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Original Article

Associations of MACC1, AGR2, and KAI1 expression with the metastasis and prognosis in head and neck squamous cell carcinoma

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Abstract: Background: Metastasis-associated in colon cancer-1 (MACC1, was firstly found in colon cancer and associated metastasis and prognosis in various cancers), anterior gradient 2 (AGR2, was considered as a valuable prognostic factor for some cancers), and Kangai 1 (KAI1, was a tumor metastasis suppressor gene) are all related to metastasis and prognosis of many cancers. However, the associations of MACC1, AGR2, and KAI1 in head and neck squamous cell carcinoma (HNSCC) are still unclear. In this study, we analyzed associations among MACC1, AGR2, and KAI1 in HNSCC, and their respective associations with clinicopathological parameters and overall survival (OS) in HNSCC. Methods: Positive expression of MACC1, AGR2, and KAI1 in 106 whole HNSCC tissue samples was detected by immunohistochemical staining. Patient's clinical data and demographics were both collected. Results: Positive rates of MACC1 and AGR2 were significantly higher, and positive rate of KAI1 was significantly lower, in HNSCC and than those in control tissues. Positive rates of MACC1 and AGR2 were positively correlated with grades of tumor, TNM stages, and lymph node metastasis (LNM) stages, and negatively with patients OS; positive rate of KAI1 was negatively associated with grades of tumor, TNM stages, and LNM stages, and the positive expression of KAI1 subgroup had significantly longer OS than did the negative KAI1 subgroup. In multivariate analysis, positive expression MACC1, AGR2, and KAI1, and tumor stages, as well as LNM stages were potential to be independent prognostic factors for OS in patients with HNSCC. Conclusions: MACC1, AGR2, and KAI1 may represent potential metastatic and prognostic biomarkers, as well as promising therapeutic targets for HNSCC.

Keywords: HNSCC, MACC1, AGR2, KAI1, prognosis, metastasis

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most frequently diagnosed cancer and with an estimated 600,000 cases every year worldwide [1]. The major risk factors are exposed to consumption of tobacco and alcohol [2, 3]. Moreover, high-risk human papilloma virus (HPV) related HNSCC (in particular HPV 16) are accounted for 25% HNSCC cases [4, 5]. Because HNSCC is usually asymptomatic in its early stages, many patients diagnosed with HNSCC in China have III to IVA stage.

Metastasis-associated in colon cancer-1 (MACC1) was originally found in colon cancer in 2009 and was considered as a valuable metastatic and prognostic biomarker for colon cancer [6]. MACC1 can control the MET gene transcriptional activity and is a key regulator of the HGF/MET signaling pathway [6-8]. MACC1 can promote tumor cells proliferation, migration, and invasion in vitro, as well as induce tumor progression and metastasis in vivo [6, 9-11]. In addition to its critical role in the prognosis and metastasis of CRC, accumulating studies also demonstrate that MACC1 is able to be considered as a useful biomarker in several other solid cancers [8, 12-15].

Anterior gradient-2 (AGR2) which was originally identified in Xenopus *laevis* can encode the human homologue of a secreted protein [16]. AGR2 is considered as a member of protein

Table 1. Patients characteristics

Patients characteristics	Frequency (n)	Percentage (%)
Age (years)		
<60	41	38.7
≥60	65	61.3
Gender		
Male	71	67.0
Female	35	33.0
Location		
Oral	37	34.9
Larynx	63	59.4
Lip	6	5.7
Size (cm)		
<2.0	48	45.3
≥2.0	58	54.7
Smoking		
No	41	38.7
Yes	65	61.3
Alcohol		
No	48	45.3
Yes	58	54.7
Grade		
Well	42	39.6
Moderate	51	48.1
Poor	13	12.3
Lymph node metastasis		
N0	69	65.1
N1	34	32.1
N2	3	2.8
TNM stages		
I	34	32.1
II	34	32.1
III	35	33.0
IV	3	2.8

N0: no regional lymph node metastasis; N1: the number of regional lymph node metastasis is no more than 3; N: the number of regional lymph node metastasis is more than 3.

disulfide isomerase (PDI) family which is found in the endoplasmic reticulum (ER) [17]. As a PDI, AGR2 is considered to participate in protein folding and maturation of client proteins [17, 18]. And ER is considered to be involved in carcinogenesis and tumor biology [19]. AGR2 can promote cell proliferation, migration, malignant transformation, and metastasis [20-22]. AGR2 is overexpressed in various types of cancers, such as breast cancers, oral cancers, lung cancers, prostate cancers, and pancreatic cancer [22-26]. AGR2 is also a useful biomarker for prognosis in clinical practice [16].

Kangai1 (KAI1), also named as CD82, is initially identified as a suppressor gene of tumor metastasis in prostate cancer cells [27]. KAI1 gene which encodes transmembrane glycoprotein of tetraspanins family (TM4SF) is located on chromosome 11P11.2. KAI1 can modulate cell membrane structure by interacting with integrin and other TM4SF proteins [28]. KAI1 is able to inhibit epithelial-mesenchymal transition (EMT) in cancer cells [29]. KAI1 can regulate cell migration, adhesion, differentiation, and invasion [12, 30]. KAI1 can strengthen intercellular adhesion and stabilize E-cadherin/β-catenin complex, but inhibit tyrosine phosphorylation of β-catenin on HGF stimulation [31]. Accumulating studies have revealed that down or lost expression of KAI1 should promote metastasis and mean an unfavorable prognosis in various types of human cancer [12].

In summary, studies of MACC1, AGR2, and KAI1 suggested that these biomarkers should be involved in tumor metastasis and progression; however, the relationships among MACC1, AGR2, and KAI1 in HNSCC have not been widely reported. In this paper, we evaluated the hypothesis that these biomarkers were mutual related and correlated with metastasis and prognosis in HNSCC.

Materials and methods

Specimens

All 106 HNSCC tissues and the corresponding "normal" tissues were collected from the Department of Pathology, the First Affiliated Hospital of Bengbu Medical College, from January 2011 to December 2012. All tissue samples were obtained with patient consent and this study was approved by the ethical committee of Bengbu Medical College and performed according to the ethical guidelines of the Declaration of Helsinki. Patients who had received preoperative anti-cancer therapy (chemo or radio-therapy or any other anti-cancer therapy) were excluded. The corresponding adjacent head and neck noncancerous tissues were removed from the same patient. All patients were collected completely demographic, clinicopathologic, and follow-up data. Overall survival (OS) was calculated from the patient's

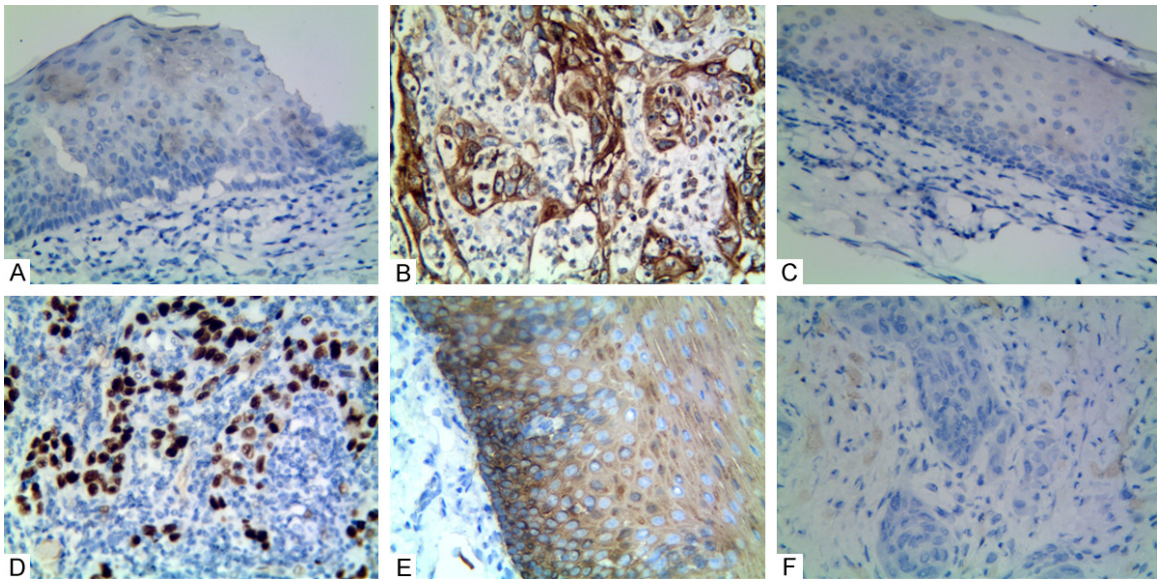

Figure 1. Expression of the patients in head and neck squamous cell carcinoma (×400 magnification). A. Negative MACC1 expression in the control normal cells. B. Positive MACC1 expression in the cytoplasm of cancer cells. C. Negative AGR2 expression in the control normal cells. D. Positive AGR2 expression in the nuclei of cancer cells. E. Positive KAI1 expression in the membrane and cytoplasm of control normal cells. F. Negative KAI1 expression in cancer cells.

surgery data to his/her death date or December 2016 (mean OS: 53.3 months; range: 22-70 months). Tumor-node-metastasis (TNM) stages and lymph node metastasis stages were evaluated in accordance with the 7th edition of the American Joint Committee on Cancer (AJCC). Tumor grades were evaluated in accordance with World Health Organization (WHO) standards. Other characteristics see **Table 1**.

Immunohistochemistry

Immunohistochemistry was conducted in accordance with the Elivision^{TM} Plus detection kit instructions (LabVision, USA). All HNSCC and the control tissues were fixed in 10% buffered formalin and then embedded in paraffin. Paraffin slices (4 μm thick) of representative HNSCC and control tissues were cut, then deparaffinized in xylene and dehydrated in a gradient concentration alcohol. All slices were washed with phosphate buffer saline (PBS, pH 7.2) for 10 min. Then incubated with 3% H_2O_2 in methanol for 10 min to block the endogenous peroxidase activity at room temperature. Subsequently placed in citrate buffer (pH 6.0) at 95°C to repair antigen for 30 min. After several washes with PBS, all slices were blocked by goat serum for 30 min, subsequently incubated with rabbit polyclonal antibody against human MACC1 (Santa Cruz Biotechnology, CA, USA) or rabbit polyclonal antibody against human AGR2 (Cell Signaling Technology, Inc., Danvers, MA, USA) or mouse monoclonal antibody against human KAI1 (Abcam Cambridge, MA, USA) at 37°C for 1 h. Finally, all slices were counterstained with hematoxylin, dehydrated, air-dried, and mounted. Negative controls were conducted by not use primary antibody from the immunohistochemistry procedure.

Calculation of staining

To calculate the immunostaining of MACC1, AGR2, and KAI1, the positive staining cells at least 10 representative high-power fields (HPF) were chosen, then the intensity and extent of positive staining cells were scored. The scores were evaluated in accordance with intensity scores (none: 1; weak: 2; moderate: 3; strong: 4) and extent scores (11% <positive cells: 1; 10% <positive cells <51%: 2; 50% <positive cells <76%: 3; 75% <positive cells: 4). The final scores that ranged 0-12 were multiplied by the intensity and extent. Scores >2 were considered as positive results. Immunostaining results were evaluated by two independently experienced pathologists who were blind to patients' demographic, clinical, and follow-data.

Table 2. The associations between expression of MACC1, AGR2, and KAI1 and clinicopathological characteristics of head and neck squamous cell carcinoma (HNSCC)

Variables	MACC1		*P*	ARG2		*P*	KAI1		*P*
	-	+		-	+		-	+	
Age			0.725			0.091			0.360
<60 years	15	26		30	11		27	14	
≥60 years	26	39		37	28		37	28	
Gender			0.845			0.958			0.714
Male	27	44		45	26		42	29	
Female	14	21		22	13		22	13	
Location			0.599			0.285			0.349
Oral	16	21		27	10		22	15	
Larynx	22	41		37	26		40	23	
Lip	3	3		3	3		2	4	
Size (cm)			0.329			0.282			0.005
<2.0	21	27		33	15		22	26	
≥2.0	20	38		34	24		42	16	
Smoking			0.090			0.105			0.126
No	20	21		22	19		21	20	
Yes	21	44		45	20		43	22	
Alcohol			0.566			0.079			0.234
No	20	28		26	22		26	22	
Yes	21	37		41	17		38	20	
Grade			<0.001			<0.001			0.003
Well	26	16		32	10		18	24	
Moderate	15	36		34	17		34	17	
Poor	0	13		1	12		12	1	
LNM			<0.001			0.002			<0.001
N0	37	32		51	18		29	40	
N1	4	30		16	18		32	2	
N2	0	3		0	3		3	0	
TNM stages			<0.001			0.001			<0.001
I	24	10		29	5		4	30	
II	12	22		21	13		24	10	
III	50	30		17	18		33	2	
IV	0	3		0	3		3	0	

Table 3. Association between expression of MACC1, AGR2, and KAI1 in HNSCC

Variable	MACC1		r	*P*	AGR2		r	*P*
	-	+			-	+		
MACC1							0.244	0.012*
-					32	9		
+					35	30		
KAI1			-0.426	<0.001@			-0.338	<0.001@
-	14	50			32	32		
+	27	15			35	7		

*: Positive association; @: Negative association.

Statistical analysis

Associations between clinical characteristics and expression of MACC1, AGR2, or KAI1 were compared using Chi-square or Fisher's exact test. Associations between MACC1, or AGR2, or KAI1 were compared using Spearman coefficient test. Effects of MACC1, AGR2, or KAI1 on OS were determined by univariate and multivariate COX regression analyses. OS was determined by the Kaplan-Meier method with log-rank test to evaluate association between MACC1, or AGR2, or KAI1 staining results or clinicopathological characteristics, using SPSS 19.0 software for Windows (Chicago, IL). A value of $P<0.05$ was considered statistically significant.

Results

There was a significant difference between the expression of MACC1, or AGR2, or KAI1 and some clinicopathological characteristics.

MACC1 positive staining was mainly located in the cytoplasm of the cancer cells. The positive expression rate of MACC1 in the HNSCC specimens (61.3%, 65/106) was significantly higher than that in the control tissues (7.5%, 8/106; $P<0.05$; **Figure 1A** and **1B**). The positive rate of MACC1 in HNSCC was positively related with TNM stages, LNM stages, and tumor grades, but not with patient's age, gender, location, size, smoking, and alcohol (**Table 2**).

AGR2 positive staining was mainly located in the nuclei of the cancer cells. Similar to MACC1, AGR2 expression was significantly higher in HNSCC tissues (36.8%, 39/67) than that in the control tissues (18.9%, 20/106; $P<0.05$; **Figure 1C** and **1D**). The positive rate of AGR2 in HNSCC was positively related with TNM stages, LNM stages, and tumor grades, but not with patient's age, gender, location, smoking, alcohol, and size (**Table 2**).

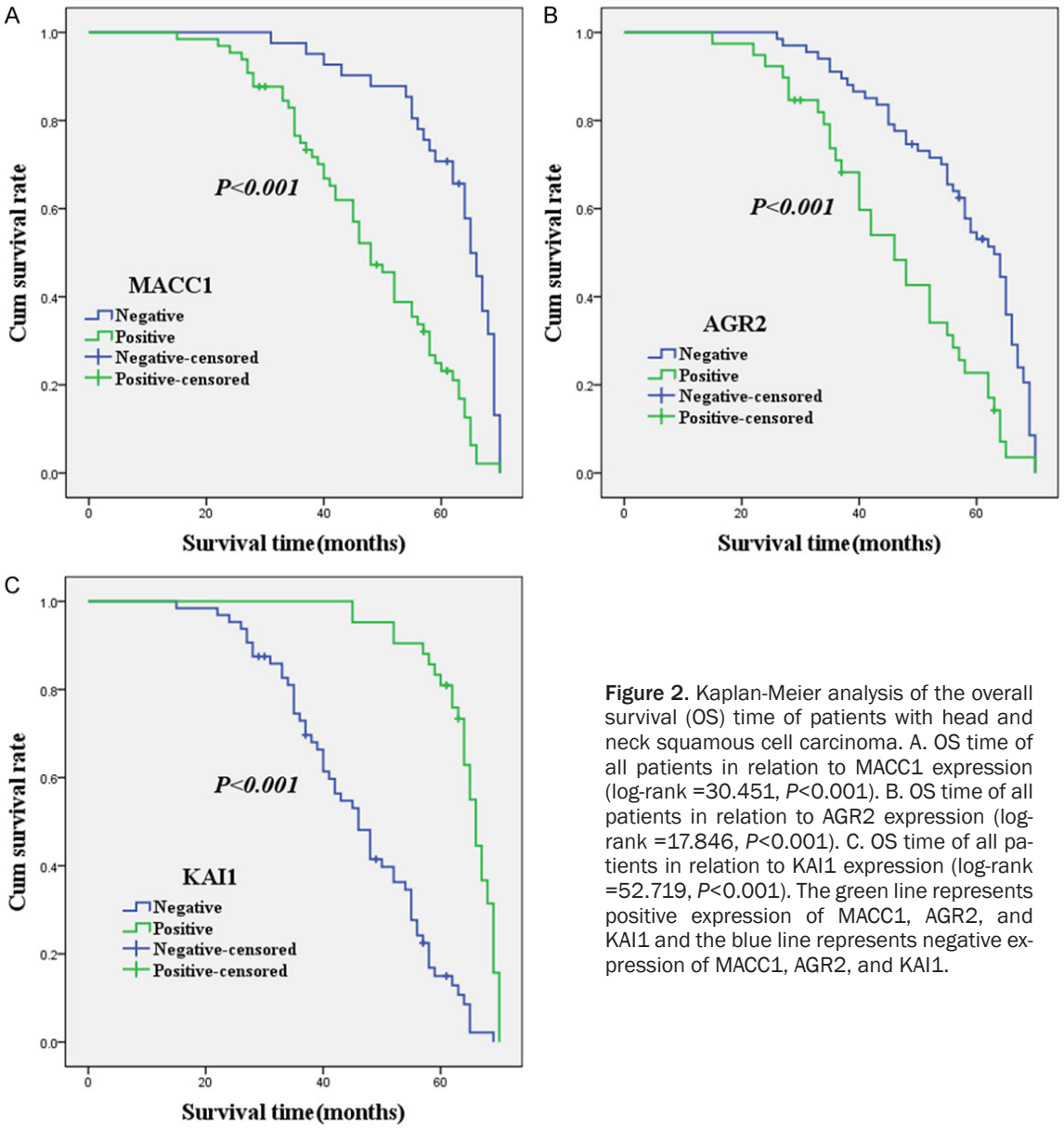

Figure 2. Kaplan-Meier analysis of the overall survival (OS) time of patients with head and neck squamous cell carcinoma. A. OS time of all patients in relation to MACC1 expression (log-rank =30.451, *P*<0.001). B. OS time of all patients in relation to AGR2 expression (log-rank =17.846, *P*<0.001). C. OS time of all patients in relation to KAI1 expression (log-rank =52.719, *P*<0.001). The green line represents positive expression of MACC1, AGR2, and KAI1 and the blue line represents negative expression of MACC1, AGR2, and KAI1.

KAI1 positive staining was mainly located in the membrane and cytoplasm of the cancer cells. The positive rate of KAI1 was significantly lower in HNSCC tissues (39.6%, 42/106) than that in the control tissues (94.3%, 100/106; *P*<0.05; **Figure 1E** and **1F**). The positive rate of KAI1 was negatively related with tumor size, grades, TNM stages, and LNM stages, but not with patient's age, gender, location, smoking, and alcohol (**Table 2**).

Associations between expression of MACC1, or AGR2, or KAI1 in HNSCC

Spearman coefficient analysis suggested that there was a negative association between KAI1 expression and that of MACC1 (r=-0.386, *P*<0.001), or AGR2 (r=-0.338, *P*<0.001) expression; and there was a positive association between MACC1 expression and AGR2 expression (r=0.204, *P*=0.036; **Table 3**).

Univariate and multivariate analyzes

Follow-up data suggested that OS was significantly lower in HNSCC patients with MACC1+ samples (46.5±13.5 months) compared with those with MACC1- (61.7±9.7 months; log-rank =30.451, *P*<0.001; **Figure 2A**). Similarly, OS of AGR2+ patients (44.5±14.2 months) was significantly shorter than those of AGR2- patients (56.9±12.2 months; log-rank =17.846,

Table 4. Results of multivariate analyses of overall survival (OS) time

Covariate	B	SE	P	HR	95% CI
MACC1	0.561	0.279	0.044	1.753	1.015-3.026
AGR2	0.661	0.290	0.023	1.937	1.096-3.423
KAI1	-0.826	0.347	0.017	0.438	0.222-0.863
TNM stages	0.568	0.289	0.049	1.765	1.002-3.110
LNM stages	1.351	0.466	0.004	3.861	1.549-9.620

P<0.001; **Figure 2B**). The OS of KAI1+ patients (64.0±6.3 months) was significantly higher than those of KAI1- patients (44.8±12.8 months; log-rank =52.719, *P*<0.001; **Figure 2C**).

Multivariate COX regression analysis indicated that positive expression of MACC1, AGR2, or KAI1, TNM stages, as well as LNM stages were independent prognostic factors of OS for HNSCC (*P*<0.05; **Table 4**).

Discussion

HNSCCs are highly heterogeneous tumors, which may influence the effectiveness of biomarkers assessment. So, prognostic and metastatic value of biomarkers should be thoroughly evaluated to ensure their effectiveness. Metastasis and recurrence are the main reason of cancer treatment failure. MACC1 is a candidate biomarker which was considered as a usefully prognostic and metastatic marker in various cancers. It was believed that MACC1 should be involved in many fundamentally biological processes of tumors, such as tumorigenicity, motility, proliferation, invasion, metastasis, as well as epithelial mesenchymal transition (EMT) [6, 7, 13, 32]. In this study, the positive expression of MACC1 in HNSCC tissues was significantly higher than that in the control tissues. Moreover, we found that overexpression of MACC1 was positively associated with tumor grade, TNM stages, and LNM stages. In univariate analysis, we found that OS of MACC1+ HNSCC patients was significantly lower than that for the MACC1- patients. Our results were similar to the previous studies of HNSCC [33, 34], which suggested that MACC1 should be considered as a useful biomarker for HNSCC.

EMT plays a critical role in normal development, inflammatory reaction, and tumor metastasis. Some studies have demonstrated that overexpression of AGR2 was associated with EMT process in tumors [35-37]. Furthermore, overexpression of AGR2 could promote tumor cells growth and metastasis and play an unfavorable role on the clinical outcome [20-26, 35]. In this study, we found that AGR2 expression in HNSCC tissues was significantly higher than that in the control tissues. Furthermore, we found that overexpression of AGR2 was positively associated with tumor grades, TNM stages, and LNM stages. Similar to MACC1, OS of AGR2+ HNSCC patients was significantly lower than that of the AGR2- patients. The same results were found in our study, which indicated that overexpression of AGR2 was closely linked to tumors progression and metastasis [20-26, 37].

Inactivation of suppressor gene of tumor metastasis plays an important role in the process of tumor metastasis. It is well known that KAI1 is a suppressor gene of tumor metastasis. KAI1 inhibits tumor metastasis by promoting cell to cell adhesion or inhibiting cell to extracellular matrix adhesion [38]. In current study, we found that the positive expression of KAI1 was significantly lower in HNSCC tissues than that in the control tissues. And its positive expression was negatively correlated with tumor size, grades, TNM stages, as well as LNM stages. In addition, Kaplan-Meier survival analysis suggested that HNSCC patients with positive expression of KAI1 had significantly higher survival time than did negative KAI1 patients. These results indicated that down or lost expression of KAI1 could accelerate tumor cells progression and metastasis, which were consistent with the previous studies [12, 27-31, 38].

In this study, multivariate COX regression analysis suggested that positive expression of MACC1, AGR2, and KAI1, TNM stages, as well as LNM stages are independent prognostic factors for HNSCC patients. Our results thus suggested that MACC1, AGR2, as well as KAI1 should be considered as useful biomarkers for HNSCC, especially in predicting metastasis and prognosis.

It is well known that the process of metastasis should include a serial of complicated process, such as inactivation of suppressor gene of tumor metastasis, activation of factors of tumor metastasis, and EMT. MACC1 can promote proliferation and metastasis of tumor cells by acti-

vating of HGF/C-MET signaling pathway [6, 7]. In this study, there was a negative association between MACC1 expression and KAI1 expression. The previous studies have demonstrated that KAI1 could form complex by bind to C-MET or inhibited activation of HGF [39, 40]. KAI1 could inhibit activation of MACC1 in order to inhibit the migration and motility of tumor cells [39, 40]. Moreover, KAI1 expression was also negatively associated with AGR2 expression. Overexpression of AGR2 can promote tumor cells proliferation, invasion, metastasis, as well as EMT. Normal expression of KAI1 can inhibit tumor cells EMT by strengthening β-catenin/E-cadherin complex [12, 31]. Aberrant expression of KAI1 may lose inhibition of tumor EMT, thus promote tumor cell invasion and metastasis. In the meantime, overexpression of MACC1 and AGR2 further promote tumor cells proliferation, invasion, metastasis, and EMT.

Conclusions

Our results suggested that aberrant expression of MACC1, AGR2, and KAI1 should be involved in the development of HNSCC. The combined detection of MACC1, AGR2, as well as KAI1 should be valuable as promising biomarker for metastasis, and thereby prognosis of HNSCC for patients.

Disclosure of conflict of interest

None.

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