Epigenetic Alterations of the *SERPINE1* Gene in Oral Squamous Cell Carcinomas and Normal Oral Mucosa

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A high level of plasminogen activator inhibitor-1 (PAI-1 or *SERPINE1*) in tumor extracts is a marker of a poor prognosis in human cancers, including oral carcinomas. However, the mechanisms responsible for the upregulation of PAI-1 in cancers remain unclear. Investigating specific PAI-1 expressing cells in oral carcinomas by immunohistochemistry, we found that PAI-1 was expressed in 18 of the 20 patients, mainly by cancer cells. Two showed PAI-1 positive stromal cells surrounding the tumor areas and five showed PAI-1 positive cells in tumor-adjacent normal epithelium. By real-time RT-PCR analysis, 17 of 20 patients with oral carcinoma were found to have between 2.5- and 50-fold increased tumor PAI-1 mRNA level, as compared with the matched tumor-adjacent normal tissues. The PAI-1 mRNA level in connective tissues from 15 healthy volunteers was similar to the level in tumor-adjacent normal tissues, but the level in epithelium was 5- to 10-fold lower. Analyzing DNA methylation of 25 CpG sites within 960 bp around the transcription initiation site of the *SERPINE1* gene by bisulfite sequencing, we did the surprising observation that both tumors and tumor-adjacent normal tissue had a significant level of methylation, whereas there was very little methylation in tissue from healthy volunteers, suggesting that tumor-adjacent normal tissue already contains transformation-associated epigenetic changes. However, there was no general inverse correlation between PAI-1 mRNA levels and *SERPINE1* gene methylation in all tissues, showing that CpG methylation is not the main determinant of the PAI-1 expression level in oral tissue.

INTRODUCTION

Oral carcinogenesis is believed to represent a multistep process driven by the accumulation of carcinogen-induced genetic and epigenetic changes. It is generally accepted that many of the cancer-associated gene changes stem from the gain, loss, or mutation of genetic information, as well as DNA methylation (Ha and Califano, 2006; Shaw, 2006). However, not only alterations of proto-oncogenes and tumor suppressor genes but also many other regulatory factors related to cell growth, migration, and proliferation are involved in tumorigenesis.

It has been demonstrated that the urokinase-type plasminogen activator (uPA) system plays an important role in tumor metastasis and invasion. The uPA system is a serine protease system with a complex pericellular organization. The main biochemical function of the system is the uPA-catalyzed proteolytic conversion of the inactive zymogen plasminogen to the active, nonspecific protease plasmin, which can catalyze degradation of many extracellular matrix proteins. The activity of uPA is inhibited by plasminogen activator inhibitor-1 (PAI-1). A high level of uPA in tumors is correlated with a poor prognosis in many cancer types, including oral cancer. Furthermore, it has been shown that high tumor PAI-1 levels, similarly to high uPA levels, predict a poor prognosis (Andreasen et al., 2000; Durand et al., 2004). PAI-1 is expressed in oral squamous cell carcinomas but not in normal oral mucosa; furthermore, PAI-1 is mainly expressed in cancer cells in oral

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carcinoma (Clayman et al., 1993; Nozaki et al., 1998; Curino et al., 2004; Lindberg et al., 2006). In this respect, oral carcinomas are different from other cancer types, such as breast, prostate, and colon cancers, in which PAI-1 is predominantly found in stromal myofibroblasts and not in the cancer cells themselves (Offersen et al., 2003; Illemann et al., 2004; Usher et al., 2005). Although it has been well-demonstrated that PAI-1 is overexpressed in human cancers, the mechanism of the altered level of PAI-1 expression in tumors is unclear.

It has become increasingly clear that epigenetic events, i.e., inheritable changes in gene function that cannot be explained by changes in DNA sequence, play an important role in development of cancer by modifying gene expression levels (Herman, 1999; Esteller and Herman, 2002). In this way, aberrant DNA methylation is important in inactivation of tumor suppressor genes and other cancer-related genes in most types of tumors, including oral carcinomas (McGregor et al., 2002). Concerning the effect of CpG methylation of the *SERPINE1* gene on regulation of transcriptional activity, we have previously found that methylation of 25 CpG sites within approximately 960 bp around the transcriptional initiation site is inversely correlated with the PAI-1 expression in five human tumor cell lines (Gao et al., 2005).

In the present study, we characterized 20 patients with oral squamous cell carcinomas and 15 healthy volunteers with respect to PAI-1 expressing cell types by immunohistochemical staining, PAI-1 mRNA levels by RT-PCR, and *SERPINE1* gene methylation status to study mechanisms behind the increased expression in oral cancers.

MATERIALS AND METHODS

Sample Preparation

Frozen surgical specimens of oral squamous cell carcinoma from 20 patients were obtained from Odense University Hospital, Denmark. The median age of the 20 patients was 63 years (range, 48–94 years); there were six women and 14 men. The location of the carcinomas was six at the tongue (presented as T1–T6), nine at the floor of the mouth (presented as F1–F9), and five at the alveolar ridge (presented as A1–A5). For all patients, a biopsy from clinically normal mucosa close to the tumor was included as well. Hematoxylin-eosin staining of the sections was performed and the ratio between cancer and normal tissues were examined on histological sections. In 14 cases, in which the cancer cells contributed 80% or more, the tissue blocks were used directly for RNA preparation. In six cases, a laser microdissection system (P.A.L.M.) was used to separate cancer cells from adjacent normal tissue, as well as to separate histologically normal epithelium from the connective tissue. Fifteen samples from normal buccal mucosa obtained from healthy volunteers served as control tissue, too. There were five males and 10 females, the median age was 36 years (range, 25 to 62 years), and one smoker. Before the following procedures for DNA/RNA preparation, epithelium in the normal tissue was separated from the stroma by incubation in 2 mM EDTA buffer (Mackenzie et al., 1979).

For all tissues, informed consent and approval by the Ethics Committee were obtained according to Danish legislation.

DNA was extracted by routine procedures using the DNeasy Kit (Qiagen, Copenhagen, Denmark). Total RNA was extracted by routine procedures using the RNeasy Mini Kit (Qiagen, Copenhagen, Denmark). On-column DNase digestion by RNA-free DNase I (Qiagen, Copenhagen, Denmark) was performed during the RNA purification procedure.

PAI-1 mRNA Expression Analyzed by Quantitative Real-Time RT-PCR

Reverse transcription reaction was performed with 2 μg DNA-free RNA, using First-strand cDNA Synthesis Kit (Amersham, Copenhagen, Denmark). Quantitative real-time RT PCR was performed as described elsewhere (Gao et al., 2005). Amplification reactions were carried out in a final volume of 25 μl containing 2× Brilliant Multiplex Master Mix (Stratagene, Copenhagen, Denmark). The comparative CT (threshold cycle) and copy numbers described in the manufacturer's protocol was used to quantitate the relative PAI-1 mRNA expression level, comparing tumor samples to corresponding normal controls.

Thermal conditions were as follows: 95°C for 10 min to activate the DNA polymerase, followed by 40 cycles of 95°C for 30 sec and 62°C for 30 sec, and 72°C for 30 sec. Amplification was performed on a Mx4000® Multiplex Quantitative PCR System (Stratagene, Copenhagen, Denmark). The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene mRNA was amplified as an internal control to normalize the data of the PAI-1 mRNA level. All PAI-1 mRNA levels were expressed relative to the PAI-1 mRNA in the cell line HT-1080, which has a high PAI-1 expression

and was run as a positive control in each reaction (Gao et al., 2005, 2008).

Immunohistochemical Staining for PAI-1 Antigen

To investigate the histological distribution of PAI-1 antigen in oral carcinomas, immunohistochemical staining was performed. Affinity-purified rabbit polyclonal antibodies as well as control rabbit anti-PAI-1 polyclonal antibodies depleted for anti-PAI-1 IgG have been described previously (Offersen et al., 2003).

Four micrometer frozen sections were prepared. The primary antibodies were diluted in 50 mM Tris, 150 mM NaCl, pH 7.6 (TBS), containing 0.25% BSA, and incubated on the sections overnight in Shandon racks (Thermo Shandon, Pittsburgh, PA) at the following concentrations: PAI-1 polyclonal antibodies (0.5 μg/ml). After 30 min at room temperature, sections were washed in 50 mM Tris, 150 mM NaCl, pH 7.6, containing 0.5% Triton X-100 (TBS-T). The primary antibodies were detected with envision reagents and anti-rabbit IgG horseradish peroxidase-conjugated polymers (DakoCytomation, Copenhagen, Denmark). Each incubation step was followed by washing with 6 ml of TBS-T. Endogenous peroxidase was blocked by incubation in 1% H_2O_2 for 15 min. Sections were developed with 3, 3′-diaminobenzidine chromogenic substrate (DAB, DakoCytomation, Copenhagen, Denmark) for 15 min and finally counterstained with hematoxylin.

Methylation Analysis

Genomic DNA was treated with sodium bisulphite as described previously (Clark et al., 1994). Bisulfite sequencing was performed as described elsewhere (Gao et al., 2005). PCR amplification was performed in 25 μl of reaction mixture containing 100 ng bisulfite-treated DNA, 10 pmol of each primer, 0.2 mM each dNTP, 1 unit of HotStarTaq DNA polymerase (Qiagen, Copenhagen, Denmark), and 1× PCR buffer. Three pairs of primers were used for amplifying the 958 bp sequence from −805 to +152, one covering CpG #1 to #7, which will be referred as the Exon1 (SeqE), one covering CpG #8 to #15, which will be referred as the proximal sequence (SeqP) and the other covering CpG #16 to #25, which will referred as the distal sequence (SeqD). The sequences of the primers were as follows: PAI-1SeqE-F, AGTTGTGT TTGGTTGTAGGGTTAAG; PAI-1SeqE-R, CT TTTCTCCTACCTAAAATTCTCAAAAA (4 to 152, 151 bp); PAI-1SeqP-F, TTTGATAATTT TATAGTGATTTGGTT; PAI-1SeqP-R CACC CACTCACTAACTCTAAAAATC (−433 to −73, 361 bp); PAI-1SeqD-F, TTTTTATTATGGT AATTTTTGGTTT; PAI-1SeqD-R, AAATTA TCAAAAATAACCTCCA TCAA (−805 to −422, 384 bp). The reaction for PAI-1SeqE was started with initial denaturation at 95°C for 10 min, followed by 35 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. Reactions for PAI-1SeqP and PAI-1SeqD were started with initial denaturation at 95°C for 10 min, followed by 35 cycles at 95°C for 1 min, 57°C for 1 min, and 72°C for 2 min. Amplified DNA was subcloned into vector pCR2.1 by using a TOPO TA cloning kit (Invitrogen, Copenhagen, Denmark) and sequenced. Eight or 10 positive clones were analyzed for each sample. The total frequency of total methylated CpGs was calculated as (number of methylated CpGs/number of totally analyzed CpGs) × 100%.

Statistical Analysis

Paired *t* test was performed for comparison of the PAI-1 mRNA expression level and FTM between tumor samples and corresponding normal tissues. Fisher's exact probability test was used for analysis the correlation between PAI-1 mRNA expression and immunohistochemical staining intensity, as well as for analysis the correlation between methylation frequency and PAI-1 mRNA levels in carcinomas. Wilcoxon rank sum test was performed for comparison the PAI-1 expression in patients with and without lymph node metastasis. The χ^2 test was performed for comparison between 4G/5G polymorphism in tumor and adjacent histologically normal samples. Student's *t* test was performed for comparison of methylation frequency between 4G and 5G alleles in both tumor and tumor-adjacent, histologically normal tissue samples.

RESULTS

Immunohistochemical Staining of PAI-1 Antigen in Oral Carcinomas Specimens

All of the 20 investigated tumors were squamous cell carcinomas. In eight cases, minor parts of histologically normal mucosa were presented adjacent to the tumor sample.

Immunohistochemical staining for PAI-1 was performed in the 20 tumor samples. Two tumors were negative and in three tumors only a few positive areas were found (Fig. 1A); a correspondingly

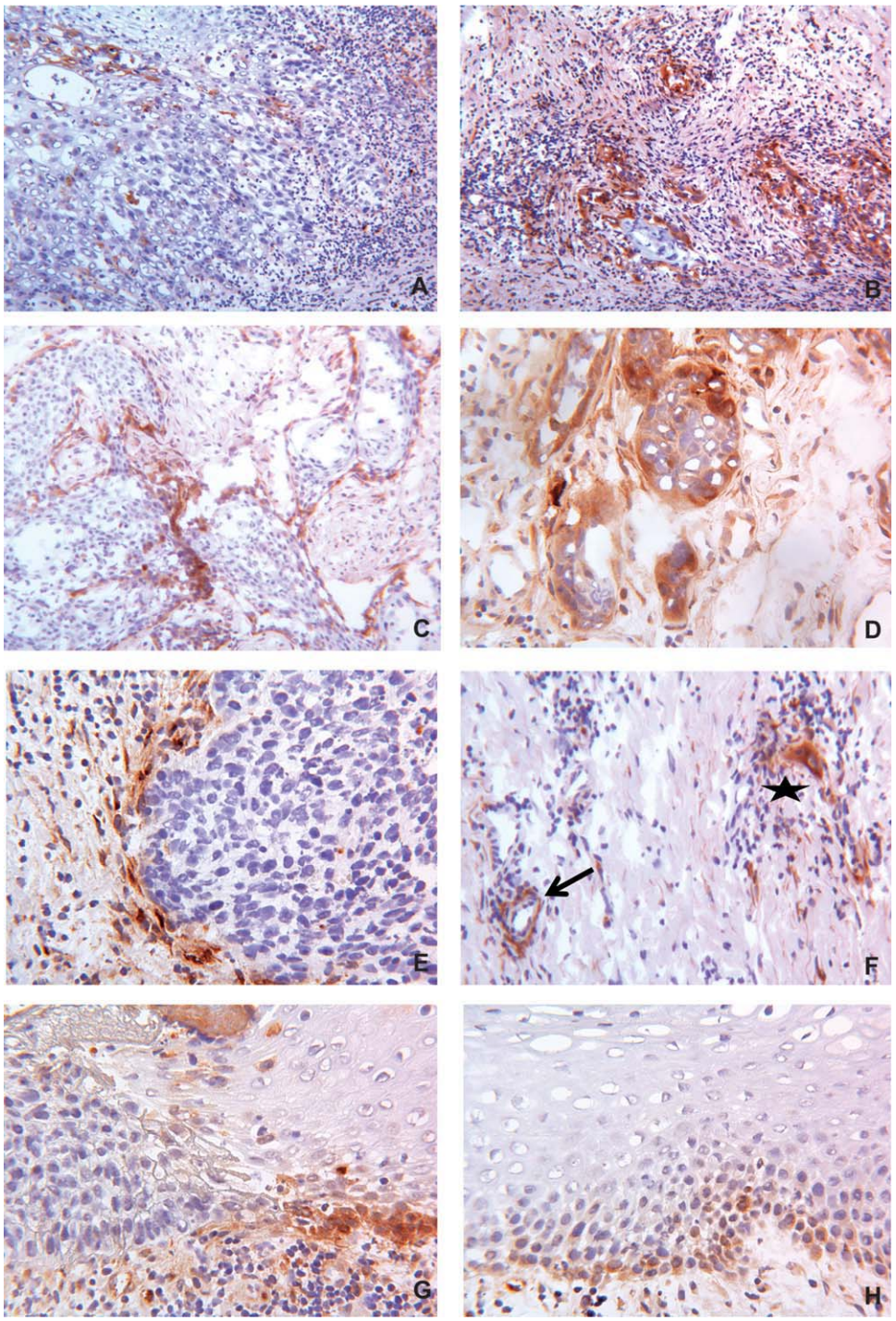

Figure 1. Immunohistochemical staining of PAI-1 expressing cell types. (A) A few cells were positive for PAI-1, but most are negative; (B) a heterogeneous distribution of PAI-1 positive carcinoma cells was demonstrated; (C, D) the positive PAI-1 staining was mainly located at the invasive front of the tumor island; (E) the staining reaction was negative in the cancer cells themselves but positive in the surrounding stroma; (F) PAI-1 expression was also seen in endothelial cells of small vessels surrounding the malignantly invasive strands (arrow) or clusters (star); (G) the positivity of PAI-1 staining was located in the subepithelial connective tissue in histologically normal epithelium, but it was negative in cancer cells with a clear borderline (the picture is from the same sample of E); (H) the positivity of PAI-1 staining was also seen in basal cells in tumor-adjacent, histologically normal epithelium.

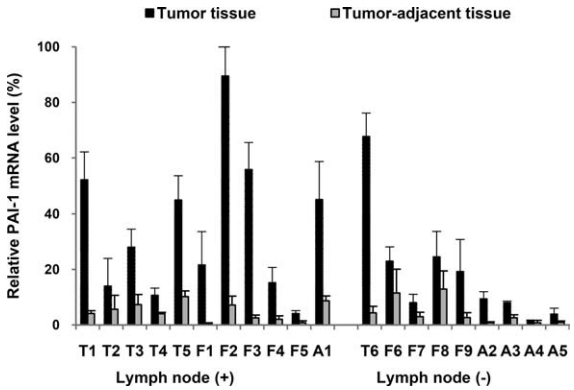

Figure 2. PAI-1 mRNA expression level in 20 oral carcinomas and matched tumor-adjacent, histologically normal tissue. PAI-1 mRNA levels were determined by Q-real time RT-PCR and normalized against the GAPDH mRNA level. The PAI-1 level was given as a percentage of the level in HT-1080 cells. The figure shows means ± SD for three independent determinations. The level in tumors (black bars) was significantly elevated compared with the level in the corresponding samples of histologically normal tissue from the same patient (gray bars; $P < 0.001$, as evaluated by a paired t test). The patients are organized into two subgroups in the figure, according to lymph node involvement. The locations of the carcinomas are indicated: tongue (T 1–6), the floor of the mouth (F 1–9), and alveolar ridge (A1–5).

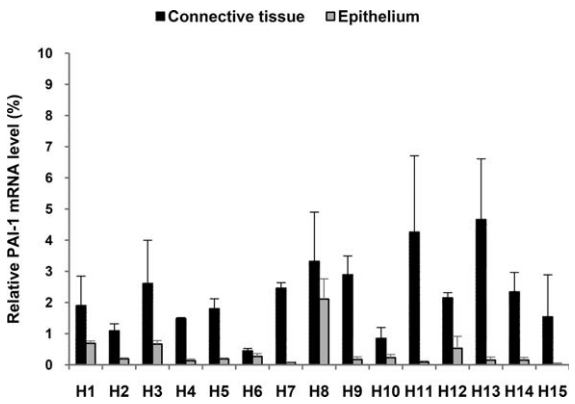

Figure 3. PAI-1 mRNA expression level in epithelium and connective tissue in samples of tissue from healthy volunteers. PAI-1 mRNA levels in epithelium and connective tissue from 15 healthy volunteers (H1–H15) were determined by Q-real time RT PCR and normalized against the GAPDH mRNA level. The PAI-1 level was given as a percentage of the level in HT-1080 cells. The figure shows means ± SD for three independent determinations. The level in the connective tissues (black bars) was higher than the level in corresponding epithelium from the same healthy volunteers (gray bars; $P < 0.01$, as evaluated by a paired t test).

low level of PAI-1 mRNA was found in these cases. In 17 patients, PAI-1 was demonstrated in the carcinoma cells. The distribution was heterogeneous, with some parts of the tumor negative and other parts positive (Fig. 1B). The positive PAI-1 staining was mainly located at the basal outer cell layer of the tumor island (Figs. 1C and1D). In two patients, PAI-1 positive cells were found in tumor stroma. In one of these cases, the staining reaction was negative in the cancer cells themselves (Fig. 1E). In five cases, PAI-1 expression was also seen in endothelial cells of small vessels surrounding the malignantly invasive strands or clusters (Fig. 1F). Normal mucosa adjacent to the tumor showed positive staining in five cases. In one of these, the positivity was located in the subepithelial connective tissue (Fig. 1G), in the rest of the cases in basal epithelial cells (Fig. 1H).

PAI-1 mRNA Levels, as Evaluated by Quantitative Real-Time RT-PCR

By quantitative real-time RT-PCR analysis, oral carcinomas from 17 of 20 patients were found to have a higher PAI-1 mRNA level than control tissue, which was tumor-adjacent, histologically normal oral tissue from the same patient. The fold increase of the PAI-1 mRNA level in the tumors, as compared with the tumor-adjacent, histologically normal tissue, was from 2.5- to 50-fold (Fig. 2). The average PAI-1 mRNA level relative to the positive control (HT-1080 cells) in the 20 tumors was 27% ± 24%, which is significantly higher than the level of 4.5% ± 3.7% in the corresponding adjacent, histologically normal tissue from the same patients ($P < 0.001$, paired t test). There was no significant difference between patients with or without lymph nodes metastasis ($P > 0.05$, by Wilcoxon rank sum test). To evaluate the correlation between PAI-1 mRNA level in tumors and the immunohistochemical staining results, patients were divided into two groups according to the median value of the PAI-1 mRNA level as well as according to the intensity and the positive area in immunohistochemical stainings for PAI-1 in the tumors. These two assays demonstrated a statistically significant correlation as analyzed by Fisher's Exact Probability Test ($P = 0.0013$).

The PAI-1 mRNA levels in epithelium and connective tissues from 15 healthy volunteers were also measured. The average PAI-1 mRNA level in epithelium was 0.38% ± 0.52%. The average PAI-1 mRNA level in connective tissue was 2.3% ± 1.2%. The two values are significantly different ($P < 0.001$, paired t test; Fig. 3). The PAI-1 mRNA level in tumor-adjacent, histologically normal tissues from cancer patients was not different from the level in connective tissues from healthy volunteers, but 5- to 10-fold higher than the level in epithelium from healthy volunteers ($P < 0.01$). Although we did not have enough tissue to do RT-PCR analysis on RNA from epithelium and connective tissue from tumor-adjacent, histologically normal tissue

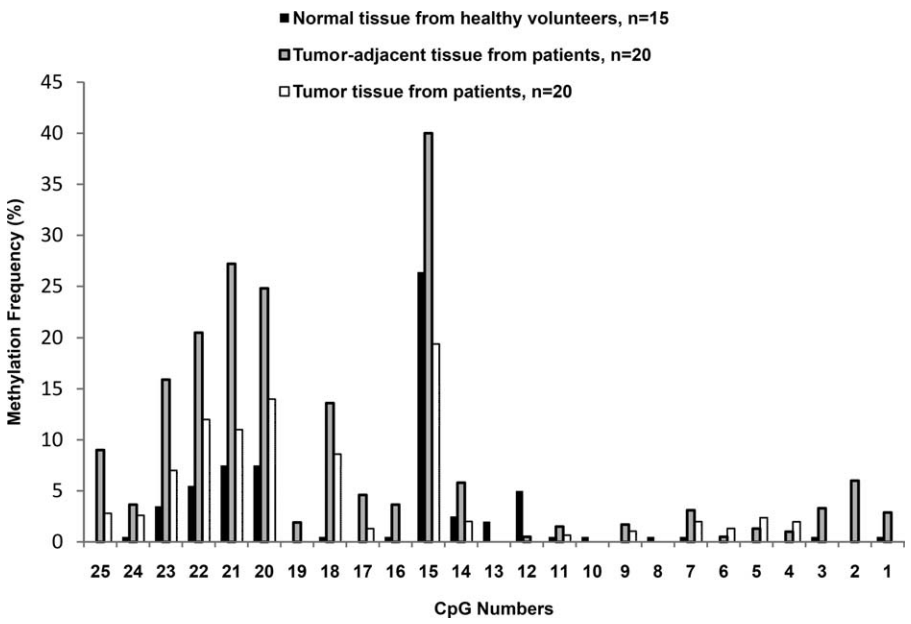

Figure 4. Total frequency of methylation at each of 25 CpG sites in the *SERPINE1* promoter in tissues from both patients and healthy volunteers. The total frequency of methylation was analyzed for the 25 CpG sites within the −805 to +152 region of the *SERPINE1* gene in 15 samples of tissue from healthy volunteers, 20 samples of tumor-adjacent, histologically normal tissue, and 20 samples of tumors. From each of the samples, eight or 10 clones of DNA were analyzed for CpG methylation. The bars represent the frequency of methylation at each particular CpG site, numbered from the 3′ end. Black bars, tissue from healthy volunteers; gray bars, tumor adjacent tissues from patients; blank bars, tumor tissues from patients.

separately, this result indicated that epithelium from tumor-adjacent, histologically normal epithelium has a higher PAI-1 mRNA level than epithelium from healthy volunteers.

The Methylation Status of the SERPINE1 Gene in Oral Tissues

Three pairs of primers were used for bisulfite sequencing of the region from −805 to +152 of the *SERPINE1* gene, containing 25 CpG dinucleotides. The three primer sets defined the distal region, between base pairs −805 to −422, covering CpG #25 to #16; the proximal region, between base pairs −433 to −73, covering CpG #15 to #8; and the exon 1 region, between base pairs 4 to 154, covering CpG #7 to #1. The methylation pattern was analyzed in the 20 paired samples of both tumor and adjacent, histologically normal tissue from patients with oral carcinoma, and 15 paired samples of both epithelium and connective tissue from healthy volunteers.

Inspecting the methylation patterns within individual samples, some recurrent patterns could be distinguished which were common to all samples (Fig. 4). The methylation frequency, defined as the number of clones methylated at a certain CpG site as a percentage of the total number of clones analyzed for this CpG site, was low for all sites in the exon 1 region (sites #1 to #7) and most of the sites in the proximal region (sites #8 to #14). In fact, the total methylation frequency was only 2.7% in this region. Interesting, however, the methylation frequency of CpG site #15, located at −405 bp, was relatively high. This site was found to be methylated in 30% out of a total of 598 individual DNA clones analyzed, including all samples from both patients and healthy volunteers. In the distal region, the methylation frequency was much higher than in the other regions, particularly at CpG sites #18, 20, 21, 22, and 23. These five sites were methylated with a total frequency of 12.3% in analyzed DNA clones.

Comparing the 8–10 clones analyzed for each tissue sample, it was evident that some clones contained methylation at multiple sites, whereas others were totally devoid of methylation, as shown by the example in Figure 5. This result may suggest that most samples contained DNA from different cell types with very different levels of methylation.

Looking for possible differences in methylation patterns between samples of different origins, we concentrated on the CpG sites with a high degree of methylation, i.e., #15, #18, #20, #21, #22, #23.

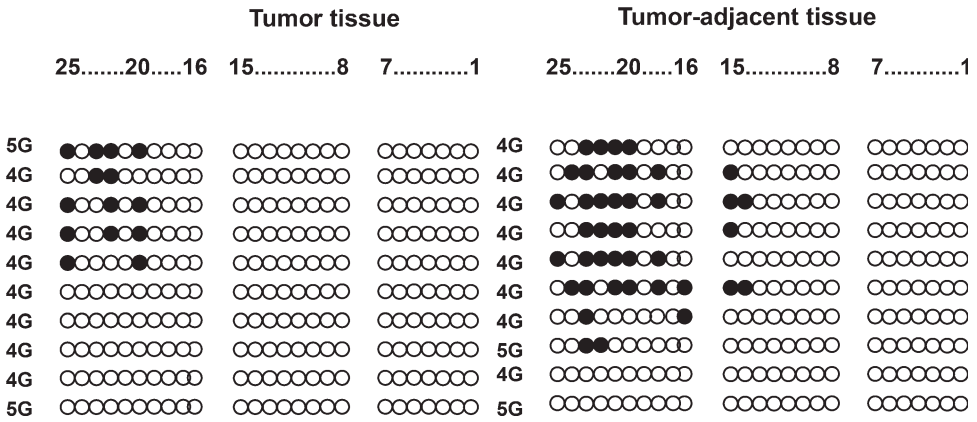

Figure 5. Example of methylation frequency in individual clones from the same tissue sample. The circles represent the single CpG sites, which were numbered # 1 to 25 from the 3′ end to 5′ end in the top row. The region covered by the 3 primers (CpG #1 to 7, exon 1 region; CpG #8 to 15, proximal region; CpG #16 to 25, distal region) are indicated. Filled circle, methylated; empty circle, unmethylated. The figure shows the methylation status for 10 single DNA clones for each primer, one for each row. The data are for patient #F3. The status of the analyzed allels with respect to the 4G/5G polymorphism is also indicated.

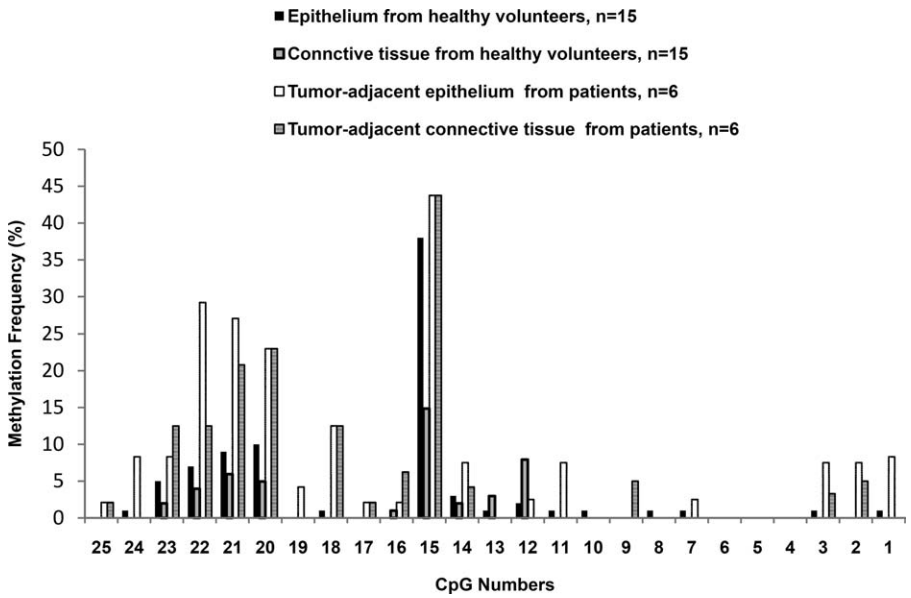

Figure 6. Methylation frequency at each of the 25 CpG sites in the *SERPINE1* promoter in epithelium and connective tissue from both healthy volunteers and in tumor-adjacent, histologically normal tissue from patients. The total frequency of methylation was analyzed for the 25 CpG sites within the −805 to +152 region of the *SERPINE1* gene in epithelium and connective tissue adjacent to tumors from six oral carcinoma patients and from 15 healthy volunteers. From each of the samples, eight or 10 clones of DNA were analyzed for CpG methylation. The columns represent the frequency of methylation at each particular CpG site, numbered from 3′ end. Black bars, epithelium from healthy volunteers; gray bars, connective tissue from healthy volunteers; blank bars, tumor adjacent epithelium from patients; bars with insert, tumor adjacent connective tissue from patients.

Interestingly, the total frequency of methylation at sites #15, #18, #20, #21, #22, #23, calculated for all samples from a specific group, was higher in tumor-adjacent, histologically normal tissue from patients than in the tumors and in control tissue from healthy volunteers (Fig. 4). Thus, CpG site #15 was found to be methylated in 19% of the tumor samples, in 40% of the samples of tumor-adjacent, histologically normal tissue, and in 26% of the samples of tissues from healthy volunteers, respectively (Fig. 4). This pattern also applied when the total frequencies of methylation for epithelium and connective tissue from tumor-adjacent, histologically normal tissue and from control samples from the healthy volunteers were counted separately (Fig. 6). Interestingly, in healthy volunteers, the total frequency of methylation at site #15 was considerably higher in epithelium than in connective tissue. Interestingly, the difference between the tissue from the

TABLE 1. Total Frequencies of Methylation in Different Types of Samples in Different Individuals

		Methylation frequency (%)									
			Three regions								
			Exon1	Proximal		Distal					
Investigated groups	No.	Total	Subtotal	Subtotal	CpG #15	Subtotal	CpG #18	CpG #20	CpG #21	CpG #22	CpG #23
Healthy volunteers											
Epithelium	15	3.4 ± 1.8	0.4 ± 0.8	6.4 ± 4.2	32 ± 22	3.5 ± 9.5	2.9 ± 5.3	7.9 ± 15	6.3 ± 14.4	4.9 ± 14	3.5 ± 10.6
Connective tissue	15	1.8 ± 2.0	0	3.6 ± 5.3	15 ± 18[a]	2.1 ± 2.4	0	4.5 ± 9.3	5.0 ± 9.2	3.2 ± 8.3	1.3 ± 5.2
Patients											
Tumor-adjacent epithelium	6	8.8 ± 4.3[b]	3.3 ± 4.1[b]	7.3 ± 3.4	44 ± 15	12 ± 11	13 ± 14[b]	23 ± 15	27 ± 13[b]	29 ± 26[b]	8.3 ± 10
Tumor-adjacent connective tissue	6	6.2 ± 2.8[c]	1.2 ± 2.9	6.6 ± 2.8	44 ± 15[c]	9.2 ± 5.8[c]	13 ± 16[c]	23 ± 19[c]	21 ± 26	13 ± 16	13 ± 14[c]
Tumor-adjacent tissue, unfractionated	14	8.2 ± 3.7	2.7 ± 4.2	5.6 ± 3.3	37 ± 21	14 ± 9.6	15 ± 14	26 ± 19	30 ± 17	20 ± 16	21 ± 22
Tumor tissue	20	3.6 ± 2.7[d]	1.6 ± 3.8	2.9 ± 3.0[d]	19 ± 22	5.6 ± 4.8[d]	8.6 ± 11	14 ± 17	13 ± 16[d]	11 ± 13	12 ± 14

For each sample, the total frequency of methylation was determined for each CpG site. The numbers show the mean ± SD for the indicated numbers of individuals. The tumor samples and the tumor-adjacent, histologically normal tissue from patients were matched samples from the same patient.

[a]Significantly lower than epithelium from healthy volunteers ($P < 0.025$).

[b]Significantly higher than epithelium from healthy volunteers ($P < 0.01$).

[c]Significantly higher than connective tissue from healthy volunteers ($P < 0.025$).

[d]Significantly lower than unfractionated tumor-adjacent, histologically normal tissue ($P < 0.01$).

TABLE 2. Comparison of Frequencies of Methylation at CpG #24 to #20 at 4G and 5G Alleles, Respectively

	Patients ($n = 20$)		Healthy volunteers ($n = 15$)	
	Tumor tissue (%)	Tumor-adjacent tissue (%)	Epithelium (%)	Connective tissue (%)
At 4G alleles	10 ± 11	24 ± 17[a]	6.9 ± 14	3.8 ± 7.1
At 5G alleles	13 ± 12	11 ± 10[b]	0.19 ± 0.73[b]	0[b]

The total frequency of methylation was determined for each of the CpG sites #20 to #24. The numbers showed the mean ± SD for the percentage of the number of methylated CpGs against the total number of CpG sites analyzed. The tumor samples and the tumor-adjacent, histologically normal tissue from patients were matched samples from the same patient. Differences between the various numbers were evaluated by Student's *t* test.

[a]Significantly higher than the corresponding value for tumor tissue.

[b]Significantly lower than the corresponding value for the 4G allele.

healthy volunteers and the tumor-adjacent, histologically normal tissue from the patients was observed for connective tissue as well as epithelium.

We also calculated the total frequency of methylation in individual samples and the corresponding means for the separate groups of samples (Table 1). The total frequency of methylation in the tumor-adjacent, histologically normal tissue from patients was significantly higher than the total frequency of methylation in the samples from the other tissues, but there was no significant difference between the other groups of samples. As far as methylation at sites #15, #18, #20, #21, #22, #23 was concerned, the same pattern as described above for the total frequency of methylation persisted.

Conclusively, there was more methylation in control tissue from the patients than in the tumors, which again had more methylation than the control tissue from the healthy volunteers.

Methylation Profiles and 4G/5G Polymorphism

A 4G/5G polymorphism is localized at position −675 of the *SERPINE1* gene. It is generally held that the 4G allele is associated with a higher transcription than the 5G allele (Ye et al., 1995). The present bisulfite sequencing analysis allowed us to determine if methylation frequencies of the CpG sites in the distal region (base pairs −805 to −422) was correlated with the occurrence of the 4G or the 5G alleles. First, using the 4G/5G polymorphism analysis, it was possible to determine if the *SERPINE1* gene was imprinted for its methylation pattern. Methylation could be seen in both 4G and 5G alleles, indicating that methylation was not imprinted (Strathdee et al., 2007). Second, the 4G allele had a higher frequency of methylation than the 5G allele. In tissue samples from healthy volunteers, the relatively low frequency of methylation was almost completely associated with the 4G allele. In tumor-adjacent, histologically normal tissue, the frequency of methylation in the 4G allele was twice as high as in the 5G allele. In contrast, there was no difference in methylation frequency between 4G (10%) and 5G alleles (13%) in the tumors (Table 2). There was no significant difference for the ratio of the number 4G to 5G alleles between normal tissue and tumors and healthy volunteers and no significant difference in the frequency of 4G carriers between patients and healthy volunteers (data not shown).

Correlation Between Gene Methylation and Expression Level of PAI-1 in Oral Carcinomas

The molecular mechanism for regulating PAI-1 expression in different cell types remains unclear. We therefore considered whether CpG methylation contributes to the differential PAI-1 mRNA expression in the various types of tissue samples analyzed here.

The tumors and the tumor-adjacent, histologically normal tissue have a higher frequency of methylation than the tissue from the healthy controls. On the other hand, the tumors and the tumor-adjacent, histologically normal tissue have a higher PAI-1 mRNA level than the tissue from the healthy controls. It is clear that we did not find the expected correlation between the frequency of methylation and the PAI-1 mRNA levels, therefore, methylation of the analyzed regions of the *SERPINE1* gene might not be decisive for the PAI-1 mRNA level. The same conclusion held true when comparing the methylation frequency and the PAI-1 mRNA levels individually among different samples from both patients and healthy volunteers, even from the same tissue type. Even if there was a remarkably lower frequency of methylation of CpG site #15 and correspondingly higher PAI-1 mRNA levels in connective tissue than in epithelium

from healthy volunteers as well as higher PAI-1 mRNA in tumor tissues than in adjacent histologically normal tissues, the correlation did not reach statistical significance. However, when we divided patients into two groups according to the median value of the PAI-1 mRNA level as well as according to the median value of methylation frequency, to compare the PAI-1 mRNA levels and the methylation frequency in the tumor samples, we did find an inverse correlation, both with respect to total methylation and methylation at CpGs #15, #18, #20, #21, #22, and #23 ($P < 0.05$, evaluated by Fisher's Exact Probability Test).

In conclusion, the results indicated that CpG methylation is not in general involved in restricting the PAI-1 expression in healthy oral mucosa, but may play a role in fine tuning the mRNA levels in tumor cells.

DISCUSSION

Our immunohistochemical staining results agree well with previous reports on PAI-1 in oral squamous cell carcinomas. Previously, overexpression of PAI-1 in oral squamous cell carcinomas was demonstrated by different analyses, including ELISA, Northern blot, and immunohistochemical staining, and most studies showed that increased expression level correlated with a poor prognosis (Nozaki et al., 1998; Hundsdorfer et al., 2005; Lindberg et al., 2006). In the present study, we also found an increased PAI-1 expression in oral carcinomas by both immunohistochemical staining and RT-PCR. We found that the prominent PAI-1 expressing cell type in oral carcinomas is the cancer cells. Our finding of PAI-1 in the cancer cells agrees well with previous reports (Yasuda et al., 1997; Nozaki et al., 1998; Lindberg et al., 2006). In fact, the latter of these studies (Lindberg et al., 2006) was done with the same affinity-purified, carefully controlled and carefully characterized antibodies used in this case. These antibodies were also used in previously reported studies of breast, colon, and prostate carcinomas (Offersen et al., 2003; Illemann et al., 2004; Usher et al., 2005), in which the predominant PAI-1 expressing cell type is the stromal myofibroblast, with some additional expression by endothelial cells, while there was little expression by the cancer cells (Offersen et al., 2003; Illemann et al., 2004; Usher et al., 2005). Thus, the cellular expression pattern for PAI-1 in oral squamous cell carcinomas is different from that in breast, colon, and prostate carcinomas.

Moreover, in addition to confirming previous immunohistochemical results, we also now found that in some oral carcinomas, PAI-1 is expressed by fibroblast-like cells in the stroma surrounding the cancer cells and not in cancer cells themselves. Moreover, we also found PAI-1 expression by tumor-adjacent, histologically normal epithelium, not reported in previous studies. In vitro studies of wound healing indicated that PAI-1 modulates the complex process of injury resolution through control of focalized plasmin-mediated matrix remodeling and cell migration. Certain "senescence-associated" genes (i.e., CDKN2A and Tp53) may actually function in wound repair and early malignant program by inhibiting proliferation but also promoting migration (Chan et al., 2001; Ploplis et al., 2004; Darbro et al., 2005; Kortlever et al., 2006; Natarajan et al., 2006). Indeed, keratinocytes at the leading edge during wound re-epithelialization are less mitotically active than cells more displaced from the motile front (Dabelsteen and Mackenzie, 1976) and have been shown to express relatively high levels of PAI-1 by other studies (Garlick and Taichman, 1994; Jensen and Lavker, 1996; Providence and Higgins, 2004; Qi et al., 2008; Shetty et al., 2008a,b). PAI-1 may also be a downstream target gene during Tp53-induced cellular senescence. The observed upregulation of PAI-1 in histologically normal mucosa adjacent to tumors may indicate cellular senescence as part of an early malignant development; this is in agreement with previous studies that have shown molecular changes in histologically normal mucosa adjacent to oral carcinomas (Kortlever et al., 2006). One recent report indicated that Tp53 protein can interact with a 70 nucleotide sequence at PAI-1 mRNA 3′-UTR region, resulting in upregulation of PAI-1 expression and mRNA stabilization (Shetty et al., 2008b).

Methylation of DNA at cytosines in CpG sequences generally suppresses gene transcription. Accordingly, we previously showed that CpG methylation of the *SERPINE1* gene 5′-flanking region is inversely correlated with PAI-1 mRNA expression in five human tumor cell lines and that inhibition of CpG methylation by 5-aza-2′-deoxycytidine treatment can induce PAI-1 mRNA expression in cells otherwise having a methylated *PAI-1* gene 5′-flanking region (Gao et al., 2005). We were therefore interested in investigating whether methylation of CpGs in the *SERPINE1* gene 5′-flanking region are implicated

in regulation of PAI-1 expression in normal and malignant oral tissue. When doing so, we made several interesting observations.

We found that the methylation patterns in both normal and malignant oral tissue are in general quite different from that previously observed in the cell lines (Gao et al., 2005). In the cell lines, the highest frequency of methylation was found at CpG sites #1 to #14 of the *SERPINE1* gene 5′-flanking region, at which sites very little methylation was observed in the tissue samples analyzed here. In contrast, the most heavily methylated positions in the tissue samples were CpGs #15 and #20 to #23. On the basis of this observation, one will have to conclude that methylation patterns in cell lines do not necessarily reflect that of the tissues from which they were derived but rather is an artifact of the culture conditions.

The second, very surprising observation concerning methylation is the difference in methylation pattern between normal oral mucosa from healthy volunteers and tumor-adjacent, histologically normal tissue from the patients. Except for CpG #15, there was very little methylation of the *SERPINE1* gene 5′-flanking region in DNA from healthy volunteers, while the region had significant methylation in tumor-adjacent, histologically normal tissue from the patients. This observation seems to indicate that the tumor-adjacent normal tissue, despite being histologically normal, has already begun an early malignant development. This conclusion is in agreement with our previous observation of aberrant methylation of both *ABO* gene and *DBCCR1* gene in such tissue (Gao et al., 2004a,b). Importantly, the increased methylation in the tumor-adjacent, histologically normal tissue also involves the connective tissue. Accepting the idea of an early malignant state of this tissue, the increased methylation would imply epigenetic changes also in stromal cells.

From our results, methylation does not seem to be a predominant determinant of the higher PAI-1 expression level in oral carcinomas: Methylation of the *SERPINE1* gene 5′-flanking region was much higher in tumor-adjacent, histologically normal tissue than in normal oral mucosa from healthy volunteers, while the expression level was similar in these two groups of tissues. Still, in some instances, methylation may be involved in fine tuning PAI-1 expression. Thus, we observed a significant inverse correlation between the expression levels and the methylation levels in the carcinoma tissue.

The 4G/5G polymorphism is located within a GC-rich sequence. It is generally agreed that the 4G allele is associated with a higher level of PAI-1 gene transcription and a higher PAI-1 level in blood plasma (Ye et al., 1995). We found methylation at both 4G and 5G alleles, and could, therefore, exclude *SERPINE1* gene methylation as an imprinted gene pattern (Strathdee et al., 2007). Furthermore, we found that the methylation frequency of CpG #24 to #20 at 4G alleles was significantly higher than that at 5G alleles, in normal tissue as well as in tumor-adjacent, histologically normal tissue, while the frequency was identical in carcinoma tissue. However, the functional significance of this difference between 4G and 5G alleles is not clear, as there is not a significant correlation between methylation and expression. Moreover, obviously, when going from normal tissue from healthy volunteers to tumor-adjacent, histologically normal tissue, methylation increases in 5G alleles as well as 4G alleles. The decrease in methylation on the 4G allele when going from to tumor-adjacent, histologically notmal tissue to tumor tissue may contribute to the increased expression if the 4G allele is the one preferentially transcribed.

Among other genes in the plasminogen activation system, methylation of the *PLAU* gene, encoding the PAI-1 target protease uPA, was previously studied extensively. The methylation pattern of the *PLAU* gene was much different from methylation of the *SERPINE1* gene. In primary breast cancer patients, full demethylation of the *PLAU* gene was seen in all grade III samples and associated with higher expression level of uPA, compared with a fully methylated gene in the normal mammary epithelial cell line HMEC (Pakneshan et al., 2004). The difference between the *SERPINE1* gene and the *PLAU* gene in this respect may be related to the fact that there is no typical CpG island in the *SERPINE1* gene promoter region, while this is the case with the uPA gene promoter (Pakneshan et al., 2004). Moreover, our studies were done by comparing tumor samples and corresponding adjacent histologically normal tissues not by comparing tumor samples and cultured cells as done in above study (Pakneshan et al., 2004).

In conclusion, we have mapped PAI-1 expressing cells in oral squamous cell carcinomas and found that in addition to the previously reported expression by cancer cells, PAI-1 is in some cases also expressed by stromal fibroblasts adjacent to the cancer cells and by histologically normal

epithelium adjacent to the tumors. We made the surprising observation that the *SERPINE1* gene is most highly methylated in tumor-adjacent, histologically normal tissue and completely different from the tissues from healthy volunteers, indicating that these cells are probably already in early malignant development. Although CpG methylation may be implicated in fine tuning PAI-1 expression, it is not the main determinant of the high PAI-1 expression in oral squamous cell carcinoma cells. Other regulatory mechanisms rather than methylation need to be investigated in different cell types in tumors in the future.

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