Histol Histopathol (2008) 23: 1213-1222

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Expression profile of metastasis-related genes in invasive oral cancers

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Summary. We used suppression subtractive hybridization (SSH) and oligonucleotide microarray to differentiate expression profiles of metastasis-related genes and to evaluate their clinical significance in patients with invasive oral cancer (OCa). Overexpression of the specific genes was confirmed by reverse transcription-PCR (RT-PCR). Cells expressing the gene were identified by immunohistochemistry in pathology specimens. Clinical correlation and significance were analyzed statistically. Using these methods, we detected increased expressions of MMP-1, -3, -7, -9, -10 and interleukin (IL)-8 in invasive OCa. Moreover, our data showed that overexpressions of MMP-1, -3, -7, -10 and IL-8 were associated with reduced survival.

Key words: Oral cancer, Matrix metalloproteinase, Prognosis

Introduction

The dramatic increase of incidence [from $7.3/10^5$(1995) to $29.0/10^5$ person-year (2005)] and death rate [from $4.0/10^5$ (1995) to $7.82/10^5$ person-year (2005)] of patients with oral cancer (OCa) over the last decade in Taiwan has attracted medical, economic and social attention (Annual Reports, 2006). The decrease of average age [from 57 (1995) to 48 year-old (2005)] and the increase of cancer deaths in men were even more striking [from $7.58/10^5$ (1995) to $14.3/10^5$ person-year (2005)] (Chen et al., 1999). Although surgery is a curative treatment for early stage OCa, most patients presented with advanced disease. The poor prognosis in patients with advanced disease is compounded with rapid growth and wide-spread metastasis, as well as with radiation-resistance, drug-resistance, and the early recurrence of cancer cells (Chen et al., 1999; Tralongo et al., 1999; Shintani et al., 2003; Ziober et al., 2006a,b). The effort of multiple therapeutic modalities, however, was not favourable for these patients (Chen et al., 1999; Ziober et al., 2006a,b). Therefore, to identify factor(s) that is/are closely associated with disease progression and can predict metastatic potential of cancer cells is important in order to initiate suitable treatment against these tumor characteristics and to increase survival and life quality of patients as well.

Advances in epidemiology and molecular biology have shown that oncogenes, such as $p21^{ras}$, c-erbB-2, c-erbB-3 and HER-2/neu (Hou et al., 1992; Kuo et al., 1995; Shintani et al., 1999; Khan et al., 2002; Caulin et al., 2004), are frequently detected in oral cancers. Moreover, risk factors in life style, e.g., betel quid chewing, smoking and alcohol consumption, are major causes that may fortuitously activate oncogene expression. These results support a general concept that although carcinogenesis is a result of cumulative genetic alterations, the disease progression, on the other hand, could be a concerted outcome of tumor-matrix interaction, as well as pathophysiological dysregulations that further augment oncogene expression and the aberrant cell growth (Wang et al., 2004). To identify and to characterize genes that may coordinately constitute a malicious ring for cancer progression, however, remain as major task for understanding the nature of the disease and to design the most effective cancer management.

By applying suppression subtractive hybridization (SSH) and microarray, Lin et al identified interesting gene expression patterns of matrix metalloproteinase (MMP) between non-metastatic and metastatic non-small cell lung cancer (NSCLC) (Lin et al., 2004). Among these, increased expressions of MMP-1, -2, -7,

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-8 and -10 were most prominent in metastatic NSCLC. Moreover, overexpressions of MMP-1 and -2 were two independent factors for patients with poor prognosis. In fact, MMP, an endopeptidase that degrades extracellular matrix (ECM), could play a critical role in the metastasis of tumor cells (Egeblad and Werb, 2002). Tumor cells that produce enzymes to break the ECM obstacle in order to invade the surrounding tissues and the distal organs may have more survival advantages (Yu and Stamenkovic, 2000; Sternlicht and Werb, 2001; Yu et al., 2002). Since MMPs are frequently up-regulated in human tumors, and MMPs have been shown to damage structural components of ECM, it is reasonable to ask whether expression of MMPs could be a prognostic index in patients with OCa. Although expression of MMP-1, -2 and -9 has been reported in oral squamous cell cancer (Nagata et al., 2003; Kim et al., 2006; Ziober et al., 2006a,b), other MMPs have not been broadly explored.

In this study, we combined SSH and microarray to determine the expression pattern of metastasis-related genes in patients with invasive OCa. The results were verified by reverse transcription-polymerase chain reaction (RT-PCR). Expression of the specific gene was determined by immunohistochemistry in pathological samples. Correlation of prognostic significance and clinicopathological parameters with the respective gene were evaluated statistically.

Materials and methods

Patients and tissue samples

From January 1990 to December 1995, 185 patients who were diagnosed as having oral cancers were enrolled in the study. Because of the distinct features of disease, patients with cancer originating from the tongue were excluded from the study. The stage of the disease was classified according to the TNM system for oral cancer (American Joint Committee for Cancer Staging, Chicago, American Joint Committee on Cancer, 1988). However, 68 patients received only radiation and/or chemotherapy, and 117 patients underwent surgical resection (with 1.5 to 2 cm margins guided by frozen section examination during operation). Tumor size, lymph node involvement, cell differentiation, lymphovascular invasion and mitotic number were evaluated in these patients. Patients with evident lymph node involvement, perineural invasion, lymphovascular permeation, extracapsular and multiple-site spreading were irradiated with 60-70 Gy at the affected areas (92 patients). Patients beyond stage III were supplemented with chemotherapy (85 patients). After treatment, patients were routinely followed every 3 to 6 months. Tumor recurrence in the head and neck and distal metastasis were identified when visual examination, whole body bone scan, magnetic resonance imaging (MRI) and computerized tomography (CT) showed suspected evidence of the disease and the disease was confirmed by a pathology tissue proof.

RNA extraction, cDNA Synthesis, SSH, Microarray and RT-PCR

RNA extraction and gene amplification have been described previously (Lin et al., 2004; Chen et al., 2006). Briefly, following RNA extraction, the first-strand cDNA was synthesized by using 3' SMART CDS primer IIA 5'-AAGCAGTGGTATCAACGCAGAUGTAC UTNB-$_1$BN-3' (*Rsa* I restriction enzyme site is underlined, NB-1B = A, G or C, and N = A, G, C or T; BD, Franklin Lakes, NJ) and AMV reverse transcriptase. The second-strand was synthesized by using SMART IIA primer 5'-AAGCAGTGGTATCAACGCAGA UGTACUGCGGG-3' and T4 DNA polymerase to form double-strand DNA (ds cDNA). An aliquot of ds cDNA was then amplified with primer 5'-AAGCAG TGGTATCAACGCAGAUGTACU-3' in a polymerase chain reaction (PCR). An equal amount of the amplified products from five patients with the same disease stage was pooled and digested with restriction enzyme Rsa I. The restriction products were ligated to two specific adaptors (Tagged-pool, http://www.bdbiosciences.com/clontech). After performing forward SSH against cDNA pool from non-tumor oral tissue (NTOT) to exclude genes that were commonly expressed in both tumor and non-tumor parts, the reaction mixtures were subjected to 35 cycles of PCR using standard procedure, denaturing at 94°C for 45 seconds, hybridizing at 56°C for 30 seconds, and elongating at 72°C for 45 seconds. The amplified products were resolved in a 2.5% agarose gel, and visualized by ethidium bromide staining to determine the efficacy of SSH. For reverse SSH, cDNA from five NTOT was used as tagged-pool.

The resultant cDNA was then labeled respectively with fluorescent nucleotides (Cy3 or Cy5), and the reaction products were hybridized to microarray slides (G4110B, human 1A, Agilent Technologies, Santa Clara, CA) to identify the genes that were up- or down-regulated in OCa and NTOT. The correlated genes and gene ontology were analyzed and clustered by GenSpring GX software. The ratio between normalized fluorescent signal intensity of OCa tissues and NTOT was measured on the individual spot of the microarray, and values of the three readings were averaged. The cut-off ratio for highly expressed genes was set at 2.0 (with 99.5% coefficient variance in a scatter plot). Expression ratio of a gene that fell between 0.5 and 2.0 was considered constitutive. The significant difference of gene expression ratio was determined by a Pearson correlation. The genes identified by microarray, which were overexpressed in more advanced OCa, were confirmed with colony membrane arrays and RT-PCR, as previously described (Lin et al., 2004; Chen et al., 2006).

After isolation and measurement of RNA yield, cDNA was synthesized by AMV reverse transcriptase using oligo random primers. An aliquot of cDNA was

subjected to 35 cycles of reaction using a GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, CA). The reaction mixture contained 1x Taq buffer (BRL, Bethesda, MD), 1.5 mM MgCl2, 2 μM dNTP, 0.25 μM of respective 3' and 5' primers, 1 U of Taq DNA polymerase, and 2 μl of cDNA. PCR was carried out according to standard procedures by denaturing cDNA template at 94°C for 30 seconds, hybridizing with primers at 56°C for 30 seconds, and elongating DNA synthesis at 72°C for 45 seconds. The amplified products were analyzed in 1.5% agarose gel, and visualized by ethidium bromide staining. Specificity of the amplified fragment was determined by DNA sequencing (Protech Technology, Taipei, Taiwan), and percent match to database of GenBank (http://HTwww.ncbi.nlm.nih.gov/blastTH).

The primer sequences are listed as follows:
sMP1: 5'-CTGGAATTGGCCACAAAGTT-3' (sense, X54925.1, nts 1327-1346)
aMP1: 5'-AAGCTGCTCTCTGGGATCAA-3' (antisense, X54925.1, nts 1786-1767)
sMP3: 5-TGCTTTGTCCTTTGATGCTG-3' (sense, NM_002422.3, nts 941-960)
aMP3: 5'-ATCGATTTTCCTCACGGTTG-3' (antisense, NM_002422.3, nts 1232-1213)
sMP7: 5'-GAGTGCCATATGTTGCAGAA-3' (sense, NM_002423.2, nts 310-329)
aMP7: 5'-TGGGGATCTCCATTTCCATA-3' (antisense, NM_002423.2, nts 772-753)
sMP9: 5'-TTGACAGCGACAAGAAGTGG-3' (sense, NM_004994.2, nts 1155-1174)
aMP9: 5'-CCTGTGTACACCCACACCTG-3' (antisense, NM_004994.2, nts 1794-1775)
sMP10: 5'-GTTAACAGCAGGGACACCGT-3' (sense, NM_002425.1, nts 1055-1074) aMP10: 5'-ACCCATATCTGTCTTCCCCC-3' (antisense, NM_002425.1, nts 1481-1462)
sIL8: 5'-GTGCAGTTTTGCCAAGGAGT-3' (sense, HTNM_000584.2TH, nts 166-185)
aIL8: 5'-CTCTGCACCCAGTTTTCCTT-3' (antisense, HTNM_000584.25TH, nts 361-342)

The primer sequences were selected by using Primer3 at HThttp://www-genome.wi.mit.edu/TH, and the sequence uniqueness was determined by using Blast at http://www.ncbi.nlm.nih.gov. For quantitative RT-PCR (qPCR), SYBR green was used to measure the difference in gene expression in a Rotor-Gene™ 3000 (Corbett Life Science, Sydney, Australia).

Immunohistochemical staining

Immunohistochemical staining was performed by an immunoperoxidase method as previously described (Lin et al., 2004; Chen et al., 2006). Briefly, immunohistochemical characterization was performed on paraffin sections of biopsy using an LSAB method (Dako, Carpenteria, CA). The primary antibodies were added at dilution of 1:50 according to supplier's manual, and incubated at 4°C overnight before processed with LSAB. The chromogenic reaction was visualized by peroxidase-conjugated streptavidin (Dako, Carpenteria, CA) and aminoethyl carbazole (Sigma, St. Louis, MO). Slides were counter-stained with Mayor's hematoxylin, and positive staining was recognized under a light microscope as crimson-red granules. Antibodies for MMP-1, -3, -7, -9 and -10 were from Chemicon International, Inc. (Temecula, CA), and those for IL-8 were from Pierce Endogen (Rockford, IL). Positive immunohistochemical staining was noted when crimson-red precipitates in the cytoplasm were detected. The slide was counterstained with Mayor's hematoxylin. Each batch had a positive lung cancer control to ensure the quality.

Slide evaluation

In each case, NTOT served as internal negative control. Slides were evaluated by two independent pathologists. An ImmunoReactive Scoring system was adapted for this study (Remmele and Schicketanz, 1993). Briefly, a specimen was considered strongly positive if more than 50% of cancer cells were positively stained; intermediate, if positive cells were around 25-50%; weak, if less than 25% but more than 10% positively stained; and negative, if less than 10% cells stained. Both strong and intermediate positive cases were classified as high expression. Weak and negative were classified as low expression (Lin et al., 2004).

Statistical analysis

Relations between metastasis and clinicopathological parameters were analyzed by Chi-Square test. Survival curves were plotted with Kaplan-Meier method (Kaplan and Meier, 1958). Statistical difference of survival was compared by a log rank test (Mantel, 1966). Statistical analysis of patient's survival was performed using GraphPad Prism4 statistical software (San Diego, CA). Multivariate analysis using Cox's hazards model was calculated by SPSS statistical software (Chicago, Ill).

Results

Expression of MMPs in oral carcinomas

To determine expression profiles of metastasis-related genes in patients with oral cancer (OCa), SSH was used to amplify the differentially expressed genes from five-pair each of cancer fraction in various stages and their non-tumor counterparts (Fig. 1A). Microarray slides were then used to identify the genes that were amplified by SSH in the respective groups. Following analyses of scatter plotting and hierarchical clustering (Fig. 1B1-B4), differentially expressed genes in different groups were identified (Table 1). Interestingly, expression of MMP-1, -3, -7, -9, and -10, kallikrein 10 (serine proteinase, normal epithelial cell specific 1),

Table 1. Metastasis-related genes with significant differential expression in oral caner compared to those in epithelial disorders of oral cavity identified by SSH and microarray.

Description of the gene	Dysp.[a]	I	II	III	IV
Metastasis-related genes					
MMP-1 (interstitial collagenase)	1.00	1.89	3.00	8.33	3.49
MMP-3 (stromelysin 1, progelatinase)	0.23	0.73	4.32	14.23	5.62
MMP-7 (matrilysin)	0.98	1.00	3.32	8.33	6.74
MMP-9 (gelatinase B, 92 kDa type IV collagenase)	0.52	0.74	2.45	12.61	6.37
MMP-10 (stromelysin 2, interstitial collagenase)	0.98	1.07	6.40	9.87	8.52
MMP-15 (membrane-inserted)	0.95	1.71	2.29	2.56	2.10
interleukin-8 (IL-8)	1.68	1.00	1.72	4.23	4.06
aldo-keto reductase 1C2 (AKR 1C2)	0.92	1.18	1.36	3.68	7.32
aldo-keto reductase 1C3 (AKR 1C3)	0	0	0	1.1	3.69
Serine proteinase inhibitor/serine proteinase					
colligin 2 (serine proteinase inhibitor clade H member 2)	3.34	2.36	1.41	0.86	0.91
kallikrein 6 (neurosin)	0.69	1.20	2.44	2.50	2.86
kallikrein 10 (serine proteinase, Normal epithelial cell specific 1)	0.88	1.66	2.33	4.03	5.77
Transcription factor/modulator					
hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor) (HIF1α)	0.52	0.57	1.89	3.24	12.1
CXCR4	1.03	0.91	1.29	1.72	2.60
14-3-3σ	0.81	1.54	0.76	0.96	3.26
14-3-3ζ	0.59	2.17	3.18	3.94	7.35
Extracellular matrices, cytokine and receptors					
transforming growth factor, beta-induced, 68 kDa (TGFBI)	0.39	1.16	2.42	3.44	4.67
vascular endothelial growth factor-A (VEGF-A)/VEGF-C	-[b]/0.76	1.17/-	1.37/-	2.54/-	3.06/-
plasminogen activator, urokinase receptor (PLAUR)	1.71	2.41	13.16	13.05	7.08
platelet/endothelial cell adhesion molecule (PECAM1, CD31)	1.09	1.00	2.59	9.23	12.95
laminin α3 (a subunit of heterotrimeric laminin 5)	0.52	0.99	1.51	1.98	3.04
laminin ß3 (a subunit of heterotrimeric laminin 5)	0.79	0.97	1.36	2.13	1.83
laminin γ2 (a subunit of heterotrimeric laminin 5)	0.67	1.14	1.19	2.73	6.50
integrin α6 (laminin receptor)	0.38	1.53	1.48	2.36	3.02

[a]Dysp.: dysplasia; [b]-: not detectable

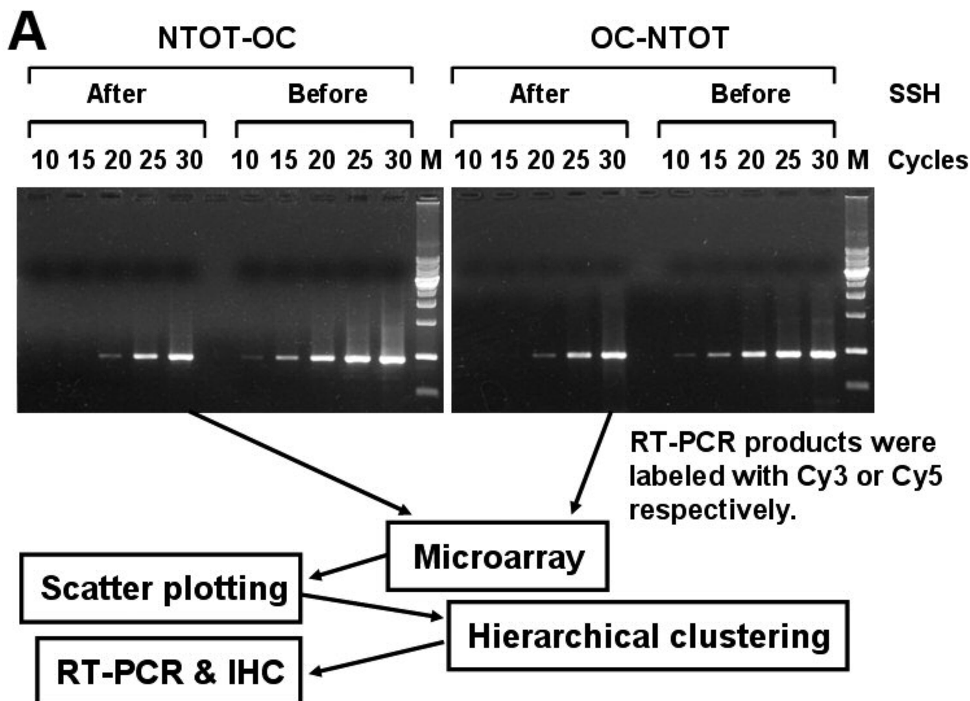

Fig. 1. Representative scheme of combining suppression subtractive hybridization (SSH) and microarray to determine gene expression patterns among biopsy samples from patients with precancerous and cancerous lesions in oral cavity, in particular, the tumor fractions and non-tumor oral tissues (NTOT) in OCa specimens. **A.** Following cDNA synthesis by SMART CDS primers, products from either NTOT or OC were divided and ligated to the respective adaptors (tester cDNA). Tester cDNA was then hybridized to driver cDNA (cDNA without adaptor) to exclude common genes. The reaction mixture was amplified by PCR to enrich differentially expressed genes. In the final run of PCR, the reaction product was separately labeled with fluorescent nucleotide Cy3 (red) or Cy5 (green), and hybridized to a microarray slide (Agilent Technologies, Taipei, Taiwan).

kallikrein 6, as well as that of IL-8, HIF1α, 14-3-3ζ, aldo-keto reductase 1C2 (AKR1C2), laminin γ2 (a laminin 5 subunit), urokinase-type plasminogen activator (uPA) receptor, vascular endothelial growth factor (VEGF) and platelet/endothelial cell adhesion molecule (PECAM1/CD31) increased markedly. Expression of

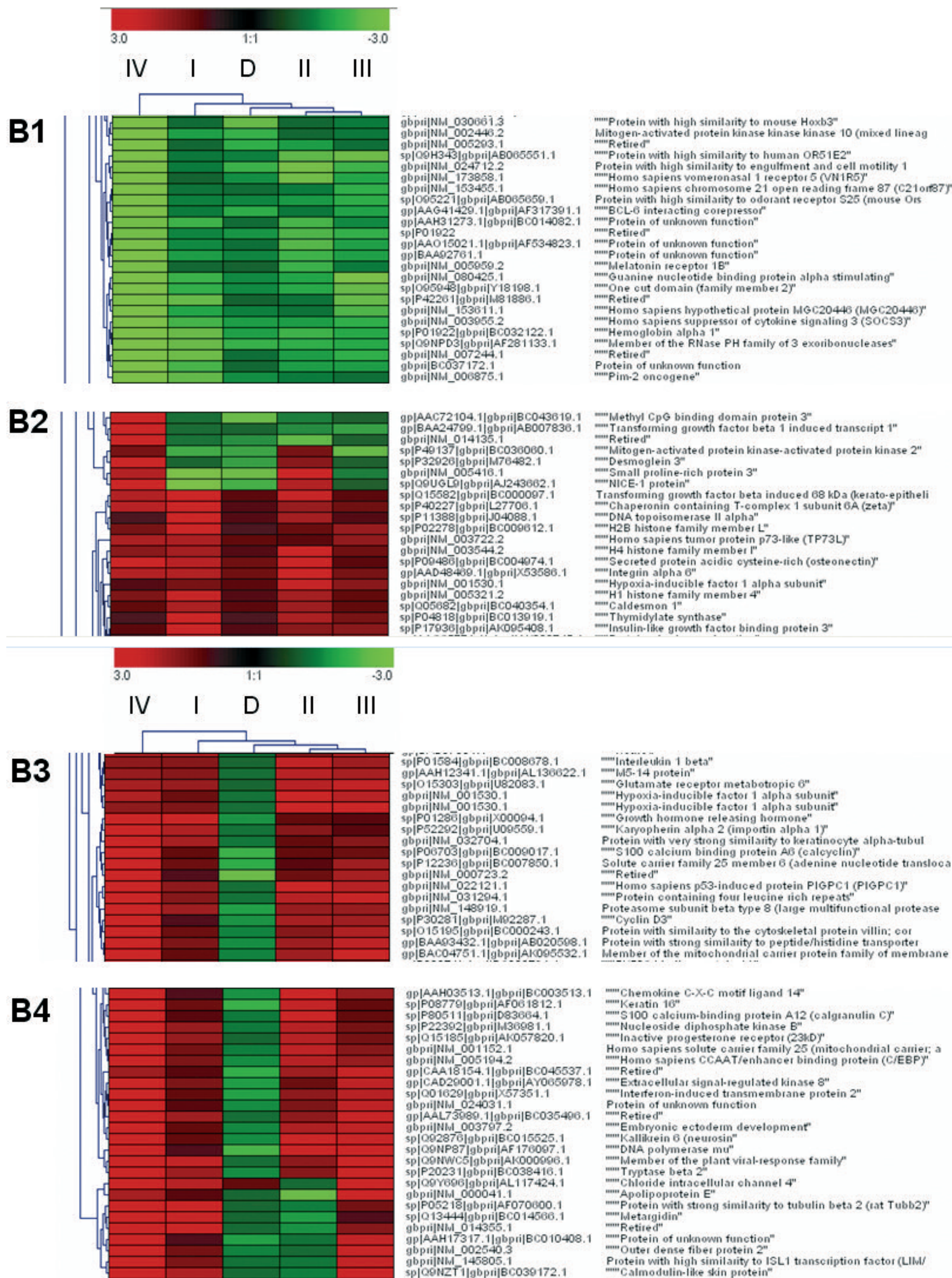

Fig. 1. B. Representative result of microarray data. The correlated genes and gene ontology were analyzed and clustered by GenSpring GX software (Agilent Technologies, Taipei, Taiwan) to map out genes that were exclusively overexpressed in OCa or in NTOT. (**B1**) Genes that were down-regulated during disease progression of OCa; and (**B2-B4**) Genes that were up-regulated during disease progression of OCa.

kallikrein 13, seladin-1 and statherin, on the other hand, reduced from early stage of oral epithelial dysplasia and totally disappeared during OCa development. In terms of disease progression, 14-3-3ζ and PLAUR increased early in stage I. Expression of MMPs correlated with that of kallikrein 10, kallikrein 6, and CD31. Elevated expressions of IL-8, however, were associated with hypoxia-inducible factor-1 alpha (HIF-1_) (Table 1). Overexpression of MMP-1, -3, -9, -10 and IL-8 was confirmed by RT-PCR (Fig. 2) with paired OCa and NTOT to validate the results of microarray. The respective change of gene expression in MMPs and IL-8 was further measured by qPCR. The difference in gene expression level was calculated from the respective CT of the specific gene between OCa and NTOT.

Clinical features of patients

There were 117 patients enrolled in this study. No patient died of surgery-related causes. The median follow-up time was 47 months, ranging from 5 to 131 months. The mean age of these patients was 51.9 years, ranging from 26 to 73 years, and male/female ratio was 5.88 (100 men and 17 women), and 85 patients (72.6%) were smokers. Eighty (68.4%) patients had evident lymph node involvement. A significant difference was found between these two groups in the following parameters: age (p<0.001), gender (p=0.021) smoking habit (p=0.03) and tumor stages (p<0.001). Male patients had a notably higher incidence of tumor metastasis than female patients. Statistical analysis further showed that expression of MMPs in tumors was correlated with betel quid chewing, tobacco smoking and tumor staging (Table 2).

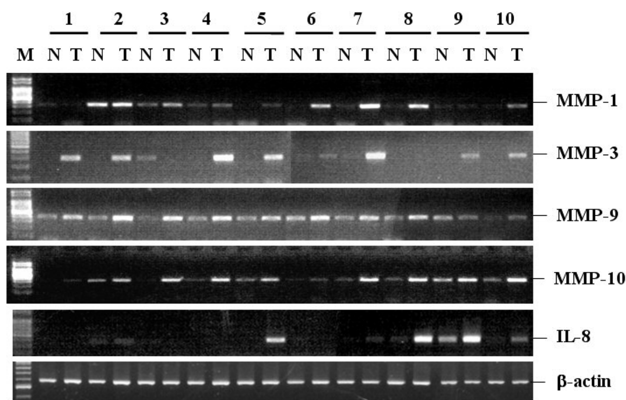

Fig. 2. Differential gene expression of MMPs and IL-8 between NTOT and tumors from OCa patients was confirmed by RT-PCR and the respective change of gene expression was determined by quantitative real-time RT-PCR. Expression patterns of MMPs and IL-8 were detected by RT-PCR. Expression of _-actin was used as a monitoring standard of RT-PCR. N: non-tumor oral tissue (NTOT), T: tumor fraction of surgical resections.

Immunohistochemical evaluation and survival analysis

Compared to negatively stained samples, MMP-1 was detected in 69 (59.0%), MMP-3 in 93 (79.5%), MMP-7 in 79 (67.5%), MMP-9 in 67 (57.3%), and MMP-10 in 83 (70.9%) of pathological specimens by indirect immunoperoxidase staining. A representative example of MMP-3 overexpression in OCa is shown in Fig. 3A. Moreover, MMP-1 was detected in 77.0% (57/74), MMP-3 in 75.6.0% (56/74), MMP-7 in 66.2% (49/74), MMP-9 in 51.4% (38/74) and MMP-10 in 71.6% (53/74) of metastatic lymph nodes. Interestingly,

Table 2. Comparison of parameters between patients with and without metastasis.

Parameter	Metastasis (n=80)	Without metastasis (n=37)	*p* value
Age (years)	50.0 ± 0.9	56.0 ± 1.5	<0.001
Gender			
Male (n=100)	73	27	0.021
Female (n=17)	7	10	
Smoking			
Smokers (n=85)	63	22	0.03
Non-smokers (n=32)	17	15	
Betel quid chewing			
Yes (n=89)	77	12	<0.001
No (n=28)	3	25	
IL-8[a]			
High (n=51)	43	8	<0.001
Low (n=66)	37	29	
MMP expression[b]			
MMP-1			
High (n=69)	59	10	<0.001
Low (n=48)	21	27	
MMP-3			
High (n=93)	76	17	<0.001
Low (n=24)	4	20	
MMP-7			
High (n=79)	63	16	<0.001
Low (n=38)	17	21	
MMP-9			
High (n=67)	51	16	0.037
Low (n=50)	29	21	
MMP-10			
High (n=83)	62	21	0.029
Low (n=34)	18	16	
IL-8			
High (n=51)	42	9	0.004
Low (n=66)	38	28	
Stage			
I (n=17)	0	17	<0.001
II (n=15)	0	15	
III (n=52)	47	5	
IV (n=33)	33	0	
Cell differentiation			
Well (n=66)	41	25	0.236
Moderate (n=45)	34	11	
Poor (n=6)	5	1	

[a, b, c]: according to immunohistochemical findings.

MMP-3 was clearly expressed on the border of each tumor nest. In the early stage, expression of IL-8 was detected in infiltrating cells of the tumor nest. In the late stages, IL-8 was detected in tumor cells (Fig. 3B).

Among the 117 patients who had surgery, though radiation and chemotherapy were commenced properly, 53 (45.3%) patients experienced tumor recurrence, and the tumor developed within 12 months of operation. Overexpression of MMP-1 (p<0.001), -3 (p<0.001) and -10 (p<0.001), as well as that of IL-8 (p=0.0012) was associated with lower survival rate (Fig. 4A-4D). The difference in cumulative survivals was significant. The difference in MMP-9 overexpression, however, was marginal (p=0.0508) (The median survival for MMP-9+ patients is 35 months, and that for MMP-9- patients is 46 months). By univariate analysis, in addition to expression of MMP-1, -3, -10 and IL-8, tumor stage, betel quid chewing habit and gender were also correlated

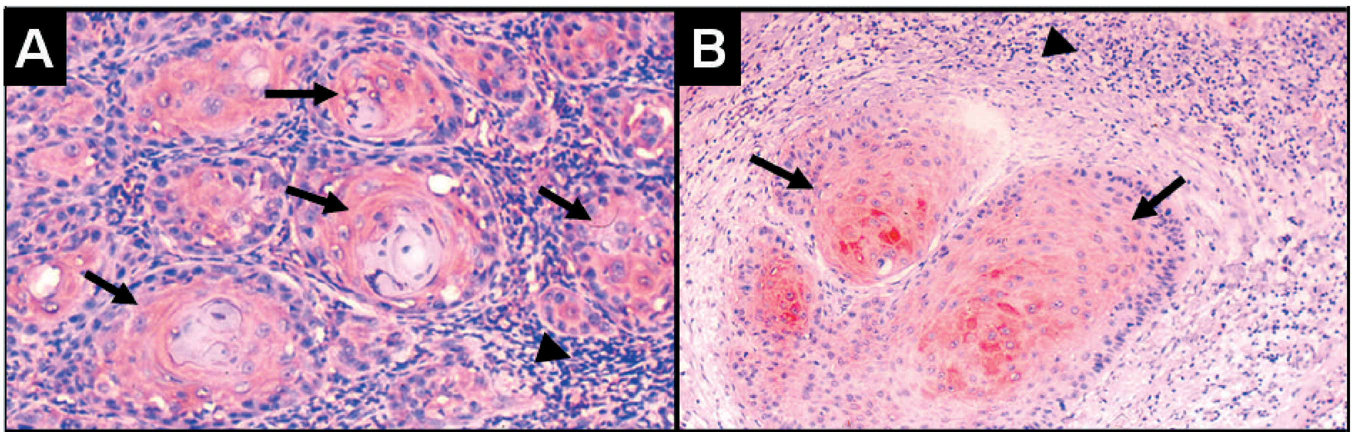

Fig. 3. Representative examples of immunohistochemical staining. **A.** Overexpression of MMP-3 was detected in OCa cells (original magnification, x 250). **B.** Overexpression of IL-8 was detected in OCa cells. Expression of MMP-3 and IL-8 was detected by immunohistochemical staining (as crimson-red precipitates in the cytoplasm) using specific antibodies. The slide was counterstained with Mayor's hematoxylin (original magnification, x 200). Arrows: tumor nest; arrowhead: tumor infiltrated lymphocytes

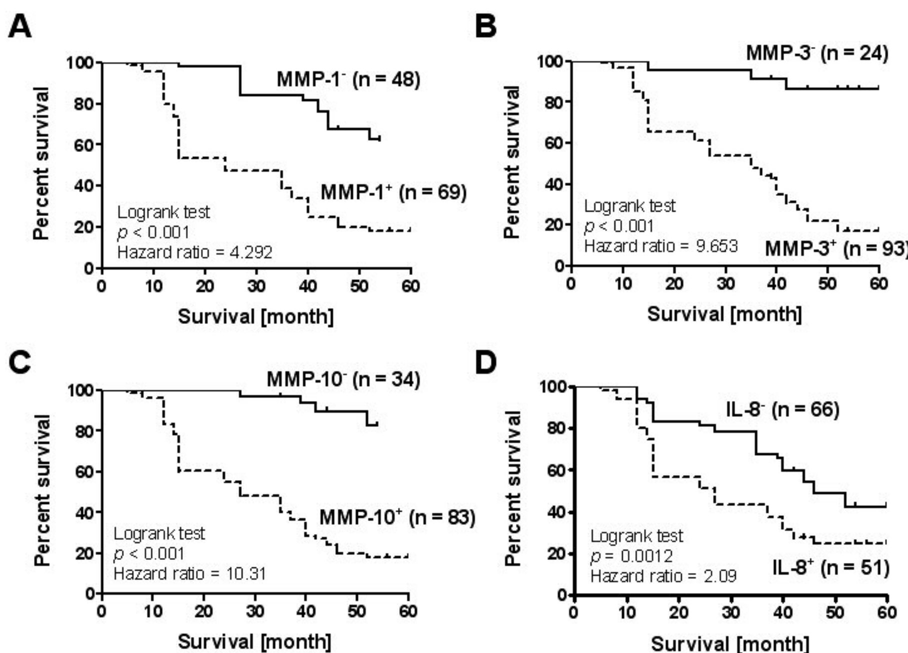

Fig. 4. Cumulative survival curves in patients with OCa. Survival curves of patients with differential gene expression in OCa cells were plotted with Kaplan and Meier method. Statistical difference of survival between patient groups divided by **A.** MMP-1 (p<0.001) (The median survival for MMP-1+ patients is 24 months, and that for MMP-1- patients is >24 months); **B.** MMP-3 (p<0.001) (The median survival for MMP-3+ patients is 35 months, and that for MMP-1- patients is >35 months); **C.** MMP-10 (p<0.001) (The median survival for MMP-10+ patients is 27 months, and that for MMP-10- patients is >27 months); **D.** IL-8 (p=0.0012) expression was compared by the log rank test (The median survival for IL-8+ patients is 27 months, and that for IL-8- patients is 46 months).

Table 3. Univariate and multivariate analyses of clinicopathological and molecular parameters in patients with OCa according to immunohistochemical findings.

Parameters	Univariate			Multivariate
	Hazard ratio	95% confidence intervals of ratio	*p* value	*p* value
Tumor stages			<0.001	0.009
Betel quid chewing	4.425	1.898 to 5.462	<0.001	0.002
MMP-1	4.292	2.409 to 6.541	<0.001	0.006
MMP-3	9.653	2.345 to 6.961	<0.001	0.001
MMP-7	2.247	1.339 to 3.680	0.002	0.032
MMP-9	1.578	0.9984 to 2.716	0.0508	0.08
MMP-10	10.31	2.843 to 7.945	<0.001	0.001
IL-8	2.091	1.393 to 3.881	0.0012	0.027

with poor prognosis. By multivariate analysis, the difference in betel quid chewing habit (p=0.002), tumor stage (p=0.009), overexpression of MMP-1, MMP-3, MMP-10 and IL-8 was significant as well (Table 3). These data indicate that these biological factors may be regulated by a common mechanism, such as betel quid chewing, which promotes cancer cell growth and metastatic potential.

Discussion

The results presented above show that overexpression of MMP-1, -3, -7, -10, and IL-8 is frequently detected and correlated with metastasis and survival in OCa patients. Patients with overexpression of MMP-1, -3, -7, -10, and IL-8 have a significantly higher incidence of early tumor recurrence which is frequently associated with poor prognosis.

Oral cancers contain a heterogeneous population of tumor cells and normal tissues. Cancer staging that is dependent upon tumor size and involvement of neighboring tissues, lymph nodes and organs, as well as clinical appearance of the patient is by far the most reliable factor for estimating patient's treatment outcome. However, cancer staging can not predict tumor cell growth, spreading or resistance to radiation and/or chemotherapies. Molecular techniques are therefore useful for delineating the intricate mechanisms of cancer progression and to improve the efficacy of therapy.

In fact, applications of microarray have identified various expression patterns of MMPs in OCa (Nagata et al., 2003; Kim et al., 2006; Ziober et al., 2006a,b). Despite racial differences, the most commonly identified MMPs were MMP-1, -3 and -9 (Nagata et al., 2003; Katayama et al., 2004; Kim et al., 2006; Ziober et al., 2006a,b). MMPs are primarily synthesized as inactive zymogens (pro-MMPs). The release of zymogen provokes serine proteinases to remove pro-peptide from pro-MMP. By showing the concomitant increase of kallikreins and MMP-1, -3, -7, -9 and -10, our data confirmed these findings (Sternlicht and Werb, 2001; Egeblad and Werb, 2002; Nagata et al., 2003; Katayama et al., 2004; Kim et al., 2006; Ziober et al., 2006a,b). Unlike their findings though, our data suggested that a differential increase of MMP expression could be a pathophysiological response to betel quid chewing. Concurrent overexpression of MMP-1, -7 and -10, in which genes are clustered within a narrow stretch on chromosome 11, indicated such a possibility, and that the synchronized activation of clustered genes could be geographical or temporal.

By a keratinocyte/fibroblast co-culture system, Ghaffari et al found that exogenously added stratifin (14-3-σ) stimulated dermal fibroblasts to express MMP-1 (collagenase-1), -3 (stromelysin-1) and -10 (stromelysin-2) (Ghaffari et al., 2006). In this study, elevated 14-3-3 ζ detected. The increased 14-3-3ζ expression was comparative to that of MMP-1, -3, -7, -9 and -10. Expression preference of 14-3-3ζ, instead of 14-3-3ζ could be OCa-specific. An *in vitro* study by Lam et al showed that insulin inhibits stratifin-mediated increase of MMP expression (Lam et al., 2004), and their data supported our results that the aberrant gene expression in OCa is influenced by pathophysiological responses. However, since no marked change of insulin, insulin-like growth factor (IGF) or IGF receptor was detected, increased MMP expression in OCa lesions may be associated with other factor(s), e.g., IGF binding proteins (Fig. 1B2).

It is therefore worth noting that mRNA of uPA receptor (uPAR, CD87), HIF-1α and IL-8 also increased in OCa specimens. In fact, plasminogen activation has been shown to play crucial roles in cancer metastasis, in which the latent proteolytic activity of uPAR was activated by uPA to facilitate tumor cell migration and invasion (Wang et al., 2006). Thus, levels of uPAR expression should be able to reflect the aggressiveness of cancer cells. Increased expression of HIF-1α was also detected in the more advanced OCa. HIF-1α is a subunit of heterodimeric HIF-1, which contains α and ß subunits (Wang et al., 1995). With normal oxygen tension, oxygen-activated prolyl hydroxylase (PHD) hydroxylates newly synthesized HIF-1α and induces degradation of the protein via ubiquitin-proteasome pathway (Ke and Costa, 2006). Under hypoxia, PHD becomes inactive, and HIF-1α is accumulated inside

cells and joins with HIF-1ß to form an HIF-1 transcription factor to trigger expression of hypoxia-inducible genes, including glycolytic enzymes, angiogenic factors [e.g., vascular endothelial growth factor (VEGF), CXC chemokine receptor 4 (CXCR4), and IL-8], and cell survival-related proteins (p21WAF1/CIP1) (Berger et al., 2003; Mizokami et al., 2006; Wang et al., 2007). Our results, in which HIF-1α, IL-8 and CD31 expressions were concomitantly elevated in the more advanced stages of OCa, supported the notion that hypoxia-mediated metabolic change via HIF-1α overexpression not only rendered cancer cells more aggressive, but also increased cisplatin resistance of cancer cells (Tanaka et al., 2005).

These data considered together with our current findings, in which the expression pattern of MMP was closely associated with those of metastasis- and inflammation-related genes, clearly indicated that the behavior of oral cancer cells could be affected by microenvironmental factors around the afflicted oral mucosa. Undoubtedly, these hypotheses remain to be verified. It is clear that the expression of MMPs and other metastasis-related genes directly or indirectly determine the severity of disease progression and, possibly, drug and radiation resistance. We are evaluating these issues in an ongoing study, in particular the *in vitro* effect of hypoxia on expression of HIF1_ and IL-8. Hopefully, this in itself can provide a better tool for determining the necessity of future therapeutic regimens to improve the efficacy of treatment and patient's quality of life as well.

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Accepted April 23, 2008