RESEARCH ARTICLE

Prognostic Value of MAC30 Expression in Human Pure Squamous Cell Carcinomas of the Lung

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Abstract

Recent evidence haas indicated that meningioma-associate protein (MAC30) exhibits different expression patterns in various tumors. However, little is known about the value of MAC30 in human squamous cell carcinoma of lung (SQCLC). The purpose of our study was to investigate the expression of MAC30 and to explore its clinical significance in SQCLC patients. A total of 156 Chinese patients diagnosed with SQCLC were selected for this study. The expression of MAC30 in all tissues was confirmed by immunohistochemical staining. Quantitative real-time PCR was performed to analyze MAC30 mRNA expression in 32 cases of SQCLC patients with corresponding non-tumor lung tissues. We observed enhanced mRNA expression of MAC30 in SQCLC as compared to control samples. Further, elevated MAC30 protein expression was strongly associated with poor tumor differentiation, TNM stage, and lymph node metastasis. In addition, we observed that patients with increased MAC30 expression demonstrated poor overall survival. Multivariate analysis explicated that increased MAC30 expression was a valuable independent predictable factor for poor tumor differentiation and short survival in SQCLC patients. Our present study suggests that MAC30 may serve as a biomarker for poor tumor differentiation and outcomes of patients with SQCLC.

Keywords: MAC30 - squamous lung cancer - metastasis - prognosis

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Introduction

Lung cancer leading an unfortunate 5-year survival rate as less than 15%, accounts for the primary cause of cancer-related death worldwide (S. & Peters, 2015; William et al., 2009). More than 75% of total lung malignancies are non-small cell lung cancer (NSCLC) at diagnosis (Jemal et al., 2008). Although there were significant developments about molecular mechanisms in lung adenocarcinoma, minor progress in survival was achieved in last decade (Chudgar et al., 2015). Furthermore, as the major histological subtype of NSCLC, the study of molecular abnormalities in SQCLC occupied the major histological subtype of NSCLC was little improved (Kenmotsu et al., 2014). Therefore, it is imperative to select valuable biomarkers for early detection and predicting prognosis through deeper molecular mechanisms in SQCLC.

Meningioma-associated protein (MAC30), also known as transmembrane protein 97 (TMEM97), acts as a member of the insulin-like growth factor-binding protein family (IGFBP) and regulates insulin-like growth factor (IGF) activity (Murphy, Pykett, Harnish, Zang, & George, 1993). MAC30 is located on 17q11.2 and observed in normal organs such as lung, brain, heart and skeletal muscles (Malhotra et al., 1999). Recent research suggests that MAC30 plays a role in cellular cholesterol and lipid metabolism (Bartz et al., 2009). Indeed, MAC30, which was originally confirmed as an elevated gene in human meningioma (Murphy et al., 1993; Sobin & Fleming, 1997), showed adverse expressions in different types of tumors (Kayed et al., 2004). Based on the evidences of the upregulation of MAC30 expression in breast, esophagus, oral, stomach and colon cancers (Kayed et al., 2004; Moparthi et al., 2007; Yan et al., 2010) in contrast to the downregulated expression in pancreatic cancers (Kayed et al., 2004), we believed that MAC30 played as a suppressor or promoter in different tumors with unknown definite functions (Murphy et al., 1993). To date, although overexpression of MAC30 associated with poor clinical outcome in NSCLC was reported (Han et al., 2013), there's no published report to clarified relationship between MAC30 expression and clinicopathological features in pure SQCLC.

In the present study, we investigated the correlation

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of MAC30 expression in clinicopathological features in SQCLC patients. Furthermore, we also assessed the value of MAC30 to predict unfavorable tumor differentiation and prognosis of patients with SQCLC.

Materials and Methods

Patients and tissue samples

A total of tumors specimens from 156 Chinese patients with SQCLC and 32 corresponding non-tumor tissues were obtained from surgical resections at Yixing people hospital affiliated Jiangsu University between June 2005 and July 2012. This study was retrospectively performed and was approved by the institutional review board of the Jiangsu University Faculty of Medicine. The informed consent obtained from all subjects was confirmed. And all participants consent to participate in the study with their information. These patients included 121 men and 35 women, ranging in age from 42 to 83 years, with a median age of 64 years. None of the patients received adjuvant chemotherapy or radiotherapy before surgery. The clinicopathological diagnosis was determined according to the World Health Organization and International Union against Cancer Tumor-Node-Metastasis (TNM) staging system (Sobin & Fleming, 1997). Two pathologists classified the tumor specimens independently and unanimous agreement was obtained. The differentiation of SQCLC was categorized as follows: well differentiated, more than 50% of obvious keratin pearl or intercellular bridge observed in tumor tissues; moderately differentiated, 20-50% of keratin pearl or intercellular bridge observed in tumor tissues; and poorly differentiated, less than 20% of keratin pearl or intercellular bridge. Surgically removed tumors and matched noncancerous tissue samples used for mRNA detection were immediately frozen in liquid nitrogen and kept at -80 °C until extraction of RNA.

Quantitative real-time RT-PCR

Total RNA from frozen tissues was isolated using Trizol reagent (Life Technologies, MD, USA) according to the manufacturer's instructions. Reverse transcription was performed on 1 μg of total RNA from each sample. Quantitative real-time RT-PCR was performed using SYBR Green (Takara, Dalian, China) on a Real-Time Quantitative Thermal Block (Biometra, Germany). The PCR primer sequences were designed according to the human MAC30 and GAPDH gene sequences reported in GenBank and were synthesized: MAC30, sense: 5'-GGCAGCAGAGGAGTAGCTTGA-3'; antisense: 5'-GCTTGCTGGCGCTAAAAGG-3'. The reactions were carried out at 95 °C for 30 s, then 35 cycles of 95 °C for 20 s, 55 °C for 15 s, and 72 °C for 20 s, and a final extension at 72 °C for 10min. Real-time PCR was used to detect the specificity of the PCR via the dissociation reaction plot subsequent examination. Data were normalized to GAPDH.

Immunohistochemistry analysis

MAC30 protein expression in 156 tumor tissue samples was confirmed by immunohistochemistry analysis, which was performed on formalin-fixed, paraffin-embedded, 3-μm-thick tissue sections. After pretreated at 60 °C for 1 h, the sections were dewaxed in xylene, hydrated, and washed in phosphate-buffered saline Tween solution (PBST). The sections were treated with 3% H_2O_2, then incubated with a polyclonal antibody against MAC30 (1:500; SC-1971, Santa Cruz, CA, USA) overnight at 4 °C. After washed by PBST for 3 times with each 15 min, the sections were incubated with corresponding second antibody at room temperature for 1 h. The results were visualized with diaminobenzidine. In each immunohistochemistry run, the negative controls were stained without primary antibody.

Two independent pathologists, who were particularly experienced in immunohistochemistry, evaluated MAC30 staining in all sections. The expression of MAC30 was quantified using a visual grading system based on the percentage of stained cells out of total number of tumor cells, and divided from 0 to 3: 0=negative, 1+, 1-30 %; 2+, 31-60 %; 3+, >60 %. The intensity of staining was graded on a scale: 0, negative; 1, weak positive; 2, moderate positive; 3, strong positive. The sums of extend score and intensity score were used to define the MAC30 expression levels, which were graded into two groups: low-level MAC30 expression (with a score≤2), and high-level MAC30 expression (with a score>2).

Statistical analysis

Relationships between MAC30 expression and clinicopathological characteristics were analyzed by the X2 test. The Kaplan-Meier method was used to test the association between MAC30 expression and overall survival. The relationship between MAC30 expression and tumor differentiation stages was assessed by the univariate and multivariate logistic regression. A cox regression model was contributed to identify whether MAC30 expression was an independent factor related to the survival. All statistical analyses were operated using SPSS version 13.0. A P value <0.05 was considered to be statistically significant.

Results

Overexpression of MAC30 mRNA in SQCLC tissues

We used real-time PCR to confirm the expression of MAC30 mRNA in 32 cases of patients with SQCLC and corresponding adjacent lung tissues. As shown in Figure 1, elevated MAC30 mRNA expression in 26 of the 32 cases was significantly described. Furthermore, the expression level of MAC30 mRNA in the SQCLC cases was over 3-fold than that in corresponding control samples. So, we conclude that overexpression of MAC30 mRNA may play important roles in the progression of SQCLC, which should not be neglected.

Immunohistochemistry assessment of MAC30 expression in SQCLC tissues

A total of 156 lung sections from patients with SQCLC were conducted to affirm the expression of MAC30 protein via immunohistochemistry (Figure 2). As classical criteria described above, the high-level MAC30 expression was

observed in 125 patients (80.1%), while 31 patients were classified as low-level MAC30 expression (19.8%) (Table1).

Relationship between MAC30 expression and clinicopathological parameters

Table 1 summarized the relationship between MAC30 expression and clinicopathological variables in SQCLC. We observed that the increased expression of MAC30 was significantly related to poor tumor differentiation, TNM stages and lymph node metastasis (P<0.05). In contrast, there was no statistical correlation between MAC30 expression and patient age, smoking status, gender and tumor classification.

Table 1. Association Between MAC30 Expression and Clinicopathological Variables of SQCLC Patients

Variables	No. (N=156)	MAC30 expression		p value
		Low (N=31)	High (N=125)	
Age (years)				0.326
<60	43	15	26	
≥60	113	16	99	
Gender				0.102
Male	121	27	94	
Female	35	4	31	
Smoking status				0.431
Non-smoker	92	15	77	
Smoker	64	16	48	
Tumor differention				0.032
Well	22	16	6	
Moderate	40	11	29	
Poor	94	4	90	
TNM stage				0.018
I- II	41	23	18	
III-IV	115	8	107	
Tumor classification				0.671
T1+T2	38	21	17	
T3+T4	118	10	108	
lymph node metstasis				0.003
No	48	19	29	
Yes	108	12	96	

Impact of MAC30 overexpression on tumor differentiation in patients with SQCLC

Most studies also showed that cellular differentiation was closely related to survival in lung cancer patients (Wang et al., 2013). Poorly differentiated lung cancers are associated with significantly shorter survival than those with moderate to well differentiation. So, it's essential to investigate the biological indication of MAC30 on tumor differentiation, which affects clinical therapies and survival of patients with lung cancers. Among the 94 SQCLC patients with poor tumor differentiation, there were 90 patients with overexpression of MAC30. Actually, except other clinicopathological parameters, overexpression of MAC30 was an independent predictor of tumor differentiation in SQCLC via univariate and multivariate logistic analysis (Table 2).

Overexpression of MAC30 predicts poor prognosis of SQCLC patients

In order to confirm whether elevated MAC30

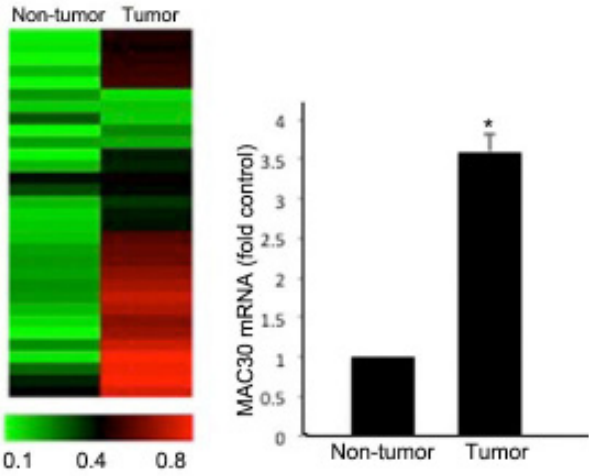

Figure 1. MAC30 mRNA Expression was Confirmed in SQCLC Tissues and Adjacent Normal Tissues Via Quantitative Real-time PCR and Normalized to GAPDH.* p<0.05

Table 2. Risk Factors for SQCLC Patients with Poor Tumor Differentiation B and SE: the Parameter Estimator of Association Coefficient and its Standard error CI Confidence Interval

Variables	Univariate analysis				Multivariate analysis			
	B	SE	95 % CI	P value	B	SE	95 % CI	P value
MAC30				0.012	1			0.009
Low	1				1.47	0.415	1.491-3.754	
High	1.32	0.301	1.335-3.153					
Age (years)				0.563				
<60	1							
≥60	1.01	0.456	0.712-1.983					
Gender				0.772				
Male	1							
Female	0.875	0.423	1.024-3.847					
Smoking status				0.483				
Non-smoker	1							
Smoker	1.15	0.571	0.535-1.763					
Tumor classification				0.191				
T1+T2	1							
T3+T4	1.038	0.51	0.376-1.793					
lymph node metstasis				0.061				
No	1							
Yes	0.867	0.491	0.517-1.753					

Table 3. Univariate and Multivariate Analysis of Prognostic Factors in 156 Patients with SQCLC

Variables	Univariate		P value	Multivariate		P value
	HR	95 % CI		HR	95 % CI	
MAC30			0.009	1		0.012
Low	1			1.143	0.512-2.015	
High	1.081	0.571-1.912				
Age (years)			0.416			
<60	1					
≥60	1.034	0.601-1.638				
Gender			0.561			
Male	1					
Female	0.867	0.501-1.942				
Smoking status			0.273			
Non-smoker	1					
Smoker	1.113	0.641-1.803				
Tumor differention			0.019			0.015
Well	1			1		
Moderate	1.126	0.561-1.792		1.237	0.602-1.998	
Poor	1.318	0.781-2.153		1.426	0.841-2.241	
TNM stage			0.008			0.013
I- II	1			1		
III-IV	1.341	0.801-2.317		1.184	0.486-1.923	
Tumor classification			0.191			
T1+T2	1					
T3+T4	1.219	0.551-1.903				
lymph node metstasis			0.013			0.007
No	1			1		
Yes	1.241	0.664-1.691		1.371	0.629-1.774	

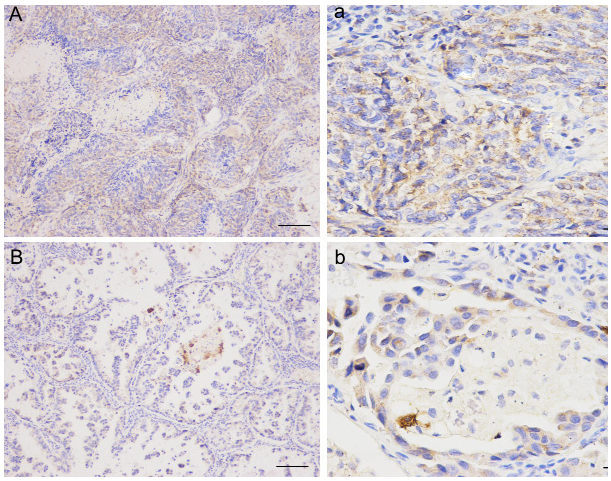

Figure 2. Representative Immunohistochemical Staining for MAC30 Expression in SQCLC. A and a: high expression of MAC30. B and b: low expression of MAC30. A and B original magnification, ×100; a and b original magnification, ×200

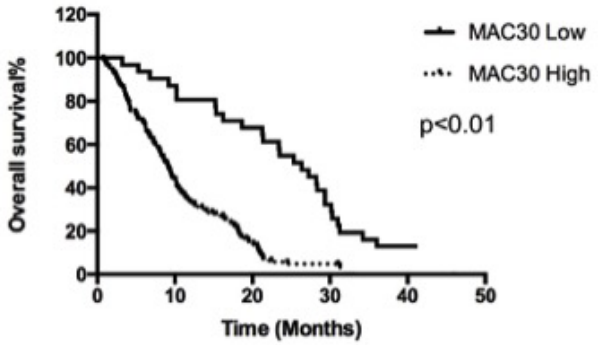

Figure 3. Kaplan-Meier Analysis for the Correlation Between Overall Survival of SQCLC Patients and MAC30 Expression.

expression is a predictor of unfavorable prognosis in SQCLC patients, we firstly used Kaplan-Meier method to analyze the association between MAC30 expression and prognosis of patients. Indeed, patients with higher MAC30 expression displayed shorter overall survival than that of patients with lower MAC30 expression (P<0.05). Absolutely, increased MAC30 expression level is correlated with unfortunate outcomes in SQCLC patients (Figure 3) (P<0.05).

Furthermore, MAC30 expression level and conventional factors were assessed via Cox's univariate and multivariate regression analysis, in order to determine whether MAC30 could act as an independent risk factor for poor prognosis in patients with SQCLC. Actually, in addition to MAC30 expression, tumor differentiation, TNM stages, and lymph node metastasis were indicated to be associated with overall survival through the univariate analysis (P<0.05). Moreover, a multivariate analysis presented that MAC30 expression, tumor differentiation, TNM stages, and lymph node metastasis were independent prognosis factors for overall survival in SQCLC patients (Table 3) (P<0.05).

Discussion

Investigations of NSCLC molecular pathogenesis have increased remarkably and principles of treatment are changed for recent years; however these improvements mainly limited to adenocarcinoma (Kim, 2013). As the most common type of NSCLC (Kenmotsu et al., 2014), SQCLC seems to be different from adenocarcinoma on response to targeted therapies (Kim, 2013). Recent research has extensively focused on genetic alterations, which have a critical role in the development of SQCLC and can become targets of anti-cancer chemotherapy ("Comprehensive genomic characterization of squamous

cell lung cancers," 2012). Unfortunately, there's no inspiring targeted therapy against SQCLC up to now. So, it's more necessary to explain the impact of MAC30 expression on clinicopathological details and prognosis in pure SQCLC.

To our knowledge, the importance of overexpression MAC30 on tumor differentiation and prognosis in SQCLC has not been emphasized. MAC30, a recently identified meningioma-associated gene, exhibits opposite expressions in various tumors (Kayed et al., 2004; Moparthi et al., 2007; Yan et al., 2010). Consisted with previous report showing the elevated MAC30 in NSCLC (Han et al., 2013), we confirmed the overexpression of MAC30 in SQCLC from our present study. Further, we analyzed the association between MAC30 expression and traditional clinicopathogical characteristics in SQCLC. We realized that the high expression of MAC30 was significantly correlated with poor tumor differentiation, TNM stages and lymph node metastasis, but not associated with age, smoking status, tumor classification and gender. Moreover, our data also showed that patients with high MAC30 expression suffered from a significantly poorer overall survival when compared with patients with low expression of MAC30. From these current data, we conclude that high MAC30, which could enhance cell proliferation of cancer cells, plays a critical role in the progression of SQCLC.

There're still bottlenecks at early diagnosis and prognosis of SQCLC. Conventional states for prognosis, such as TNM stage, lymph node status, and tumor differentiation are insufficient. Therefore, it's more essential to investigate the valuable and individual manner to evaluate the development and prognosis of SQCLC. With the evidence of MAC30 overexpression in lymph node metastatic tissues, zhang et al reported the association between elevated MAC30 expression and poor prognosis in colon cancer (Zhang et al., 2006). And Yan et al demonstrated the prognostic role of MAC30 overexpression on lymph node metastasis in oral squamous cell carcinoma (OSCC) (Yan et al., 2010) and epithelial ovarian cancer (Yang et al., 2013). Fortunately, the earlier research about MAC30 in human malignances guides us into the new expectation on MAC30 with lung cancer. Although Han et al have demonstrated the prognostic value of MAC30 in NSCLC (Han et al., 2013), the prognostic utility of MAC30 expression in SQCLC remains unclear. In our recent study of SQCLC patients, we confirmed that elevated MAC30 expression was a predictor for poor tumor differentiation, which shows a greater ability for invasion and hence correlates with horrific prognosis (Tsutani et al., 2014). Moreover, the multivariate analysis showed that MAC30 expression was an independent prognostic biomarker for overall survival of patients with SQCLC. Our data suggest that there's a potential correlation between pathogenesis and development of SQCLC with overexpression of MAC30, which significantly indicated worse tumor differentiation and survival of patients.

MAC30 with diverse expression exhibited the promotion and suppression in different human tumors. Unfortunately, as a recent developed gene, the mechanism of MAC30 in tumor initiation and progression is still unclear. Recent study suggested that MAC30 expression was induced by BRCA1 (Atalay, Crook, Ozturk, & Yulug, 2002), which is the prognostic factor of NSCLC (Zhao, Zhang, Du, & Gu, 2014). Moreover, as an important regulator in progression of SQCLC (Fan et al., 2015), p53 also mediates MAC30 expression levels (Kannan et al., 2001). Indeed, it's encouraged to investigate the explicit mechanisms of MAC30 in SQCLC via analyzing the relationship between MAC30 and other genes.

In conclusion, this study demonstrated the overexpression of MAC30 in a large proportion of patients with SQCLC and closely associated with progression and poor prognosis in SQCLC. MAC30 expression level appears to be an important potential biomarker for tumor differentiation and survival for SQCLC patients. Whatever, from our present data, we suggest that MAC30 may be an attractive therapeutic target for SQCLC. To solve the problem, further studies will be required to confirm the molecular function of MAC30 in SQCLC.

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