Expression of Four Histone Lysine-methyltransferases in Parotid Gland Tumors

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Abstract. *Methylation of histones is one of the important "epigenetic" mechanisms associated with the transcriptional silencing and/or activating of tumor suppressor genes. To assess whether epigenetic phenomena could be involved in salivary gland carcinogenesis, the expression levels of four histone lysine-methyltransferases (HMT) were investigated, in both pleomorphic adenoma and the adjacent normal tissue of the parotid glands. The expression levels of three HMTs, SETB1, Eu-HMTase and SET08, were higher in tumor tissues. On the contrary, DOTL1 presented a lower expression level in the tumor tissues than in the corresponding normal tissues. These data suggest that the HMTs may be involved in the differentiation of pleomorphic adenoma, probably through chromatin structural changes, and indicates that the study of the epigenetic mechanism which modulates the variation in the methylation profile of histones may be useful to obtain information concerning those genes involved in tumor transformation in human parotid glands.*

Pleomorphic adenoma (PA), the most common benign tumor of the human salivary glands (1, 2), is an epithelial tumor of complex morphology, possessing both epithelial and myoepithelial elements embedded in a mucopolysaccharide stroma (3). PA is the most frequent neoplasm arising in the parotid glands (4) and represents almost 50% of all neoplasms (5); in addition, the rate of malignant change with potential metastasis has been reported to be 2-3% (1, 2).

It is well known that the transformation from normal to cancer cells can be caused by mutations in the molecular pathways that control cell proliferation, differentiation and survival. In particular, carcinogenesis is a multistep process resulting from mutation in genes that maintain genetic stability in normal cells (6). Many authors have indicated that cancer, besides genetic and cytogenetic processes, also results from "epigenetic" mechanisms acting on transcriptional regulation and gene expression, which interfere with gene stability (7). In this context, it has been demonstrated that histones, apart from their essential role in chromatin remodeling, are also involved in transcriptional regulation and gene expression through "epigenetic changes" (8, 9). In particular, the methylation of lysine residues of histone H3 and H4 is one of the major mechanisms associated with the transcriptional silencing and/or activation of tumor suppressor genes (10). Interestingly, the epigenetic mechanisms which involve histone modification do not involve mutation of the DNA itself, although they are inheritable in the short term and can be reversed by small molecules used in cancer therapy strategies (10). Therefore, the study of genes implicated in histone methylation status may provide insights into cancer development.

Little information has so far been reported concerning epigenetic alterations in salivary gland tumors. To assess whether the epigenetic mechanisms which produce histone modification may be involved in the differentiation of salivary gland tumors, the levels of expression of four histone lysine-methyltransferases (HMT) were evaluated, in the PA of parotid glands and in adjacent normal tissue from the same patients.

Materials and Methods

Tissues. Ten patients, affected by pleomorphic adenoma of the parotid gland, were selected for this study. After formal parotidectomy, two samples of surgical specimen were taken for

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Table I. *Primer sequences used for PCR.*

Primer	Sense	Antisense
SETDB1 (498 bp)	met 1: 5'-gacagtagctctgaggacgaatct-3'	met 2: 5'-gcatagagccagacctgattc-3'
DOT1L (528 bp)	met 3: 5'-ccgtgccatcgacagcat-3'	met 4: 5'-gctccttcagctggtgatcc-3'
SET08 (540 bp)	met 5: 5'-gaactcagttacacatcacgaagtca-3'	met 6: 5'-ggtgatctcgatgaggtccc-3'
Eu-HMTase1 (491 bp)	met 7: 5'-cgcaagaccatgccga-3'	met 8: 5'-cgcctgctctgcaccat-3'
GAPDH (463 bp)	gap 1: 5'-gtggatattgttgccatcaatga-3'	gap 2 : 5'-tgatggcatggagtgtggtca-3'

each patient: tumoral and clinically healthy salivary tissue harvests around the margins of the specimen, at least at 1 cm from the tumor. In all cases, the classification of the tissue was made at the time of surgery by the surgeon (co-author G.C.). Histological tests were carried out routinely on the samples and the margins were found to be tumor-free. Finally, the samples were quickly frozen by immersion in liquid nitrogen and were stored at –80˚C until RNA extraction.

This protocol was approved by an Institutional Review Board and patients gave their informed consent.

RNA extraction. Total RNA from normal and neoplastic tissues was prepared according to Spranger *et al.* (11). The tissues were homogenized with a solution (10 ml/g tissue) containing 4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl and 0.1 M 2-β-mercaptoethanol. After extraction with phenol:chloroform:isoamyl alcohol (50:49:1) and precipitation with isopropanol, the resulting RNA was washed with 70% ethanol and dissolved in sterile water. The RNA was then treated with DNase-RNase-free and, after the sub-sequential extraction with phenol:chloroform:isoamyl alcohol (50:49:1), it was precipitated with ethanol and the pellet was dissolved in sterile water and quantified.

Histone methyltransferase selection. Four mRNAs, coding for different human HMTs involved in gene silencing, were selected from the NCBI gene bank database: SETDB1 (accession No: NM_012432), DOT1L (accession No: NM_032482), SET08 (accession No: NM_020382) and Eu-HMTase1 (accession No: NM_024757). Specific oligonucleotide primers were designed to amplify approximately 500 bp of each cDNA coding region (see Table I).

RT-PCR, cloning and sequencing. First-strand cDNA was synthesized using 5 μg total RNA extracted from normal and neoplastic tissues, 40 ng random exameric primer (Promega Corp., Madison, USA) and 200 U Superscript III™ (Invitrogen, Paisley, UK) in a total volume of 20 μl, according to the manufacturer's instructions (Invitrogen, Paisley, UK). Then, 4.0 μl of this cDNA template was used for the PCR (25 μl volume) with 1.5 mM $MgCl_2$, 1xPCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl), 0.2 mM dNTP, 2.0 U *Taq* DNA polymerase (Promega Corp.) and 15 pmol of specific oligonucleotide primers. The same cDNA template (1.5 μl) was also used, as a control, to amplify an appropriate region of glyceraldehyde-3-phosphate dehydrogenase cDNA (GAPDH, accession No NM_002046) with specific oligonucleotide primers (see Table I). Amplifications of the HMT cDNAs were carried out for 35 cycles and the GAPDH cDNA for 30 cycles. Both amplifications were as follows: 94˚C for 1 min, 60˚C for 1 min and 72˚C for 1 min. The amplification products were electrophoresed on 1% agarose gel in 1xTBE buffer.

The amplification products were purified by a QIAGEN gel extraction kit (QIAGEN, Hilden, Germany) and were cloned into the pGEM-T Easy vector according to the manufacturer's instructions. The inserts were sequenced on both strands using [^{35}S]ATP (1000 Ci/mmol, Amersham) by the dideoxy chain termination method (12). The nucleotide sequences were compared to the human sequences in the NCBI gene bank database to verify the specificity of the cloned products.

cDNA probes. Ten micrograms of each plasmid containing the four HMT cDNAs, as well as of the plasmid containing GAPDH cDNA, were digested with the restriction enzyme *Eco*RI and separated by electrophoresis. The obtained cDNA inserts were purified and labelled with [α-^{32}P]dATP and [α-^{32}P]dCTP (3000 Ci/mmol, Amersham) using the random priming labelling kit (Amersham).

Southern blot analysis. The amplification products, obtained by RT-PCR as described above, were electrophoresed on 1% agarose gels in 1xTBE buffer and transferred to Hybond-N$^+$ filters (Amersham) by overnight capillary blotting. The filters were incubated in prehybridization solution (5x SSC, 5x Denhardt's, 100 μg/ml sonicated salmon sperm DNA, 5 mM EDTA at pH 8.0, 0.5% SDS and 50 mM sodium phosphate at pH 7.0) for 1 h at 65˚C and were hybridized with respective radiolabelled cDNAs ($0.5x10^6$ c.p.m./ml) at 65˚C overnight. The filters were washed twice for 30 min at 65˚C in 0.2xSSC and 0.1% SDS, and were finally exposed to X-ray film (Kodak X-OMAT AR). Successively, the amount of cpm for each band was obtained using an Instant Imager (Packard). The cpm values obtained for the product of GAPDH were used to normalize the corresponding value product of the four HMTs.

Statistics. The data were expressed as means±SD. The Gaussian distribution of continuous variables was assessed by the Shapiro-Wilk normality test and the statistical analysis was performed by the Student's *t*-test. Probability levels <0.05 were considered statistically significant.

Results

Gene expression levels of histone lysine methyltransferases. The HMT gene transcription levels were studied in pleomorphic adenoma and in samples of normal parotid gland tissues adjacent to the tumor. The presence of the expected DNA fragment size was confirmed for GADPH (483 bp), SETDB1

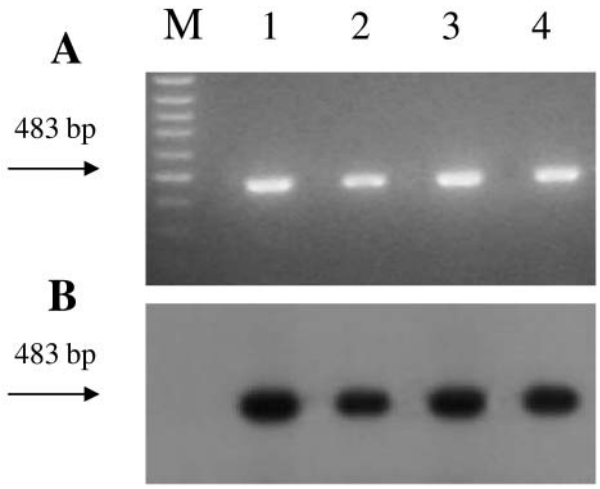

Figure 1. *Analysis of transcript of human GADPH by RT-PCR and Southern blot hybridization. Total RNAs extracted from human neoplastic and adjacent normal parotid gland tissues were subjected to RT-PCR amplification using the primer indicated in Table I. The results shown are relative to two specimens. Lanes 1, 3 normal parotid gland; Lanes 2, 4 pleomorphic adenoma. The PCR products were revealed by ethidium bromide (A) and by Southern blot analysis using the cDNA of GADPH as the probe (B).*

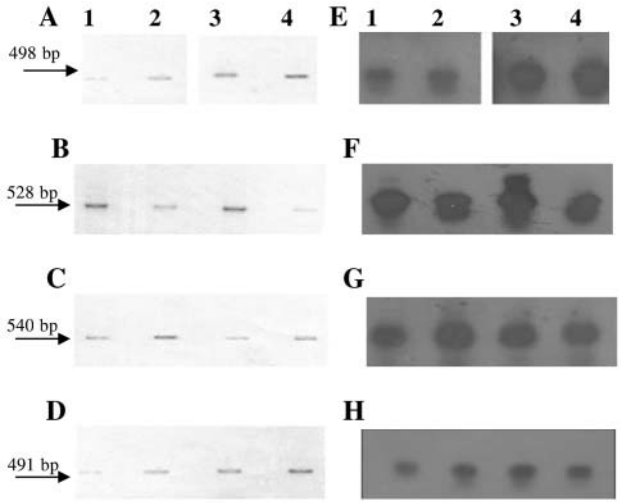

Figure 2. *Analysis of transcripts of human HMTs by RT-PCR and Southern blot hybridization. Total RNAs extracted from human neoplastic and adjacent normal parotid gland tissues were subjected to RT-PCR amplification using the primer indicated in Table I. The results shown are relative to two specimens. A) SETDB1 on normal parotid gland (lanes 1, 3) and pleomorphic adenoma (lanes 2, 4); B) DOT1L on normal parotid gland (lanes 1, 3) and pleomorphic adenoma (lanes 2, 4); C) SET08 on normal parotid gland (lanes 1, 3) and pleomorphic adenoma (lanes 2, 4); D) Eu-HMTase1 on normal parotid gland (lanes 1, 3) and pleomorphic adenoma (lanes 2, 4). The PCR products were revealed by ethidium bromide (left panels) and by hybridization with cDNA probe (right panels).*

(498 bp), DOT1L (528 bp), SET08 (540 bp) and Eu-HMTase1 (491 bp) by carrying out a RT-PCR on cDNA prepared from normal parotid gland tissue (data not shown) with the specific oligonucleotide primers (Table I). The five DNA fragments obtained were eluted from gel, cloned and sequenced to confirm the specificity of the PCR products. These DNA inserts were subsequently used in Southern blot analyses to determine the HMT transcript levels in normal and neoplastic tissues compared to the level of the transcript for the constitutive gene GADPH. RT-PCR was then carried out on normal and neoplastic tissues surgically removed from ten patients assayed for non-degraded RNA with the GADPH RT-PCR. The RT-PCR product for GADPH, carried out with the specific oligonucleotide primers (Table I) on two specimens analyzed by agarose, are reported in Figure 1A. The corresponding Southern blot hybridized with the 483-bp fragment described above confirms the specificity of the PCR fragment (Figure 1B). In addition, the amount of radioactivity present in each band was evaluated in cpm using an Instant Imager (Packard Instrument Company, Meriden, CT, USA) for all the performed experiments. These values were averaged and used to normalize the data in cpm obtained from the four HMTs from the corresponding control and neoplastic tissue samples.

The RT-PCR products for the four HMTs, carried out with the specific oligonucleotide primers (Table I), on two specimens are reported in Figure 2. Both ethidium bromide colorations (Figure 2 A-D) and the corresponding Southern blots (Figure 2 E-H) hybridized with the specific cDNA probe are reported. For all the performed experiments, the amount of radioactivity for each HMT DNA product was evaluated in cpm as described above, averaged and, finally, each value was normalized compared to the GADPH value obtained for the corresponding control and neoplastic tissues.

The ratio of the HMT/GADPH transcription level obtained on ten specimens and performed in duplicate experiments is reported in Figure 3. The results indicated that the SETB1, SET08 and EuHMTase1 transcripts were consistently increased in the neoplastic tissues, while the DOT1L transcript consistently decreased compared to the control parotid gland.

Discussion

PA is an epithelial tumor of complex morphology, possessing both epithelial and myoepithelial elements embedded in a mucopolysaccharide stroma (3). PA is the most common benign tumor of human salivary glands (1, 2) and is the most frequently occurring tumor of the parotid glands (4). To date, the majority of studies on the PA of parotid glands are prevalent at morphological and

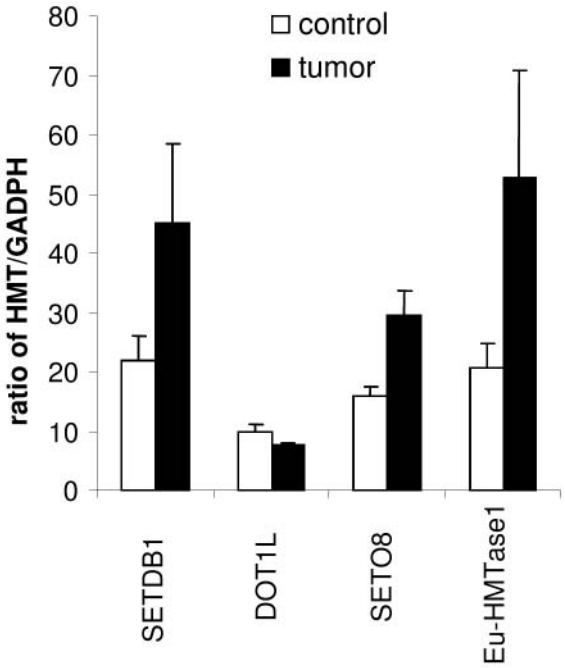

Figure 3. *Quantification of HMT PCR results. The ratio of the transcription level of HMT versus GADPH are shown on the y-axis. In the neoplastic tissue, the DOT1L transcript level was lower than in the normal tissues ($p<0.05$), while the SETDB1, SET08, Eu-HMTase1 transcripts were higher than in normal tissue, with statistical significance ($p<0.05$).*

histochemical levels, while very few studies have been undertaken at the molecular level (5). Recently, Kainuma *et al.* (13) indicated that cDNA microarray may be useful for the pre-operative diagnosis of the salivary gland tumor; in addition, Maruya *et al.* (14) suggest that gene expression screening of salivary gland neoplasms may be used to identify molecular markers of potential histogenetic and clinical significance. Moreover, the study of the expression profiles of those genes involved in potential genetic and epigenetic events of biological significance provide information to help in understanding tumorigenesis (15).

It is well known that negative regulation of gene transcription is an important strategy in establishing and maintaining cell-specific gene expression patterns; the transformation from normal cells to cancer cells is often determined by the re-expression of some silencing genes (6). In recent years, it has become apparent that one of the emerging prevention and therapy targets for cancer is the reversal of aberrant gene silencing mediated by epigenetic mechanisms associated with transcriptional repression (16, 17). In this context, epigenetic mechanisms, which involve DNA methylation and the aberrant pattern of histone modifications, are alterations in gene expression in an inheritable manner without directly altering the genome (15).

Experimental evidence indicated that the epigenetic inactivation of genes crucial for the control of normal cell growth may determine carcinogenetic transformation (18). For many of these genes, it was shown that their re-expression in tumor cells can result in the suppression of tumor cell growth or altered sensitivity to existing anticancer therapies (16, 17).

In particular, the histone acetylation and methylation status have emerged as key players in the repression or inactivation of genes and chromosomal domains through the silencing of some tumor-suppressing genes (9). In addition, there is evidence that the methylation of specific arginines or lysines in histones H3 and H4 is an important phenomenon which acts on the expression of genes involved in carcinogenetic transformation (19, 20).

As reported by Gibbons (21), the histone methyltransferase EZH2 is highly expressed in metastatic prostate cancer and its level of expression is correlated with the degree of aggressive cancer. However, forced overexpression of EZH2 led to an increase in the rate of cell proliferation and reduction of the expression of this enzyme reduced proliferation of prostatic cancer cells (21).

Here, the expression levels of four HMTs involved in gene silencing, as evaluated in the human parotid glands of ten patients with PA, are reported and compared to the expression levels observed in adjacent normal tissue from the same patient.

HMT are a class of enzymes that methylate lysine residues of histones H3 and H4, in particular, SETB1 and Eu-HMTase methylate histone H3 on lysine 9, SET08 methylate histone H4 on lysine 20 and DOT1L methylate histone H3 on lysine 79. It has also been demonstrated that the majority of HMT contain a conserved catalytic core called the SET domain, which shares sequence homology with an independently described sequence motif, the PR domain (22). Intact PR or SET sequences are required for tumor suppression function (22). It is interesting to note that the above four HMT chosen from the data bank are involved in gene silencing and, among them, DOT1L lacks the SET domain (22).

Our results, obtained from RT-PCR and Southern blot analysis, showed that three out of the four HMT considered (SETB1, Eu-HMTase and SET08) showed a higher level of expression in the tumor tissues compared to those observed in the normal tissues (Figure 3). On the contrary, the expression level of DOT1L was lower in PA as compared to that observed in the normal tissues (see Figure 3).

In summary, the four HMT considered show different expression patterns in PA compared to normal tissues, suggesting that these enzymes may contribute to the differentiation of the parotid tumor through chromatin structural changes that modulate gene regulation. Nevertheless, further experiments are needed to obtain

more quantitative data to assess the direct involvement of HMT in salivary gland tumors. The data presented here suggest that the variation in the methylation profile of histones H3 and H4 is an epigenetic mechanism that may contribute to activating or silencing those genes involved in tumor transformation in human parotid glands. In concluson, the evaluation of the expression level of HMT may add to our knowledge of tumor transformation in salivary glands, considering that changes in the histone pattern as well as in DNA methylation are used as markers in cancer.

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