Overexpression of MAC30 in the Cytoplasm of Oral Squamous Cell Carcinoma Predicts Nodal Metastasis and Poor Differentiation

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Overexpression of MAC30 in the cytoplasm of oral squamous cell carcinoma predicts nodal metastasis and worse differentiation

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Abstract

The meningioma associated protein **(**MAC30) was highly expressed in several types of tumors including esophageal, gastric and colon tumors as compared to the normal tissues. The MAC30 expression has been gradually increased from the normal colorectal mucosa to the primary colorectal cancer and to the metastatic colorectal cancers in the lymph nodes. The expression was related to the patient's survival of colorectal cancers. However, there is no study of MAC30 in oral squamous cell carcinoma (OSCC). We, in this study, investigated MAC30 expression in OSCC and further analyzed associations of the MAC30 expression with clinicopathological variables in OSCCs. MAC30 expression was immumohistochemically examined in 20 normal oral mucosa specimens and 43 OSCCs. Expression of meningioma associated protein (MAC30) in the cytoplasm was remarked increased from the normal oral epithelial cells to the primary oral squamous cell carcinoma (OSCC). Frequency of the strong cytoplasmic staining was significantly higher in the primary OSCC as compared to the normal oral mucosa (51% vs 20%, p=0.019). Furthermore, the frequency of MAC30 expression in primary tumors of the patients with lymph node metastasis was higher than those without metastasis (65% vs 35%, P=0.048), and the frequency of MAC30 expression in worse differentiated tumors was higher than those with better differentiated ones (90% vs 39%, P=0.005). Overexpression of MAC30 in the cytoplasm of OSCC may predict nodal metastasis and worse differentiation.

Keywords: MAC30; OSCC; Metastasis; Differentiation

Introduction

The meningioma associated protein (MAC30) is a member of the insulin-like growth factor binding protein family and regulates IGF activity. The MAC30 gene is located on 17q11.2, having a small segment of similarity to an apical gut membrane poly protein of *Haemonchus contortus* to olfactory receptor 30 of *Mus musculus* and to cytochrome b in several organisms. However, there is no real sequence homology to any human gene[1].

MAC30 mRNA has been expressed in a variety of normal tissues, especially in testicular and gastric tissues. mRNA levels of the MAC30 are significantly increased in breast and colon cancers, however decreased in pancreatic and renal cancers. MAC30 protein is expressed in various normal tissues, such as esophageal, gastric, pancreatic and colon tissues, mainly in the mucosal cells and acinar and islet cells of the pancreas. In line with the MAC30 mRNA transcription, the expression of MAC30 protein were significantly increased in esophageal, gastric and colon cancers, but was weak expression or absent in pancreatic cancer cells of primary tumors and metastases[2]. Recently, our research group found that MAC30 cytoplasmic expression was significantly increased from distant normal mucosa to primary tumor and to metastasis, and was positively correlated with infiltrated growth pattern, phosphatase of regenerating liver (PRL) and tended to be positively correlated with Ki-67. The MAC30 cytoplasmic expression in metastatic lymph node indicated poor survival[3, 4]. These findings indicated that it may play a role in the development of tumor, promoting tumor progression, invasion and metastasis, and as prognostic factor.

Studies have shown that several molecular factors are not only involved in OSCC development but also treatment response of OSCC patients[5 - 8]. The $p33^{ING1b}$ protein, as a

TP53-linked tumor suppressor, is one of the ING1 gene products [9,10], and was found to be related to melanoma, acute lymphoblastic leukemia, seminoma, papillary thyroid and breast carcinoma [10-12], as well as the oral squamous cell carcinoma (OSCC)[13-15]. Our previous study indicated that it was related to the metastasis of OSCC [16].

Particularly interesting new cysteine-histidine rich protein (PINCH) is an adapter protein consisting primarily of five LIM domains, and the gene is located on chromosome 2q12.2. The PINCH is up-regulated in the stroma of different tumors [17-23]. Furthermore, PINCH has been considered as a prognostic factor in patients with colorectal cancer [17], and has been related to lymph node metastasis of OSCCs [21]. These indicate that PINCH plays an important role in tumor development, and predicting the ability of invasion and metastasis.

The present study aimed to determine MAC30 expression in OSCC compared to normal oral mucosa, and further to analyse the relationship of MAC30 expression in OSCC with clinicopathological variables including patients' gender, age, tumor location, size, lymph node statues and grade of differentiation and biological factors including PINCH and $p33^{ING1b}$.

Patients and methods

Patients

Formalin-fixed paraffin-embedded tissues were obtained from the Department of Pathology of the First Hospital of Hebei Medical University, Shijiazhuang, Hebei Province, China. There were 43 OSCCs including 20 gingiva, 13 tongue and 10 buccal carcinomas. None of the patients had received preoperative radiotherapy or chemotherapy. The patients' gender, age, tumor size, location, lymph node status, and differentiation were obtained from surgical and /or pathological records at the Hospital. Mean age of the patients was 54 yeas (from 26 to 80 years). The submandible and neck lymph nodes were taken for determining metastasis. All patients included in this study have given consent for the material taken for scientific research. According to WHO classification, differentiation was graded as well, moderately, poorly differentiated and undifferentiated. Well and moderately differentiated tumors were grouped as better differentiation and poorly differentiated and undifferentiated tumors as worse differentiation. There were 20 normal oral mucous samples from orthodontic surgical operations. The samples were free from pre-cancer and cancer as determined by histological examination. All slides including normal specimens and tumors were reconfirmed by two pathologists (Zhu Z.L, Yang Y.H).

Data of PINCH [21] and $p33^{ING1b}$ [16] expressions, determined by immunohistochemistry, were from our previous study carried out at our laboratory. Among 57 OSCCs for PINCH staining, 30 cases were matched with the present cases for MAC30 analysis, among 49 OSCCs for $p33^{ING1b}$ staining, 27 cases were matched with the present cases for MAC30 analysis, t he rest of cases did not have enough tissue for

sectioning for MAC30 immunostaining. PINCH and $p33^{ING1b}$ expression was graded as negative (none or <5% of positive cells) or positive, based on the intensity of PINCH staining in stromal cells and $p33^{ING1b}$ staining in the nuclear and cytoplasm of normal and tumors cells.

Immunohistochemical staining and evaluation

The protocol for immunohistochemical staining was followed our laboratory[24]. The sections (5μm) were incubated at 60°C over night, deparaffinized in xylene, and rehydrated with graded ethanol and distilled water. In order to exposed masked epitopes, the sections were boiled in 0.01 M citrate buffer (Ph 9.0) in a high pressure cooker for 2 min, and then kept at room temperature for 30 minutes. In order to block the endogenous peroxidase activity, the sections were incubated with 3% H_2O_2-methanol for 20 minutes and washed with phosphate buffered saline (PBS, pH 7.4). Then the sections were further treated with protein block solution for 10 minutes in order to avoid non-specific binding of the antibody. After removing the blocking solution, the sections were incubated with a monoclonal antibody MAC30 1-19 [2] in 1:5 in dilution, diluted in antibody diluent (DAKO) at 4^oC overnight. Then a biotinylated anti-rabbit IgG antibody (Fuzhou Maixin Biology Technology Limited Company, Fuzhou, Fujian Provence China) was applied for 30min following by an incubation of an avidin-biotin-peroxidase (Fuzhou Maixin Biology Limited Company) complex for 30 min. The sections were rinsed in PBS between the incubation steps. The perxidase reaction was developed by use of diaminobenzidine (Beijing Zhongshan Biology Technology Limited Company, Beijing, China) for 8 min. After counterstaining with hematoxylin, the sections were dehydrated

and mounted. The sections known to stain positively were included as negative and positive controls. For negative controls, the sections incubated with PBS instead of the primary antibody were not stained, whereas positive controls were stained.

The slides were microscopically examined and scored independently by two investigators (Zhu Z.L, pathologist, and Wang D.W, stomatologist) without any clinical or pathological information. To avoid artifacts, the areas with poor morphology, margins of the sections, and necrosis were not considered. In the present study, we examined the staining of epithelial cells, tumor cells and stroma of tumor. Intensity of cytoplasmic staining was graded as negative (no positive cells), weak, moderate and strong staining. The nuclear staining was graded as negative (5%), 5-50%, 50-75% and >75%, regardless of the staining intensity. In cases with discrepant results, a consensus score was reached after re-examination.

Statistical analysis

The Chi-square method was used to test the relationship of the frequencies of MAC30 expression in normal oral mucosa and OSCC, as well as the relationship between MAC30 expression and clinicopathological variables or biological variables. All *P* values cited were two-sided and *P* values < 0.05 were judged as statistically significant.

Results

MAC30 expression was examined in 20 normal mucosa samples and 43 OSCCs. MAC30 cytoplasmic expression in the normal mucosa and primary tumor were shown in Fig. 1.

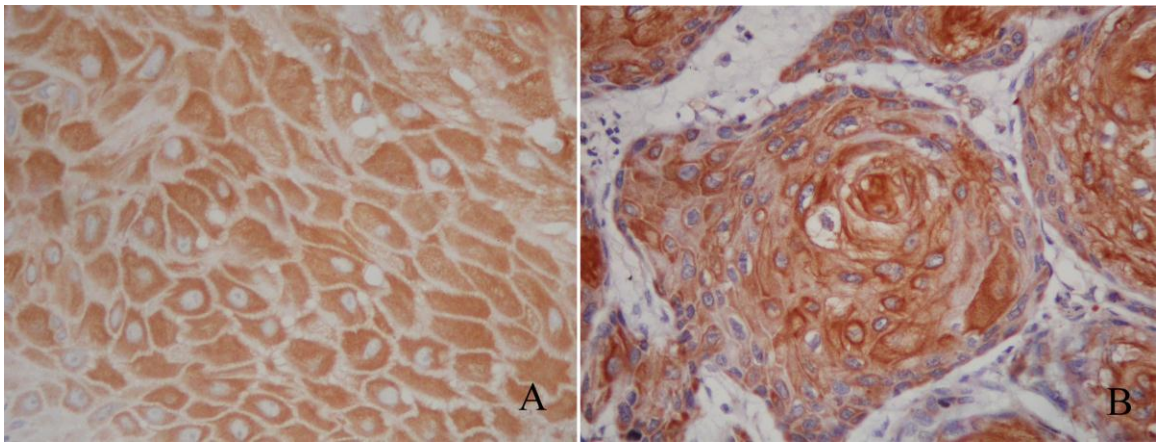

Figure1. *Cytoplasmic expression of MAC30 in normal oral epithelial cells (A) and primary oral squamous cell carcinoma cells (B).*

According to the similarities of the clinicopathological features, OSCC cases showing negative and weak MAC30 immunostaining were grouped as the weak group, and cases showing moderate and strong MAC30 immunostaining were grouped as the strong group.

Strong cytoplasmic expression of MAC30 was observed in 20% (4/20) of normal mucosa specimens, and 51% (22/43) of primary tumors. The rate of MAC30 cytoplasmic expression was significantly higher in the primary tumors than that in normal mucosa (p=0.019). The frequency of MAC30 expression in primary tumors of the patients with lymph node metastasis was higher than those without metastasis (65% vs 35%, P=0.048), and the frequency of MAC30 expression in worse differentiated tumors was higher than those with better differentiated ones (90% vs 39%, P=0.005, Table 1). MAC30

expression was not significantly correlated with patients' gender, age, tumor location and size (P>0.05, Table 1).

Table 1 The relationship between MAC30 expression and clinicopathological variables in oral squamous cell carcinoma

Variables	*n*	Strong MAC30 (%)	*P*-value
Gender			0.28
Male	20	12(60)	
Female	23	10(43)	
Age (years)			0.27
≤ 50	18	11(61)	
>50	25	11(44)	
Tumor location			0.70
Gingival	20	12(60)	
Tongue	13	6(46)	
Buccal	10	4(80)	
Tumor size (cm)			0.67
≤2	15	7(46)	
>2	28	15(33)	
Lymph node status			0.048
Non-metastasis	20	7(35)	
Metastasis	23	15(65)	
Differentiation			0.005
Worse	10	9(90)	
Better	33	13(39)	

We also examined the relationship between cytoplasmic expression of MAC30 and PINCH and $p33^{ING1b}$ which studied in our previous paper in OSCCs. There was no relationship of MAC30 with PINCH and $p33^{ING1b}$ (p>0.05, data not shown).

There was one tumor with weak staining in the nuclear, and two tumors with weak stromal staining.

Discussion

MAC30 was first described to be overexpressed in meningiomas, and altered expression was also found in different types of human tumors[1, 2, 25]. MAC30 gene is expressed in a broad spectrum of normal tissues, such as the brain, lung, heart, skeletal muscle, testes, ovary and pregnant uterus [25]. MAC30 was expressed at moderate levels in normal pancreatic acinar cells and low levels in most pancreatic cancer cells. In contrast to pancreatic cancer, the expression of MAC30 was stronger in breast, stomach and colon cancers than the corresponding normal tissues [3, 4, 25,] , these indicate that MAC30 plays an importance role in various types of malignancies.

In the present study, we found that the positive frequency of strong cytoplasmic expression of MAC30 significantly increased from normal mucosa to primary tumor (20% vs 53%, *P*=0.02), which was in line with the results observed in esophageal, gastric, breast, stomach and our previous study in colorectal cancer [2, 3], these findings further confirmed that MAC30 may play a role in the development of malignancies including OSCC, perhaps acting as an oncogene.

Our previous study in colorectal cancers showed that MAC30 cytoplasmic expression was increased from distant normal mucosa to primary tumor and to metastasis, the frequency of strong cytoplasmic expression of MAC30 in cases with lymph node metastasis was significantly higher than in non-metastatic cases (65% vs 35%, *P*=0.048). Taken together, these findings suggest a role for MAC30 in promoting metastasis. MAC30 might be a new biomarker for predicting the lymph node metastasis

of OSCC and can provide voluble information for the surgical treatment. Because lymph node metastasis status in the neck decides the operation styles, that is whether needing to resect the neck lymph node. So searching for predictor to identify whether patients have lymph node metastasis before operation is main clinical objective. If MAC30 expression of preoperative biopsy of the primary tumors, especially in the sentinel lymph node (SLN) of the neck was positive, together with other biomarkers' positive expression such as PINCH and P33^{ING1b} [21, 16], which were also related to lymph node metastasis of OSCC, and the tumor's size, location, we would be able to decide whether we need resection of the lymph node of neck before the operation. It can also help clinician to decide whether radiotherapy or chemotherapy is needed after the operation. MAC30 negative staining of the primary tumors, especially in the SLN perhaps suggests a good survival. It is interesting to design a study to examine MAC30 expression in preoperative biopsy samples from the same patients to determine whether the MAC30 expression is related to metastasis of lymph node of neck, cancer recurrence, and eventually patient's outcome. Since some molecular factors, such as thymidylate synthase (TS), cyclin-dependent kinases (CDKs) and GLUT family members, may be related to treatment response in head and neck cancer patients [5-8], it is great interesting to further study MAC30 in relation to certain treatments of OSCC patients in order to see if MAC30 expression has any impact in chemo- or/and radio-therapeutic strategies.

In the present study, we found that the frequency of strong cytoplasmic expression of MAC30 in the cases with worse differentiation was significantly higher than in better differentiation cases (90% vs 39%, p=0.005), this showed that MAC30 was related with the differentiation of OSCC. As we know that MAC30 mRNA is expressed as a

non-erythropoietic gene in the fetal liver, but not in the adult liver. This suggested a possible role in growth and differentiation of liver. We would propose that as OSCC developing, especially in the worse differentiated tissues, the MAC30 gene then began to turn on as in the fetal liver and functioned as a regulation protein to guide the differentiation of the oral squamous carcinoma cells, so the MAC30 cytoplasmic expression was up-regulated especially in the worse differentiated tumors which was like the early stage of the fetal liver. That MAC30 is involved in differentiation rather than proliferation is also supported by various observations. MAC30 mRNA expression is down-regulated in human fibroblast in response to serum [26] and MAC30 is down-regulated by c-jun N-terminal kinase antisense oligonucleotides (JNK2AS) in human PC3 prostate carcinoma cells, in conjunction with growth suppression and induction of apoptosis in this cells [27].

Conclusion

In the present study, we found that MAC30 was related to the development, invasiveness, and lymph node metastasis in the OSCC. Overexpression of MAC30 in the cytoplasm of OSCC may predict nodal metastasis and worse differentiation.

Conflict of interest

The authors declare that they have no conflict of interest.

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