Quantitative analysis of *P16* gene CpG methylation in Uyghur patients with cervical squamous cell carcinoma and its relationship with HPV16 infection

J.-X. Cheng[1,2], M. Yuan[1], A.-L. Li[1], P. Zhou[1], G.-Q. Shen[1] and Y. Zhang[2]

[1]Department of Gynecology,
Cancer Hospital Affiliated to Xinjiang Medical University, Urumchi, China
[2]Department of Gynecology, Xiangya Hospital of Central South University,
Changsha, China

Corresponding author: Y. Zhang
E-mail: yizhangcn@yeah.net

ABSTRACT. The methylation of CpG sites in the promoter region of the *P16* gene in Uyghur patients with cervical squamous cell carcinoma (CSCC) was quantitatively analyzed and its relationship with human papillomavirus 16 (HPV16) infection was explored. Cervical samples were collected from 20 Uyghur patients with CSCC and 20 Uyghur controls. Matrix-assisted laser desorption ionization-time of flight mass spectrometry was applied to detect methylation of CpG sites in the promoter region of the *P16* gene; polymerase chain reaction was performed to assess HPV16 infection in the 2 groups. Among the 16 CpG sites in the *P16* gene promoter region, the methylation level of the CpG1-2 and CpG 6 sites, as well as the HPV16 infection rate, was higher in the CSCC group than in the control group (P < 0.05). There was no significant correlation between *P16* CpG methylation and HPV16 infection in Uyghur patients with CSCC. The *P16* gene

CpG1-2 and CpG 6 hypermethylation and HPV16 infection, which are independent of each other, play an important role in cervical squamous cell carcinogenesis in Uyghur patients.

Key words: Cervical squamous cell carcinoma; Uyghur; *P16* gene; Methylation of CpG sites; HPV16

INTRODUCTION

The morbidity of cervical cancer is the highest among all gynecological malignant tumors in China, particularly in Xinjiang, where morbidity is as high as 459-562 per million Uyghur women. The mortality rate of cervical cancer in Sinkiang ranks first among Chinese ethnic minorities and is a serious threat to the health and life of Uyghur women. Previous studies have demonstrated that human papillomavirus (HPV) infection is the main cause of cervical cancer and that 94.31% of Uyghur patients with cervical cancer have HPV16 infection. However, a single virus infection is insufficient to cause disease, and disorders in the DNA methylation level and pattern are observed with the occurrence and development of cervical cancer (Ushijima, 2005). Deletion, mutation, and methylation of the *P16* gene in a wide variety of human tumor tissues have been widely studied in many fields. We used matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) to detect the methylation content of each CpG site in the promoter region of the *P16* gene in cervical samples from Uyghur patients with cervical squamous cell carcinoma (CSCC) and Uyghur controls; polymerase chain reaction (PCR) was performed to detect the HPV16 DNA fragment. We explored the relationship between *P16* CpG methylation, HPV16 infection, and CSCC in Uyghur women to identify early warning indicators of CSCC by studying the pathogenesis of CSCC in Uyghur patients.

MATERIAL AND METHODS

Clinical data

Cervical tissue samples were collected from 20 Uyghur patients with early-stage CSCC, who underwent surgery at the Cancer Hospital of Sinkiang, the First Affiliated Hospital of Shihezi University, or the Third Affiliated Hospital of Shihezi University between October 2009 and March 2010. Four cases were in stage Ia, 13 cases in stage Ib1, and 3 cases in stage IIa, all of whom had not received chemotherapy or radiotherapy before surgery. Cervical epithelial tissue samples were also obtained from 20 Uyghur control subjects with non-cervical benign gynecological diseases who underwent panhysterectomies in the corresponding period. All materials were collected with the patients' consent and were confirmed by pathology. All fresh specimens were immediately rinsed with 0.9% normal saline *in vitro* to wash away the blood and mucus on the surface and were stored at -80°C. This study was conducted in accordance with the Declaration of Helsinki and with approval from the Ethics Committee of the Xiangya Hospital of Central South University. Written informed consent was obtained from all participants.

Primer design

EpiDesgner (http://www.epidesigner.com) was used to design *P16* gene primers to amplify the 326-base pair (bp) fragment; HPV16 primers were designed based on a previous study (zur Hausen, 2000) (Table 1).

Table 1. Primer sequences.

Primer	Primer sequence	Amplified fragment
P16 gene	Forward 5'-aggaagagagGTGGGGAGGAGTTTAGTTTTTTTTT-3'	326 bp
P16 gene	Reverse 5'-agggagaaggctCCACCCTCTAATAACCAACCAAC-3'	
HPV16	Forward 5'-GACCCAGAAAGTTACCACAG-3'	268 bp
HPV16	Reverse 5'-CACAACGGTTTGTTGTATTG-3'	

DNA extraction and sulfite process

An SK1252 genomic DNA extraction kit (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.; Shanghai, China) was employed to isolate DNA from cervical samples according to the manufacturer protocol, followed by agarose gel electrophoresis, and then photographs were taken under an ultraviolet gel imaging system. No DNA degradation, RNA, or protein residues were detected. Bisulfite-treatment EZ DNA methylation kits (Sequenom Inc.; San Diego, CA, USA) served as a reference for performing DNA modification and purification through sulfite treatment according to the manufacturer instructions.

PCR amplification and *in vitro* transcription

After using the MassCLEAVE kit (Sequenom Inc.) to amplify the target gene fragment on 384-well plates using PCR, shrimp alkaline phosphatase was added to remove free nucleotides in the reaction system. The PCR products were then subjected to *in vitro* transcription and T cleavage, following by purification by adding clean resin. After centrifugation, the samples were stored until further analysis.

Methylation detection and mass spectrogram analysis

A MassARRAY Nanodispenser (Sequenom Inc.) was used to remove 22 μL reaction liquid from a 384-well plate and placed onto a chip containing the matrix; a MassARRAY mass spectrometer was employed to collect mass spectral data; data were analyzed using the EpiTYPER software (Sequenom Inc.).

HPV16 DNA detection

The HPV16 DNA fragment was detected using PCR in a total reaction volume of 25 μL, including 17.75 μL ddH$_2$O, 2.5 μL 10X PCR buffer (Mg^{2+}), 2 μL dNTPs, 0.25 μL *Taq* DNA polymerase, 1 μL both forward and reverse primers, and 0.5 μL DNA template. Reaction conditions were: amplification at 94°C for 10 min for early degeneration, followed by 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. For final electrophoretic

observation, 5 µL PCR product was applied to 2% agarose gel electrophoresis, and then photographs were acquired under an ultraviolet gel imaging system.

Statistical analysis

Data were analyzed using the SPSS16.0 software (SPSS, Inc.; Chicago, IL, USA); hierarchical clustering analysis was performed using the Cluster 3.0 and Treeview softwares. The nonparametric rank sum test (Mann-Whitney U test) was used for correlation analysis. The χ^2 test was employed to analyze HPV16 infection in CSCC and control groups. $P < 0.05$ was considered to be statistically significant.

RESULTS

P16 gene detection

The *P16* gene and β-globin were amplified by PCR using cervical samples from 20 patients with CSCC and 20 controls; the target fragment was 326 bp (Figure 1), indicating that the proper samples had been obtained. These samples were used for subsequent methylation tests.
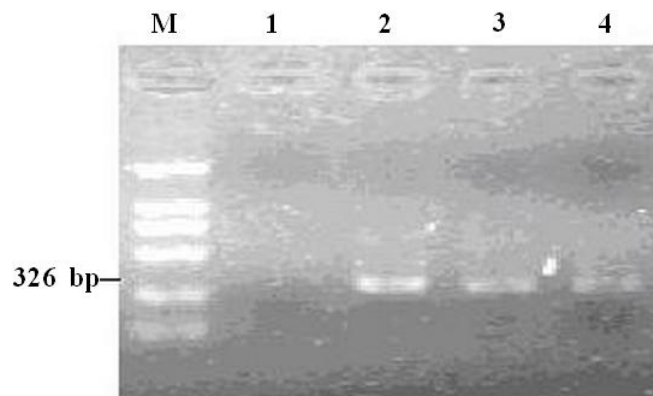

Figure 1. Electrophoresis of the *P16* gene following PCR. *Lane M* = DL 2000 DNA marker; *lane 1* = control; *lanes 2-4* = PCR-amplified bands.

Methylation level of each CpG site in the *P16* gene

Two-way clustering analysis was performed for mass spectrometric data acquired using the MassARRAY EpiTYPER mass spectrometer. This apparatus can accurately locate each CpG site in a gene fragment and reveal its specific methylation status. In this experiment, the promoter region of *P16* included 74 sites, 16 of which were studied according to the characteristics of primer design. A total of 40 samples were investigated in our study; the amplicon of each sample contained 16 CpG sites, which were divided into 12 CpG units after T cleavage. Each CpG unit included single or multiple CpG sites. There were 480 CpG units in all, among which 405 (84.4%) CpG units were analyzed. The CpG sites of *P16* showed varying

methylation levels. Two-way clustering analysis was used to determine the distribution trend of methylation for 12 CpG units from *P16* in the CSCC group (N = 20) and the control group (N = 20). A clustering analysis diagram (Figure 2) showed that the total methylation level was higher in the CSCC group than in the control group.
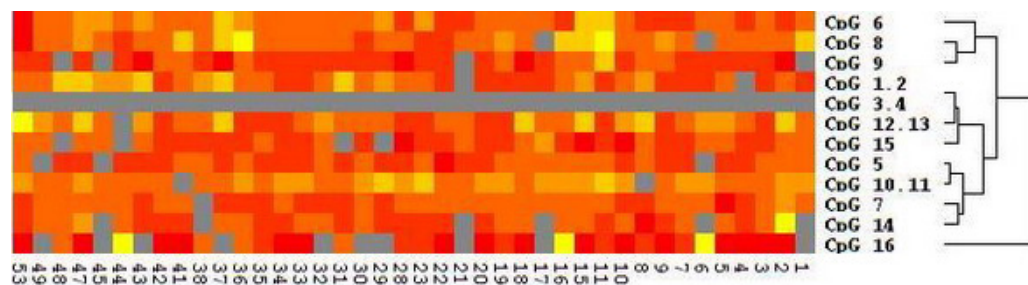
Figure 2. Cluster analysis of *P16* gene CpG methylation sites in 40 cervical tissues. The abscissa indicates each sample, *lanes 1-23* = non-cancerous tissues; *lanes 28-53* = CSCC tissues. The ordinate indicates each CpG site, while colors represent the methylation status of CpG islands. Light gray indicates CpG sites unanalyzed, and yellow and red indicate 100 and 0% methylation rates, respectively.

Comparison of the *P16* gene CpG methylation in cervical samples between groups

The methylation level of the CpG1-2 and CpG6 sites was higher in the CSCC group than in the control group (P < 0.05), and no statistical difference was observed at other sites (P > 0.05, Table 2).

Table 2. Comparison of the *P16* promoter methylation status of CpG islands between the cervical squamous cell carcinoma (CSCC) and control groups.

CPG unit	CpG site	CSCC group		Control group		P
		N	means ± SD	N	means ± SD	
Unit1	CpG1-2	20	0.1259 ± 0.0099	18	0.0772 ± 0.0083	0.001
Unit2	CPG3-4	-	-	-	-	-
Unit3	CPG5	18	0.0717 ± 0.0052	19	0.0758 ± 0.0072	0.501
Unit4	CpG6	20	0.1075 ± 0.0075	20	0.0910 ± 0.0093	0.029
Unit5	CpG7	19	0.0742 ± 0.0054	20	0.0870 ± 0.0067	0.121
Unit6	CpG8	20	0.1045 ± 0.0120	18	0.1289 ± 0.0141	0.250
Unit7	CpG9	18	0.0444 ± 0.0062	18	0.0667 ± 0.0091	0.062
Unit8	CpG10-11	19	0.1311 ± 0.0058	19	0.1389 ± 0.0066	0.344
Unit9	CpG12-13	19	0.1132 ± 0.0118	20	0.1060 ± 0.0096	0.822
Unit10	CpG14	18	0.0789 ± 0.0057	16	0.0850 ± 0.0133	0.794
Unit11	CpG15	16	0.0625 ± 0.0066	20	0.0650 ± 0.0079	0.860
Unit12	CpG16	13	0.1054 ± 0.0705	17	0.0924 ± 0.0587	0.710

Comparison of HPV16 infection between groups

The HPV16-positive rate was higher in the CSCC group than in the control group (χ^2 = 10.101, P < 0.05, Table 3). Results of HPV infection inspection in both groups are shown in Table 3 and Figure 3.

Table 3. Determination of HPV infection in both groups.

	HPV-positive	HPV-negative	Sum	HPV-positive rate (%)
CSCC group	16	4	20	80
Control group	6	14	20	30
Sum	22	18	40	55
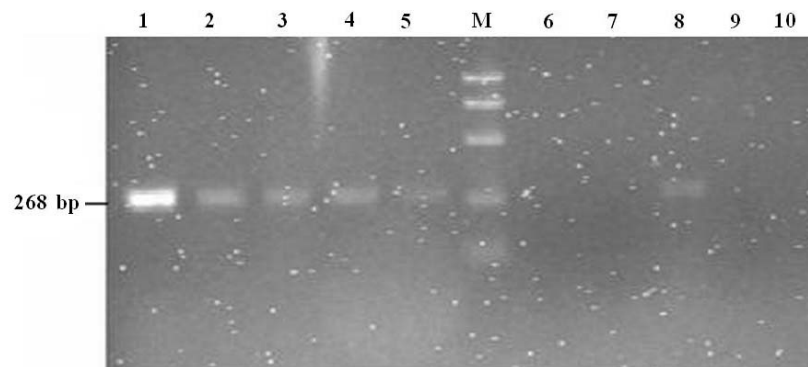
Figure 3. Electrophoresis of HPV16 PCR products. *Lane M* = DNA ladder; *lanes 1-5* = CSCC group; *lanes 6-10* = control group.

Correlation analysis between *P16* gene CpG methylation sites and HPV16 infection in the CSCC group

Correlation analysis revealed no significant correlation between methylation of CpG sites in the promoter region of *P16* and HPV16 infection status (+/-) in Uyghur patients with CSCC ($P > 0.05$, Table 4).

Table 4. Relationship between *P16* gene CpG methylation sites and HPV16 infection in the CSCC group.

CpG sites	HPV (+)		HPV (-)		Wiloconxon *W*	P
	N	Means ± SD	N	Means ± SD		
CpG1-2	16	0.1213 ± 0.0114	4	0.1400 ± 0.0212	162.0	0.569
CpG5	15	0.068 ± 0.0055	3	0.0900 ± 0.0116	129.0	0.104
CpG6	16	0.1025 ± 0.0081	4	0.1275 ± 0.0165	156.5	0.269
CpG7	15	0.0747 ± 0.0066	4	0.0725 ± 0.0075	37.5	0.798
CpG8	16	0.1025 ± 0.0141	4	0.1125 ± 0.0236	164.0	0.701
CpG9	15	0.0460 ± 0.0071	3	0.0367 ± 0.0120	22.0	0.435
CpG10-11	15	0.1333 ± 0.0667	4	0.1255 ± 0.0125	29.5	0.284
CpG12-13	15	0.1067 ± 0.1376	4	0.1375 ± 0.0202	137.0	0.192
CpG14	15	0.0760 ± 0.0065	3	0.0933 ± 0.0067	129.0	0.103
CpG15	12	0.0592 ± 0.0080	4	0.0725 ± 0.1109	94.5	0.360
CpG16	13	0.1054 ± 0.0705	0	-	-	-

No results were obtained after analyzing methylation test of 16 CpG sites in 4 HPV-negative samples.

DISCUSSION

DNA methylation is a type of epigenetic modification characterized by the addition of a methyl group to the C5 position of the cytosine in the 5'-CpG-3' dinucleotides through

the action of DNA methyltransferase. Methylated CpG dinucleotides can inhibit the expression of certain genes by gathering transcription inhibitors or by blocking the combination of activating transcription factors (Kim et al., 2003). In the human genome, non-methylated CpG sites are not evenly distributed. By contrast, they generally accumulate locally, forming some regions with high GC and CpG dinucleotide content, such as the CpG island, which plays important roles in maintaining normal cellular functions, genetic imprinting, embryogenesis, and human tumorigenesis (Rodenhiser and Mann, 2006). However, approximately 70-80% of total CpG dinucleotides are methylated. Park et al. (2007) studied 13 genes from 8 types of human malignant tumors and found that abnormal methylation of some CpG islands is commonly observed in many histopathological types of human malignant tumors, and each histopathological type corresponds to one specific methylation pattern. For instance, in multiple myeloma tissue, DNA methylation regulated a relatively low number of genes (Jung et al., 2012), whereas research on cervical diseases has indicated that the methylation frequency of some genes may increase with increasing cervical disease grade, and that the number of methylated genes may also elevate with increasing cervical intraepithelial neoplasias grade (Henken et al., 2007; Shivapurkar et al., 2007; Lai et al., 2010; Lim et al., 2010). Thus, because of the frequency and severity of CSCC in the Uyghur population, we propose using CpG methylation as an early indicator of CSCC.

The *P16* gene, located on chromosome 9p21, is a cancer suppressor gene that directly affects the cell cycle. It is 8.5 kb long and includes 3 exons (1, 2, 3) and 2 introns. Abnormal methylation of the *P16* gene is involved in the occurrence and development of many types of human tumors (Rocco and Sidransky, 2001). Lee et al. (2012) discovered that the *P16* gene methylation is related to the histological grade of invasive breast cancer and the condition of estrogen and progestin receptors. Wang et al. (2013) considered *P16* gene methylation to be very important, as it is closely associated with the metastatic and histological features of papillary thyroid carcinoma. A previous study by Huang et al. (2011) demonstrated that abnormal methylation of the *P16* gene promoter is an early event of cervical cancer, which may promote the progression from precancerosis to cancer. In this study, we used MALDI-TOF MS to examine multiple CpG sites and obtain methylation data for specific methylation sites. The advantages of this technique include its high accuracy, high sensitivity, high analysis speed, wide measuring range, high resolution, and small sample volume, which helps in performing high throughput DNA methylation measurements. Using this technique, we examined 40 cases of CSCC and observed varying levels of methylation of the CpG locus in the *P16* gene. The methylation rate of the CpG1-2 and CpG6 loci in the promoter region of *P16* in the CSCC group of Uyghur patients was higher than that in the control group, and CpG1-2 and CpG6 were considered to be different sites in Uyghur CSCC. Our results not only revealed abnormal methylation in the promoter region of the *P16* gene in Uyghur patients with CSCC, but also demonstrated that methylation differences among loci are closely related to the occurence of CSCC. Therefore, abnormal methylation in the promoter region of the *P16* gene may be an effective indicator of CSCC (Bolanca et al., 2010; Huang et al., 2011) and provides a basis for targeted therapy against *P16* methylation.

HPV infection is the primary pathogenic factor of cervical cancer; of the high-risk HPV types, E6 and E7 have been shown to function as oncoproteins by inhibiting the activity of p53 and Rb (Dyson et al., 1989; Scheffner et al., 1990). The methylation level in cervical cancer correlates with HPV16 infection in cervical cells (Patel et al., 2012), and HPV16 virus integration

is related to the development of cervical carcinogenesis (Mazumder et al., 2011). In our previous study, the HPV-positive rate reached up to 73.56% in cervical intraepithelial neoplasias and cervical cancer; a study examining the cervical cancer spectrum in Xinjiang Uyghur women demonstrated that HPV16 accounted for the highest proportion in Uyghur cervical cancer. This study showed that the detectable rate of HPV16 in the CSCC group was higher than in the control group, further indicating the high infection rate of HPV16 in Xinjiang Uyghur CSCC patients.

This study also demonstrated that in the CSCC group, the methylation of 16 loci in the *P16* gene was not related to HPV16 infection. This not consistent with the results of Ishikawa et al. (2006), which may result from differences in experimental methods. The Ishikawa study used methylation-specific PCR, through which only methylation could be detected, while in our study, MALDI-TOF MS was used to simultaneously and quantitatively measure multiple sites over a wider range of comparison. In this study, the methylation of 16 of 74 sites within the promoter region of the *P16* gene was studied, and therefore further studies are required to determine whether additional methylation sites are associated with HPV16.

In conclusion, our study demonstrated that the *P16* gene CpG1-2 and CpG6 hypermethylation and HPV16 infection, which are independent of each other, play an important role in cervical squamous cell carcinogenesis in Uyghur patients. It has been suggested that HPV16 methylation is correlated with the degree of cervical carcinogenesis (Ding et al., 2009). Additional studies are needed to resolve this issue.

ACKNOWLEDGMENTS

REFERENCES

Bolanca IK, Sentija K, Simon SK, Kukura V, et al. (2010). Estimating clinical outcome of HPV induced cervical lesions by combination of capsid protein L1 and p16INK4a protein detection. *Coll. Antropol.* 34: 31-36.

Ding DC, Chiang MH, Lai HC, Hsiung CA, et al. (2009). Methylation of the long control region of HPV16 is related to the severity of cervical neoplasia. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 147: 215-220.

Dyson N, Howley PM, Munger K and Harlow E (1989). The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* 243: 934-937.

Henken FE, Wilting SM, Overmeer RM, van Rietschoten JG, et al. (2007). Sequential gene promoter methylation during HPV-induced cervical carcinogenesis. *Br. J. Cancer* 97: 1457-1464.

Huang LW, Pan HS, Lin YH, Seow KM, et al. (2011). P16 methylation is an early event in cervical carcinogenesis. *Int. J. Gynecol. Cancer* 21: 452-456.

Ishikawa M, Fujii T, Saito M, Nindl I, et al. (2006). Overexpression of p16 INK4a as an indicator for human papillomavirus oncogenic activity in cervical squamous neoplasia. *Int. J. Gynecol. Cancer* 16: 347-353.

Jung S, Kim S, Gale M, Cherni I, et al. (2012). DNA methylation in multiple myeloma is weakly associated with gene transcription. *PLoS One* 7: e52626.

Kim J, Kollhoff A, Bergmann A and Stubbs L (2003). Methylation-sensitive binding of transcription factor YY1 to an insulator sequence within the paternally expressed imprinted gene, Peg3. *Hum. Mol. Genet.* 12: 233-245.

Lai HC, Lin YW, Huang RL, Chung MT, et al. (2010). Quantitative DNA methylation analysis detects cervical intraepithelial neoplasms type 3 and worse. *Cancer* 116: 4266-4274.

Lee JJ, Ko E, Cho J, Park HY, et al. (2012). Methylation and immunoexpression of p16(INK4a) tumor suppressor gene in primary breast cancer tissue and their quantitative p16(INK4a) hypermethylation in plasma by real-time PCR. *Korean J. Pathol.* 46: 554-561.

Lim EH, Ng SL, Li JL, Chang AR, et al. (2010). Cervical dysplasia: assessing methylation status (Methylight) of CCNA1, DAPK1, HS3ST2, PAX1 and TFPI2 to improve diagnostic accuracy. *Gynecol. Oncol.* 119: 225-231.

Mazumder ID, Singh RK, Mitra S, Dutta S, et al. (2011). Genetic and epigenetic changes of HPV16 in cervical cancer differentially regulate E6/E7 expression and associate with disease progression. *Gynecol. Oncol.* 123: 597-604.

Park SY, Kim BH, Kim JH, Cho NY, et al. (2007). Methylation profiles of CpG island loci in major types of human cancers. *J. Korean Med. Sci.* 22: 311-317.

Patel DA, Rozek LS, Colacino JA, Van Zomeren-Dohm A, et al. (2012). Patterns of cellular and HPV 16 methylation as biomarkers for cervical neoplasia. *J. Virol. Methods* 184: 84-92.

Rocco JW and Sidransky D (2001). p16(MTS-1/CDKN2/INK4a) in cancer progression. *Exp. Cell Res.* 264: 42-55.

Rodenhiser D and Mann M (2006). Epigenetics and human disease: translating basic biology into clinical applications. *CMAJ* 174: 341-348.

Scheffner M, Werness BA, Huibregtse JM, Levine AJ, et al. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* 63: 1129-1136.

Shivapurkar N, Sherman ME, Stastny V, Echebiri C, et al. (2007). Evaluation of candidate methylation markers to detect cervical neoplasia. *Gynecol. Oncol.* 107: 549-553.

Ushijima T (2005). Detection and interpretation of altered methylation patterns in cancer cells. *Nat. Rev. Cancer* 5: 223-231.

Wang P, Pei R, Lu Z, Rao X, et al. (2013). Methylation of p16 CpG islands correlated with metastasis and aggressiveness in papillary thyroid carcinoma. *J. Chin. Med. Assoc.* 76: 135-139.

zur Hausen H (2000). Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. *J. Natl. Cancer Inst.* 92: 690-698.