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The study of *p16* and *p15* gene methylation in head and neck squamous cell carcinoma and their quantitative evaluation in plasma by real-time PCR

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Abstract

Epigenetic silencing of the *p16* and *p15* genes by promoter methylation are commonly observed in human epithelial malignancies, including head and neck squamous cell carcinomas (HNSCC). In this study, a methylation-specific polymerase chain reaction (MSP) was used to evaluate the methylation status of the *p16* and *p15* genes in 73 HNSCC surgical specimens. *p16* and *p15* gene methylation was also examined in 29 paired metastatic lymph nodes and 29 paired histologically, normal resection margin mucosae. The quantity of cell-free methylated *p16* and *p15* DNA in the plasma samples of 20 HNSCC patients and 24 healthy controls was also examined using a fluorescence-based real-time PCR assay. The frequencies of *p16* and *p15* methylation in the primary tumour were 49% and 60%, respectively. Concordant methylation of *p16* and *p15* in tumour samples and metastatic lymph nodes was found in 59 and 38% of cases, respectively. A significantly higher prevalence of *p15* methylation was found in histologically-normal surgical margin epithelia of HNSCC patients with chronic smoking and drinking habits compared with non-smokers and non-drinkers. In addition, methylated *p16* and *p15* DNA levels were significantly higher in the plasma of HNSCC patients (mean 56 copies/ml plasma and 65 copies/ml plasma, respectively) compared with normal controls (mean 6 copies/ml plasma and 16 copies/ml plasma, respectively). In conclusion, promoter methylation of the *p16* and *p15* genes is involved in the pathogenesis of HNSCC and may be related to chronic smoking and drinking. The differential levels of methylated *p16* and *p15* DNA in plasma might be potential useful markers in screening high-risk populations for early HNSCC and monitoring their treatment response.

Keywords: *p15*; *p16*; Tumour suppressor genes; HNSCC

1. Introduction

Both the p16 and p15 proteins are inhibitors of cyclin-dependent kinases that prevent the cell going through the G1/S phase transaction. Inactivation of p16 and p15 are important steps in cancer development [1]. Down-regulation of p16 and p15 expression are found in many cancers [2,3]. We have previously reported a high frequency (48%) of p16 downregulation in a study of 225 head and neck squamous cell carcinomas [4]. Transcriptional silencing of these inhibitors is frequently associated with methylation of 5′ CpG islands [5,6]. Methylation of *p16* has been found in precancerous oral dysplastic tissues, adjacent non-neoplastic areas of gastric carcinoma and ulcerative colitis [7–10]. The role of p15 is less well documented.

Methylated *p16* is present in bronchial epithelia before the clinical evidence of lung cancer in chronic smokers [11]. It therefore suggests the potential of using methylated DNA as a tumour marker in cancer screening, monitoring chemoprevention and the treatment of cancer. Apart from examining cells, the other possible source of detection of these tumour markers is in the absorbed tumour DNA in peripheral blood. Elevation

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of cell-free circulating DNA is observed in cancer patients [12,13]. The circulating DNA shares many properties of the cancerous DNA and it is suggested that it originates mostly from the cancerous tissues [14,15]. By using a methylation-specific polymerase chain reaction (MSP), several methylated genes have been detected in the peripheral blood of patients with head and neck, liver, lung, oesophageal, gastric and colorectal cancers [16–21]. Methylated DNA of cancer-specific genes in the peripheral blood therefore becomes a promising tumour marker that might be useful in screening for early cancer in high-risk populations and for the early detection of sub-clinical residual disease non-invasively after treatment. Since the methylated promoter DNA in the circulation reflects the total body content of methylated cancer cells, we hypothesise that the quantity of cell-free circulating methyated promoter DNA in cancer patients should be higher than in normal controls or patients in remission after treatment. The quantitative evaluation of methylated DNA by real-time PCR might therefore be more sensitive and specific than non-quantitative PCR in differentiating cancer patients from healthy individuals. In this study, we evaluated the role of *p16* and *p15* methylation in head and neck squamous cell carcinomas (HNSCC); in primary tumours, metastatic lymph nodes and surgical resection margins. The quantity of methylated *p16* and *p15* DNA in the plasma of HNSCC patients was also evaluated using a real-time fluorogenic PCR approach.

2. Patients and methods

2.1. Tissue and plasma samples

In this study, 73 primary HNSCC, 29 matched metastatic lymph nodes, 29 matched morphologically normal resection margin mucosae, and 20 matched peripheral blood samples from patients before treatment were evaluated. All specimens were obtained at the University of Hong Kong Medical Center. The tumour specimens were graded according to the TNM classification of the Union International Contre le Cancer (UICC). There were 61 male and 12 female patients. The median age was 63 years (range 28–92 years). The median duration of follow-up of patients who were alive without tumour at the last follow-up was 45 months. The tumour sites of these 73 HNSCC included oral cavity ($n=33$), oropharynx ($n=8$), hypopharynx ($n=21$), and larynx ($n=11$). There were 3 T1 (2N0, 1N1), 21 T2 (8N0, 5N1, 7N2,1N3), 24 T3 (10N0, 4N1, 9N2, 1N3) and 25 T4 (8N0, 3N1, 12N2, 2N3) tumours. 3 patients had distant metastasis at the time of primary surgery and were treated for palliation, and the other 70 patients had primary surgical treatment with curative intent. None of these patients had prior chemotherapy or radiotherapy. Seven tonsils taken from tonsillectomy specimens of chronic tonsillitis patients were also included as normal controls.

All patients had surgical treatment. The tumour specimens were taken from the surgical specimens. The normal resection margin mucosae were collected a minimum of 1.5 cm away from the tumour edge after resection of the tumour. Sections that were frozen intraoperatively and subsequent paraffin sections were used to confirm the normal histology of these mucosae [22]. Peripheral blood was collected from 20 HNSCC patients prior to surgery and also from 24 healthy volunteer donors to be used as normal controls. The whole blood sample was first centrifuged at 400 *g* for 10 min at room temperature. The plasma and buffy coat layer were then separately transferred to another plain tube. An additional centrifugation at 1000 *g* for 10 min was performed on the plasma fraction to remove the remaining cellular components. Written consent was obtained from all of the patients before the specimen collection and the study was approved by the University ethics committee.

2.2. Methylation-specific polymerase chain reaction (MSP)

Tissue DNA was extracted using a conventional phenol-chloroform protocol. MSP was performed as described by Herman and colleagues and also in our previous publication [23,24]. Sodium bisulphide modification was carried out using a CpGenomeTM DNA Modification Kit (Intergen, New York, USA). Universal methylated human male genomic DNA (Intergen, New York, NY, USA) was used as the positive control. Genomic DNA purified from the peripheral blood of a healthy voluntary donor was used as a negative control. A blank control containing all the PCR components except sample DNA was also included. 'Hot start' PCR was carried out using AmpliTaq Gold polymerase (Perkin-Elmer Corp., Foster City, USA). The primer sequences and PCR conditions were previously described in Ref. [23]. A total of 40 cycles was used for both the *p15* and *p16* genes. PCR products were resolved in a 4%-agarose gel followed by ethidium bromide staining.

2.3. Quantitative analysis of cell-free methylated p16 and p15 in the plasma

Fluorescence-based Methylight assay was performed as previously described in Ref. [25]. Plasma DNA was extracted from 400 μl plasma using the QIAamp Blood and Tissue Kit (Qiagen, Hilden, Germany). All extracted DNA was then eluted into 100 ul water and was subjected to a sodium bisulphide modification. Bisulphide-modified DNA was finally eluted in 40 ul water and then 5 ul was used for each real-time PCR reaction. For

absolute quantification, the standard curve was generated by serial diluting the universal methylated human male genomic DNA (Intergen, New York, USA) and modified together with the sample DNA. A conversion factor of 6.6 pg DNA equivalent to two copies of the gene (*p15* or *p16*) was used in the calculation of the gene copy number of the PCR reaction. The concentration of the methylated DNA in plasma is expressed as copy number per ml of plasma with the following formula:

$$\text{The concentration of plasma methylated gene (copy/ml)} = Q/V$$

Q = copy number of methylated gene derived from the standard curve and V = the amount of plasma in ml being used in the Methylight assay (0.05 ml in this study).

The GenBank accession number (amplicon location), sense primer sequence, reverse primer sequence and fluorogenic probe sequence were as follows: (1) *p16*, NM_000077 (66-133), 5′-TGGAGTTTTCGGTTGA TTGGTT-3′, 5′-AACAACGCCCGCACCTCCT-3′ and 5′-FAM-ACCCGACCCCGAACCGCG-TAMRA-3′; (2) *p15*, S75756 (350-430), 5′-AGG AAG GAG AGA GTG CGT CG-3′, 5′-CGA ATA ATC CAC CGT TAA CCG-3′, and 5′-FAM-TTAACGACACTCTTCCCTT CTTTCCCACG-TAMRA-3′. All the reactions were performed in 96-well plates (Perkin-Elmer Corp., USA) and monitored by PE Applied Biosystems 7700 Sequence Detector (Perkin-Elmer Corp., USA). In each run of the quantitative PCR, the samples were tested in duplicate.

2.4. Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 7.5. All P values are two-sided. $P < 0.05$ was considered statistically significant.

3. Results

3.1. p16 *and* p15 *gene methylation in primary tumour and normal control samples*

Fig. 1 shows the representative MSP results of the primary tumours. Of the 73 HNSCC primary tumour specimens, methylation of the *p16* and *p15* genes was detected in 36 (49%) and 44 (60%) primary tumours, respectively. Methylation of both the *p16* and *p15* genes was found in 24 (33%) of the tumour samples. 56 (77%) tumours showed methylation of either *p16* or *p15*. No significant correlation was found between *p16* and *p15* methylation (Chi-square, $P = 0.271$). There was no significant correlation of the *p16* and *p15* methylation status with the clinicopathological parameters including tumour grade, stage, T stage, nodal metastasis (defined by either pathological node-positive stage or nodal recurrence) and nodal stage (Chi-square test, all $P > 0.05$). There was no significant correlation of survival with the promoter methylation status of both *p16* (Log-rank, $P = 0.1392$) and *p15* (Log-rank test, $P = 0.0735$). Methylation of *p16* was not detected in any of the 7 normal tonsils. Promoter methylation of *p15* was found in 1/7 (14%) of the normal tonsils.

3.2. p16 *and* p15 *methylation of metastatic tumour in lymph nodes of the neck*

There were 29 matched primary tumours and metastatic lymph nodes for the methylation studies. Methylation of the *p16* promoter was found in 41% (12/29) of the metastatic lymph nodes. A concordant pattern of *p16* promoter methylation pattern was found in 59% (17/29) matched primary tumours and metastatic nodes (five concordant methylated *p16* and 12 concordant unmethylated *p16*).

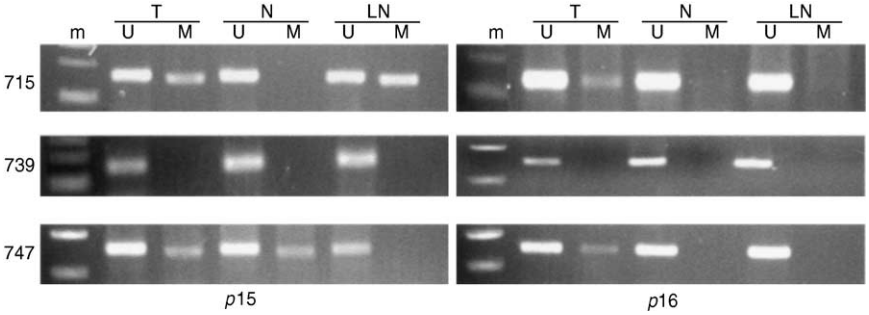

Fig. 1. Methylation analysis of *p16* and *p15* genes in HNSCC patients. Representative MSP products of *p16* and *p15* genes of 3 patients (cases 715, 739 and 747). Genomic DNA was extracted from the tumour (T), paired normal resection margin (N) and paired metastatic lymph node (LN). 50 ng of the bisulphite-modified DNA was subjected to MSP analysis. Lane U indicates the presence of unmethylated genes; Lane M indicates the presence of methylated genes. Due to the contaminating normal cells, all tissues shows the presence of unmethylated *p16* and *p15* genes on the 4% agarose gel.

Methylation of the *p15* promoter was found in 38% (11/29) of the metastatic lymph nodes. A concordant *p15* methylation pattern was found in 44% of the matched primary tumours and metastatic nodes (four concordant methylated *p15* and 7 concordant unmethylated *p15*).

3.3. p16 *and* p15 *gene methylation in histologically-normal resection margin mucosae*

There were 29 matched histologically normal resection margin epithelia available for the methylation studies. Methylation of the *p16* and *p15* promoters was found in five (17%) and 18 (62%) of the histologically normal resection margin epithelia, respectively. Methylation of *p16* and *p15* in the normal resection margins was not correlated with age ((*t*-test, $P = 0.803$ and 0.279, respectively), gender (Fishers test, $P = 0.553$ and 1, respectively) and local recurrence (Fishers test, $P = 0.320$ and 0.483, respectively).

3.4. *Methylation of* p16 *and* p15 *in the normal resection margin epithelia of patients with chronic smoking and drinking habits*

Of the 29 patients in which resection margin mucosae were evaluated, 23 were chronic cigarette smokers. The median starting age of smoking was 23 years old (range 5–42 years). The median duration of smoking was 40 years (range 2–65 years). The median number of cigarettes smoked per day was 20 (range 10–60). Promoter methylation of *p16* was found in 17% (4/23) of the chronic smokers and 17% (1/6) of the non-smokers (odds ratio = 1.009, 95% Confidence Interval (CI) 0.674–1.511). Methylation of the *p15* promoter was found in 35% (8/23) of the chronic smokers and 0% (0/6) of the non-smokers (odds ratio = 1.533, 95% CI 1.138–2.067). Chronic smokers had a significantly higher frequency of *p15* promoter methylation in their normal adjacent epithelia.

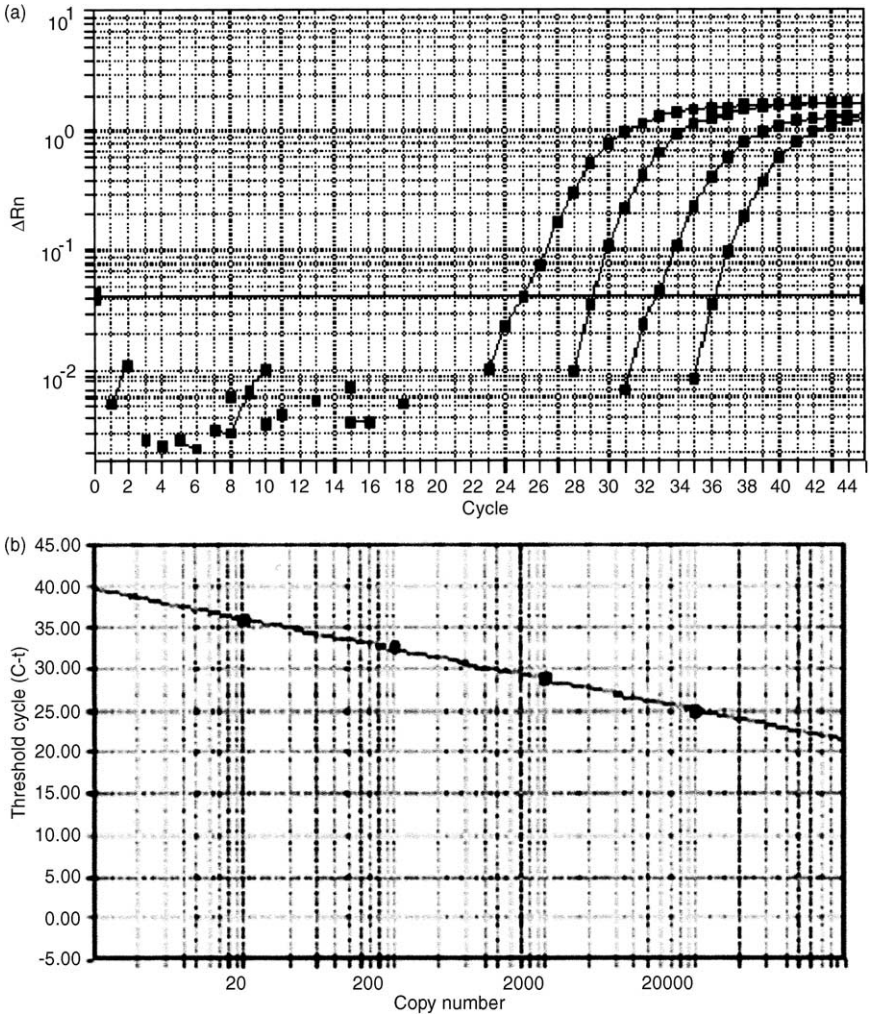

Fig. 2. Detection of hypermethylated DNA by Methylight assay. (a) Amplification plot of serial-diluted Universal Methylated DNA against PCR cycles; (b) standard curve plot with threshold cycle against the quantity of methylated DNA measured as copy number.

Of these 29 patients, 15 were chronic drinkers. The median starting age of drinking was 43 years old (range 5–64 years). The median duration of drinking was 20 years (range 4–51 years). The concentration of alcohol drunk ranged from 5 to 40%. The median total daily amount of alcohol drunk was 69 ml (range 10–256 ml). Methylation of *p16* was found in 27% (4/15) normal epithelia of the chronic drinkers and 7% (1/14) of the non-drinkers (odds ratio = 1.266, 95% CI 0.903- 1.775). Methylation of *p15* was found in 53% (8/15) of the normal resection margin epithelia of the chronic drinkers, but was not found in any of the non-drinkers (odds ratio = 2.143, 95% CI 1.247–3.681). Chronic drinkers had a significantly higher frequency of *p15* methylation in their histologically normal adjacent resection margin epithelia.

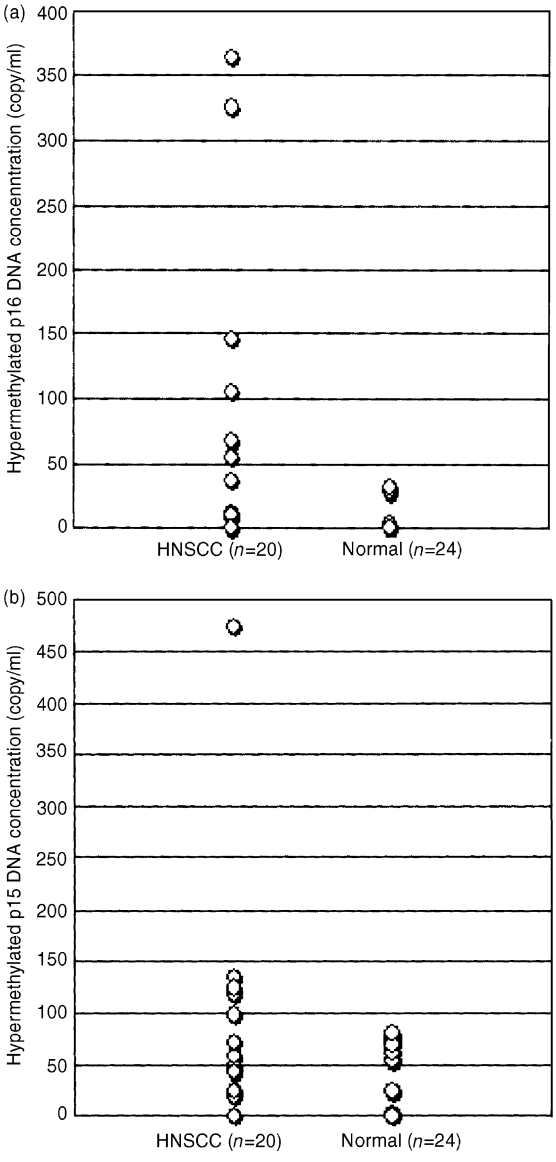

Fig. 3. Concentration of methylated *p16* and *p15* in plasma. (a) Comparison of methylated *p16* concentrations in the plasma of HNSCC patients and normal controls; (b) comparison of methylated *p15* concentration in the plasma of HNSCC patients and normal controls.

3.5. *Plasma concentration of methylated* p15 *and* p16 *promoter DNA in HNSCC patients*

Peripheral blood plasma samples were collected from 20 HNSCC patients before operation and 24 normal healthy donors as controls. The quantity of methylated gene copy was examined by the Methylight approach (Fig. 2) and the concentration was expressed as gene copy number/ml plasma.

Methylated *p16* in plasma was found in 13/20 (65%) of the HNSCC patients and 4/20 (20%) of the healthy controls. The mean concentration of methylated *p16* was 56 copies/ml (range 0–364 copies/ml) in the HNSCC patients and 6 copies/ml (range 0–32 copies/ml) in the healthy controls (Fig. 3a). The mean concentration of methylated *p16* in plasma of HNSCC patients was significantly higher than in the control group (*t*-test, $P = 0.016$).

Methylated *p15* in plasma was found in 12 (60%) HNSCC patients and 12/24 (50%) of the healthy controls. The mean concentration of methylated *p15* was 65 copies/ml (range 0–474 copies/ml) in the HNSCC patients and 16 copies/ml (range 0–80 copies/ml) in the healthy controls (Fig. 3b). The mean concentration of methylated *p15* in the plasma of HNSCC patients was significantly higher than in the control group (*t*-test, $P = 0.0037$).

4. Discussion

Chromosome 9p21, the location of the *p16* and *p15* genes, is frequently altered in HNSCC and pre-malignant lesions [26,27]. In the present study, the high frequency of aberrant *p16* and *p15* methylation in both primary tumours and metastatic lymph nodes suggested that epigenetic alterations of *p16* and *p15* genes could be one of the mechanisms involved in multi-step HNSCC carcinogenesis. In order to address whether *p15* and *p16* methylation are involved in the early stage of development of HNSCC, we performed a methylation analysis on the adjacent normal epithelia of HNSCC patients. These normal mucosae were taken from the resection margin at least 1.5 cm from the tumour edge. This distance has been shown in our previous histological analysis to be free of cancer [22]. We could identify the presence of methylated *p16* and *p15* in the histologically-normal epithelia of HNSCC patients. Similar results on *p16* methylation have been reported in patients with normal gastric epithelia adjacent to gastric cancers [28]. The presence of *p16* and *p15* methylation in normal mucosa indicated their possible involvement in field cancerisation phenomenon of the upper aerodigestive tract and early stages of carcinogenesis [29].

Chronic smoking and drinking are predisposing factors for the development of HNSCC [29,30]. Methylation of

p16 is associated with smoking in non-small lung cancer [31]. Methylation of *p16* was also found in 35% of the sputum of smokers without lung cancer, and may be a useful tumour marker for the screening of high-risk populations [8]. Our results are compatible with studies of premalignant lesions of other groups, in which high a incidence of *p16* methylation (75%) has been reported in head and neck premalignant lesions [32]. Our results suggested that methylation of both *p16* and *p15* in normal tissues could be due to the chronic exposure of carcinogens present in tobacco and alcohol. Thus, hypermethylated *p16* and *p15* might be a useful molecular marker in screening high-risk populations undergoing chemopreventive treatments and monitoring of their responses.

From the results of this study, methylation of both *p15* and *p16* was found in invasive cancers and metastatic lymph nodes. We postulate that the quantity of circulating methylated DNA might be higher in cancer patients when compared with healthy individuals. By using a quantitative PCR approach, we demonstrated that methylated *p16* and *p15* DNA are present in the plasma of both cancer patients and normal controls. However, patients with HNSCC had significantly higher concentrations of both *p16* and *p15* methylated DNA in their plasma compared with healthy individuals. Our results suggest that quantitative measurement of plasma methylated *p16* and *p15* gene concentrations might be useful in the future for cancer screening and monitoring treatment response. However, prospective collection of blood specimens from cancer patients and a large scale population screening study are necessary to clarify the potential of methylated *p16* and *p15* as tumour markers in HNSCC.

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