Original Article
Correlation between high-risk human papillomavirus (HR-HPV) infection, DNA methyltransferase 1 (DNMT1) expression, and death-associated protein kinase (*DAPK*) methylation status in cervical lesions

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Abstract: The aim of this study was to investigate the correlation between high-risk human papillomavirus (HR-HPV) infection, expression of DNA methyltransferase 1 (DNMT1) protein, and methylation of death-associated protein kinase (DAPK) promoter in the development of cervical lesions. We included 175 patients at various stages of cervical lesions in our study. HR-HPV infection was investigated using surface Plasmon resonance method, immunocytochemistry assay was used to detect the expression of DNMT1 and the methylation status of DAPK was determined using methylation-specific polymerase chain reaction (PCR). The positive expression rates of DNMT1 protein in normal, cervical intraepithelial neoplasia (CIN)1, CIN2, CIN3, and squamous cell carcinoma (SCC) group were 5.71%, 57.14%, 62.86%, and 76.67%, respectively. The positive expression rates of DAPK methylation in normal, CIN1, CIN2, CIN3, and SCC group were 2.86%, 11.43%, 14.29%, 45.71%, and 60.00%, respectively. In terms of association with the lesion progression, DNMT1 protein expression and DAPK methylation demonstrated an increasing trend (*P*<0.05). During the development of cervical lesions, high-risk HPV, DNMT1 protein expression, and DAPK gene methylation were positively correlated with each other (*P*<0.05). DAPK methylation may be completed under the action of DNMT1, which might play a synergistic role with HR-HPV persistent infection in cervical cancerization.

Keywords: Cervical cancer, cervical exfoliated cells, DNA methyltransferase 1, death-associated protein kinase, methylation, human papillomavirus

Introduction

Squamous cell carcinoma of the uterine cervix remains a leading cause of morbidity and mortality worldwide, with more than 85% of the cases occurring in developing countries [1]. Infection with high risk human papillomavirus (HR-HPV) is necessary for the development of cervical cancerization. HPV infection is common, especially in young women, and frequently shows a transient course [2]. Following the HR-HPV infection, cervical cancer develops through pre-cancerous lesions (cervical intraepithelial neoplasia graded 1 to 3 (CIN1-3) and finally, progresses to invasive cervical cancer. The molecular mechanisms involved in the progression of HPV infections to cervical cancer are poorly understood.

Epigenetic changes, including histone modifications, nucleosome occupancy and positioning, protein and non-coding RNA interactions, as well as direct DNA modifications, are important mechanisms for the development of tumors [3]. In cervical lesions, DNA methylation has gained the most attention. DNA methylation is catalyzed by DNA methyltransferases (DNMTs), which catalyze the covalent addition of methyl groups to cytosines in the CpG dinucleotide. The DNMT1, known as the "maintenance methyltransferase" is mostly used by cells to maintain a stable DNA methylation status through cell division. Aberrant expression of DNMT1 can lead to abnormal DNA methylation, especially hypermethylation of tumor suppressor genes leading to the silencing of tumor suppressor genes, contributing to uncontrolled cell growth

[4]. Abnormal methylation of promoters of tumor suppressor genes is common in cervical cancer, and the analysis of DNA methylation as a prognostic biomarker is under intense investigation [5]. Death-associated protein kinase (DAPK) is a protein kinase associated with apoptosis, forward-induced apoptosis. Recent studies have shown that methylation of *DAPK* is a potential biomarker for the identification of premalignant lesions that have a high risk to evolve into invasive cervical cancer [6]. However, the direct relationship between methylation of *DAPK* and DNMT1 in cervical cancerization has not been described before.

To evaluate the potential role of HR-HPV, DNMT1 and methylation of DAPK in the progression of CIN to cervical cancer, our study tested the expression of DNMT1 in a range of cervical exfoliated cells obtained from normal, CIN1-3 stage and diagnosed cervical cancer patients. Our findings shed light on the probable mechanism of *DAPK* methylation induced by abnormal expression of DNMT1, which may provide a new insight to block CIN progress to cervical cancer.

Methods

Subjects

Cervical exfoliated cells were obtained from 175 women with a median age of 32 years (range 30-50) through colposcopic examinations between January and December of the year 2013. According to the results of cervical biopsy pathologic diagnosis was categorized into four study groups, each with 35 patients namely CIN1-3 and squamous cell carcinoma (SCC); the control group of 35 cases was negative for intraepithelial lesion or malignancy. According to the 2009 International Federation of gynecology and Obstetrics (FIGO) stage standard: 21 patients with cervical squamous cell carcinoma were categorized as Ia stage, 6 patients as Ib stage, and 8 patients as IIa stage. According to the pathological classification: 10 patients demonstrated high differentiation, 13 patients demonstrated middle differentiation, and 2 patients exhibited low differentiation. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Shanxi Medical University Second Hospital. Written informed consent was obtained from all participants.

All participants were Han Chinese living in Shanxi for more than five years and were confirmed by two independent pathological examinations. No patient had received radiotherapy or chemotherapy before. After obtaining written informed consent from all participants, cervical exfoliated cells were stored in the Thinprep cytologic test (TCT) cell preservation solution at room temperature, until further analysis.

Surface Plasmon resonance (SPR)

Genomic DNA was extracted using Genomic DNA Fast Extract kit (Bodhisattva Ka Beijing Medical Technology Co. Ltd., Beijing City, China) form the remaining TCT cell preservation solution, and measured at 260 nm, 280 nm absorbance (OD value) by a UV spectrophotometer. The OD260/OD280 (R) reflects the concentration levels of DNA i.e. if R<1.8, the solution contains organic contamination; 1.8<R<2.0, the DNA quality is better; R>2.2, DNA has hydrolyzed to single nucleotides in the solution. In this study, the extracted DNA was detected by UV spectrophotometer, R-values between 1.8 to 2.0 indicated that the DNA had a high-level purity and little protein contamination. Qualified DNA solution was detected for HPV-DNA using HPV genotyping kit (Beijing gold Bodhisattva Jia Medical Technology Co., Beijing City, China); the test results were interpreted using the biosensor chip reading device.

Immunocytochemistry

The TCT remaining cell preservation solution was used to make a thin smear using Thinprep2000 automated cell preparation instrument. Microscope identification was done to select the satisfactory smears with as recommended by the TBS diagnostic system (revised in 2001). This system was modified by using the Streptavidin/Peroxidase series kit (Zymed Laboratories, South San Francisco, CA, USA); diaminobenzidine chromogenic, instead of primary antibody in PBS buffer was used as a negative control. The positive expression of DNMT1 protein was in the nucleus, and a small part of the cell cytoplasm. The DNMT1 protein can be dyed tan or yellow granular. As a secondary scoring method for the criteria of immunohistochemistry results, we randomly selected five regions with high magnification on each cell slide and observed for the number of positive cells. The criteria for evaluation of positive
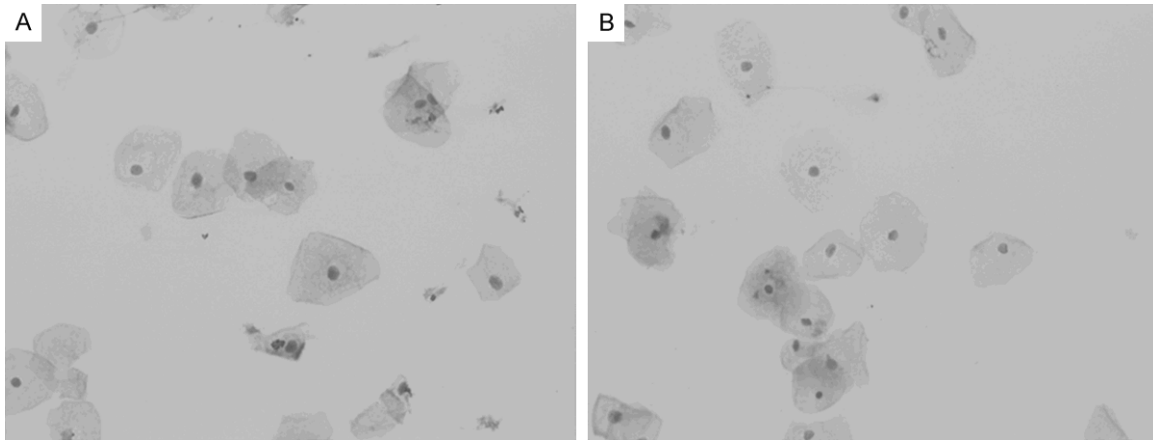
Figure 1. The expression of DNMT1 protein. A: Positive expression of DNMT1 protein ×400; B: Negative expression of DNMT1 protein ×400.

cells was as follows: zero point for positive cells ≤5%; one point for 6% to 25% positive cells; two points for 26% to 50% positive cells; three points for 51% to 75% positive cells and four points for positive cells >75%. The staining intensity grading scores ranged as follows: zero points for colorless; one point for pale yellow; two points for yellow or dark yellow and three points for brown or tan. Positive results were interpreted if the product of two points on a slide was ≥1.

Methylation specific PCR (MSP)

The qualified DNA was used to make the bisulfite modification using methylation specific PCR kit (Beijing days grace Gene Technology Co., Beijing City, China). The primer was amplified by ready-to-use PCR Kit 2.0 (days grace gene Technology Co., Beijing City, China), and the PCR products was analyzed by electrophoresis through SDS-PAGE gel preparation kit (Beijing Soledad Technology Co., Beijing City, China). Methylation criteria was classified as: permethylation, if the methylated primers amplified target fragment and unmethylated primers did not amplify the objective fragment; partially methylated (hemimethylation), if the target bands of methylated primers and unmethylated primers were amplified and non-methylation, if the unmethylated primers amplified the target band and methylated primers did not amplify.

Statistical analysis

Statistical analyses were conducted using the SPSS 13.0 software package (SPSS Inc., IL, USA). The Chi-square test and trend Chi-square test were used to describe a correlation between HR-HPV, DNMT1, *DAPK* methylation and clinical features. Pearson correlation analysis was used to describe a correlation between HR-HPV, DNMT1 and *DAPK* methylation. $P< 0.05$ was used as the criterion for statistical significance.

Results

High-risk HPV infection

175 cases of cervical lesions in cervical exfoliated cells in each phase of high-risk HPV infection were analyzed for HPV-DNA. The high-risk type HPV positive rate was 81.14%, the differences among these groups was statistically significant ($\chi^2=35.224$, $P<0.001$). In terms of the lesion progression, HPV infection rate is also showed an increasing trend (χ^2 trend $=30.658$, $P<0.05$).

DNMT1 expression

The expression of DNMT1 protein in this experiment was 53.71%. In various types of cervical lesions the positive expression rates of DNMT1 protein have significant difference ($\chi^2=36.339$, $P<0.05$). The DNMT1 protein expression rate showed increasing trend with the extent of the lesion progression, (χ^2 trend $=26.993$, $P<0.05$) (**Figure 1**).

DAPK methylation

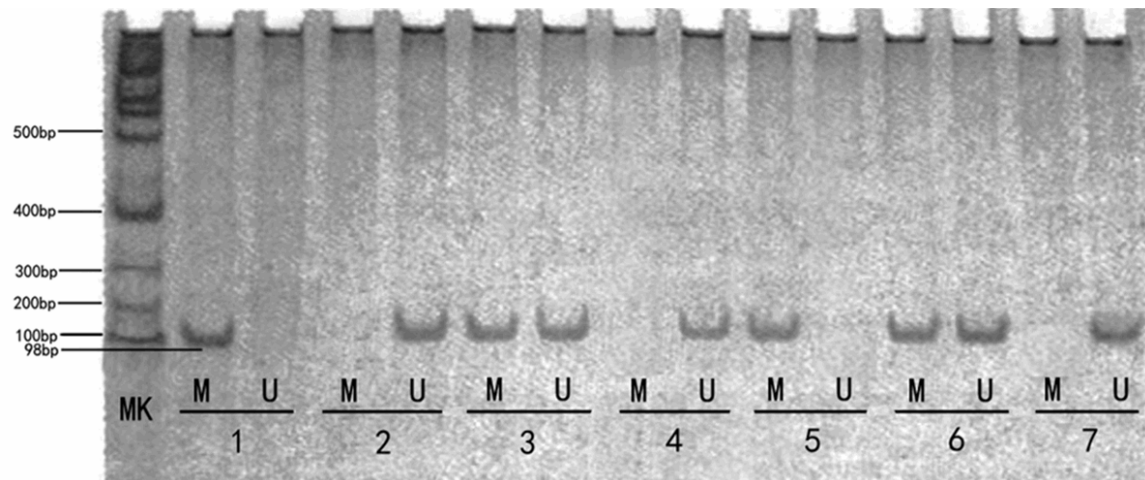The methylation rate of the promoter region of *DAPK* gene in CpG island was 26.86%, which

Figure 2. DAPK methylation in cervical exfoliated cells. 1, 2 for SCC group; 3, 4 for CIN3 group; 5 for CIN2 group; 6 for CIN1 group; 7 for normal control group; M for methylation, U for non-methylation.

Table 1. The consequences of high-risk HPV infection, DNMT1 expression and DAPK methylation in cervical exfoliated cells

	Total number	HR-HPV-positive n (%)	P-value	DNMT1-positive n (%)	P-value	DAPK-positive n (%)	P-value
Normal control	35	16 (45.71)	<0.05	2 (5.71)	<0.05	1 (2.86)	<0.05
CIN1	35	26 (74.28)		20 (57.14)		4 (11.43)	
CIN2	35	32 (91.43)		22 (62.86)		5 (14.29)	
CIN3	35	33 (94.29)		23 (65.71)		16 (45.71)	
SCC	35	35 (100.00)		27 (77.14)		21 (60.00)	

correlated with the extent of progression of the lesions. The *DAPK* gene methylation rates were 3.33%, 10.00%, 13.33%, 46.67% and 60.00%. There was significant difference among the groups (χ^2=38.523, *P*<0.05), and showed an increasing trend (χ^2 trend =34.278, *P*<0.05) (**Figure 2**; **Table 1**).

Correlation analysis

Through Pearson correlation analysis, HR-HPV infection was related to DNMT1 protein expression and *DAPK* gene methylation in the progression of cervical lesions (r=0.340, 0.291, *P*<0.01), the expression of DNMT1 protein and the methylation of *DAPK* gene was also associated (r=0.291, *P*<0.001).

Discussion

Cervical cancer is the most common gynecological cancer, 85% of all the new cases per year present in the developing countries. Cervical cancer incidence and mortality in China is accounted for one third of all the cases. Numerous studies show almost all of CIN and cervical cancer patients (more than 96%) with high-risk HPV infection [6]. For cervical cancer, cervical lesions secondary to HPV infection is the main factor. Among other factors, sexually active individuals, smoking, early start of sex life, sexually transmitted diseases and other related factors synergies progressive disease process. The development stages for cervical cancer include CIN1, CIN2, CIN3 and carcinoma in situ, invasive cervical cancer stages. The CIN has two different endings, only some of the lesions in the high-risk HPV long-term and persistent infection develop advanced lesions and invasive cancer. However, the molecular mechanisms involved in the progression of low-grade CIN to cervical cancer are yet poorly understood. Hence exploring the synergistic factors of HR-HPV is vital for blocking the progression of CIN to cervical cancer.

The genotyping of HPV-DNA has revealed more than 120 kinds of this pathogen. According to the size of its virulence, they are divided into two kinds namely high-risk and low-risk. Low-risk type mainly causes exogenous wart lesions,

the flat warts class lesions and CIN1 at genital tract, peri anal skin, and the lower part of the vagina. These generally do not progress to cancer. The high-risk types can lead to CIN2, CIN3 lesions and cervical cancer [7]. In this study, plasma resonance technology could detect 16 HR-HPV type (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 81), the positive rates of infection were 45.71% in the normal control group, 74.28% in the CIN1 group, 91.43% in the CIN2 group, 94.29% in the CIN3 group, and 100.0% in the SCC group. The HR-HPV infection positive rate gradually increased with the progress of cervical lesions, and they are most similar to the results reported in the literature [6]. Nevertheless, the ratio of cervical lesions developing into cancer is much lower than HPV infection rate in the population, and the majority of HPV-positive patients will be negative in about one year, meaning that in addition to HPV infection, there are other synergistic mechanisms that play an important role in the occurrence and progression of cervical lesions.

Current epigenetics research has mainly concentrated on the aberrant methylation of the tumor suppressor gene promoter CpG island. As the DNMT causes abnormally high methylation of the CpG island at the 5' end of tumor suppressor gene, it causes a change in the spatial structure of the chromatin leading to structural alterations and silencing of the expression of tumor suppressor genes resulting in tumorigenesis [8]. Epigenetic changes are the key events in the early tumor formation [9]. As the core content of epigenetics, DNA methylation affects the gene expression pattern and plays an important role in tumor development, closely associated with silencing of tumor suppressor gene transcription [10]. Especially in recent years, several methylation inhibitor compounds have been approved by the FDA for the treatment of certain cancers and show promise as effective anticancer drugs in the future [11]. Currently, the change in DNA methylation patterns has become the focus of attention in the process of cancer development. DNA methylation is a type of DNA modification generally associated with transcriptional silencing, including the global DNA hypomethylation in non-promoter regions and the hypermethylation of CpG islands in the promoter regions. Where the former cause the activation of proto-oncogene and the latter

cause the silence of tumor-suppressor gene transcription [12]. The core of the present study focused on the promoter regions of CpG island methylation leading to tumor suppressor gene transcription silencing. Accumulating evidence suggests that alterations of DNA methylation are involved even in the early and precancerous stages [13].

Apoptosis is genetically controlled programmed cell deaths. Under normal circumstances, the proliferation and apoptosis of cells are in dynamic equilibrium. Once the balance is disturbed, the cells show infinite proliferation state, which may lead to cancer. Apoptosis related protein kinase, DAPK is a calcium/calmodulin-dependent serine/threonine kinase and has an important and regulatory role in many signaling pathways [14]. The *DAPK* located in the chromosome 9q34.1 is a calcium/calmodulin dependent kinase. As a kind of apoptosis positive phase adjustment factor, *DAPK* participates in multiple apoptosis pathways including the p19ARF/p53, Fas, TNF-α and γ-INF induced pathways [15], and maintained the steady state of dynamic balance between the proliferation and apoptosis of tissue cells [16]. Promoter methylation of the tumor suppressor gene, *DAPK*, has been reported for several cancers including cervical cancer [17]. However, a link with HR-HPV persistence has not been reported previously but there is a plausible mechanism for a causal relationship. Our study found that the methylation positive rate of *DAPK* gene promoter in cervical cells was 2.86% in the normal control group; 11.43% in the CIN1 group; 14.29% in CIN2 group; 45.71% in CIN3 group and 60.00% in the SCC group. With the development of cervical lesions, the *DAPK* methylation rate has shown an increasing trend. Additionally, *DAPK* gene methylation and HR-HPV infection were positively correlated in the process of cervical cancerization (r=0.291, *P*<0.01), suggesting that hypermethylation of DAPK gene promoter region may cause synergistic effect with HR-HPV persistent infection in the process of cervical cancerization.

Accumulating studies have shown that the promoter methylation of the tumor suppressor gene is closely related with the activity of DNMTs [18]. The DNMT1 is the major enzyme responsible for maintenance of the DNA methylation pattern and often referred to as mainte-

nance methyltransferase. DNMT1 is composed of 1616 amino acids, the relative molecular weight of its protein is 190000, its gene is positioned at 19q13.3~p13.2 and the full length of its cDNA is 5434 bp. It is the main type of mammalian DNMT, and is highly expressed in proliferating cells [19]. It is generally believed that DNMT1 gene expression increased in cancer cells prior to the change of the high methylation suppressor gene, suggesting the increasing activities of DNMT1 is a characteristic early molecular event from precancerous conditions to malignant progression [13]. Sawada et al. [20] has found that the DNMT1 protein expression score was increased even in low-grade CIN ($P<0.0001$) in comparison with histologically normal squamous epithelium. It was further increased in higher-grade CIN ($P<0.0001$ compared to low-grade CIN), then the DNMT1 protein expression score remained at a plateau in microinvasive carcinoma (Stage IA, $P=0.0690$ compared to higher-grade CIN) and then decreased with cancer invasion (Stage IB or more, $P=0.0176$ compared to Stage IA). Au Yeung et al. [21] further examined the functional role of DNMT1 in HPV-16 E6 associated cervical cancer development and found that knockdown of E6 in HPV-16 positive human cervical carcinoma SiHa and CaSki cells led to the increase in p53, repression of DNMT1 protein and promoter activity, suggesting that HPV-16 E6 may act through p53/DNMT1 to regulate the development of cervical cancer. Consistent with the domestic and foreign reports, our study found that in cervical cells, DNMT1 positive expression rate were 5.71% for the normal control group; 57.14% for CIN1 group; 62.86% for CIN2 group; 65.71% for CIN3 group; 77.14% for the SCC group, and with the progress of cervical lesions, the rate of DNMT1 positive expression is gradually increased. However, whether the increasing activity of DNMT1 participated in the methylation of DAPK and a link with HR-HPV persistence has not been reported previously. In our study, we found the DNMT1 expression; *DAPK* gene methylation and HR-HPV persistent infection were positively correlated with each other, suggesting *DAPK* methylation may be completed under the action of DNMT1, which may play a synergistic effect with HR-HPV persistent infection in the cervical cancerization.

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Disclosure of conflict of interest

None.

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References

[1] Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010; 127: 2893-2917.

[2] Couture MC, Page K, Stein ES, Sansothy N, Sichan K, Kaldor J, Evans JL, Maher L and Palefsky J. Cervical human papillomavirus infection among young women engaged in sex work in Phnom Penh, Cambodia: prevalence, genotypes, risk factors and association with HIV infection. BMC Infect Dis 2012; 12: 166.

[3] Shen H and Laird PW. Interplay between the cancer genome and epigenome. Cell 2013; 153: 38-55.

[4] Bashtrykov P, Jankevicius G, Smarandache A, Jurkowska RZ, Ragozin S and Jeltsch A. Specificity of Dnmt1 for methylation of hemimethylated CpG sites resides in its catalytic domain. Chem Biol 2012; 19: 572-578.

[5] Tornesello ML, Buonaguro L, Giorgi-Rossi P and Buonaguro FM. Viral and cellular biomarkers in the diagnosis of cervical intraepithelial neoplasia and cancer. Biomed Res Int 2013; 2013: 519619.

[6] Kalantari M, Osann K, Calleja-Macias IE, Kim S, Yan B, Jordan S, Chase DM, Tewari KS and Bernard HU. Methylation of human papillomavirus 16, 18, 31, and 45 L2 and L1 genes and the cellular DAPK gene: Considerations for use as biomarkers of the progression of cervical neoplasia. Virology 2014; 448: 314-321.

[7] McGraw SL and Ferrante JM. Update on prevention and screening of cervical cancer. World J Clin Oncol 2014; 5: 744-752.

[8] Fang J, Zhang H and Jin S. Epigenetics and cervical cancer: from pathogenesis to therapy. Tumour Biol 2014; 35: 5083-5093.

[9] Zoratto F, Rossi L, Verrico M, Papa A, Basso E, Zullo A, Tomao L, Romiti A, Lo Russo G and Tomao S. Focus on genetic and epigenetic events of colorectal cancer pathogenesis: implications for molecular diagnosis. Tumour Biol 2014; 35: 6195-6206.

[10] Nazarian R and Jazirehi AR. Epigenomics and targeted therapy in cancer. Epigenomics 2014; 6: 571-575.

[11] Heerboth S, Lapinska K, Snyder N, Leary M, Rollinson S and Sarkar S. Use of epigenetic drugs in disease: an overview. Genet Epigenet 2014; 6: 9-19.

[12] Schubeler D. Function and information content of DNA methylation. Nature 2015; 517: 321-326.

[13] Kanai Y and Hirohashi S. Alterations of DNA methylation associated with abnormalities of DNA methyltransferases in human cancers during transition from a precancerous to a malignant state. Carcinogenesis 2007; 28: 2434-2442.

[14] Bovellan M, Fritzsche M, Stevens C and Charras G. Death-associated protein kinase (DAPK) and signal transduction: blebbing in programmed cell death. FEBS J 2010; 277: 58-65.

[15] Zhang W, Stabile LP, Keohavong P, Romkes M, Grandis JR, Traynor AM and Siegfried JM. Mutation and polymorphism in the EGFR-TK domain associated with lung cancer. J Thorac Oncol 2006; 1: 635-647.

[16] Benderska N, Ivanovska J, Rau TT, Schulze-Luehrmann J, Mohan S, Chakilam S, Gandesiri M, Ziesche E, Fischer T, Söder S, Agaimy A, Distel L, Sticht H, Mahadevan V and Schneider-Stock R. DAPK-HSF1 interaction as a positive-feedback mechanism stimulating TNF-induced apoptosis in colorectal cancer cells. J Cell Sci 2014; 127: 5273-5287.

[17] Flatley JE, Sargent A, Kitchener HC, Russell JM and Powers HJ. Tumour suppressor gene methylation and cervical cell folate concentration are determinants of high-risk human papillomavirus persistence: a nested case control study. BMC Cancer 2014; 14: 803.

[18] Subramaniam D, Thombre R, Dhar A and Anant S. DNA methyltransferases: a novel target for prevention and therapy. Front Oncol 2014; 4: 80.

[19] Peng DF, Kanai Y, Sawada M, Ushijima S, Hiraoka N, Kitazawa S and Hirohashi S. DNA methylation of multiple tumor-related genes in association with overexpression of DNA methyltransferase 1 (DNMT1) during multistage carcinogenesis of the pancreas. Carcinogenesis 2006; 27: 1160-1168.

[20] Sawada M, Kanai Y, Arai E, Ushijima S, Ojima H and Hirohashi S. Increased expression of DNA methyltransferase 1 (DNMT1) protein in uterine cervix squamous cell carcinoma and its precursor lesion. Cancer Lett 2007; 251: 211-219.

[21] Au Yeung CL, Tsang WP, Tsang TY, Co NN, Yau PL and Kwok TT. HPV-16 E6 upregulation of DNMT1 through repression of tumor suppressor p53. Oncol Rep 2010; 24: 1599-1604.