcinoma of the head and neck) means 2~4 fold higher risk of developing squamous cell carcinoma in any localization. Among the exogenous factors in the literature, tobacco and alcohol mention are the most important etiological factors for the development of squamous cell carcinoma in the head and neck region (El-Husseiny et al., 2000; Schantz and Yu, 2002; Llewellyn et al., 2003; Cooper et al., 2004; Dalla Vecchia et al., 2004; Figuero Ruiz et al., 2004). It seems not only alcohol abuse but also tobacco was an independent risk factor for the development to be a squamous cell carcinoma. Studies showed that it was dependent on the amount and concentration of the alcohol to be taken to an increasing surface damage of the mucosal epithelium, and thereby facilitated the absorption of carcinogenic components of tobacco smoke (van der Waal, 1996, 1999; La Vecchia et al., 1997; Moreno-López et al., 2000; Llewellyn et al., 2003; Dalla Vecchia et al., 2004). Another cause for the development of laryngeal squamous cell carcinoma in the Asian and African region, seems to be the consumption of betel nut (Maier et al., 1999; Maier and Tisch, 1999; Moreno-López et al., 2000; Jeng et al., 2001). The pathogenesis of squamous cell carcinoma, especially in the oral cavity, are persistent mechanical irritation, eg by poorly fitting dentures. Also, a lack of oral hygiene in general seems have a potentially supportive influence on the carcinogenesis of oral squamous cell carcinoma (Moreno-López et al., 2000). Other potentially beneficial factors are the Ches ionizing radiation, nutritional deficiencies (especially vitamin A and iron deficiency) and viral infections for the development of squamous cell carcinoma in the head and neck areas. In over 85% of people suffering from a nasopharyngeal carcinoma, patients had positive antibodies against the Epstein-Barr virus. Recent studies also demonstrate the role of human papilloma virus (HPV) in oral tongue cancer (Elango et al., 2011) and laryngeal squamous cell carcinoma.

Therefore, this study was to investigate the prognostic role of Antigen KI-67 (Ki-67) and G1/S-specific cyclin-D1 (cyclin-D1) in patients with LSCC.

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Table 1. Surgery Factors

Factors	N	%
Preservation of larynx	67	72.83
Neck dissection	78	84.78
Reconstruction		
Pectoralis major myocutaneous flap	21	22.83
Esophago-gastric anastomosis	4	4.35
Free jejuna transfer	2	2.17

Materials and Methods

Patients

From Jan 2000 to Aug 2005, 92 patients, who were diagnosed as LSCC in Qilu hospital, were included in this study. Their age ranged from 36 to 79 years, with a mean of 55.4 years. Of all the patients,their clinical history datas were collected (including therapy, date of recurrence or the occurrence of metastases and eventual death date). The management they received included surgery and postoperative radiation therapy. Surgery factors are shown in Table 1. This study was conducted in accordance with the declaration of Helsinki. And all patients had signed the consent for the use of collected data without disclosure of personal identity. The study protocol was also approved by the ethics committee of Qilu Hospital.

Postoperative Follow-up

After curative resection, follow-up included a history and physical examination, electronic laryngoscope examination every 3 months. Every 8 months computed tomography (CT) or magnetic resonance imaging (MRI) was performed to verify recurrence or not. Postoperative radiation was routinely recommended to reduce the recurrence risk. The follow-up ended October 2011. The median follow-up time was 45.5 months (range: 3–73 months).

Immunohistochemical staining

To stain the nuclear antigen Ki-67 immunohistochemically, the monoclonal mouse anti-human antibody MIB-1 (DAKO) was used as a marker against the Ki-67 antigen. The Ki-67 antigen is a large nuclear protein (345, 395 kDa) whose expression is strongly associated with cell proliferation (Scholzen and Gerdes, 2000). The fact that the Ki-67 protein is detected in all active phases of the cell cycle (G (1), S, G (2) and in mitosis), but is missing in resting cells (G (0)). It makes an excellent marker for the so-called "growth fraction" to determine a cell population. The staining with the MIB-1 antibodies generally followed the recommendations of the manufacturer DAKO, being used for the visualization of the reaction, a standardized, indirect immunoperoxidase method (EnVision kit). In the case of Ki-67 antibody, a dilution of the primary solution was 1:400. The sections a so-called epitope-retrival were subjected in the steamer at a pH of 9.0 prior to staining.

To stain the nuclear antigen cyclin-D1 immunohistochemically, the rabbit monoclonal anti-human antibodies clone SP4 (Thermo Fisher Scientific GmbH). The cyclin-D1 protein is one of the key proteins for monitoring the cell cycle. It exerts its effect in conjunction with cdk4 and/or cdk6 in which it phosphorylates the Rb protein. Putative protooncogene is overexpressed in a variety of human neoplasms. For the visualization of the reaction, in turn, the standardized indirect immunoperoxidase method was used (EnVision kit). In the case of cyclin-D1 antibody, a dilution of 1:25 was chosen for the primary solution. The so-called epitope-retrival sections were subjected in the steamer at a pH of 9.0 prior to staining.

The labeled nuclei index was calculated as the percentage of labeled nuclei out of the total number of tumor cells counted, and graded in the following manner: 0 (negative staining); 1+ (low staining or <20%); 2+ (moderate staining or 20-50%); and 3+ (strong staining or >50%). 0 and + are considered as low expression and 2+ and 3+ are regarded as high expression.

Statistical analysis

The relationships between the various pathological-anatomical, immunohistochemical and clinical chemistry findings were examined using chi-square tests (2 each group). In all experiments, the prognosis for patients was chosen as the endpoint for the simultaneous analysis. For the calculation of survival times, survival patients were classified as aborted experiments. The presentation of cumulative survival curves according to the method of Kaplan-Meier. For the statistical evaluation of differences between the cumulative survival curves depending on the marker, a log-rank test and applied analysis of variance (ANOVA) was performed. A p-value < 0.05 was considered to indicate statistical significance. Data were analyzed with the SPSS 18.0 statistical software package (SPSS Inc.).

Results

The expression of cyclin-D1 was shown in Table 2. The expression of cyclin-D1 was dectected in 60 (65.2%) out of 92 tumor samples. There was a statistically significant correlation between the Cyclin-D1 expression and TNM stage ($P = 0.029$). However, no statistical relationship was found between Cyclin-D1 expression and other variables (gender, age, stage of cancer, histological differentiation,

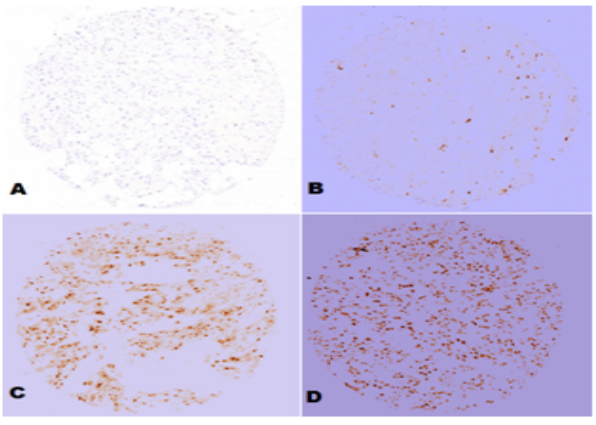

Figure 1. The Negative (A), Weak (B), Moderate (C) and Severe (D) Expression of Cyclin-D1 after Immunohistochemical Staining of the Tumor Samples

Table 2. Clinicopathologic Variables Associated with Different Expression Patterns of Cyclin-D1 or Ki67

Variables	n	Expression of Ki67		P	Expression of cyclin-D1		P
		High	Low		High	Low	
Gender							
Male	78	47	31	0.428	36	42	0.469
Female	14	10	4		5	9	
Age							
≤60	48	31	17	0.446	20	28	0.559
>60	44	25	19		21	23	
TNM stage							
I+II	21	11	10	0.304	5	16	0.029
III+IV	71	46	25		36	35	
Histologic Grade							
Well + Moderate	44	24	20	0.234	16	28	0.13
Poor	48	32	16		25	23	
Smoking history							
Current and former	71	45	26	0.605	33	38	0.497
Never	21	12	9		8	13	
Alcohol history							
Current and former	65	41	24	0.731	31	34	0.349
Never	27	16	11		10	17	

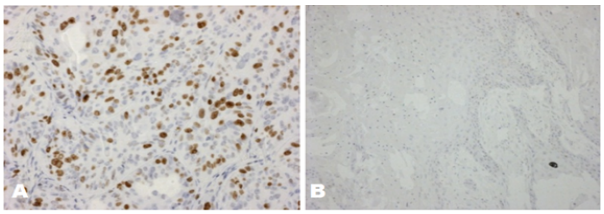

Figure 2. The Positive (A) and Negative (B) Expression of Ki-67 after Immunohistochemical Staining of the Tumor Samples

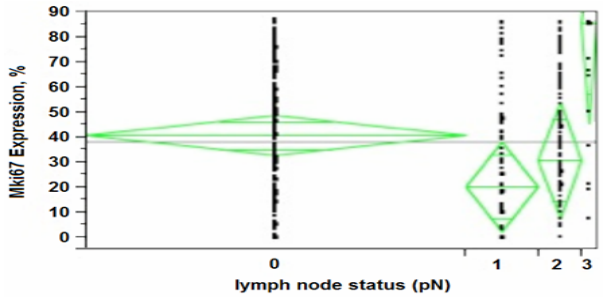

Figure 3. The Correlation of Ki-67 Expression with the Lymph Node Status Using Anova/t-test

anatomical site, smoking history and alcohol consumption history). Examples of the cyclin-D1 expression after immunohistochemical staining of the tumor samples were shown in Figure 1.

A typical example for Ki-67 expression patterns in the samples was shown in Figure 2. Ki67 overexpression is not associated with the advanced stage, gender, age, histological differentiation, anatomical site, smoking history and alcohol consumption history (Table 2). A statistically significant correlation was found between lymph node status and the expression of Ki-67 ($p = 0.025$). When a lymph node status $pN = 0$, a mean value of Ki-67 expression was 40.2% (SD = 3.853%, n = 25). And the Ki-67 expression was 20% (SD = 8.787 %, n = 6) at $pN = 1$, 30.3% (SD = 11.343%, n = 3) at $pN = 2$ and 85% (SD =19.647%, n = 1) at $pN = 3$ expression. The details were shown in Figure 3.

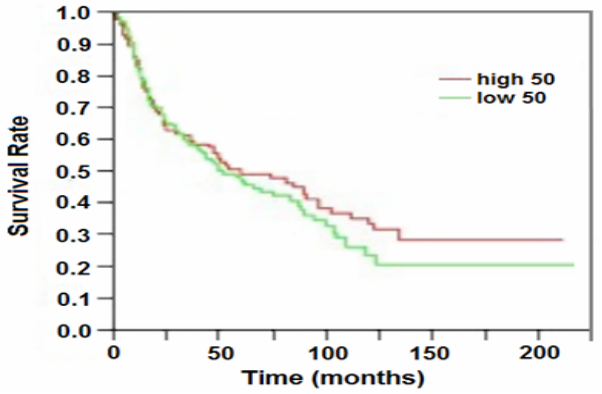

Figure 4. Kaplan-Meier Survival Analysis According to Ki-67 Expression

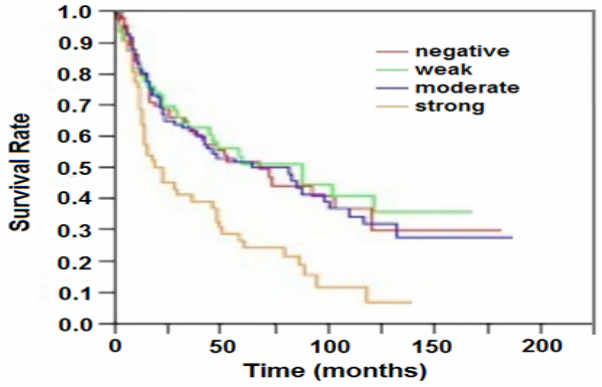

Figure 5. Kaplan-Meier Survival Analysis According to the Cyclin-D1 Expression

Possible associations of Ki-67 expression with the survival of the patients were initially investigated. The first group of "low 50" was with an expression below 50%, and the second group of "high 50" included all samples with a Ki-67 expression greater than 50%. The results of this analysis were presented in Figure 4. There was no statistical correlation between Ki-67 expression and survival time ($p = 0.457$).

Furthermore, there was a statistical correlation between the cyclin-D1 expression and survival rate of patients ($p = 0.0004$), shown in Figure 5. There were 22 cases with negative cyclin-D1 expression, 29 cases with low, 29 cases with moderate and 12 cases with a strong cyclin-D1 expression.

Discussion

The studies showed that a cyclin-D1 overexpression was found (moderate and strong expression) in 41 cases and a weak or negative expression in 51 cases. The results demonstrated that the cyclin-D1 expression were frequent changed in the laryngeal squamous cell carcinoma. In the literature, for example, Kyomoto et al. (1997) reported 53% of cases, and Ishiguro et al. did 37.5% of cases (Ishiguro et al., 2003), who investigated the overexpression of cyclin in the cases of pharyngeal and oral carcinomas by overexpression of cyclin. Angadi et al. found the overexpression in 70.7% oral cavity cancers (Angadi and Krishnapillai, 2007). Hofele et al. showed

the overexpression in 39% of oral cavity carcinomas. Mahdey et al. (2011) found that cyclin D1 amplification may differ in different subsites of oral squamous cell carcinoma (tongue vs cheek). In summary, therefore, the fluctuations in the expression of cyclin-D1 are most likely to the different composition of the tumor collectives (oral cavity, pharynx, larynx, respectively).

Further, it was investigated whether the cyclin-D1 expression were significantly associated with tumor phenotype factors, such as TNM stage, Histologic Grade and lymph node status. The cyclin-D1 expression was significantly associated with TNM stage. Several studies have shown a correlation of cyclin-D1 overexpression to tumor thickness, to lymph node status and to the grading (Kyomoto et al., 1997; Fujii et al., 2001; Miyamoto et al., 2002; Ishiguro et al., 2003; Myo et al., 2005; Yu et al., 2005).

The expression of Ki-67 was significantly associated with the lymph node status ($p = 0.0252$). Several researches have focused on the significance of Ki-67 in squamous cell carcinoma of head and neck (Girod et al., 1998; Sittel et al., 1999; Stoll et al., 2000; Bettendorf and Herrmann, 2002; Carinci et al., 2002; Myoung et al., 2006). This heterogeneous result showed the correlation of Ki-67 expression to tumor phenotype factors. However, there is a correlation of Ki-67 expression for lymph node status (Myoung et al., 2006), whereas other studies reported no statistical association between Ki-67 expression and tumor phenotype factors (Bettendorf and Herrmann, 2002). Thus, Ki-67 seems to have no distinct meaning as a marker for head and neck squamous cell carcinoma.

The results of this study emphasize those changes in the expression of cyclin-D1 in laryngeal squamous cell carcinoma. However, it turns out in particular a significant fluctuation of the results with respect to the sampling point of the cancers. Regarding the markers studied here, the results of cyclin-D1 and Ki-67 are clearly. The quite heterogeneous statements about the importance of cyclin-D1, Ki-67 may therefore be due to a generalization of the cancers as head and neck cancers. In general, different methods for the detection of cyclin-D1 amplification are available, such as Southern blotting, polymerase chain reaction (PCR) and also applied fluorescence in situ hybridization (FISH) (Akervall et al., 1997; Wang et al., 1999; Fujii et al., 2001; Ishiguro et al., 2003). This reflects the differences in the methods directly contradict the results in the literature.

A low cyclin-D1 expression in patients with laryngeal squamous cell carcinoma is associated with the longer survival. An increase in survival probability is because that cyclin-D1 primarily is expressed in neoangiogenesis of newly formed endothelial cells. In this study, it was shown that the low cyclin-D1 expression is associated with the long survival. This opportunity represents a new therapeutic approach to hypopharyngeal squamous cell carcinoma and requires a further and deeper research.

In summary, our findings suggested that the low cyclin-D1 expression was correlated with the high survival rate. And low cyclin-D1 might be a prognostic biomarker for patients with hypopharyngeal squamous cell carcinoma.

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