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H3K4 histone methylation in oral squamous cell carcinoma

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Methylation of specific lysine residues in histone tails has been proposed to function as a stable epigenetic marker that directs biological functions altering chromatin structure. Recent findings have implicated alteration in heterochromatin formation as a contributing factor in cancer development. In order to verify whether changes in the overall level of H3K4 histone methylation could be involved in oral squamous carcinoma, the levels of H3K4me1, me2 and me3 were measured in oral squamous carcinoma, leukoplakias and normal tissues. The levels of H3K4me2 and me3 were significantly different in oral squamous cell carcinoma in comparison with normal tissue: the level of H3K4me2 was increased while that of H3K4me3 decreased. No significant differences could be found between the two types of tissues in the level of H3K4me1. A similar trend was found in the leukoplakias that appeared more like the pathological than normal tissue. These results support the idea that alteration of chromatin structure could contribute to oncogenic potential.

Keywords: H3K4, histone methylation, oral squamous carcinoma

INTRODUCTION

Evidence accumulated over the past few years suggests that post-translational modifications of histone amino termini constitute a "histone code" that directs a variety of processes involving chromatin (Jenuwein & Allis, 2001). Histone methylation represents the most recently recognized component of the histone code. Methylation of specific lysine residues in histone tails has been proposed to function as a stable epigenetic marker that directs biological functions ranging from transcriptional regulation to heterochromatin assembly (Völkel & Angrand, 2007). Methylation of a specific lysine residue (at position 4 and 9 in the amino acid chain) in histone H3 has been shown to be involved in the regulation of chromatin structure (Lachner & Jenuwein, 2002).

H3K9 methylation is generally associated with transcriptionally inactive heterochromatin, while K4 methylation is associated with transcriptionally active euchromatin (Lachner & Jenuwein, 2002). Lysine methylation on the ε-nitrogen can occur as mono-, di-, or trimethylated forms.

Histone methylation is reversible and dynamically regulated as suggested by the identification of the H3K4-specific histone demethylase LSD1 (Shi *et al.*, 2004) and the H3K36me2-specific histone demethylase JHDM1 (Tsukada *et al.*, 2006). Both LSD1 and JHDM1 demethylate only di- or monomethylated histones while some enzymes of the jumonji class can demethylate also me3 (Völkel & Angrand, 2007). So far all known lysine histone methyltransferases with the exception of Dot1/DOT 1L contain a conserved methyltransferase domain, termed SET (Su(var)3-9, Enhancer-of-zeste, Trithorax) domain, of about 130 amino acids. Recently many connections have been reported between SET-domain proteins that might have a lysine methyltransferase activity and cancer, suggesting that this enzymatic activity is implicated in carcinogenesis.

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Abbreviations: SET, Su(var)3-9, Enhancer-of-zeste, Trithorax; OSCC, oral squamous cell carcinoma; H3K4, lysine four of histone H3; A.U., arbitrary units; DTT, dithiothreitol; H, oral healthy mucosae; L, oral leukoplakias; C, oral squamous cell carcinomas; PMSF, phenylmethylsulfonyl fluoride.

Probably these SET-domain proteins will join several other families of chromatin modifying enzymes (acetylases, deacetylases and kinases) that are already implicated in cancer (Kouzarides, 1999). There are examples in which the appearance of a methyl group on either lysines or arginines has been correlated with genes being activated or repressed. One such case is the cyclin E promoter, whose activity is repressed by H3K9 methylation in the G1 phase of the cell cycle (Nielsen *et al.*, 2001).

Oral squamous cell carcinoma (OSCC) is one of the ten most common cancers. It is more common in men and its prevalence increases with age. Similarly, among all potentially malignant lesions and conditions of the oral mucosa, oral leukoplakia (OL) is the most common and extensively studied (Van der Waal *et al.*, 1997; Reibel, 2003). Biological markers that can help to identify the lesions with an aggressive phenotype and worse prognosis need to be studied for both type of oral lesions.

The aim of this work is to characterize the oral squamous carcinoma at a molecular level, measuring the total amount of different forms of H3K4 methylated histone. Our working hypothesis is based on the possibility that levels of mono-, di- and trimethylation of H3K4 are different in cancerous oral tissues compared to normal tissues. To this aim we measured the content of K4-methylated H3 histone in healthy, potentially malignant, and cancerous oral tissues in order to evaluate their usefulness as markers of the proliferation status.

MATERIALS AND METHODS

Acid-soluble protein extraction. Oral tissue (10–50 mg) was washed with PBS (0.137 M NaCl, 1.76 mM KH_2PO_4, 2.7 mM KCl, 10.14 mM Na_2HPO_4, pH 7.4) and then treated with 7 volumes of lysis buffer (10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 1.5 mM PMSF). Tissue was manually ground with a sterile pestle. After this treatment HCl was added to a final concentration of 0.2 M and the samples were kept in ice for 30 min. The samples were centrifuged at 13 000 r.p.m. for 10 min at 4°C. Protein concentration of the supernatant was determined by BioRad assay (Bradford, 1976) that involves the addition of an acidic dye to protein solution and subsequent measurement at 595 nm with a spectrophotometer. Protein concentration of the supernatant was verified by Coomassie staining of sodium dodecyl sulphate (SDS)/polyacrylamide gels.

Nine different specimens were analyzed for each: oral healthy mucosae (H), oral leukoplakias (L) and oral squamous cell carcinomas (C). Only histologically confirmed samples were used. An analogous procedure was performed to extract acid-soluble proteins from HeLa cells.

Experiments were undertaken with the understanding and written consent of patients and the protocol was approved by a Bioethical Committee.

Western blotting analysis. Acid-soluble proteins (10 μg) from oral tissue were resolved by electrophoresis on 12% SDS/polyacrylamide gels and transferred to nitrocellulose membranes (Hybond-C, Amersham). Acid-soluble proteins (5 and 10 μg) extracted from HeLa cells were used as a quantitative standard in Western blotting analysis. Polymers of cytochrome *c* from monomers to heptamers were used as molecular mass standards, as reported in Fig. 1 (Panel A). Three different primary antibodies were used in independent experiments to detect methylated histone H3 in the protein samples: anti-monomethyl Histone H3(K4), anti-dimethyl Histone H3(K4), and anti-trimethyl Histone H3(K4), all purchased from Upstate. The anti-monomethyl one was diluted 1:250 in 5% dry milk in TBS (10 mM Tris/HCl pH 7.5, 150 mM NaCl) with 1% BSA and 0.05% Tween 20 and incubated for 1 h; the anti-dimethyl antibody was diluted 1:10 000 in 3% dry milk in PBS and incubated for 90 min; the anti-trimethyl one was diluted 1:250 in 5% dry milk in TBS and incubated for 1 h. Incubation was always performed at room temperature. Membranes were then washed two times with water and incubated with peroxidase-conjugated monoclonal antibody anti-rabbit IgG (Sigma) at a dilution of 1:5000. Detection was performed using the ECL plus chemiluminescent kit (Amersham), as described by the manufacturer. Film scanning was done on a Gel DOC 1000 system (BioRad) and the chemiluminescence was quantified by Molecular Analist 2.1 version software (BioRad). The methylation level of H3 histone was reported as arbitrary units (A.U.) that represent the ratio between the band intensity in the samples and that in the HeLa cell extract.

As a negative control experiments were repeated skipping the step of membrane incubation with primary antibodies and no bands were revealed (not shown).

RESULTS

Histone methylation is an important epigenetic mark that allows transcriptional regulation. Particularly, H3 lysine 4 histone methylation is involved in transcriptional activation in several organisms, including humans. In the present work, levels of H3K4 mono-, di- and trimethylation were analysed in oral mucosae of individuals affected by carcinoma or leukoplakia and in clinically healthy individuals as control. Characteristics, risk factors and levels

Table 1. Characteristics, risk factors and H3K4 mono-, di- and trimethylation levels of specimens analysed in this study.

N.D. means not determined.

Healthy patients (H)

Individuals	Gender	Age	Smoking	Alcohol	Localization	Diagnosis	Clinical stage	Candida	HPV DNA	MONO	DI	TRI
1	M	28	20/d	none	gingiva	healthy	-	none	Negative	51	21	70
2	M	39	10/d	none	gingiva	healthy	-	none	Negative	45	31,5	70
3	F	62	none	250cc/d	gingiva	healthy	-	C. albicans	N.D.	28	37	100
4	F	45	none	none	tongue	healthy	-	none	Positive	49	18	66
5	M	58	ex smoker	none	gingiva	healthy	-	C. albicans	Negative	36	38	88
6	F	55	15/d	350cc/d	buccal mucosa	healthy	-	none	N.D.	28	9	86
7	F	37	none	none	gingiva	healthy	-	none	N.D.	31	12	130
8	M	53	20/d	none	tongue	healthy	-	none	N.D.	49	39	105
9	M	46	20/d	none	buccal mucosa	healthy	-	none	Negative	40	25	122

Patients with oral leukoplakia (L)

Individuals	Gender	Age	Smoking	Alcohol	Localization	Diagnosis	Clinical stage	Candida	HPV DNA	MONO	DI	TRI
1	M	44	10/d	none	buccal mucosa	leukoplakia non homogeneous	-	none	Negative	83,7	93	28,9
2	M	57	20/d	none	buccal mucosa	leukoplakia	-	C. albicans	Negative	100	100	16
3	F	46	30/d	none	buccal mucosa	leukoplakia	-	C. albicans	N.D.	60	94	12
4	M	58	40/d	250cc/d	tongue	leukoplakia	-	C. albicans	Negative	78	73	14
5	F	50	none	none	gingiva	leukoplakia	-	C. albicans	Negative	70	120	16
6	F	43	30/d	none	buccal mucosa	leukoplakia	-	C. albicans	N.D.	96	60	24
7	M	83	none	none	palate	proliferative verrucous leuko	-	C. albicans	Negative	74	71	43
8	F	61	10/d	none	tongue	leukoplakia	-	C. tropicalis	Negative	87	104	51
9	F	55	20/d	none	palate	leukoplakia	-	none	Negative	105	122	55

Patients with squamous cell carcinoma (C)

Individuals	Gender	Age	Smoking	Alcohol	Localization	Diagnosis	Clinical stage	Candida	HPV DNA	MONO	DI	TRI
1	M	67	none	250cc/di	tongue	SCC	I	C. albicans	Negative	39,5	58,8	57
2	M	74	none	none	tongue	SCC	I	C. famata	Negative	43	65	29
3	F	75	none	none	buccal mucosa	SCC	IV	C. albicans	Negative	23	74	59
4	M	70	none	500cc/di	tongue	SCC	IV	C. tropicalis	Negative	40	43	27
5	F	80	none	none	tongue	SCC	III	none	Positive	40	79,4	59
6	F	61	30/d	none	floor	SCC	IV	C. cerevisiae	N.D.	39,8	28	58
7	M	72	20/d	none	lip	SCC	III	C. albicans	Negative	33	38	77
8	M	43	20/d	150cc/di	buccal mucosa	SCC	III	C. albicans	N.D.	50	69	74
9	M	63	20/d	750cc/di	floor	SCC	IV	C. kefyr	Negative	47	74	74

of H3K4 mono-, di- and tri-methylation of the specimens analysed in this study are reported in Table 1, but it is difficult to correlate the data of methylation with these parameters because of the small number of samples.

In order to measure levels of H3K4 methylation, Western blotting experiments were performed using specific antibodies. It is important to note (Fig. 1, Panel A) that only one band of the expected molecular mass was found in all samples when anti-mono or anti-dimethyl H3K4 antibodies were used. Anti-trimethyl H3K4, instead, revealed one band of a high molecular mass and, in some cases, small amounts of multiple bands with various lower molecular masses. These results suggest that trimethylated H3K4 histone showed a tendency to form polymers/complexes in the experimental conditions used.

Results for three individuals are reported in Fig. 1 (Panel A).

Original data on the other eight healthy individuals, eight patients with leukoplakia and eight patients with carcinoma are reported in Fig. 1 (Panel B). The histograms of the average values of mono-, di- and trimethylation levels in carcinomas, leukoplakias and healthy mucosae are reported in Fig. 2.

The methylation levels of H3 histone are reported in arbitrary units (A.U.), comparing the band intensity of the analysed samples with the bands revealed in the histone preparation from HeLa cells on the same films. Carcinomas and leukoplakias show the same behaviour of H3K4 methylation in comparison with the healthy oral mucosae, although not the same absolute values. In particular, they both show an increased dimethylation and an even more evident reduced trimethylation level compared to healthy mucosae. On the other hand, only carcinomas showed no significant differences in monomethylation level in comparison to healthy mucosae.

DISCUSSION

The ε-amine group of certain lysine residues in histones can undergo methylation that can occur

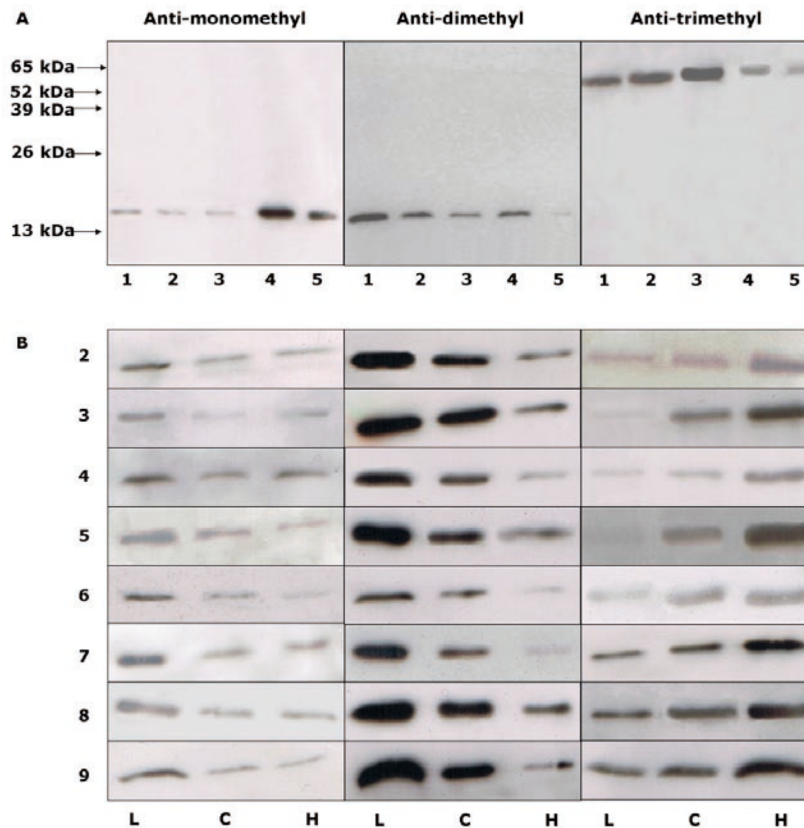

Figure 1. Western blotting of acid-soluble proteins from specimens of individuals 1 (Panel A) and other eight individuals (2–9, Panel B).
Panel A. Lanes 1 from leukoplakias; lanes 2 from carcinomas; lanes 3 from healthy mucosae; lanes 4 and 5 from HeLa cells (10 μg and 5 μg, respectively). Panel B. leukoplakias (L); carcinomas (C); healthy mucosae (H).

once (mono-methylation) or several times (di- or trimethylation); each level of modification can have different biological effects depending on the residue and context. For example, trimethylation of lysine 4 in histone H3 (H3K4me3) occurs virtually in all active genes whereas H3K9me3 occurs in compact pericentromeric heterochromatin which is transcriptionally inert (Völkel & Angrand, 2007). Moreover, high levels of H3K4 trimethylation appeared to be associated with the 5′ regions and showed a strong positive correlation with transcription rates, active polymerase II occupancy and histone acetylation (Rutherburg *et al.*, 2007). In contrast, patterns of dimethyl H3K4 differ significantly between yeast and vertebrate chromatin: in *Saccharomyces cerevisiae*, dimethylated H3K4 appears to spread throughout genes and is associated with a transcriptionally ''poised'' as well as active state (Pokholok *et al.*, 2005), whereas in vertebrates, the majority of H3K4 dimethylation colocalizes with H3K4 trimethylation in discrete zones (Bernstein *et al.*, 2005). Several interactors of methylated H3K4, including the basal transcription factor TFIID, have recently been identified and it has been demonstrated that they bind to the target *via* different domains (Rutherburg *et al.*, 2007; Vermeulen *et al.*, 2007).

Methylation of histone lysines is catalyzed by several histone-specific methyltransferases that belong to a novel family whose members share a conserved catalytic motif known as the SET domain; this domain has been demonstrated to play a tumor-suppress function (Kimkc & Huang, 2003). Some enzymes, such as SET8, possess narrow substrate specificities, while SET7/9 has a broader substrate specificity and can methylate several targets (Völkel & Angrand, 2007).

Almost all histone lysine methylations are removable. Two classes of lysine demethylases have recently been identified: the LSD1/BHC110 class (which removes H3K4me1 and me2) and the jumonji class (which removes H3K4me2 and me3, H3K9me2 and me3, and H3K36me2 and me3). The LSD1-mediated demethylation reaction was surprisingly specific; i.e., recombinant LSD1 demethylates lysine 4 of histone H3, but not other methylated arginines or lysines (Bannister & Kouzarides, 2005). Unlike LSD1, the JmjC domain demethylases do not require protonated nitrogen in the substrate; in this way they are capable of demethylating not only mono- and dimethylated but also trimethylated lysine residues. In this context, it is interesting to note that JHDM1 and JHDM2 both demethylate only mono- and

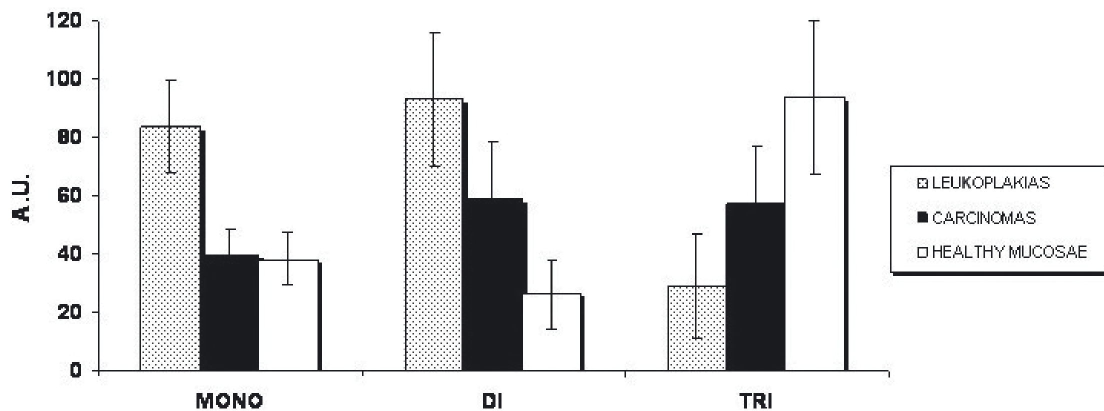

Figure 2. Levels of H3K4 mono-, di- and trimethylation in leukoplakias, carcinomas and healthy mucosae. Methylation levels are reported in arbitrary units (A.U., see text). The reported results are relative to averages for 9 different specimens (±S.D.).

dimethylated lysine, although they are chemically compatible with the reversal of lysine trimethylation (Völkel & Angrand, 2007).

Recent findings have implicated alterations in heterochromatin formation as a contributing factor in cancer development (Fraga & Esteller, 2005). Specifically, a significant reduction of H3K9me3 and H4K20me3 levels, which are hallmarks of heterochromatin, has been correlated with tumorigenesis (Fraga *et al.*, 2005). Therefore, aberrant regulation of the demethylases controlling these methylation marks could contribute to the oncogenic potential. For instance, the *JMJD2C/GASC1* gene is amplified in squamous cell carcinoma (Yang *et al.*, 2000). Recently a transcriptional repressor of tumor suppressor genes has been implicated in breast cancer, as a histone demethylase enzyme (PLU-1) that has the ability to reverse the trimethyl H3K4 modification state (Yamane *et al.*, 2007).

Mutations or translocations of histone methyltransferases have been directly linked to prostate, breast and hematopoietic cancers, emphasizing the importance of histone methylation balance *in vivo*. For instance, the misregulation of MLL (H3K4 methylase), hDOT-1L (H3K79 methylase), and NSD-1 (H3K36 methylase) has been linked to human hematopoietic cancers and these data emphasize the need to maintain methylation balance at H3K4, 36 and 79 (Okada *et al.*, 2005).

The data reported in this paper demonstrated that the overall levels of H3K4me2 and me3 are significantly different in oral squamous cell carcinoma in comparison with cells of the healthy tissues; the level of H3K4me2 is increased while that of H3K4me3 decreased. In contrast, no significant differences between the two types of tissues are found in the level of H3K4me1. The same trend in di- and trimethylation was found in the leukoplakias that, in this respect, appeared more similar to the pathological status than to the healthy tissue. These data on the similarity of the leukoplakia with cancer at level of H3K4 methylation support the idea that a high number of leukoplakia are premalignant lesions. It is interesting to understand why leukoplakias show more pronounced changes in methylation than cancer tissues but it is possible that this data are mainly linked to a higher homogeneity of such tissue alterations in comparison with that of carcinomas.

The data on the level of H3K4me2 are in agreement with the results obtained by immunohistochemical analysis on squamous cell cancer and displastic oral lesions (Piyathilake *et al.*, 2005).

In conclusion, on the basis of the reported results, methylation of lysine 4 of histone H3 is affected in oral squamous cancer.

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