SPANDIDOS PUBLICATIONS

# Down-regulated expression of *SERPIN* genes located on chromosome 18q21 in oral squamous cell carcinomas

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**Abstract.** Serpins (serine protease inhibitors) are known as a diverse family of protease inhibitors; however, various other biological activities including tumor suppression, have been recently reported for these molecules. To clarify whether members of the serpin family are involved in OSCC (oral squamous cell carcinoma), global gene screening using microarray analysis was performed with OSCC-derived cell lines. A trend toward diminished expression was shown for some *SERPIN* genes located on 11q12-q13.1 and 18q21. mRNA expression of *SERPIN* genes at these chromosome regions was therefore analyzed using real-time quantitative RT-PCR (qRT-PCR) in 55 OSCC samples and matched normal tissue. Statistically significant decreases in expression were found for *SERPINB12* (P=0.001), *SERPINB13* (P=0.001), *SERPINB4* (P=0.042), *SERPINB3* (P<0.001), *SERPINB11* (P<0.001), *SERPINB7* (P=0.021) and *SERPINB2* (P=0.018). All of these genes are located on 18q21, the known location of the serpin gene cluster. The results strongly suggest that this chromosome region plays a crucial role in OSCC. Some serpin members in the region might be involved in tumor suppression, or there might be unidentified tumor suppressor genes within or near the chromosome region.

## Introduction

Serpins (serine protease inhibitors) are the largest and most broadly distributed superfamily of proteins containing a conserved domain. Most serpins inhibit serine proteases; however, some inhibit caspases or papain-like cysteine proteases (1,2). Several members of the group show non-inhibitory activities, functioning as hormone transporters, molecular chaperones or tumor suppressors (3-6). SERPINB5 (maspin) was first identified as a tumor suppressor protein in human breast cancer, and the diminished expression of this protein has been frequently observed in advanced human breast cancer specimens (6). Correlations between SERPINB5 expression and clinical features have been previously reported; however, both up- and down-regulation have been observed in various tumors, and some investigators have noted its paradoxical expression (7-10). In oral carcinoma, Xia *et al* suggested that higher SERPINB5 expression was significantly associated with better overall survival (11). Furthermore, down-regulated expression of the *SERPINB13* (*headpin/hurpin*) gene has been demonstrated in head and neck malignancies (12-14). Both *SERPINB5* and *SERPINB13* genes are located on chromosome 18q, which is the location of the serpin gene cluster and other members of serpin family. Frequent genetic alterations have been observed in this chromosomal region in head and neck malignancies (15-20). We previously reported that loss of heterozygosity (LOH) at 18q21 was observed in 43.8% of oral squamous cell carcinomas, and that this was significantly correlated with the clinicopathological features (21). These studies suggest that serpin family members located on chromosome 18q play crucial roles and might act as tumor suppressors in oral squamous cell carcinomas.

The aim of this study was to investigate expression profiles of serpin family members in OSCC and to clarify the relationship between their expression profiles and clinical features.

## Materials and methods

*Cell culture*. The OSCC-derived cell lines used in this study were HSC2, HSC3 and CA9-22 (Human Science Research Resources Bank, Osaka, Japan); plus H1 and Sa3 (provided by Dr S. Fujita, Wakayama Medical University, Wakayama, Japan). All OSCC-derived cell lines were cultured in Dulbecco's modified Eagle medium/F-12 HAM (Sigma-Aldrich Co., St. Louis, MO, USA) supplied with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich) and 50 U/ml penicillin and streptomycin (Sigma-Aldrich). All cell lines were incubated at 37˚C in a humidified atmosphere with 5% $CO_2$.

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*Key words:* serpin, oral squamous cell carcinoma, chromosome 18

Table I. Clinicopathological features of oral squamous cell carcinoma cases examined in the study.

| Profiles of cases | No. |
|---|---|
| Age (year old) | |
| Range | 46-84 |
| Mean | 63.9 |
| Gender | |
| Male | 37 |
| Female | 18 |
| Differentiation | |
| Well | 31 |
| Moderately | 23 |
| Poorly | 1 |
| Tumor size (T) | |
| T1 | 4 |
| T2 | 23 |
| T3 | 10 |
| T4 | 18 |
| Regional neck metastasis (N) | |
| N0 | 17 |
| N1 | 10 |
| N2a | 4 |
| N2b | 16 |
| N2c | 8 |
| Distant metastasis (M) | |
| M0 | 55 |
| M1 | 0 |
| Stage classification | |
| I | 4 |
| II | 21 |
| III | 0 |
| IV | 30 |

Table II. Specific primer sequences used in real-time quantitative RT-PCR analysis.

| Target gene | Primer sequence |
|---|---|
| *SERPINB6* | |
| (F) | 5'-GGCACGCAGTACTTGCTTAGGA-3' |
| (R) | 5'-GCTACCCAGGTGTTTATGTG-3' |
| *SERPING1* | |
| (F) | 5'-GCAAGGGACCTGCTTCTATTAGC-3' |
| (R) | 5'-AGAGAAAGCTGCAAAAAGTGGTTT-3' |
| *SERPINB5* | |
| (F) | 5'-GTGGTTGGCACTAGACTGG-3' |
| (R) | 5'-CAAGGTAACGTGAGCACTTTAATC-3' |
| *SERPINB12* | |
| (F) | 5'-GACAAGAGATTAACTTCTGG-3' |
| (R) | 5'-CTTGTTTTCATTCGCATTTA-3' |
| *SERPINB13* | |
| (F) | 5'-GGCATAGGCTTTACTGTCACATCC-3' |
| (R) | 5'-CAAGACTGGAAATGCAAGTGAGAC-3' |
| *SERPINB4* | |
| (F) | 5'-GCCAAGGTCCTGGAAATACCA-3' |
| (R) | 5'-TTCCATCAATTTCTCAGCAGTGA-3' |
| *SERPINB3* | |
| (F) | 5'-GGTTACAGAGGAGGGAGCAGAA-3' |
| (R) | 5'-GGGTGATTACAATGGAACTCTTCA-3' |
| *SERPINB11* | |
| (F) | 5'-TACCCTCAGCATTGCCAACA-3' |
| (R) | 5'-CAGTTTGCAACCTGGCTTGATA-3' |
| *SERPINB7* | |
| (F) | 5'-CACTGGTGACTTGACCCTTCCT-3' |
| (R) | 5'-GGTGAGACACATGGTGGTAGAATG-3' |
| *SERPINB2* | |
| (F) | 5'-CAGATCCAGAAGGGTAGTTA-3' |
| (R) | 5'-CAGACTTCTCACCAAACAGCTT-3' |
| *SERPINB10* | |
| (F) | 5'-AGCTGAATGAGTGGACCAG-3' |
| (R) | 5'-TCCTGAGAAATCAGCTTTGC-3' |
| *SERPINB8* | |
| (F) | 5'-CGATCCCCTGACAAAGCTG-3' |
| (R) | 5'-TTAGCTTCCTTAAACATCAT-3' |

*RNA extraction from OSCC-derived cell lines and OSCC samples*. Total RNA was extracted from each cell line and tissue samples using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Each extracted RNA sample was stored separately at -80˚C until use.

*DNA microarray analysis of OSCC-derived cell lines*. Double-stranded cDNA was synthesized from 20 $\mu$g of total RNA from each cell line and tissue samples using the Superscript Choice system (Invitrogen Life Technologies). A biotin-labeled *in vitro* transcription reaction was carried out using the cDNA template (Enzo Bioarray, Farmingdale, NY, USA). According to the manufacturer's protocols (Affymetrix, Santa Clara, CA, USA), 7 $\mu$g of cRNA was fragmented and added to the hybridization mixture. Expression profiles were created using the Human Genome U 133 Plus 2.0 arrays containing 54675 probe sets (Affymetrix). Arrays were stained with phycoerythrin-streptavidin antibody followed by a second staining with phycoerythrin-streptavidin and then scanned using Affymetrix Gene Chip Operating Software 1.1 (Affymetrix).

Expressions of *SERPIN* genes determined with DNA microarray analysis.

| Symbol | Genbank | Chromosome location | Affymetrix no. | Relative value (vs. human normal oral keratinocyte) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | H1 | Sa3 | KB | HSC2 | HSC3 |
| *SERPINC1* | BC022309 | 1q23-q25.1 | 1554491_a_at | 5.33 | 1.25 | 0.87 | 3.61 | 3.49 |
| *SERPINE2* | AI359165 | 2q33-q35 | 227487_s_at | 1.15 | **0.20** | **0.10** | 1.13 | 0.23 |
| *SERPINI1* | NM_005025 | 3q26.1 | 205352_at | 1.03 | 0.36 | 0.94 | 0.85 | **0.13** |
| *SERPINI2* | NM_006217 | 3q26.1-q26.2 | 207636_at | 0.79 | **0.11** | 0.29 | 0.48 | 0.59 |
| *SERPINB1* | NM_030666 | 6p25 | 212268_at | 0.46 | 0.75 | 1.06 | 0.61 | 1.13 |
| *SERPINB9* | AI986192 | 6p25 | 242814_at | 0.39 | 0.63 | **0.10** | **0.13** | 0.37 |
| *SERPINB6* | AW275007 | 6p25 | 1556950_s_at | 0.67 | 0.65 | 0.90 | 0.46 | 0.89 |
| *SERPINE1* | BC020765 | 7q21.3-q22 | 1568765_at | 0.73 | 1.26 | 0.36 | 3.01 | 1.96 |
| *SERPING1* | NM_000062 | 11q12-q13.1 | 200986_at | **0.06** | 0.26 | **0.07** | **0.09** | **0.10** |
| *SERPINH1* | NM_004353 | 11q13.5 | 207714_s_at | 1.04 | **0.20** | 1.36 | 5.64 | 2.06 |
| *SERPINA10* | NM_016186 | 14q32.13 | 220626_at | 1.05 | 0.65 | 0.41 | 0.62 | 0.51 |
| *SERPINA6* | NM_001756 | 14q32.13 | 206325_at | 2.55 | 1.46 | 1.39 | 0.52 | 1.54 |
| *SERPINA2* | NM_006220 | 14q32.13 | 208531_at | 0.38 | 0.39 | **0.16** | 0.61 | 0.34 |
| *SERPINA1* | T62088 | 14q32.13 | 230318_at | 2.36 | 0.80 | 1.36 | 0.45 | 2.67 |
| *SERPINA11* | AY185496 | 14q32.13 | 1553499_s_at | 1.84 | 2.04 | 1.40 | 1.59 | 1.86 |
| *SERPINA4* | NM_006215 | 14q32.13 | 213874_at | 0.85 | 1.06 | 0.78 | 0.57 | 1.11 |
| *SERPINA3* | NM_001085 | 14q32.13 | 202376_at | 0.47 | 8.93 | 0.79 | 3.09 | 0.28 |
| *SERPINF2* | NM_000934 | 17p13.1 | 205075_at | 2.49 | 1.45 | 1.94 | 1.81 | 0.72 |
| *SERPINF1* | NM_002615 | 17p13.1 | 202283_at | **0.19** | 0.40 | 0.30 | 1.79 | 0.60 |
| *SERPINB5* | NM_002639 | 18q21.3 | 204855_at | **0.05** | 0.27 | **0.01** | 0.26 | **0.03** |
| *SERPINB12* | NM_080474 | 18q21.3 | 1553057_at | 6.44 | 3.00 | 4.89 | 0.31 | 4.50 |
| *SERPINB13* | AF169949 | 18q21.3-q22 | 211362_s_at | 0.35 | **0.15** | 0.20 | 0.36 | 0.61 |
| *SERPINB4* | U19557 | 18q21.3 | 210413_x_at | 0.39 | 0.57 | 0.44 | 1.26 | 1.02 |
| *SERPINB3* | U19556 | 18q21.3 | 209719_x_at | 0.29 | 0.43 | 0.22 | 0.99 | 0.59 |
| *SERPINB11* | NM_080475 | 18q21.3 | 1552463_at | 0.73 | 0.38 | 0.67 | 0.76 | 0.88 |
| *SERPINB7* | NM_003784 | 18q21.33 | 206421_s_at | **0.04** | **<0.01** | **<0.01** | **0.14** | 0.21 |
| *SERPINB2* | NM_002575 | 18q21.3 | 204614_at | **<0.01** | **<0.01** | **<0.01** | 0.63 | 1.41 |
| *SERPINB10* | NM_005024 | 18q21.3 | 214539_at | **0.16** | 0.34 | 0.54 | 0.62 | 1.28 |
| *SERPINB8* | NM_002640 | 18q21.3 | 206034_at | 0.23 | **0.05** | 0.26 | 0.36 | 0.36 |
| *SERPIND1* | NM_000185 | 22q11.21 | 205576_at | 8.84 | 2.75 | 0.66 | 13.10 | 2.01 |
| *SERPINA7* | NM_000354 | Xq22.1 | 206386_at | 1.06 | 1.47 | 1.16 | **0.15** | 1.41 |

Values <0.2 are shown in bold.

*Tissue samples*. Fifty-five pairs of samples were obtained at the time of surgery performed at Chiba University Hospital between 1998 and 2006. The paired samples consisted of primary OSCC and normal oral epithelial tissue. All patients provided informed consent according to a protocol reviewed and approved by the institutional review board of Chiba University. Postoperative follow-up data were collected until April 2008 or until death, metastasis or local recurrence. Histopathologic diagnosis of each tumor specimen was performed by the Department of Pathology, Chiba University Hospital, according to the International Histological Classification of Tumors. All OSCC samples were histologically confirmed and checked to ensure the presence of tumor in >80% of each specimen. Clinicopathological features of OSCC cases examined in the present study are shown in Table I.

*mRNA expression analysis using real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)*. Among a total of 31 genes, 11 *SERPIN* genes were selected by the expression profiles determined by DNA microarray analysis and were subjected to real-time qRT-PCR analysis. Before cDNA synthesis, residual genomic cDNA was removed from the total RNA using DNAse I treatment (DNA-free, Ambion, Austin, TX, USA). The nucleotide sequences of gene-specific primers for qRT-PCR analysis are shown in Table II. Before use, the sequences of gene-specific primers were

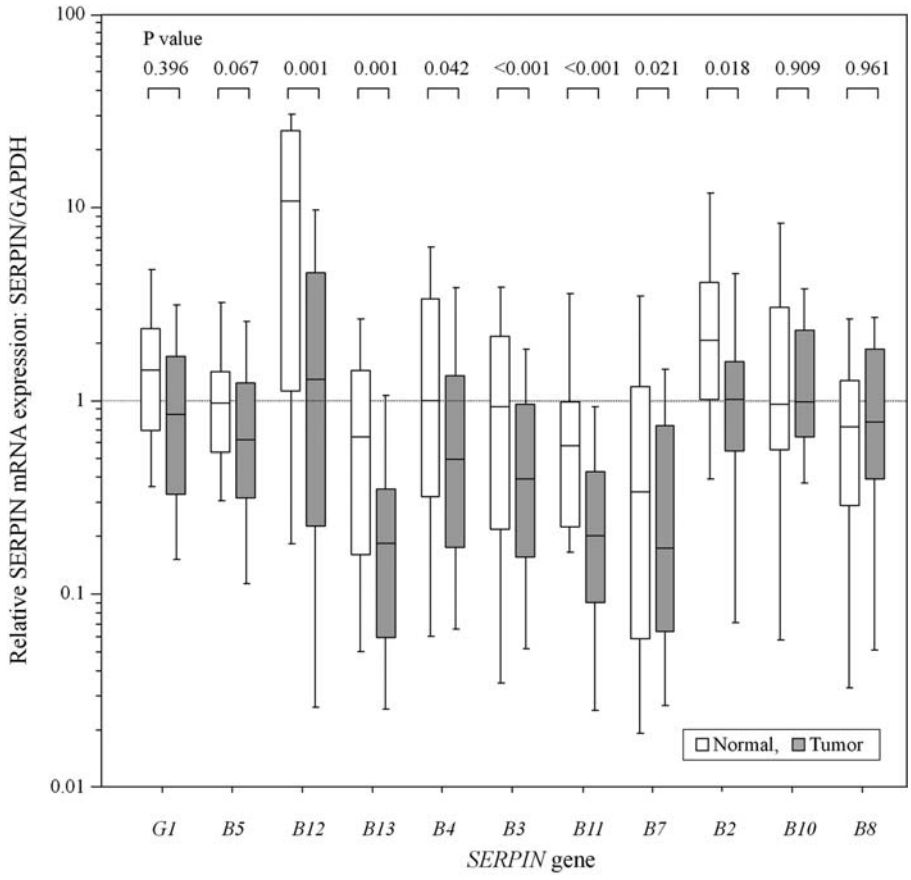


Figure 1. Comparison of mRNA expressions of *SERPIN* genes in primary OSCC samples and normal oral epithelial tissues. Statistically significant down-regulation of *SERPINB12*, *SERPINB13*, *SERPINB4*, *SERPINB3*, *SERPINB11*, *SERPINB7* and *SERPINB2* was observed in OSCC samples when compared with normal tissues.

checked with the Primer3 program (available at http:www-genome.wi.mi.edu/cgi-bin/primer/primer3_www.cgi), to avoid amplification of genomic DNA or pseudogenes. The qRT-PCR analysis was performed using the LightCycler FastStart DNA Master SYBR Green I kit (Roche, Mannheim, Germany). Details are described in our previous report (22). The transcript amount for each of the 11 genes of the serpin superfamily was estimated from the respective standard curves and normalized to the *GAPDH* transcript amount determined in corresponding samples using primers specific for the *GAPDH* genes (sense, 5'-CATCTCTGCCCCCTCTG CTGA-3'; antisense, 5'-GGATGACCTTGCCACAGCCT-3'). Amplified products were analyzed by agarose gel electrophoresis to ascertain size and purity.

*Network analysis and canonical pathway analysis*. Gene accession numbers and the results of global gene screening using microarray analysis were imported into IPA software (Ingenuity Systems, Redwood City, CA, USA). This software categorized the genes based on location, cellular components, and reported or suggested biochemical, biologic and molecular functions. The identified genes were also mapped to genetic networks available in the Ingenuity database (Ingenuity Systems) and then ranked by score, which reflected the probability that a collection of genes equal to or greater than the number in a network could be achieved by chance alone. A score of 3 indicated a 1/1000 chance that the focus genes were in a network because of chance. Therefore, scores ≥3 had a 99.9% confidence level of not being generated by chance alone.

*Statistical analysis*. Differences of the mRNA expression levels of *SERPIN* genes between normal tissues and OSCC samples were analyzed using paired t-tests and correlations between mRNA expression levels of *SERPIN* genes and clinicopathologic features were analyzed using the Fisher's exact test. Disease-free rate was defined as the percentage of patients for a given time interval between tumor treatment and detection of the first locoregional recurrence, distant metastasis or both, or the date of last follow-up, whichever occurred first. Disease-free rate was analyzed using the Kaplan-Meier analysis and differences in disease-free rates between groups with high or low expression of *SERPINB11* were compared using the log-rank test. P-values <0.05 were taken to indicate statistical significance.

## Results

*SERPIN gene expressions in OSCC-derived cell lines using microarray analysis*. Global gene screening of OSCC cell lines was carried out with DNA microarray analysis. Expressions of *SERPIN* genes in OSCC cell lines compared with normal controls are summarized in Table III. Gene expression was down-regulated in 3 or more OSCC cell lines for

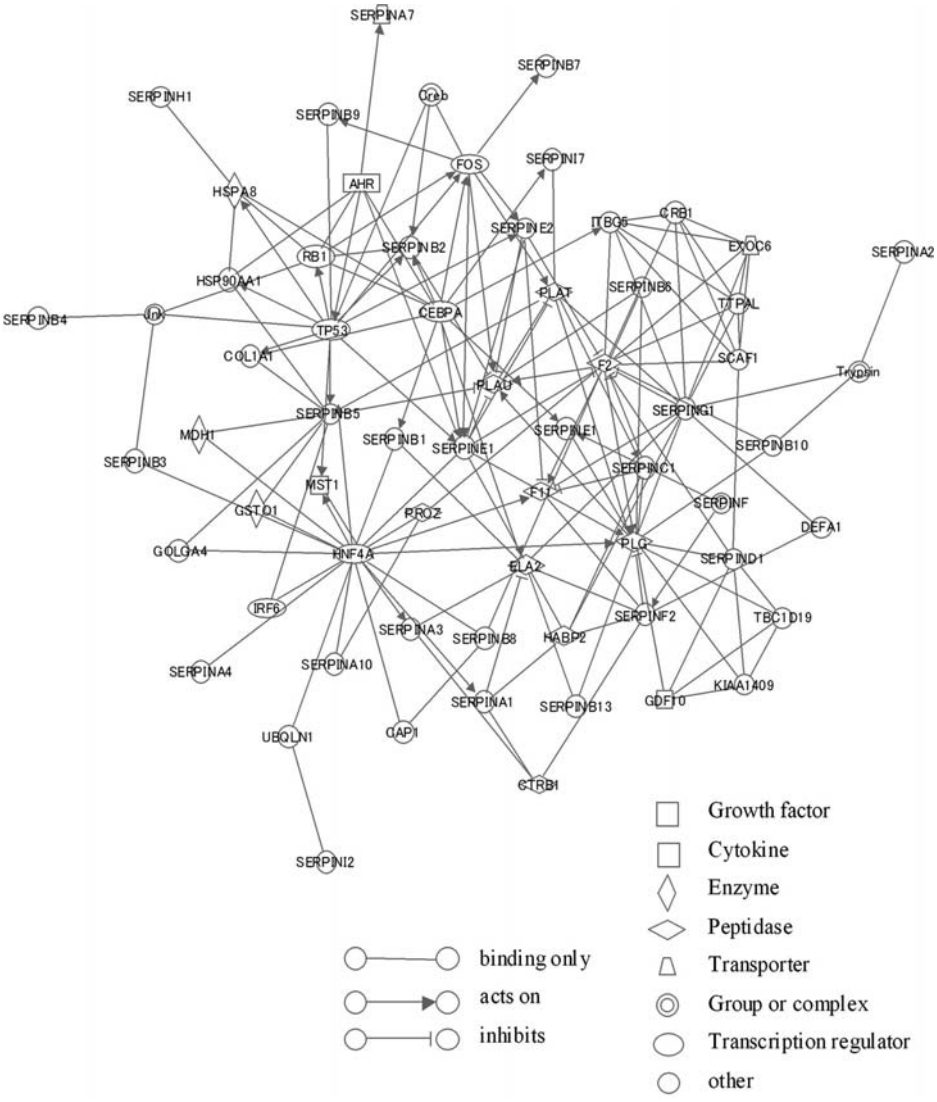


Figure 2. Network of molecules related to serpin family members identified by IPA software. Gene accession numbers and the results of global gene screening using microarray analysis were imported into the IPA software. The identified network shows the classification of molecules and their interactions.

*SERPING1*, *SERPINB5*, *SERPINB7* and *SERPINB2*. Of these genes, *SERPINB5*, *SERPINB7* and *SERPINB2* are known to be located on 18q21.3, thus, the data strongly suggested that this chromosomal region is crucial for the development of OSCC. We therefore performed further examinations to validate the expression profiles of *SERPIN* genes located on 18q21.3.

*mRNA expression of SERPIN genes in OSCC samples*. To confirm the expression profiles of *SERPIN* genes, mRNA expression levels were determined by qRT-PCR analyses in 55 OSCC samples obtained from patients with primary OSCC. Compared with matched normal tissues, mRNA expression levels of *SERPINB12* (P=0.001), *SERPINB13* (P=0.001), *SERPINB4* (P=0.042), *SERPINB3* (P<0.001), *SERPINB11* (P<0.001), *SERPINB7* (P=0.021) and *SERPINB2* (P=0.018) were significantly decreased in OSCC samples (Fig. 1).

*Genetic network analysis and canonical pathway analysis for the serpin family*. Genetic network analysis including SERPIN family members was performed using IPA software. The analysis suggested that there were two networks of molecules related to the serpin family (Table V), and the biological interactions between these molecules are summarized in Fig. 2. The figure shows binding of SERPINB3 or SERPINB4 with jnk, which is a member of the MAP kinase family and involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation and development. In addition FOS, which can form the transcription factor complex AP-1 and can regulate cell proliferation, differentiation and transformation, acted on SERPINB7 and SERPINB9. TP53 acted on SERPINB5 and SERPINB2. RB1, a tumor suppressor, is also included in the network. The data suggest that members of the serpin family might have a close relationship with some of the tumor-related molecules. SERPINB11 and SERPINB12 are not shown in the network because few reports have so far been published on these molecules.

*Relationship between clinical factors and SERPIN gene expression*. The relationship between clinical factors and mRNA expression of serpin family members was assessed.

Table IV. Correlation between mRNA expressions of *SERPIN* genes and clinical classification.

A. T classification

| Gene | Tumor size | No. of cases | | P-value[a] |
|---|---|---|---|---|
| | | Highly expressive, T/N ratio >1 (%) | Lower expressive, T/N ratio <1 (%) | |
| *SERPINB12* | T1 or T2 | 7 (26) | 20 (74) | >0.999 |
| | T3 or T4 | 7 (25) | 21 (75) | |
| *SERPINB13* | T1 or T2 | 7 (26) | 20 (74) | >0.999 |
| | T3 or T4 | 8 (29) | 20 (71) | |
| *SERPINB4* | T1 or T2 | 12 (44) | 15 (56) | 0.2695 |
| | T3 or T4 | 8 (29) | 20 (71) | |
| *SERPINB3* | T1 or T2 | 12 (44) | 15 (56) | 0.2695 |
| | T3 or T4 | 8 (29) | 20 (71) | |
| *SERPINB11* | T1 or T2 | 7 (26) | 20 (74) | >0.999 |
| | T3 or T4 | 7 (25) | 21 (75) | |
| *SERPINB7* | T1 or T2 | 7 (26) | 20 (74) | 0.1625 |
| | T3 or T4 | 13 (46) | 15 (54) | |
| *SERPINB2* | T1 or T2 | 7 (26) | 20 (74) | 0.1625 |
| | T3 or T4 | 13 (46) | 15 (54) | |

[a]P-values were calculated with Fisher's exact analysis.

B. N classification.

| Gene | Regional neck metastasis | No. of cases | | P-value[a] |
|---|---|---|---|---|
| | | Highly expressive, T/N ratio >1 (%) | Lower expressive, T/N ratio <1 (%) | |
| *SERPINB12* | N (-) | 3 (18) | 14 (82) | 0.3440 |
| | N (+) | 12 (32) | 26 (68) | |
| *SERPINB13* | N (-) | 4 (24) | 13 (76) | 0.7542 |
| | N (+) | 11 (29) | 27 (71) | |
| *SERPINB4* | N (-) | 8 (47) | 9 (53) | 0.2290 |
| | N (+) | 11 (29) | 27 (71) | |
| *SERPINB3* | N (-) | 9 (53) | 8 (47) | 0.1478 |
| | N (+) | 12 (32) | 26 (68) | |
| *SERPINB11* | N (-) | 2 (12) | 15 (88) | 0.3026 |
| | N (+) | 12 (31) | 27 (69) | |
| *SERPINB7* | N (-) | 5 (30) | 12 (70) | 0.5491 |
| | N (+) | 16 (42) | 22 (58) | |
| *SERPINB2* | N (-) | 5 (29) | 12 (71) | 0.5545 |
| | N (+) | 15 (40) | 23 (60) | |

[a]P-values were calculated with Fisher's exact analysis.

 Continued.

C. Differentiation.

| Gene | Differentiation | No. of cases | | P-value[a] |
|---|---|---|---|---|
| | | Highly expressive, T/N ratio >1 (%) | Lower expressive, T/N ratio <1 (%) | |
| *SERPINB12* | Well | 10 (32) | 21 (68) | 0.3800 |
| | Moderate or Poor | 5 (21) | 19 (79) | |
| *SERPINB13* | Well | 9 (29) | 22 (71) | 0.7706 |
| | Moderate or Poor | 6 (25) | 18 (75) | |
| *SERPINB4* | Well | 12 (39) | 19 (61) | 0.7808 |
| | Moderate or Poor | 8 (33) | 16 (67) | |
| *SERPINB3* | Well | 13 (42) | 18 (58) | 0.1480 |
| | Moderate or Poor | 5 (21) | 19 (79) | |
| *SERPINB11* | Well | 12 (39) | 19 (61) | 0.0371 |
| | Moderate or Poor | 3 (13) | 21 (87) | |
| *SERPINB7* | Well | 13 (42) | 18 (58) | 0.5835 |
| | Moderate or Poor | 8 (33) | 16 (67) | |
| *SERPINB2* | Well | 14 (45) | 17 (55) | 0.2718 |
| | Moderate or Poor | 7 (29) | 17 (71) | |

[a]P-values were calculated with Fisher's exact analysis.

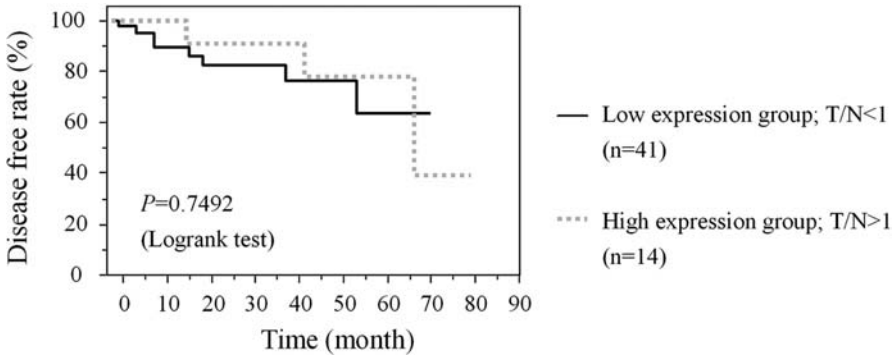


Figure 3. Comparison of disease-free rates with respect to *SERPINB11* mRNA expression status in OSCC. The overall disease-free rates in relation to the level of *SERPINB11* gene expression were determined by Kaplan-Meier analysis. OSCC cases were divided into high expression (T/N ratio >1.0) and lower expression (T/N ratio <1.0) groups. Disease-free rates tended to be decreased in the lower expression group; however, no statistically significant difference was observed.

There was no significant association between the T/N ratio of *SERPIN* gene expression and the T classification or N classification or other clinicodemographic characteristics (Table IVA and B). However, T/N ratios of gene expression in *SERPIN* genes that were down-regulated in tumor tissues tended to decrease in moderate or poorly differentiated tumors compared with well-differentiated tumors (Table IVC). In particular, significantly diminished mRNA expression was found for *SERPINB11* (P=0.0371). Disease-free rate was estimated by Kaplan-Meier analysis and compared with *SERPINB11* expression in OSCC cases. Cases were divided into high expression (T/N ratio >1.0) and lower expression (T/N ratio <1.0) groups, and disease-free rates were compared. Disease-free rates in the lower expression group tended to be decreased, although no statistically significant difference was observed (Fig. 3).

## Discussion

Previous studies have demonstrated that *SERPINB5* and *SERPINB13* have crucial roles as tumor suppressor genes; it is therefore reasonable to speculate that other serpin members

Table V. Networks of molecules related to the serpin family.

| Network | Molecules in Network | Score | Focus molecules | Top functions |
|---|---|---|---|---|
| 1 | AHR, CAP1, CEBPA, COL1A2, Creb, CTRB1, FOS, GOLGA4, GSTO1, HNF4A, HSP90AA1, IRF6, Jnk, MDH1, MST1, PLAT, PLAU, PROZ, RB1, SERPINA1, SERPINA3, SERPINA4, SERPINA7, SERPINA10, SERPINB3, SERPINB4, SERPINB5, SERPINB7, SERPINB8, SERPINB9, SERPINE1, SERPINE2, SERPINI2, TP53, UBQLN1 | 33 | 14 | Cardiovascular disease, hematological disease, organismal injury and abnormalities |
| 2 | CEBPA, CRB1, DEFA1 (includes EG:1667), ELA2, EXOC6, F2, F11, GDF10, HABP2, HSPA8, ITGB5, KIAA1409, PLAT, PLAU, PLG, RB1, SCAF1, SERPINA2, SERPINB1, SERPINB2, SERPINB6, SERPINB10, SERPINB13, SERPINC1, SERPIND1, SERPINF, SERPINF1, SERPINF2, SERPING1, SERPINH1, SERPINI1, TBC1D19, Trypsin, TTPAL | 30 | 13 | Cardiac pulmonary embolism, cardiovascular disease, respiratory disease |

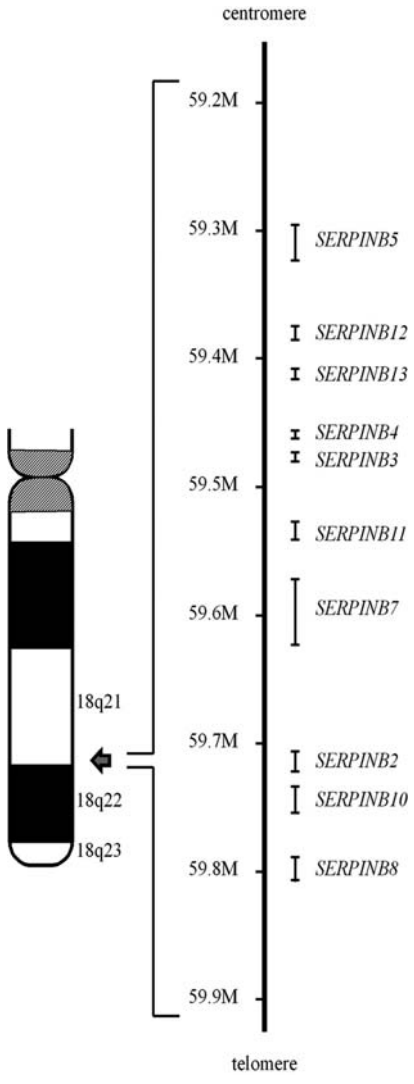


Figure 4. Schema of chromosome 18. The figure shows the location of the SERPIN gene cluster in chromosome region 18q21.

that have conserved conformations might possess similar activities in oral carcinomas. Therefore, in the present study, overall gene expression profiling was performed by DNA microarray analysis. This revealed that down-regulation of SERPIN genes was frequently observed in chromosome 11q12-q13.1 and 18q21.3. Real-time qRT-PCR analysis was then performed to validate the expression profiles of serpin members located at these chromosome regions in OSCC samples (see also Fig. 4).

Statistically significant down-regulations of mRNA expression were found for *SERPINB12* (18q21.3), *SERPINB13* (18q21.3-q22), *SERPINB4* (18q21.3), *SERPINB3* (18q21.3), *SERPINB11* (18q21.3), *SERPINB7* (18q21.33) and *SERPINB2* (18q21.3). Interestingly, these serpins belong to the clade B serpin group, suggesting that their conserved common characteristics should be closely associated with tumorigenesis in oral carcinoma. Unlike the majority of serpin members that exist in the serum and are involved in blood coagulation, fibrinolysis and inflammation, clade B serpins have predominantly intracellular functions. The present pathway analysis revealed that clade B serpins are closely related to various molecules including cancer-related molecules. Thus, the data suggest that clade B serpin members play crucial roles in tumor suppression through mechanisms such as signal transduction pathways for cell proliferation or differentiation.

Serpin members were originally identified as protease inhibitors; however, our data strongly suggest that serpin members other than SERPINB5 or SERPINB13 have tumor suppressive activities. Recently, De Koning *et al* demonstrated that down-regulation of SERPINB13 expression was positively associated with poor clinical outcome in human head and neck squamous cell carcinomas, and that SERPINB13 may act as an important protease inhibitor involved in the progression of head and neck squamous cell carcinomas (23). In the present study, mRNA expression of *SERPINB11* was significantly diminished in poorly or moderately differentiated

ses when compared with well-differentiated cases. s also a trend toward worse outcome in the group with lower expression of *SERPINB11*. This suggests that SERPINB11 is extremely important in the clinical setting and might have potential as a biomarker of OSCC.

Askew *et al* reported that single nucleotide polymorphisms in the scaffold induce conformational changes of SERPINB11, which result in the loss of trypsin inhibitory activity (24). SERPINB11 has not yet been reported as a tumor suppressor, but our data suggest that it could potentially possess the tumor suppressive function and may be particularly associated with cell differentiation. The present data revealed that down-regulated expression was frequently observed for serpin members located at 18q21, suggesting that this chromosome region is crucial in oral carcinomas.

Region 18q21 is one of the well-known instable chromosome regions in oral carcinomas, at which genetic alterations such as LOH or microsatellite instability have been frequently reported, indicating that there may be unidentified tumor suppressor gene in this region (16,18,20,25). Previously, we found a high frequency of LOH (43.8%) at 18q21 in OSCCs. In contrast, *DCC* (deleted in colorectal carcinoma) and *DPC4* (deleted in pancreatic carcinoma 4) gene, both located at 18q21, were not detected (21). These findings and our present data strongly suggest that further investigations are essential to clarify whether members of the serpin family located at 18q21 have tumor suppressive activities in oral carcinomas.

In conclusion, the present data revealed that mRNA expressions of clade B *SERPIN*s clustering at 18q21 are significantly down-regulated in OSCC. In particular, *SERPINB11* mRNA expression was associated with the level of differentiation, suggesting that it might be important in this aspect. Although tumor suppressive activity has not been indicated for these serpins with the exception of SERPINB5 and SERPINB13 in OSCC, other serpins might function as tumor suppressors. Furthermore, there might be additional unidentified tumor suppressor genes in this chromosome region.

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