Aberrant promoter hypermethylation of the CHFR gene in oral squamous cell carcinomas

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Abstract. Recent studies have shown that promoter hyper-methylation of tumor suppressor genes is an important factor in carcinogenesis of several human organs. The purpose of this study was to examine the methylation status of CHFR, a novel cell cycle regulatory gene, in both primary oral cancer tumors and the adjacent normal mucosa, and to clarify the relation between the methylation status and expression of the CHFR-related chromosomal passenger protein Aurora-A. The methylation status of the CHFR gene was examined by the methylation-specific PCR (MSP) in 49 primary oral squamous cell carcinomas (OSCC) and 6 OSCC cell lines. In 13 cases, the adjacent normal oral mucosal tissues were also examined. Normal oral mucosa from 18 healthy volunteers was used as the control. The mRNA level of Aurora-A and CHFR in OSCC cell lines was investigated by real-time RT PCR and the protein expression of Aurora-A in certain tumor samples was confirmed by immunohistochemistry. Aberrant promoter methylation of the CHFR gene was detected in 34.7% (17 of 49) of OSCC cases. As for the 13 OSCC cases with paired cancerous and adjacent normal tissues, promoter hyper-methylation of the CHFR gene was detected in 46.1% (6 of 13) of the cancerous tissues. In contrast, promoter hypermethylation of the CHFR gene was recognized in only 7.7% (1 of 13) of the surrounding normal mucosa. No hypermethylation of the CHFR gene was detected in healthy volunteers. Only one OSCC cell line shows hypermethylation of the CHFR gene with concurrently silenced mRNA expression, however, Aurora-A was expressed abundantly in all cell lines. Furthermore, there is no significant relationship between methylation status of the CHFR gene and Aurora-A protein expression in OSCC. Hypermethylation of the CHFR gene was detected in a certain part of OSCC cases whereas it had very low frequency in adjacent normal oral tissues. Although further study is needed, Aurora-A gene expression seems to be independent from methylation status of the CHFR gene in OSCC.

Introduction

Squamous cell carcinoma accounts for ~90% of oral cancer (1). Oral squamous cell carcinoma (OSCC) is the eleventh most common types of human cancer worldwide (2). In Japan, OSCC is relatively common, accounting for >9,600 new patients in 2001 and ~5,600 deaths in 2005 (3). In OSCC, the promoter hypermethylation of many tumor suppressor genes has been reported (4,5).

CHFR, checkpoint with fork head-associated and RING finger, is a recently identified gene, localized to chromosome 12.q24.33. CHFR encodes FHA domain and RING finger domain, which functions as an important checkpoint protein early in G2-M transition. The mitotic checkpoint genes (6), which prevent entry into cell cycle, are rarely inactivated in human cancer. However, the CHFR gene, which is a checkpoint that delays entry into metaphase in response to mitotic stress, is inactivated owing to lack of expression or by mutation (7). CHFR works as an E3 ubiquitin ligase and induces the proteasome-dependent degradation of Plk1 (8) and Aurora-A (9). Mouse embryonic fibroblasts (MEF) derived from *Chfr* knockout mice model show elevated protein levels of Aurora-A and display chromosome abnormalities (9). The inactivation of the CHFR gene may upregulate these mitotic kinases and are overexpressed in human malignant tumors (10). In normal human cells, CHFR are delayed in entering mitosis in the presence of mitotic stress.

CHFR is frequently downregulated in human cancers, mostly owing to the hypermethylation of its promoter region. CHFR downregulation has been found in primary cancers or in the established tumor cells of various origins, such as the lung (11), colon (12), esophagus (13), liver (14), and stomach (15). The frequency of CHFR methylation varied in each primary tumor. Lung cancer showed 19% of CHFR methylation, colorectal cancer 26-37%, esophageal cancer 16-24%, hepatocellular cancer 35%, gastric cancers 30-52%.

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However, CHFR was not hypermethylated in ovarian cancer. Moreover, the aberrant methylation of CHFR appears to be a good molecular marker with which to predict the sensitivity of several cancers to microtubule inhibitors (16,17). The loss of CHFR mRNA expression is a consequence of promoter methylation, suggesting a tumor suppressor role for this gene in oral carcinogenesis.

Aurora-A protein kinase is required for the maintenance of spindle bipolarity and the accurate completion of chromosome segregation. A portion of Aurora-A localizes to centrosomes and nearby spindle microtubules, where it rapidly exchanges with cytoplasmic pools. Mechanistically, Aurora-A plays a role in the recruitment and regulation of proteins at centrosomes (18). Aurora-A is located in the 20q13 breast cancer amplicon and is also overexpressed in colorectal, pancreatic and gastric tumors (19). Aurora-A expression in cancer is often associated with gene amplification, genetic instability, poor histologic differentiation, and poor prognosis (20).

We therefore hypothesize that hypermethylation of CHFR in OSCC could serve as a potential biomarker for clinical diagnosis and monitoring treatment effect. Furthermore, it is not clear wheather in OSCC CHFR works as ubiquitin ligase of Aurora-A *in vitro* and in human tumor samples. It prompted us to investigate CHFR promoter hypermethylation in OSCC and to clarify the relationship between CHFR methylation and Aurora-A status.

Materials and methods

Patients and cell lines. Forty-nine primary oral squamous cell carcinomas were collected from patients who underwent surgery at Department of Oral and Maxillofacial Surgery, Gifu University Hospital from 2001 to 2003. Informed consent was obtained from all patients prior to tissue acquisition. The adjacent normal tissue was also collected from surrounding mucosa in 13 out of the 49 OSCC cases. Immediately after resection, the specimens were obtained and snap-frozen at -20˚C and stored until use.

Normal oral epithelium was collected from 18 healthy volunteers (mean age: 32.5 years). Six human OSCC cell lines, HSC2, HSC4, SAS, Ca9-22, SCC9 and SCC25 were obtained from Cancer Cell Repository, Tohoku University. These cell lines were maintained in a culture in RPMI-1640 (Sigma, USA) supplemented with 10% fetal bovine serum in a 5% CO_2 incubator at 37˚C.

DNA extraction and bisulfite modification. Genomic DNA was isolated from tissue specimens and cell lines by standard phenol and chloroform extraction. Sodium bisulfite modification of genomic DNA was performed using the EZ DNA methylation gold kit (Zymo Research, USA), which integrates DNA denaturation and sodium bisulfite modification processes into a single step followed by rapid in-column desulphonation and DNA clean-up, according to the manufacturer's instructions.

Methylation-specific PCR (MSP). Methylation-specific PCR (MSP) was carried out with the following oligonucleotide primers, which were designed to be specific to either methylated or unmethylated DNA after sodium bisulfite modification as described above (11).

Methylated DNA-specific primers were 5'-ATATAAT ATGGCGTCGATC-3' (forward) and 5'-TCAACTAATC CGCGAAACG-3' (reverse). Unmethylated DNA-specific primers were 5'-ATATAATATGGTGTTGATT-3' (forward) and 5'-TCAACTAATCCACAAAACA-3' (reverse). PCR amplification consisted of 1 cycle at 94˚C for 10 min, 40 cycles of 94˚C for 1 min, 58˚C for 1 min and 72˚C for 1 min (methylated); and 94˚C for 1 min, 53˚C for 1 min and 72˚C for 1 min (unmethylated).

The resultant PCR products were separated on 3% agarose gel. CpGenome Universal Methylated DNA (Chemicon International, USA) was used as a positive control, which is enzymatically methylated human male genomic DNA. Normal human blood DNA was used as positive control for unmethylated status.

Real-time PCR (Reverse-transcriptase PCR). Total RNA was isolated from cell lines using TRIzol Reagent (Invitrogen, USA). cDNA was synthesized by using SuperScript III First-Strand (Invitrogen). Real-time PCR was performed with a Light-Cycler instrument system (Roche, Germany) using SYBR Premix Ex Taq (Takara Biochemicals, Japan) according to the manufacturer's instructions.

CHFR primer sequences were 5'-GAGAGGGGCAGTTT TGCTC-3' (forward) and 5'-CTGTGGTTTTCCCAGCAGCA-3' (reverse). Aurora-A primer sequences were 5'-GAAGCA ATTGCAGGCAACCA-3' (forward) and 5'-CTTTACCCAG AGGGCGACCA-3' (reverse). GAPDH primer sequences were 5'-TGGTATCGTGGAAGGACTCATGAC-3' (forward) and 5'-ATGCCAGTGAGCTTCCCGTTCAGC-3' (reverse).

The PCR protocol consisted of 1 cycle at 95˚C for 10 min, 45 cycles at 95˚C for 10 sec, 60˚C for 10 sec, and 72˚C for 6 sec. GAPDH was amplified as the internal marker. The expression value of CHFR and Aurora-A was normalized by GAPDH in each cell lines. The relative expression was quantitatively determined.

Immunohistochemistry of Aurora-A. Immunohistochemical staining was carried out with a monoclonal antibody for Aurora-A. It was raised and affinity-purified as described previously (21). Paraffin-embedded sections were dewaxed in xylene and rehydrated in a graded series of ethanol. After blocking endogenous peroxidase and biotin, the sections were incubated overnight with the primary antibody at 4˚C (the antibody was diluted 100-fold). Next, the sections were incubated with a link anti-mouse and anti-rabbit Ig Dako LSAB2 System, Peroxidase (Dako, Denmark) at room temperature for 10 min and Streptavidin HRP. Each incubation was followed by 3 washes with PBS. After staining with hematoxylin, the sections were examined under a light microscope.

Results

Aberrant hypermethylation of CHFR in primary OSCC. Patients clinical characteristics are shown in Table I. In primary OSCC, aberrant promoter hypermethylation of the CHFR gene was detected in 34.7% (17 of 49). (Fig. 1) The relation among

Table I. Clinicopathological features and CHFR methylation status of oral squamous cell carcinoma patients.

Case no.	CHFR methylation status[a]	Region[b]	Histpath[c]	TNM[d]	Stage[d]	Age	Gender	Lymph node metastasis[e]
1	-	Tongue	SCC(w)	Unknown	Unknown	70	M	-
2	-	Buccal Mucosa	SCC(m)	T3N0M0	III	74	M	-
3	-	Mand. Gingiva	SCC(w)	T1N0M0	I	71	F	-
4	-	Maxi. Gingiva	Verrucous	T2N0M0	Unknown	78	F	-
5	+	Tongue	SCC(w)	T2N0M0	II	79	F	-
6	+	Maxi. Gingiva· Buccal	SCC(w)	T3N0M0	III	69	F	-
7	-	Oral floor	SCC(w)	T4N3M0	IV B	70	M	+
8	-	Maxi. Gingiva	SCC(w)	T4N1M0	IV A	75	M	+
9	-	Mand. Gingiva	SCC(w)	T2N0M0	II	81	F	-
10	-	Maxi. Gingiva· Buccal	SCC(w)	T3N0M0	III	81	M	-
11	-	Mand. Gingiva	SCC(w)	T4N0M0	IV A	63	M	-
12	+	Tongue	SCC(m)	T3N0M0	III	54	M	-
13	-	Mand. Gingiva	SCC(w)	T2N1M0	III	65	Unknown	+
14	-	Tongue	SCC(w)	T2N0M0	II	73	F	-
15	-	Gingiva· Buccal	SCC(w)	Unknown	Unknown	Unknown	F	-
16	-	Tongue	SCC(w)	T1N0M0	I	61	M	-
17	-	Mand. Gingiva	SCC(w)	T2N0M0	II	75	F	-
18	+	Mand. Gingiva	SCC(m)	T4N2bM0	IV A	79	F	+
19	-	Oral floor	SCC(m)	T2N0M0	II	60	F	-
20	-	Tongue	SCC(w)	Unknown	Unknown	Unknown	F	-
21	+	Buccal Mucosa	SCC(w)	T4N0M0	IV A	86	F	-
22	-	Mand. Gingiva	SCC(w)	T3N0M0	III	70	M	-
23	+	Mand. Gingiva	SCC(w)	T3N0M0	III	79	M	-
24	+	Buccal Mucosa	SCC(w)	T3N0M0	III	79	M	-
25	-	Oral floor	SCC(w)	T3N0M0	III	79	M	-
26	+	Mand. Gingiva	SCC(w)	T4N0M0	IV A	43	M	-
27	-	Maxi. Gingiva	SCC(w)	T3N2M1	IV C	79	M	+
28	+	Mand. Gingiva	SCC(w)	T3N1M0	III	79	F	+
29	-	Tongue	SCC(w)	T1N0M0	I	81	F	-
30	-	Mand. Gingiva	SCC(w)	T2N1M0	III	66	M	+
31	+	Unknown	SCC(w)	Unknown	Unknown	Unknown	F	-
32	-	Tongue	SCC(m)	T3N1M1	IV C	60	M	+
33	+	Palate	CIS	TisN0M0	0	69	M	-
34	-	Mand. Gingiva	SCC(w)	T2N1M0	III	73	M	+
35	-	Tongue	SCC(w)	Unknown	Unknown	Unknown	F	-
36	+	Mand. Gingiva	SCC(w)	T4N2bM0	IV A	53	F	+
37	+	Mand. Gingiva	SCC(w)	T3N0M0	III	85	F	-
38	+	Buccal Mucosa	SCC(w)	T1N0M0	I	73	F	-
39	+	Buccal Mucosa	SCC(w)	T4N0M0	IV A	87	F	-
40	-	Oral floor	CIS	TisN0M0	0	Unknown	M	-
41	-	Tongue	SCC(w)	T4N1M0	IV A	71	M	+
42	-	Oral floor	SCC(w)	T4N0M0	IV A	61	M	-
43	-	Tongue	SCC(m)	T1N0M0	I	79	F	-
44	+	Unknown	SCC(w)	Unknown	Unknown	75	F	-
45	-	Tongue	SCC(w)	T2N0M0	II	50	M	-
46	-	Tongue	SCC(w)	T1N0M0	I	53	M	-
47	-	Mand. Gingiva	SCC(w)	T4N0M0	IV A	79	F	-
48	+	Mand. Gingiva	SCC(m)	T4N1M0	IV A	71	M	+
49	-	Buccal Mucosa	SCC(m)	T4N0M0	IV A	62	M	-

[a]+, methylated; -, unmethylated. [b]Maxi., maxillary; Mand., mandibular. [c]SCC(w), well-differentiated SCC; SCC(m), moderately-diferentiated SCC; CIS, carcinoma *in situ*; Verrucous, verrucous carcinoma. [d]TNM and Stage, staged by International Union Against Cancer. [e]+, with nodal involvement; -, without nodal involvement.
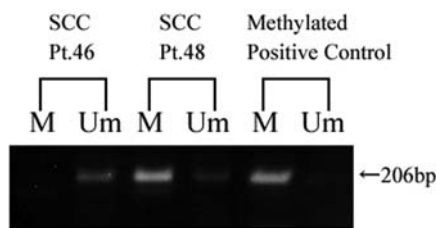
Figure 1. Methylation analysis of CHFR by methylation-specific PCR in primary oral cancer. M, methylated DNA-specific amplification; Um, unmethylated DNA-specific amplification.
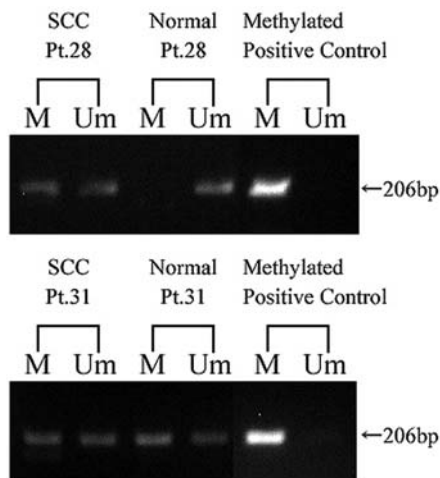

Figure 2. Methylation analysis of CHFR by methylation-specific PCR in primary oral cancer and adjacent normal tissue. M, methylated DNA-specific amplification; Um, unmethylated DNA-specific amplification.

Table II. CHFR methylation status and clinical variables.

| Variables | CHFR methylation status | | |
	M(+) n=17	M(-) n=32	P-value
Site of tumor			
Tongue	2	11	
Gingiva	8	12	0.1327
Buccal mucosa	5	4	0.4358
Oral floor	0	5	0.0467[a]
Palate	1	0	
Tumor size			
T1+T2	3	15	0.0334[a]
T3+T4	12	13	
Clinical stage			
I+II	3	11	0.2162
III+IV	11	16	
Age			
<70	5	10	0.7638
≥70	11	18	
Range	43-87	50-81	
Average	72.5	70.0	
Histological grade			
Well	13	25	
Moderate	3	5	0.8591
Verrucous	0	1	
CIS	1	1	
Lymph node metastasis			
N=0	13	24	0.9093
N≥1	4	8	
Gender			
Male	6	19	0.0831
Female	11	12	

[a]Significantlly different by χ^2 test.

the state of the methylation, clinical stage, tumor size, the part, the lymph node metastasis, gender, and the age is shown in Table II. There was a significant difference between methylated and unmethylated groups regarding tumor size. Moreover, the frequency of CHFR hypermethylation of OSCC in buccal mucosa was significantly higher than in tongue. However, no significant difference was seen in the other clinical features.

As for the 13 OSCC cases with paired cancerous and the adjacent normal tissues, promoter hypermethylation of the CHFR gene was detected in 46.1% (6 of 13) of the cancerous tissues. This methylation rate is similar to that of overall primary OSCC. In contrast, as for the surrounding normal mucosa, promoter hypermethylation of CHFR gene was recognized only in one case (1 of 13). (Fig. 2 and Table III). As a result, the frequency of the hypermethylation of CHFR in a normal mucosa was extremely lower than primary OSCC. There were no hypermethylation of CHFR gene detected in healthy volunteers.

Aberrant hypermethylation of CHFR in OSCC cell lines and gene expression level of CHFR and Aurora-A. Aberrant promoter hypermethylation of the CHFR gene was detected in Ca9-22 and SCC25 cell lines. Ca9-22 had no unmethylated specific band so that genomic DNA was fully methylated and genomic DNA was partially methylated in SCC25. The CHFR methylation was not detected in other OSCC cell lines (Fig. 3). CHFR promoter hypermethylation was detected in two of six

OSCC cell lines (33.3%), so this methylation frequency was in accordance with that of primary OSCC samples.

We performed real-time RT-PCR to assess the mRNA expression level of CHFR and Aurora-A (Fig. 4). CHFR mRNA in Ca9-22 cell line was not expressed by quantitative real-time PCR. The remaining five cell lines showed detectable levels of CHFR mRNA expression. Aurora-A mRNA expression was also detected in all cell lines. There is a dissociation of the expression between CHFR mRNA and Aurora-A mRNA in Ca9-22 cell line. Compared with other
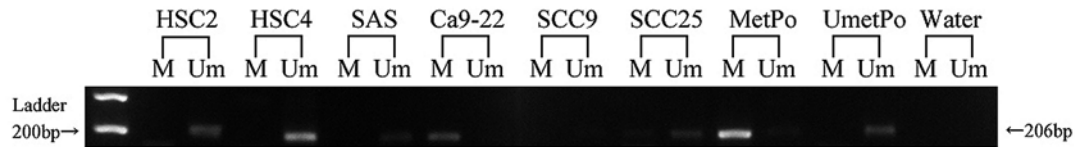
Figure 3. Methylation analysis of CHFR by methylation-specific PCR in OSCC cell lines. M, methylated DNA-specific amplification; Um, unmethylated DNA-specific amplification. MetPo, methylated positive control; UmetPo, unmethylated positive control.

Table III. Methylation status of primary OSCC and adjacent normal tissue.

Case no.	Primary OSCC	Normal mucosa
25	U	U
26	**M**	U
27	U	U
28	**M**	U
29	U	U
30	**M**	U
31	**M**	**M**
32	U	U
34	U	U
36	**M**	U
37	**M**	U
38	**M**	U
39	U	U

M, methylated; U, unmethylated.

Figuer 4. Quantification of CHFR and Aurora-A mRNA in OSCC cell lines using LightCycler system. Bars indicate expression levels normalized to those of GAPDH. The results are expressed as mean ± standard deviation.

cell lines, overexpression of Aurora-A mRNA in Ca9-22 cell line may be influenced by the uncontrolled condition of CHFR.

Immunohistochemistry of Aurora-A in human OSCC samples. Using an affinity-purified polyclonal rabbit antiserum recognizing human Aurora-A protein, a cytoplasmic Aurora-A e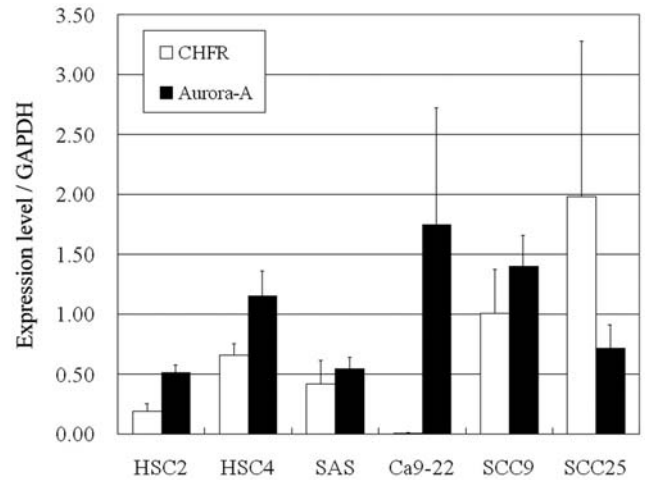xpression was detected in all of the 9 OSCC tumor samples (Table IV). There is no significant difference between Aurora-A expression by immunohistochemistry and CHFR hypermethylation status. All the tumor tissues from OSCC showed positive staining of Aurora-A in their cytoplasm independent of CHFR hypermethylation status.

Discussion

In the present study, the frequency of CHFR methylation varies in different sites compared to other organs primary

Table IV. Immunohistchemical staining status of Aurora-A.

Case no.	CHFR status	Region	Histopathology	Aurora-A staining	
				Cytoplasma	Nuclear
22	U	Gingiva	SCC(w)	+	-
23	M	Gingiva	SCC(w)	++	+
24	M	Buccal mucosa	SCC(w)	++	+
25	U	Oral floor	SCC(w)	++	±
29	U	Tongue	SCC(w)	+	-
30	U	Gingiva	SCC(w)	+++	-
32	U	Tongue	SCC(m)	++	-
36	M	Gingiva	SCC(w)	++	+
38	M	Buccal mucosa	SCC(w)	+	+

M, methylated; U, unmethylated. SCC(w), well-differentiated SCC; SCC(m), moderately-diferentiated SCC.

tumor. For example, nasopharyngeal carcinoma has high frequency of CHFR methylation at 61.1% (22). In contrast, breast cancer has low level of CHFR methylation at 0.9% (23) and ovarian cancer has no methylation (24). CHFR promoter hypermethylation of primary OSCC was 34.7% in our study. In primary head and neck squamous cell carcinomas, the frequency of CHFR methylation has been reported to be ~30% (25) and 25% (26). These results were consistent with our data.

Regarding the relationship between CHFR methylation and clinicopathological characteristics, our results indicate that the frequency of CHFR methylation occurs in T3-T4 squamous cell carcinoma was significantly higher than in T1-T2 squamous cell carcinoma. Thus, our results imply that the OSCCs in advanced stage have more frequent methylation in the promoter region of CHFR. It was also reported that the methylation of CHFR is seen only in the stage IV case of head and neck cancer (26). Therefore, when the cancer progresses, CHFR methylation occurs with high frequency. Moreover, our results indicate CHFR methylation occurs in buccal mucosa SCC significantly more frequently than in tongue SCC. Thus, location-specific occurrence of CHFR methylation is considered in OSCC.

It has also been reported that CHFR methylation correlated to its differentiation of the tumor histology for endometrium cancer (16). Our results, however, shows no relationship between the CHFR methylation status and differentiation degree of the SCC histology. A significant difference according to gender has been reported, female esophageal cancers were more frequently methylated than male (27). Our results also showed a tendency of CHFR methylation in female more frequently than male. The relationship between CHFR expression and clinical characteristics may be different among cancer types. As a result of CHFR methylation, mRNA expression of CHFR is silenced. In breast cancer, it has been reported that there is an association between CHFR mRNA expression and the tumor size (28) and it is in agreement with our data. Previous studies have revealed that the loss of CHFR expression by aberrant methylation may predict the responsiveness of human cancers to microtubule inhibitors such as taxane (29). Cancer cells silencing CHFR are sensitive to microtubule inhibitors, which are considered as a result from impaired checkpoint function. Therefore, the detection of CHFR methylation status may be used in clinical application and in curative effect judgment.

As for DNA methylation of other tumor supressor genes in OSCC, various genes have been investigated previously. CHFR is one of the cell cycle regulatory genes. The other cell cycle regulating genes such as p15 and p16 (4) have been extensively studied and the promoter hypermethylation is common in OSCC, however no significant correlation with clinicopathological characteristics or prognosis has been observed. In contrast, p14 hypermethylation has been related to good prognosis in two studies (30,31). Moreover, E-cadherin hypermethylation was ~50% and it was related to histological grade and poor survival (32). There was hypermethylation in 27% of DAPK, a gene that takes part in apoptosis, and its hypermethylation is related to the metastasis in lymph nodes (33). Hypermethylation of the RECK gene is ~50% in the cancerous tissue and is correlated with recurrence-free survival and overall survival (34). In contrast, MGMT, a DNA-repair gene, has high frequency of hypermethylation with 50% or more but there is no significant relation with clinicopathological characteristics (35).

In this study, we showed that the promoter hypermethylation of the CHFR gene occurred frequently in the primary cancerous tissues but rarely in the adjacent normal mucosa. As a result, hypermethylation of CHFR in the surrounding normal tissue of only one case is concurrent with in the cancerous tissue of the same patient. There was no case showing hypermethylation only in the normal tissues. The concept of 'Field Cancerization' that Slaughter advocated in OSCC in 1953 (36) became an important mechanism in permeation and metastasis. Field cancerization takes part in the formation of second primary tumor (37) and is important in the secondary expansion of the tumor. Methylation of CHFR in the surrounding normal tissue was rare in our results and the concept of 'Field cancerization' is not reflected in CHFR methylation in the occurrence of OSCC. However, there were several studies on high frequency of DNA methylation in certain tumor suppressor or related genes such as p16, MGMT (35), and RECK (34) detected in surrounding normal tissues associated with cancerous tissue. Further work is needed to clarify this concept for field cancerization of OSCC.

It is well accepted that CHFR works as a ubiquitin ligase of Aurora-A and it has been reported that CHFR regulates Aurora-A (9). However, this relationship was not confirmed by our investigation. CHFR hypermethylation is common even in primary OSCC tumors but not in surrounding normal mucosa whereas Aurora-A gene expression seems to be independent from methylation status and mRNA expression of the CHFR gene in OSCC. Additional study is required to determine the potential contribution of the CHFR promoter hypermethylation in oral carcinogenesis.

Acknowledgements

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