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Hypermethylation of two consecutive tumor suppressor genes, *BLU* and *RASSF1A*, located at 3p21.3 in cervical neoplasias

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Abstract

Objectives. Although initiated by human papillomavirus (HPV), cervical carcinogenesis demands other cofactors to shape its natural course. Epigenetic effects such as DNA methylation, are considered to contribute to carcinogenesis process.

Methods. The methylation status of *BLU* and *RASSF1A*, as well as the HPV infection status, were assessed in a full spectrum of cervical neoplasia, including 45 low-grade squamous intraepithelial lesions (LSIL), 63 high-grade squamous intraepithelial lesions (HSIL), 107 squamous cell carcinomas (SCC), 23 adenocarcinomas (AC), and 44 normal control tissues.

Results. The *BLU* was methylated in 76.9% of SCC, 57.4% of HSIL, 20.0% of LSIL and 12.5% of normal tissues ($P<0.001$). The *RASSF1A* was methylated in 15% of SCC, 17.5% of HSIL, but not in LSIL or normal tissues ($P<0.001$). In AC, 43.5% of patients showed *BLU* methylation and 26.1% *RASSF1A* methylation, significantly higher than the corresponding control frequencies of 12.5% ($P=0.005$) and 0% ($P=0.001$), respectively. There was an insignificant trend toward loss of *BLU* methylation with advancing clinical stages of SCC (84.8%, 67.7%, and 63.6% in stages I, II, and III/IV, respectively; $P=0.08$). Patients with LSIL infected with high-risk HPV showed a higher rate of *BLU* methylation than those without HPV (38.8% vs 9.1%, respectively; $P=0.057$). The methylation of *RASSF1A* was inversely related to HPV infection in patients with HSIL/SCC ($P=0.003$).

Conclusions. These results suggest that the methylation of *BLU* and *RASSF1A* genes is associated with cervical carcinogenesis, which could be clinically important in the future molecular screening of cervical neoplasia.

Keywords: Cervical cancer; HPV; BLU; RASSF1A; Methylation

Introduction

Cervical cancer is one of the major causes of death of women worldwide [1]. Infection with oncogenic human papillomavirus (HPV) is the most significant risk factor in its etiology. With advances in detection methods and when tumor tissue integrity is maintained, HPV DNA can be detected in virtually all cases of cervical cancer [2]. While persistence of HPV infection is detrimental in the development of high-grade squamous intraepithelial lesions (HSIL) and cancer [3], the immediate cervical lesion of HPV infection is low-grade squamous intraepithelial lesions (LSIL). In follow up, about 60% of LSIL regress, 30% persist, 5–10% progress to HSIL, and less than 1% become cervical cancer [4]. The molecular mechanism underlying the persistant viral infection and the long term, inefficient carcinogenesis process remains mostly elusive [5]. Environmental or genetic factors other than HPV may also play a decisive role in the malignant conversion of cervical keratinocytes and the progression to cancer [6,7].

Genetic changes with resultant genomic instability have long been recognized as an important mechanism in cervical

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carcinogenesis. Several molecular genetic studies have identified a few frequent alleleic loss or loss-of-heterozygosity chromosomal sites, indicating the loss of tumor suppressor genes (TSGs) in the development of cervical cancer [8–11]. In addition to genetic changes, epigenetic alterations such as DNA methylation and histone modifications are recognized as important driven forces of cancer [12–14]. Methylation of cytosine is widely found in mammal genomes in the context of the palindromic sequence 5′-CpG-3′. Most CpG dinucleotide pairs are methylated except at some areas called "CpG island" where the methylation is developmentally controlled. CpG islands are CpG-rich areas of approximately 1 kb, usually located in the vicinity of genes and often found near the promoter of widely expressed genes [15,16]. Controlled by DNA methyltransferases, global DNA hypomethylation and site-specific hypermethylation have been reported as the hallmark of cancer [12]. It has become increasingly apparent that DNA hypermethylation with subsequent epigenetic silencing of TSGs through chromatin remodeling is associated with loss of function, which may constitute the second hit of the "two hit" hypothesis, providing a selective advantage during carcinogenesis [12,13,17]. The epigenetic silencing of tumor suppressor genes by DNA hypermethylation is commonly seen in human cancers, including cervical cancer [18–21].

Our previous work and that of others have shown that the deletion of chromosome 3p21, which has been reported in several cancers, is a frequent event in cervical carcinogenesis [22–24]. The deletions are usually monoalleleic, and no mutations of the remaining allele in several candidate genes could be identified, indicating epigenetic changes such as hypermethylation may be responsible for the total shut down of the candidate tumor suppressor genes. Several TSGs are known to occur on chromosome 3p, among which are two recently characterized genes, *BLU* and *RASSF1A* [25–28].

BLU and *RASSF1A* are two tandemly head-to-tail liked genes located within a 25 kb region at 3p21.3. The present study was undertaken to examine the association between the methylation of *BLU* and *RASSF1A* promoters and cervical neoplasia of different severities. The association between the methylation phenotypes and HPV genotypes was also investigated.

Materials and methods

Patients

A hospital-based case-control study was conducted. The study included patients with LSIL ($n=45$), HSIL ($n=63$), invasive squamous cell carcinoma (SCC; $n=107$), or adenocarcinoma (AC; $n=23$) of the uterine cervix, diagnosed and treated at the Tri-Service General Hospital, Taipei, Taiwan, between 1993 and 2000. Cytological, histological, and clinical data for all patients were panel-reviewed by a group of staff members including colposcopists, cytologists, and pathologists, to reach a final diagnosis. All patients were investigated and treated with a standard protocol for cervical neoplasia at the same hospital. Controls were recruited from healthy women undergoing routine Pap screening during the same period. Exclusion criteria included previous pregnancy, chronic or acute viral infection, a history of cervical neoplasia, skin or genital warts, an immune-compromised state, other cancers, and operations on the uterine cervix. Specimens from subjects were collected and genomic DNA was extracted with an established protocol for tissue banking as previously described [29]. The concentration of DNA was determined by the PicoGreen fluorescence absorption method, and its quality was checked by agarose gel electrophoresis. Cervical scrapings of controls, LSIL and HSIL, and tumor tissues from SCC and AC were used in the present study. The study was approved by the Institutional Review Board of the Tri-Service General Hospital. Informed consent was obtained from each patient and control subject.

Bisulfite modification, methylation-specific PCR (MS-PCR) and bisulfite sequencing

Of the genomic DNA, 1 μg was bisulfite modified using the DNA modification Kit (Chemicon, Ternecula, CA, USA) according to the manufacturer's recommendations. The final precipitate was eluted in 70 μl of pre-warmed (55°C) TE buffer. MSP was performed according to Herman et al. [47]. In short, 1 μl of modified DNA was amplified using MSP primers (Table 1) that specifically recognized either the unmethylated or methylated RASSF1A and *BLU* gene sequence after bisulphite conversion [25,30]. Methylation-specific PCR was done in a total volume of 25 μl, containing 1 μl modified template DNA, 1.5 pmol of each primer, 0.2 mmol/L deoxynucleotide triphosphates and 1 unit Gold *Taq* DNA polymerase (PE Applied Biosystems, Foster City, CA). MSP reactions were subjected to initial incubation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, and annealing at the appropriate temperature for 30 s and 72°C for 30 s. Final extension was done by incubation at 72°C for 5 min. Normal DNA from human peripheral blood was bisulphite modified to serve as a control for the unmethylated promoter sequence. Normal human DNA was treated in vitro with SssI methyltransferase (New England Biolabs, Beverly, MA, USA) in order to generate a positive control for methylated alleles [31]. Amplification products were visualized on 2.5% agarose gel containing ethidium bromide and illuminated under UV light. All MSP data were done on at least two independent modifications of DNA with PCR cycles of 35. Signals that were weaker than 1:10 dilution of in vitro methylated DNA with unmethylated peripheral blood DNA and signals that were not detectable in repeated experiments were scored as negative of methylation. Bisulfite-treated genomic DNA was amplified using primers for human *RASSF1A* and *BLU* (Table 1). Amplified PCR product was purified and cloned into pCR4-TOPO vector (Invitrogen, Carlsbad, CA). DNA sequencing was performed on at least 5 individual clones using the 377 automatic sequencer (Applied Biosystems, Foster City, CA, USA).

Re-expression of BLU and RASSF1A mRNA by 5′-aza-2′-deoxycytidine treatment in cervical cancer cells

The methylation status of *BLU* and *RASSF1A* was tested in HeLa and CaSki cervical cancer cell lines by MSP. The re-expression of *BLU* and *RASFF1A* in cervical cancer cell lines after treatment with 10 μM of 5′-aza-2′-deoxycytidine (Sigma Chemical Co.) for 4 days was assessed by RT-PCR. Total RNA was

Table 1
Primers and conditions used in the present study

Primers	Sequence 5′→3′	Amplicon size (bp)	Annealing temperature (°C)
MSP			
RASSF1A MF	CGAGAGCGCGTTTAGTTTCGTT	192	58
RASSF1A MR	CGATTAAACCCGTACTTCGCTAA		
RASSF1A UF	GGGGGTTTTGTGAGAGTGTGTTT	204	58
RASSF1A UR	CCCAATTAAACCCATACTTCACTAA		
BLU MF	GCGGGTTAGAGATTCGTTC	231	55
BLU MR	TCGAAACCGAAATCCGACG		
BLU UF	GGTGGGTTAGAGATTTGTTT	235	53
BLU UR	ATATCAAAACCAAAATCCAACA		
Bisulfite sequencing			
RASSF1A BGS1	AGTTTTTGTATTTAGGTTTTTATTG	465	55
RASSF1A BGS2	CTACCCCTTAACTACCCCTTCC		
BLU BGS1	GGGGTTATTTTTATTTTTGTGTAGG	406	53
BLU BGS2	ACAACAATTCCAAATCTCCCATAT		