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Correlation of matrix metalloproteinase-2, -9, tissue inhibitor-1 of matrix metalloproteinase and CD44 variant 6 in head and neck cancer metastasis*

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Abstract: This study aimed to explore the molecular mechanism in tumor invasion and metastasis. The expression of matrix metalloproteinase-2, -9 (MMP-2, MMP-9), tissue inhibitor-1 of matrix metalloproteinase (TIMP-1), cell adhesion molecule 44 variant 6 (CD44v6), HER2/neu and p53 was investigated in 154 patients with head and neck squamous cell carcinoma (SCC) by ABC and ImmunoMax immunohistochemical method. Their clinical relevance and correlation were analysed. The expression of MMP-2, MMP-9, TIMP-1, CD44v6, HER2/neu and p53 was found in cancer cells in 87.01%, 85.71%, 68.18%, 98.05%, 55.19% and 50.65% cases respectively. Linear regression and correlation analysis revealed that there was close positive relationship ($P<0.05$) between the expression of MMP-2 and MMP-9, TIMP-1 and CD44v6, HER2/neu and MMP-9, MMP-2 and p53. Up-regulation of MMP-2 was accompanied by advanced T stage ($P<0.01$). There was also a trend of MMP-2 expression being related with tumor metastasis. Increased expression of HER2/neu was found in patients with tumor recurrence ($P<0.05$). The expression of TIMP-1 was higher in laryngeal cancer than that in pharyngeal cancer, and higher in keratinizing and non-keratinizing SCC than that in basaloid SCC ($P<0.05$). These findings suggested that MMP-2 and MMP-9, HER2/neu and MMP-9, MMP-2 and p53 had a coordinate function in aggression of tumor; that MMP-2 had a more important function than MMP-9 in tumor invasion and metastasis; and that HER2/neu might serve as a biomarker for poor prognosis in HNSCC.

Key word: Head and neck cancer, Matrix metalloproteinase-2, -9 (MMP-2 and MMP-9), Tissue inhibitor-1 of matrix metalloproteinase (TIMP-1), Cell adhesion molecule 44 variant 6 (CD44 v6), HER2/neu, p53

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INTRODUCTION

Head and neck cancer squamous cell carcinoma (HNSCC) is the fifth most frequent malignant neoplasm world-wide; and is characterized by local invasion, dissemination to local lymph node and frequent recurrence at the primary site of tumour. Despite improvements in diagnosis and treatment of head and neck cancer, including surgery, irradiation and chemotherapy, 5-year or overall survival of these patients remained unchanged over the past 30 years (Parker *et al.*, 1997).

Invasion and metastasis continue to be the greatest barrier to cancer cure. Several steps are required in development of malignant tumour and its invasion and metastasis, involving proteolytic degradation of basement membrane and extracellular matrix (ECM), altered cell adhesion and physical movement of tumour cells. The first critical phase of tumour invasion and metastasis is the enzymatic degradation of the ECM (Liotta, 1986). Some of the main proteolytic enzymes degradating ECM are the Matrix Metalloproteinases (MMPs) which are also involved in cell invasion, migration and angiogenesis. MMPs comprise a family of zinc-dependent proteolytic enzymes, which collectively can degrade most, if not all, components of the basement membrane and ECM with overlapping specificity. There are currently at least 22 known human MMPs, with new members still being discovered. Among these,

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MMP-2, MMP-9 are particularly important in tumor invasion and metastasis, because they can degrade type IV collagen, which is a main structural component of the basement membrane. Increased expression, including mRNA and protein of MMP-2 and -9, has been detected in numerous solid organ cancer types, such as breast cancer, gastric cancer, colon cancer (Curran *et al*., 2000). Production and activity of MMPs can be regulated by tissue inhibitors of MMPs (TIMPs), cytokines, other proteases and growth factors, oncogenes, hormones and tumor promoters. To date, four TIMPs (TIMP-1 to TIMP-4) have been identified, which are the major natural inhibitors of MMPs and appear to block or retard MMP precursor activation. The ability of cancer cells to invade the surrounding stroma may depend on the balance of the synthesis of proteases and their inhibitors (Overall, 1994).

Cell adhesion molecule 44 variant 6 (CD44 v6) is an integral membrane glycoprotein, which serves as a major receptor of the leukocyte marker (or cell adhesion receptor) for hyaluronan (HA), a constituent of the extracellular matrix (Günthert *et al*., 1991). The human CD44 gene consists of at least 20 exons. CD44 v4-v7 play an important role in malignant transformation and metastasis. Cancer cells likely utilize a combination of CD44 properties to adhere to blood vessels; and are involved in tumor metastasis (Ostwarld *et al*., 1997). Understanding the function of CD44 in tumor aggression, then applying the knowledge gained to repel the tumor could severely inhibit the spread of the cancer.

Numerous publications reported finding of P53 gene mutations in the majority of human tumors. In fact, no human tumor type is without of p53 mutations, although differences had been seen in the relative frequencies and patterns of nucleotide substitutions (Theillet, 1993). p53 should be considered as a clinically relevant molecular marker in future clinical trials, and as a potentially useful biomarker for molecular epidemiological studies for further exploration of the correlation between the expression of p53 and clinical features.

The HER2/neu gene which exists in both proto-oncogenic and oncogenic forms, is located on the long arm of chromosome 17q and encodes a 185-KD protein (Akiyama *et al*., 1986). The HER2/neu protein is a transmembrane growth factor receptor, and a component of a four-member family of closely related growth factor receptors, including EGFR or HER-1; HER-2; HER-3 and HER-4 (Lupu *et al*., 1987). Initial studies evaluating the relationship between HER2/neu and invasion of breast cancer were focused on the identification of gene amplification in human tumor samples. Enhanced expression of HER2/neu is now known to be involved in many human cancers. It is necessary to ascertain the expression of HER2/neu and its association with prognosis in HNSCC.

In this study, the expression of MMP-2, -9, TIMP-1, CD44v6, HER2/neu and p53 was evaluated by immunohistochemistrial staining, the relationship between their expression and clinical features, and the correlation of these factors were analysed.

MATERIALS AND METHODS

From the file archive of the Department of Otorhinolaryngology in Lübeck Medical University, a total of 154 histologically examined and reportedly documented surgical specimens of HNSCC over a 10-year period (from 1989 – 1999) were evaluated. None of the patients had previously received irradiation or chemotherapy. Of the 154 patients, 21 were women, 133 were men. The mean age was 59.45 ± 10.71 years (range 24 – 92 years). The data collected for each patient included age, gender, drinking of wine, smoking, primary tumor site, primary tumor size (T_{1-4}), cervical node status (N_0 – N_3), distant metastasis (M_{0-1}), overall TNM stage (I-IV) (according to the UICC 1997 classification) and histological grade of tumor differentiation (G_{1-3}) and histological types, follow-up results. Our investigation was confined to oropharyngeal SCC, hypopharyngeal SCC and laryngeal SCC. Histological types of squamous cell carcinoma included non-keratinizing, keratinizing and basaloid SCC. All of the patients were followed up for average of 4.49 ± 1.98 years (range 2.50 – 10 years).

Antibodies

The monocloned antibodies, MMP-2, MMP-9, TIMP-1, CD44v6 and p53 were mouse immunoglobins IgG1; the polycloned antibody-HER2/

Table 1 Primary antibodies and pretreatment

Antibody(Ab)	clone of Ab		dilution	pretreatment
MMP-2	4 D3	mono, mouse	1:40	20' MW/CS immuno-Max
MMP-9	2 C3	mono, mouse	1:400	20' MW/CS immuno-Max
TIMP-1	7-6C1	mono, mouse	1:25	10' Protease K
CD44v6	VFF-7	mono, mouse	1:100	15' MW/CS
HER2/neu		poly, rabbit	1:200	15' MW/CS + 15' RT
P53	PAB 1801	mono, mouse	1:50	20' MW/CS

MW-microwave, RT-room temperature.

neu was rabbit anti-human immunoglobin IgG1. (NOVO Castra Laboratories Ltd, UK)(table 1).

Immunohistochemistry

Tumor tissue samples were fixed in 10% buffered formalin and embedded into paraffin in a routine manner as surgical pathology specimens. Two to three cm paraffin-embedded sections were deparaffinized in three changes of xylol and rehydration by a gradient of ethanol. Slides were treated for antigen retrieval with microwave (0.01 mol/L citric acid buffer, pH 6.0), and 25% protease K. Three steps of the avidin-biotin-peroxidase complex (ABC) reaction were performed. Additionally, some slides were accompanied by immunoMax method (Merz *et al.*, 1995) in 7.5% H_2O_2 for 10 minutes to block endogenous peroxidase activity. All slides were placed in humidified chambers and incubated with the primary antibodies for 30 minutes, followed by incubation with 1:250 diluted biotinylated rabbit anti-mouse immunoglobin G1 antiserum for 30 minutes. After washing with Tris-buffer, some slides for use in immunoMax method were incubated with ABC complex / HRP (DAKO, A/S Glostrup, Denmark) for 20 minutes, then incubated with biotinyl-tyramine [1:100 diluted with Tris-buffer + 35% H_2O_2 (1:1000 diluted with Tris-buffer)] for 10 minutes. In the last step, all slides were incubated with ABC complex /AP (DAKO, A/S Glostrup, Denmark) for 30 minutes (Table 1). After washing, immunoreaction was colorefully developed with APAAP (chromogenic alkaline phosphatase substrate solution) for 30 minutes. After counterstaining with hematoxylin for 3 minutes, these specimens were observed under a light microscope.

Control test: Each antibody in different tissue samples was choosen for positive control, colonic adenocarcinoma for MMP-2, MMP-9, TIMP-1 and p53, breast cancer for HER2/neu, and tonsil tissue for CD44v6, on the basis of a definite positive reaction.

The primary antibodies were substituted by normal rabbit serum for monoclonal antibody or normal mouse serum for polyclonal antibody as a negative control.

Evaluation of staining results: Fields were randomly selected throughout each slide and the average 10 representative fields each slide were examined by two qualitified doctors. Semi-quantitative four grades were determined. − no positive cancer cells; + 0 − 25% tumor positive cell but faintly perceived; ++ 25 − 80% tumor positive cell from weak to moderate staining; +++ > 80% tumor positive cell and strong staining.

Statistical analysis: All obtained data were processed by SPSS version 9.0 software. Chi-square test and Fisher's exact test were used to evaluate the relationship between the expression of MMP-2, MMP-9, TIMP-1, CD44v6, HER2/neu, p53 and all clinical features. The correlation of the expression of MMP-2, MMP-9, TIMP, CD44v6, HER2/neu and p53 was analysed by multiple linear regression. Kaplan-Meier method was used to generate the actual survival curve, and the predicted expression of MMP-2, MMP-9, TIMP-1, CD44v6, HER2/neu, p53 was analysed by Log Rank test. $P < 0.05$ were considerated significant.

RESULTS

Expression of MMP-2, MMP-9, TIMP-1, CD44v6, HER2/neu and p53 in primary HNSCC

MMP-2 expression was found at significant

levels in 134 of 154 (87.01%) cases with HNSCC (Fig.1). The immunoreaction was confined to the cytoplasm, partly to the membrane of cells, and present in a large proportion of the cancer cells, and in some stromal cells surrounding cancer nests, and endothelial cells (Fig.2a).

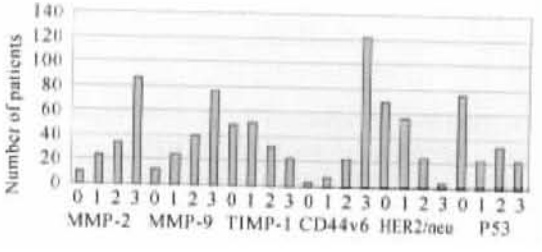

Fig.1 Expression of MMP-2, MMP-9, TIMP-1, CD44v6, HER2/neu and P53 in primary HNSCC. 0: −; 1:+; 2: ++; 3: +++.

Immunoreactivity for MMP-9 was shown in most of cases (132/154, 85.71%) with HNSCC (Fig.1). The staining was located in the cytoplasm, partly in membrane of cells. The distribution was found mainly in cancer cells, and some in stromal cells surrounding cancer nests (Fig.2b).

The expression of TIMP-1 was revealed in 105 of 154 cases (68.18%), only 22 cases (14.29%) strongly stained (Fig.1). The immunoreaction found within the cytoplasm was very obvious in both cancer cells and stromal cells surrounding cancer nests (Fig.2c).

Overexpression of CD44v6 was shown in nearly all cases (151/154, 98.05%), 122 cases (79.22%) were strongly positive (Fig.1). The staining was confined to the membrane of cells and present in cancer cells and normal surface epithelial cells, in particular, strong in the spinous layers of normal stratified squamous epithelium, but keratin pearls and keratin layer of the epithelium were negative (Fig.2d).

The expression of HER2/neu was shown in 85 of 154 cases (55.19%), Few cases (4/154, 2.60%) were strongly positive (Fig.1). The staining was confined to the membrane of cells and mainly present in cancer cells, but not in normal cells and keratin pearls (Fig.2e).

The expression of p53 was shown in 78 of 154 cases (50.65%), only 22 cases (14.29%) were strongly positive (Fig.1). The immunoreaction was confined to the nucleus in all cancer cells (Fig.2f).

Relationship of the expression among MMP-2, MMP-9, TIMP-1, CD44v6, HER2/neu and p53

The relationship of the expression among MMP-2, MMP-9, TIMP-1, CD44v6, HER2/neu and p53 was analysed by multiple linear regression. There was a very close linear positive correlation between the expression of MMP-2 and MMP-9, TIMP-1 and CD44v6, HER2/neu and MMP-9, p53 and MMP-2, $P < 0.01$, $P < 0.05$, $P < 0.01$, $P < 0.01$ (Table 2).

Table 2 Analysis of multiple linear regression and correlation between the expression of MMP-2, MMP-9, TIMP-1, CD44v6, HER2/neu and P53

Correlative item	*N*	Regression equation	*F*	*P*
MMP-9 and MMP-2,	154	MMP-9 = 0.262MMP-2 + 2.321	10.580	0.001
MMP-9 and Her2/neu	154	MMP-9 = 0.261HER2/neu + 2.720	7.642	0.006
MMP-2 and p53	154	MMP-2 = 0.151p53 + 2.494	3.976	0.002
TIMP-1 and CD44v6	154	TIMP-1 = 0.265CD44v6 + 1.195	4.250	0.041

Relationship between the expression of MMP-2, MMP-9, TIMP-1, CD44v6, HER2/neu, p53 and clinical features

The expression of MMP-2 was higher accompanied with the advanced *T* stage, $P = 0.025 < 0.05$, the lowerest rate was found in MMP-2-negative patients with T1 (1/154, 0.64%), the highest in MMP-2-overexpression patients (30/154, 19.49%) with T4. There was a trend that over-expression of MMP-2 was related with tumor metastasis, $P = 0.056$. The percent of MMP-2 expression was 100% in patients with distant metastasis (Table 3).

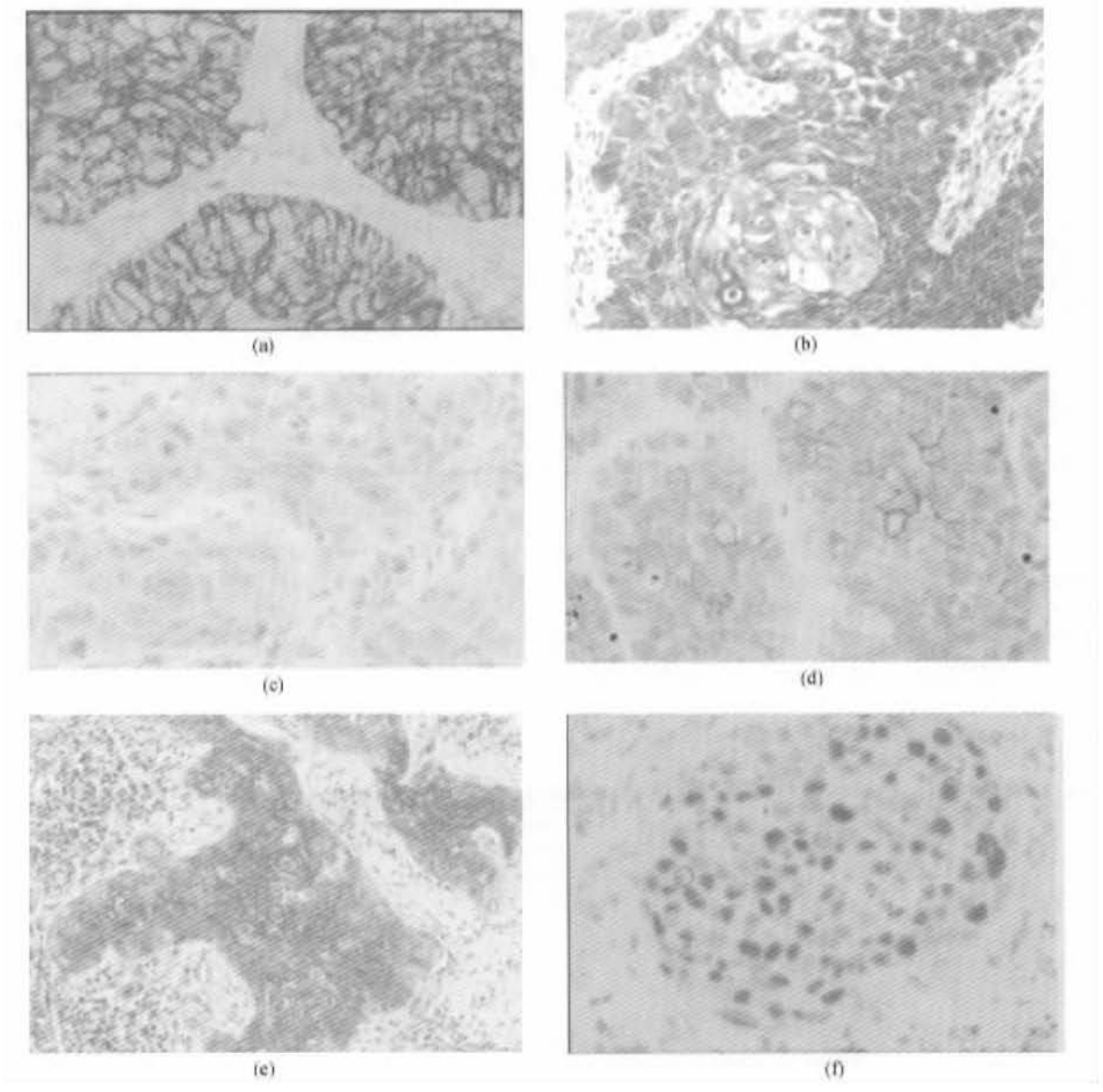

Fig.2 Immnohistochemical demonstration (a) MMP-2 in a case with moderately differentiated, basaloid squamous cell carcinoma in the glottic larynx. A strong expression of MMP-2 is confined to the cytoplasm both in cancer cells and stromal cells (original x400); (b) MMP-9 in a case with moderately differentiated, basaloid squamous cell carcinoma in the subglottic larynx. A strong expression of MMP-9 is confined to the cytoplasm both in cancer cells and stromal cells (original x400); (c) TIMP-1 in a case with moderately differentiated, keratinized squamous cell carcinoma in the subglottic larynx. A moderately positive staining of TIMP-1 is located in the cytoplasm of cancer cells (original x400); (d) CD44v6 in a case with poorly differentiated squamous cell carcinoma in the hypopharynx. A moderately positive staining of CD44v6 is located mainly in the membrane of cancer cells (original x400); (e) HER2/neu in a case with moderately differentiated, keratinized squamous cell carcinoma in the oropharynx. A strongly positive staining of HER2/neu is located mainly in the membrane of cancer cells (original x200); (f) p53 in a case with moderately differentiated, non-keratinizing squamous cell carcinoma in the subglottic larynx. A moderately positive staining of p53 is confined to the nucleus of cancer cells, not to normal tissue cells (original x400).

Table 3 TNM stage and the expressions of MMP-9, MMP-2, TIMP-1, CD44v6, HER2/neu and p53

	N	MMP-9				MMP-2				TIMP-1				CD44v6				HER2/neu				p53			
		−	+	++	+++	−	+	++	+++	−	+	++	+++	−	+	++	+++	−	+	++	+++	−	+	++	+++
T1	23	1	3	8	11	1	2	10	10	6	11	3	3	0	0	3	20	8	10	5	0	13	3	4	3
T2	47	2	8	11	26	2	5	6	34 *	20	13	7	7	3	4	5	35	21	17	7	2	27	6	9	5
T3	30	2	6	8	14	1	9	7	13	7	11	7	5	0	2	4	24	12	10	6	2	11	8	8	3
T4	54	7	8	13	26	6	7	11	30	16	16	15	7	0	1	10	43	28	29	7	0	25	5	13	11
N0	65	5	14	17	29	2	10	14	39	17	27	12	9	0	1	8	56	28	28	8	1	35	10	12	8
N1	14	2	1	3	8	1	3	2	8	4	4	4	2	0	1	3	10	6	7	1	0	7	1	3	3
N2	69	4	9	19	37	7	9	16	37	27	19	14	29	3	5	9	52	32	21	13	3	33	10	17	9
N3	6	1	1	1	3	0	1	2	3	1	1	2	2	0	0	2	4	3	0	3	0	1	1	2	2
M0	148	11	25	38	24	10	23	30	85	49	47	31	21	3	7	20	118	66	54	24	4	72	22	33	21
M1	6	1	0	2	3	0	0	4	2	0	4	1	1	0	0	2	4	3	2	1	0	4	0	1	1

$X^2 = 19.057$, $P = 0.025 < 0.05$

The expression of TIMP-1 was significantly higher in the group of laryngeal SCC (54/70, 77.14%) than in pharyngeal SCC (51/84, 60.71%), $P = 0.022 < 0.05$. The over-expression of HER2/neu was found in oropharyngeal SCC (37/56, 66.07%) compared to hypopharygeal SCC (11/28, 39.29%), $P = 0.018 < 0.05$. There was statistically significant difference between p53 expression and different tumor sites, the highest in subglottic SCC (8/9, 88.88%), then glottic SCC (21/36, 58.33%), supraglottic SCC (9/25, 36%), oropharyngeal SCC (29/56, 51.79%), hypopharyngeal SCC (11/28, 39.29%), $P = 0.045 < 0.05$ (Table 4).

Table 4 Expression of MMP-2, MMP-9, TIMP-1, CD44v6, HER2/neu and p53 in different sites of head and neck cancer

	N	MMP-9		MMP-2		TIMP-1		CD44v6		HER2/neu		p53	
		−	+	−	+	−	+	−	+	−	+	−	+
Oro-P	56	5	51	4	52	20	36	2	54	19	37	27	29
Hypo-P	28	2	26	3	25	13	15	1	27	17	11 *	17	11
supra-G	25	4	21	2	23	6	19 *	0	25	14	11	16	9 *
Glottis	36	1	35	0	36	7	29	0	36	15	21	15	21
Sub-G	9	0	9	1	8	3	6	0	9	4	5	1	8

$P < 0.05$. Oro-P: oropharynx, Hypo-P: hypopharynx, Supra-G: supraglottis, Sub-G: subglottis.

The p53 expression was higher in patients 60 years old or over (46/70, 60.53%) than those under 60 years old (32/75, 42.67%), $P = 0.038 < 0.05$ (Fig. 8). The expression of CD44v6 was higher in smokers than in non-smokers, $P = 0.020 < 0.05$ (Fig. 9). There was no statistical difference between the expression of MMP-2, MMP-9, TIMP-1, CD44v6, HER2/neu, p53 and drinking wine, $P > 0.05$.

There was no statistical difference between the TIMP-1 expression and gender, age, T stage, lymph node status, distant metastasis, $P > 0.05$. There was no statistical difference between the expression of MMP-9, CD44v6 and clinical features, $P < 0.05$.

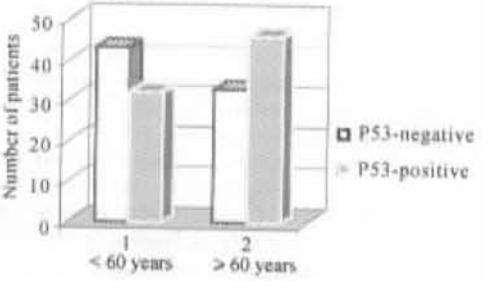

Fig. 8 The p53 expression is higher in patients 60 years old or over (46/70, 60.53%) than those under 60 years old (32/75, 42.67%), $P = 0.038 < 0.05$.

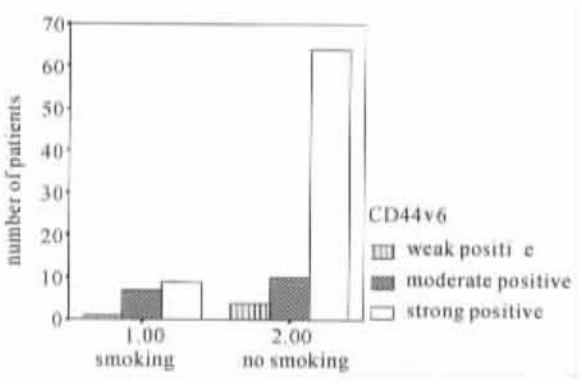

Fig. 9 The expression of CD44v6 was higher in smokers than in non-smokers, $P = 0.020 < 0.05$.

Relationship between the expression of MMP-2, MMP-9, TIMP-1, CD44v6, HER2/neu, p53 and histological grade and types

Table 5 shows that the frequency of TIMP-1 expression was higher in the non-keratinizing group (38/54, 70.37%) and keratinizing group (59/82, 71.95%) than in the basaloid SCC group (8/18, 44.44%), $P = 0.027$ and $P = 0.047$ respectively. No relationship was found between the expression of MMP-2, MMP-9, CD44v6, HER2/neu, p53 and histological grade and types, $P > 0.05$.

Table 5 Histological findings and expression of MMP-9, MMP-2, TIMP-1, CD44v6, HER2/neu, p53

	N	MMP-9		MMP-2		TIMP-1		CD44v6		HER2/neu		p53	
		−	+	−	+	−	+	−	+	−	+	−	+
Histological stage													
G1	2	0	2	0	2	0	2	0	2	1	1	2	0
G2	86	9	77	4	82	26	60	2	84	37	49	41	45
G3	66	3	63	6	60	23	43	1	65	31	35	33	33
Histological types													
N-K	54	6	48	3	51	16	38	1	53	25	29	31	23
K	82	6	76	5	77	23	59*	1	81	36	46	36	46
B	18	0	18	2	16	10	8*	1	17	8	10	9	9

* $P = 0.048 < 0.05$. N-K: non-keratinizing SCC, K: keratinizing SCC, B: basaloid SCC.

Relationship between the expression rate of MMP-2, MMP-9, TIMP-1, CD44v6, HER2/neu, p53 and outcome of treatment

There were 107 cases (69.48%) without tumor recurrence, 36 (23.38%) cases with tumor recurrence, 11 (7.14%) cases death. The average time of recurrence was 2.26 ± 1.13 years (range 1-4 years). Increased expression of HER2/neu was found in tumor recurrence compared to those without tumor recurrence, $P = 0.018 < 0.05$ (table 6). There was no significant difference in relationship between the expression rate of MMP-2, MMP-9, TIMP-1, CD44v6 and outcome of treatment (no-recurrence, recurrence and death), $P > 0.05$.

Table 6 Clinical outcome of treatment and expression of MMP-9, MMP-2, TIMP-1, CD44v6, HER2/neu and p53

	N	MMP-9		MMP-2		TIMP-1		CD44v6		HER2/neu		p53	
		−	+	−	+	−	+	−	+	−	+	−	+
No R	107	8	99	8	99	36	71	3	104	53	54	57	50
R	36	2	34	2	34	11	25	0	36	10	26*	16	20
Death	11	2	9	0	11	2	9	0	11	6	5	3	8

* $P = 0.018 < 0.05$. No R: no recurrence, R: recurrence.

There was no statistical difference between overall survival time and expression of MMP-9, TIMP-1, MMP-2, CD44v6, HER2/neu, p53 by Log Rank test, $P = 0.101$, $P = 0.569$, $P = 0.505$, $P = 0.775$, $P = 0.286$, $P = 0.0504$, respectively (Fig. 10). There was a trend that up-regulation of p53 was associated with a decreased probability of survival, $P = 0.0504$.

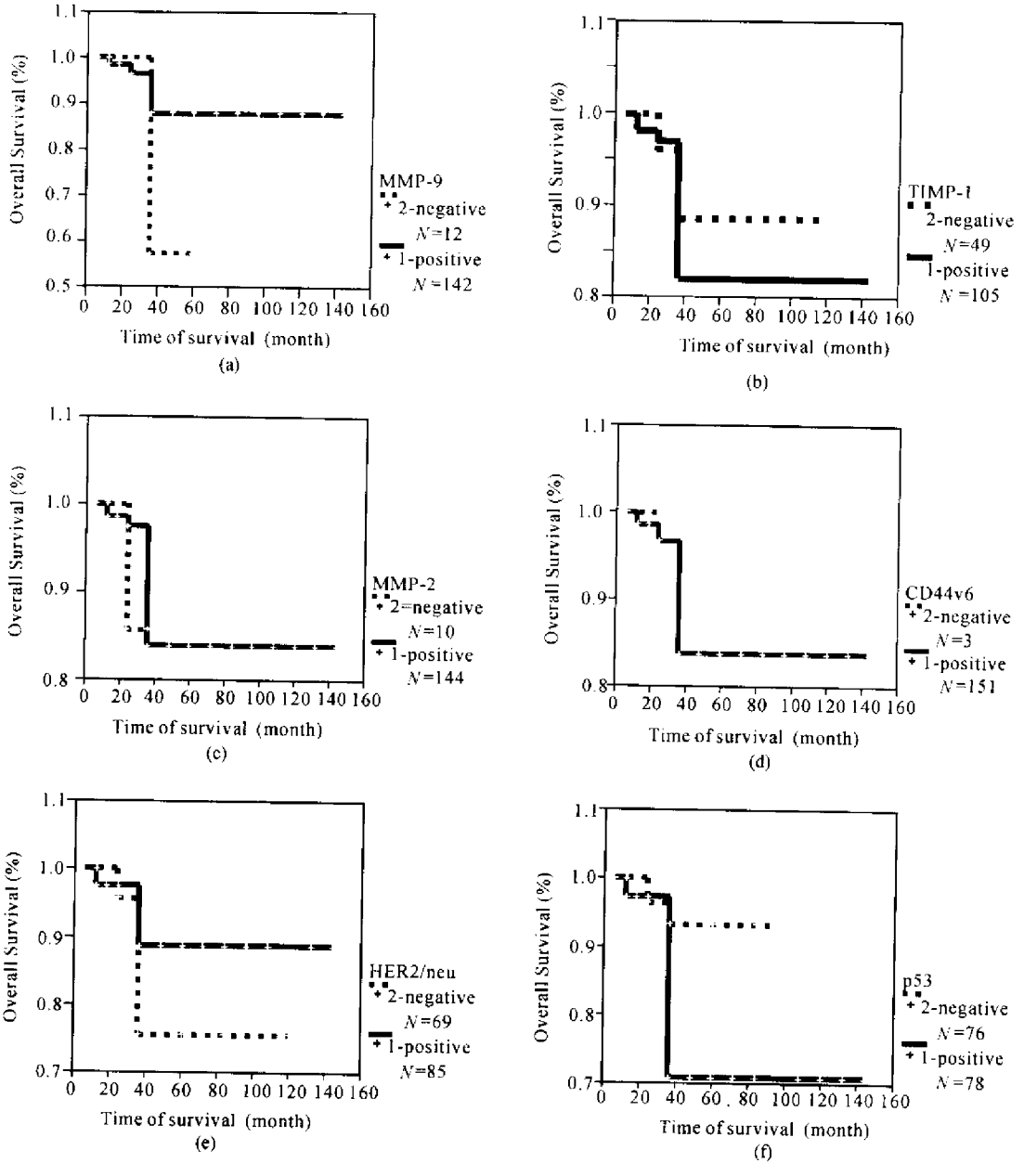

Fig. 10 Overall survival for patients with and without the expression of MMP-9, TIMP-1, MMP-2, CD44v6, HER2/neu, p53. There was no statistical difference between the overall survival and the expression of all factors by Log-Rank test, (a) $P = 0.101$; (b) $P = 0.569$; (c) $P = 0.505$; (d) $P = 0.775$; (e) $P = 0.286$; (f) $P = 0.0504$, respectively.

DISCUSSIONS

Our data showed that overexpression of MMP-2 and MMP-9 occurred in the majority of HNSCC, which provided a higher positive rate than that was revealed by using in situ hybridization technique in oral SCC (Hong *et al*., 2000), in hypopharyngeal carcinoma (Miyajima *et al*., 1995). MMP-2, -9 was expressed in a

large proportion of tumor cells, the stromal cells and endothelial cells in HNSCC. Some authors reported that the expression of MMP-2 and MMP-9 mRNAs was revealed in stromal cells but not detectable in nests of human breast cancer (Brummer *et al.*, 1999), colon cancer (Ornstein *et al.*, 1999). It was suggested that cancer cells not only secreted MMP-2, -9, but also induced stromal cells surrounding cancer nests to produce these proteinases by secreting some paracrine substances. The expression of MMP-2, -9 in endothelial cells may be involved in proteolytic degradation of basement membrane and ECM in vessels, then cancer cells invading the blood circulation by the lymphatic system or directly metastasizing into the distant organs; which may be involved in angiogenesis and facilitate the growth of second tumor.

The up-regulation of CD44v6 was shown in nearly all cases (98.05%), most of the cases were strongly immunoreactive. Down-regulated expression of CD44v6 in premalignant epithelial changes with early features of invasion occurred (Bahar *et al.*, 1997). So, CD44v6 may help to show the risk of transformation of benign or precancerous lesion to cancer. In the study, the expression of CD44v6 was higher in smokers than that in non-smokers, $P < 0.05$. It seemed that smoking could induce the over-expression of CD44v6 to increase cancer cell-cell or cell-ECM adhesion, which is involved in tumor invasion.

The study revealed a closely linear positive relationship between the expression of MMP-2 and MMP-9, $P < 0.01$. As we know, tumor invasion and metastasis require proteolysis of extracellular matrix macromolecules, MMP-2 and MMP-9 are the main enzymes that degrade the extracellular matrix (Brummer *et al.*, 1999). It seemed that both of them have a coordinate function in tumor aggression. Up-regulation of MMP-2 and MMP-9 was a good indicator of the invasion and metastatic potential of HNSCC. There was significant correlation between the over-expression of MMP-2 and advanced T stage, $P < 0.05$. MMP-2 was expressed in 100% of cases with later stage of lymph node metastasis (N3) and distant metastasis. There may be a trend that the MMP-2 expression was associated with tumor metastasis. No significant difference was found by comparing the MMP-9 expression with clinical stage, $P > 0.05$. So MMP-2 may play a more important role than MMP-9 in tumor invasion and metastasis in HNSCC.

It was interesting that TIMP-1 expression had a linear positive correlation with CD44v6 expression, $P < 0.05$. Additionally, there was no relationship between the expression of MMP-2, -9 and TIMP-1, $P > 0.05$. Recent study demonstrated that CD44v6 was related with a proteolytic form of MMP-9 on the cell surface of mouse mammary carcinoma and human melanoma; which indicated that CD44 serves to anchor MMP-9 on the cell surface and define a mechanism for CD44-mediated tumor invasion. Possibly, the correlation between CD44 and MMP-9 provided a link between cell adhesion to ECM, ECM degradation and HA metabolism (Yu *et al.*, 1999). As a functional inhibitor of tumor invasion and metastasis, TIMP-1 should play an important function in regulating the activity and inhibiting fully activated individual MMPs. The imbalance between TIMP-1 and MMPs activities would result in ECM destruction and tumor invasion. So, TIMP-1 expression may be reflective of a secondary event and be a response to elevated production of CD44v6 expression, otherwise, tumor cells may be more invasive. Based on the study, there was no statistically correlation between the expression of TIMP-1 and CD44v6 and clinical stage. It is suggested that TIMP-1 and CD44v6 may serve as independent prognostic factors in HNSCC.

Also we found there was a close linear positive correlation between the HER2/neu expression and the MMP-9 expression, $P < 0.01$. Tan *et al.* (1997) detected higher enzyme activities of type IV collagenases (MMP-9 and MMP-2) accompanied by up-regulation of HER2/neu in human breast cancer cell lines (Tan *et al.*, 1997). Furthermore, up-regulation of MMP-9 (not MMP-2, and together with down-regulation of TIMP-1) via an autocrine EGFR (an 82% sequence homology with HER2/neu proteins in the tyrosine residues at the C-domains) signaling pathway may play an important role in the invasive behavior of HNSCC cells (O-charoentat *et al.*, 2000). It is suggested that the overexpression of HER2/neu in human cancer cells (inluding HNSCC) may enhance the metastatic potential and invasive ability, which was accompanied by increased secretion of basement membrane-degrading MMPs. So the relationship be-

tween MMP-9 and HER2/neu provides an interesting object of further research in vivo with reference to tumor invasion and metastasis.

HER2/neu expression was increased in the group of tumor recurrence as compared to the group of no-recurrence, $P < 0.05$. Similar reports proved that the overexpression of HER2/neu oncoprotein was significantly correlated with shorter overall survival and lymph node stage and metastasis in oral SCC (Xia *et al*., 1997). The HER2/neu expression and positive lymph node status were significantly associated with distant metastasis in supraglottic squamous cell carcinoma (Weinstein *et al*., 1996). Tantawy *et al*. (1999) found a significant relationship between the overexpression of HER2/neu and cartilage invasion, it was demonstrated that overexpression of HER2/neu oncoprotein was related to the aggressiveness of tumors with high capability of invading laryngeal cartilages, while, the overexpression of HER2/neu was not significantly related to the clinical data (Tantawy *et al*., 1999). Forth-six percent of patients with HER2/neu gene amplification developed recurrence and 38% died of their diseases, whereas only 28% of those patients without amplification developed recurrence and 13% died of their diseases (Ro *et al*., 1989). So, the overexpression of HER2/neu oncoprotein may affect the growth of tumor cells themselves, contribute to neovascularization of solid tumor and enhance metastatic potential and invasive ability of tumors. The expression of HER2/neu may serve as a biomarker of poor prognosis in HNSCC.

In our cases, the expression of p53 mutational gene was found in 78 of 154 cases (50.65%), which compared well with the rate of 44% – 73% in previous reports (Raybaud-Biogene *et al*., 1996). Also, it was found that the expression of MMP-2 and p53 revealed a close correlation by multiple linear regression analysis, $P < 0.01$. Combined with the study, there was a trend that up-regulation of p53 was associated with a decreased probability of survival, $P = 0.0504$. This correlation indicated that: i) The up-regulation of p53-mutation gene may induce cancer cells to produce MMP-2. ii) MMP-2 can promote tumor invasion by degrading ECM, while, the mutation of p53 gene might stimulate tumor angiogenesis and tumor progression (Vermeulen *et al*., 1996). Therefore, the co-ordinate function of p53 and MMP2 may enhance tumor metastatic potential, the co-expression of p53 and MMP-2 should be considerated as a poor prognostic marker in HNSCC.

We found the expression of TIMP-1 was higher in laryngeal cancer (77.14%) than in pharyngeal cancer (60.71%), $P < 0.05$. The possible explaination may be that TIMP-1 plays a major function of counteracting tumor invasion and metastasis induced by MMPs in larynx rather than in pharynx, so, prognosis of laryngeal cancer is usually better than that of pharyngeal cancer.

The expression of TIMP-1 was higher in keratinizing SCC (71.95%) and non-keratinizing SCC (70.37%) than that in basaloid SCC (44.44%), $P < 0.05$, which accorded with the finding that TIMP-1 mRNA was detected in well differentiated epidermoid head and neck cancer (Polette *et al*., 1996). It is suggested that down-regulation of TIMP-1 provides lower ability of inhibiting MMPs and enhances tumor metastasis in basaloid SCC, which may be more aggressive in HNSCC.

CONCLUSIONS

MMP-2 and MMP-9 have a co-ordinate function to enhance tumor invasion and metastastic potential, while, MMP-2 plays a more important role in tumor invasion and metastasis than MMP-9. Increased expression of HER2/neu may serve as a biomarker of poor prognosis in HNSCC. The co-expression of p53 and MMP-2 should be considerated as a poor prognostic marker in head and neck squamous cell carcinoma. TIMP-1 and CD44v6 may be considered as independent prognostic factors in head and neck squamous cell carcinoma. TIMP-1 appears to be a crucial factor inhibiting tumor invasion and metastasis in laryngeal or no-basaloid cancer.

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