Biomarkers, 2008; 13(6): 597–606

informa
healthcare

Effect of aberrant promoter methylation of *FHIT* and *RASSF1A* genes on susceptibility to cervical cancer in a North Indian population

M. KAUSAR NEYAZ[1,2]*, R. SURESH KUMAR[1]*, SHOWKET HUSSAIN[3], SAMAR H. NAQVI[4], INDU KOHAAR[1], NISHA THAKUR[1], VEENA KASHYAP[5], BHUDEV C. DAS[3], SYED AKHTAR HUSAIN[2], & MAUSUMI BHARADWAJ[1]

[1]*Divisions of Molecular Genetics & Biochemistry,* [3]*Molecular Oncology and* [5]*Cytopathology, Institute of Cytology and Preventive Oncology (ICMR), I-7, Sector 39, Noida, India,* [2]*Department of Biosciences, Faculty of Natural Sciences, Jamia Millia Islamia, New Delhi, India and* [4]*Genetix Biotech Asia Pvt. Ltd., New Delhi, India*

Abstract

As current evidence suggests the involvement of epigenetic modification of tumour suppressor genes in human cancer, we investigated the aberrant promoter methylation of *FHIT* and *RASSF1A* genes in human papillomavirus (HPV)-mediated cervical cancer in Indian women. We analysed 60 cervical cancer tissue biopsies of different clinical stage and histological grading and 23 healthy control samples with normal cervical cytology. Methylation-specific polymerase chain reaction (MSP) was performed to analyse the methylation status of *FHIT* and *RASSF1A* genes and confirmed by sequencing. Both patients and controls were screened for HPV infection and 98% of the HPV-infected cases showed positivity for HPV type 16. Aberrant promoter methylation of the *FHIT* gene was found in 28.3% (17/60) of cases and of the *RASSF1A* gene in 35.0% (21/60) of cases; promoter methylation of both the genes was found in 13.3% (8/60) of cervical cancer cases. Methylation was significantly ($p < 0.01$) associated with the cervical cancer cases compared with controls. None of the 23 controls was found to be methylated in either of these genes. This is the first study indicating a correlation between the promoter methylation of *FHIT* and *RASSF1A* genes and the clinical stage and histological grading of cervical carcinoma in Indian women. Future studies are underway to examine the practical implications of these findings for use as a biomarker.

Keywords: *Methylation-specific polymerase chain reaction (MSP), human papillomavirus (HPV), fragile histidine triad (FHIT), RAS association domain family 1A (RASSF1A), squamous cell carcinoma (SCC)*

(Received 23 November 2007; accepted 25 March 2008)

Correspondence: Mausumi Bharadwaj, Division of Molecular Genetics & Biochemistry, Institute of Cytology & Preventive Oncology (ICMR), I-7, Sector 39, Noida 201301, India. Tel: 91-95120-2579471. Fax: 91-95120-2579473. E-mail: mausumi02@yahoo.com, bharadwajm@icmr.org.in
*Both authors contributed equally.

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.1080/13547500802078859

Introduction

Carcinoma of the uterine cervix is the second leading gynaecological malignancy worldwide (Vizcaino et al. 2000, Jemal et al. 2002), but it is the most common cancer among women in India (Das et al. 2000). Clinicoepidemiological and molecular biological data established a causal link between 'high-risk' human papillomavirus (HR-HPV) infection and cervical cancer (Durst et al. 1983). Although, HPV infection appears to play an important role in the development of cervical cancer, several lines of experimental evidence suggest that HPV infection alone is insufficient for transforming a normal cell to a malignant one (zur Hausen & Rosl 1994). This can be accounted for in the involvement of genetic and epigenetic factors either independently or in conjunction with HPV infection. Therefore, these factors may be implicated in the development of cervical cancer.

Epigenetic modification within the promoter regions of many genes has been established as a critical event in the process of tumorigenesis. There are several reports revealing silencing of tumour suppressor gene activity by DNA hypermethylation which leads to development of cancer (Jones & Baylin 2002). The pattern of such promoter methylation has been noted to be non-random, while certain genes are commonly methylated in some tumour types and others are not (Costello et al. 2000, Esteller et al. 2001). Therefore, aberrant promoter methylation of tumour suppressor genes and its association with loss of gene function is an important mechanism for carcinogenesis. On the other hand, there are also several reports showing DNA hypomethylation of oncogenes in cancers (Hanada et al. 1993).

The fragile histidine triad (*FHIT*) gene of human origin is a tumour suppressor gene for many cancers including cervical cancer (Han et al. 2004). This gene has been found to be deleted/mutated/methylated in various cancers as well as in cancer cell lines with an ultimate reduction in, or lack of, protein expression (Lea et al. 2004). But RAS association domain family 1A (*RASSF1A*) is rarely inactivated by mutations but has been suggested as a target tumour suppressor gene on the basis of its frequent inactivation through promoter hypermethylation and loss of heterozygosity in a variety of primary human cancers (Cohen et al. 2003). As the short arm of chromosome 3 is one of the frequently deleted chromosomal arms, epigenetic changes in chromosome 3p loci genes including *FHIT* and *RASSF1A* together have been implicated in the development of cervical cancer in one study on a Korean population (Choi et al. 2007).

In the present study, we used the methylation-specific polymerase chain reaction (MSP) method (Herman et al. 1996) to analyse the methylation status of *FHIT* and *RASSF1A* genes in HPV-mediated cervical cancer in Indian women. We analysed the extent of the methylation pattern and correlated it with different clinical stages/ histological grades of cervical cancer.

Material and methods

Study subjects

A total of 60 cases of histologically confirmed cervical cancer and 23 control samples of normal hysterectomy specimens from patients without cancer were used in the present study. The patients were recruited from the Department of Gynaecology, Jawaharlal Nehru Medical College & Hospital, Aligarh. The histological type and

grade of the cancer samples were classified according to the WHO criteria. The tumour stage and histological grade were determined according to the criteria laid down by International Federation of Obstetrics and Gynecology (FIGO). Ten samples were stage Ib, 27 were stage IIa, 19 were stage IIIb and four samples were stage IVa. According to histological grade, 21 samples were of well-differentiated squamous cell carcinoma (WDSCC), 30 samples were of moderately differentiated SCC (MDSCC) and nine samples were of poorly differentiated SCC (PDSCC). The mean age of the patients was 47.35 ± 12.40 years.

Written consent was obtained from all the participants and the study was carried out in accordance with the principles of the Helsinki Declaration. The study was approved by the Ethics Committee of the Institute.

DNA extraction and HPV detection

Genomic DNA was extracted from fresh cervical tissue biopsy samples (patients) and normal cervical hysterectomy specimens from patients without cancer (controls) by a standard method using proteinase K followed by phenol/chloroform/isopropanol treatment (Sambrook et al. 1989). HPV diagnosis was performed by PCR amplification using consensus primers MY09 and MY11 (Manos et al. 1989) and further typing was done by PCR using type-pecific primers for HPV 16 and HPV18 (Saiki et al. 1988).

Methylation-specific PCR and DNA sequencing

The two genes (*FHIT* and *RASSF1A*) at chromosome 3p, which have been found to be methylated in the promoter region in other cancers (Kuzmin et al. 2002, Yang et al. 2002), were considered for analysis in cervical cancer. The methylation status of these genes was determined using MSP according to the method employed by Herman et al. (1996). The primers and PCR condition for MSP assays were derived from Maruyama et al. (2002).

Briefly, 2μg of genomic DNA was treated with sodium bisulfite and incubated for 16 h at 50°C. The modified DNA was purified using the Wizard DNA purification kit (Promega, Madison, WI, USA), desulphonated with NaOH, precipitated with ethanol and resuspended in water. Modified DNA was stored at −20°C until used. Primers were employed specifically for both unmethylated and methylated cytosine (Riichiroh et al. 2002). Genomic DNA from control tissue of 23 healthy subjects along with water blanks were used as negative controls for the methylated genes. PCR products were resolved by electrophoresis on 6% native polyacrylamide or 2% agarose gels stained with ethidium bromide.

After detection of PCR products with methylated primers for *FHIT* and *RASSF1A* genes, 15% of the samples were employed for direct sequencing reactions using the Amplicycle sequencing kit (Perkin-Elmer, Norwalk, CT, USA).

Statistical analysis

Statistical analyses were performed using statistical software Graph Pad Instat Version 3.0. The frequency distribution with respect to the methylation status of these two genes among cases and controls was statistically analysed using the Fisher's exact test.

The significance of this test was considered as two-tailed. Differences were considered statistically significant for $p < 0.05$.

Results

HPV prevalence

In the study cohort, 88.33% (53/60) of the cases and 4.34% (1/23) of normal healthy controls showed positivity for HPV DNA sequence. Out of the HPV-positive cases, 98% (52/53) were infected with HPV type 16 while only one patient (1.8%) was found to be positive for HPV type 18. One (4.34%) HPV-positive healthy control was found to be infected with HPV type 16.

Methylation status of FHIT and RASSF1A promoter regions

We analysed the methylation pattern of *FHIT* and *RASSF1A* promoter regions in cervical cancer cases and controls. Figure 1 depicts a representative example of the MSP products analysed on native polyacrylamide gel for *FHIT* and *RASSF1A* genes and 15% of the samples were confirmed by sequencing. Both the techniques revealed similar results (data not shown). Aberrant promoter methylation of the *FHIT* gene was found in 28.3% (17/60) of cases and of the *RASSF1A* gene in 35.0% (21/60) of cases; promoter methylation of both genes was found in 13.3% (8/60) of cervical cancer cases. Table I summarizes the methylation of the specific genes and of both

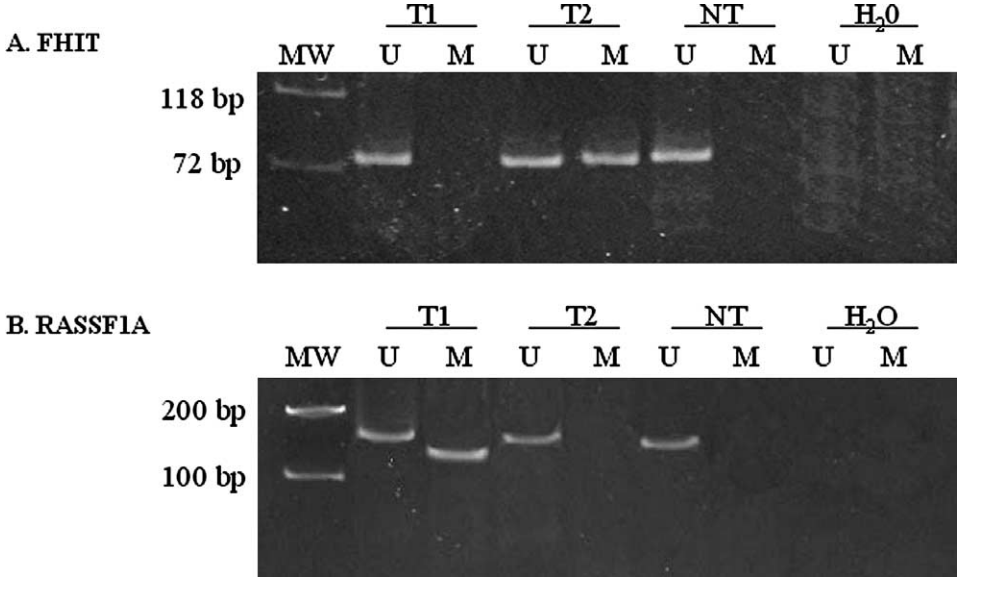

Figure 1. Methylation-specific PCR (MSP) of promoter regions of *FHIT* and *RASSF1A* genes in cervical cancer cases. (A) MSP of *FHIT* promoter regions. MW, molecular weight DNA marker ($\Phi \times 174$ *Hae*III). (B) MSP of *RASSF1A* promoter regions. MW, molecular weight DNA marker (100 bp DNA ladder). T1 and T2 are cervical cancer tissue samples. A visible PCR product in lane U indicates the presence of unmethylated gene promoter; the presence of product in lane M indicates the presence of promoter methylated sequences. In each case DNA from normal cervical tissue (NT) was used as negative control. A water-only reaction was also used as a negative PCR control.

Table I. Methylation status of *FHIT* and *RASSF1A* genes according to clinical staging and histological grading in cervical cancer.

	No. of cases	*FHIT* Methylated (%)	*RASSF1A* Methylated (%)	Both *FHIT and RASSF1A* Methylated (%)
Clinical stage				
Ib	10	2 (20.0)	3 (30.0)	0 (0)
IIa	27	8 (29.6)	11 (40.7)	5 (18.5)
IIIb	19	4 (21.0)	5 (26.3)	2 (10.5)
IVa	4	3 (75.0)	2 (50.0)	1 (25.0)
Total	60	17 (28.3)	21 (35.0)	8 (13.3)
Histological grade				
WDSCC	21	3 (14.3)	6 (28.5)	1 (4.76)
MDSCC	30	9 (30.0)	11 (36.0)	5 (16.6)
PDSCC	9	5 (55.0)	4 (44.0)	2 (22.22)
Total	60	17 (28.3)	21 (35.0)	8 (13.3)

genes together in different clinical stages and histological grades of cervical cancer. None of the 23 normal cervical tissues (controls) showed methylation in either of these two genes (Table II).

The incidence of methylation of the *FHIT* gene was 20.0% (2/10) in stage Ib and 29.6% (8/27) in stage IIa, whereas it was 21% (4/19) in stage IIIb and 75.0% (3/4) in stage IVa of cervical cancer cases (Table I). The frequency of methylation was 14.3% (3/21) in histological grade WDSCC, 30% (9/30) in MDSCC and 55% (5/9) in PDSCC (Table I). The frequency distribution of methylated versus unmethylated *FHIT* revealed that methylation was significantly ($p < 0.01$) associated with the cervical cancer cases compared with controls. Similar results ($p < 0.01$) were obtained for the incidence of methylated *FHIT* versus total gene (methylated + unmethylated) (Table II).

Likewise, for the *RASSF1A* gene, the incidence of methylation was 30.0% (3/10) in stage Ib cases, 40.7% (11/27) in stage IIa cases, 26.3% (5/19) in stage IIIb cases and 50.0% (2/4) in stage IVa cases (Table I). Also, the frequency of methylation was 28.5% (6/21) in WDSCC, 36% (11/30) in MDSCC and 44% (4/9) in PDSCC (Table I). The frequency distribution of methylated versus unmethylated *RASSF1A* and methylated versus total gene (methylated + unmethylated) revealed that methylation

Table II. Methylation status of *FHIT* and *RASSF1A* genes in cervical cancer cases and controls.

Gene	Methylation status	Cases *n* (%)	Controls *n* (%)	*p*-Value	OR (95% CI)
FHIT	Methylated	17 (28.3)	0	$<0.0^a$	1.535 (1.287–1.831)[a]
	Unmethylated	43 (71.7)	23 (100)	0.01^b	1.383 (1.211–1.580)[b]
RASSF1A	Methylated	21 (35.0)	0	$<0.01^a$	1.590 (1.313–1.925)[a]
	Unmethylated	39 (65.0)	23 (100)	$<0.01^b$	1.383 (1.211–1.580)[b]
FHIT + *RASSF1A* (Both genes)	Methylated	8 (13.3)	0	0.09^a	
	Unmethylated	52 (86.7)	23	0.19^b	

OR, odds ratio; CI, confidence interval; *p*-value, probability from the Fisher's exact test comparing the methylation status for controls and cases; [a]comparing the frequency of methylated versus unmethylated gene among cases and controls; [b]comparing the frequency of methylated versus total (methylated and unmethylated) gene among cases and controls.

wass significantly ($p < 0.01$) associated with cervical cancer cases compared with controls (Table II).

However, aberrant methylation of both genes together was not found in any patients with stage Ib cancer, whereas the frequency was 18.5% (5/27) for stage IIa, 10.5% (2/19) for IIIb and 25% (1/4) for stage IVa (Table I). Similarly, only one sample of WDSCC grade (1/21; 4.76%) was found to be methylated in both genes. However, the frequency of methylation was found to be 5 (5/30; 16.6%) for MDSCC and 2 (2/9; 22.2%) for PDSCC. The frequency distribution of methylation versus unmethylation and methylation versus total for both genes taken together showed no statistically significant association for cases compared with controls (Table II).

The *RASSF1A* gene was found to be more frequently methylated compared with the *FHIT* gene in the studied cohort. Aberrant promoter methylation in at least one gene was detected in 63% (38/60) of cases, and it was found to be statistically significant ($p < 0.01$). Although no significant correlation was established between methylation status and clinical staging for either of the genes a trend was observed with the histological grade of cervical cancer (Table I).

Discussion

Extensive screening programmes have lowered the incidence of cervical cancer but it is still the second most common cancer among women worldwide (Parkin et al. 2005), and the most common cancer among women in resource-poor countries including India. In spite of the fact that several other risk factors are involved, including early age of marriage, promiscuity, smoking, use of contraceptives, persistent HR-HPV infection is considered to be the principal aetiological factor. From epidemiological data on cervical cancer patients from 25 countries, Bosch (2003) demonstrated that HPV types 16 and 18 are present in more than 70% of cervical cancer cases. However, in India, the prevalence of HPV type 16 is found to be particularly high (~90%) while occurrence of HPV 18 varies from 3 to 20%, followed by other high-risk types such as HPV 45, 33, 35, 52, 58, 59 and 73 (Das et al. 1992, Franceschi et al. 2003, Sowjanya et al. 2005, Kailash et al. 2006, Das et al. 2008). In our previous study and also in the present study, HPV type 16 alone was found to be as high as 98% while the remainder was HPV 18; no other high-risk HPV types were detected (Kohaar et al. 2007). In India, 85–90% of cervical cancer cases are SCC and only 10–15% of cases are adenocarcinoma. Interestingly, in India HPV 16 is the most prevalent type both in SCC and in adenocarcinoma while, globally, HPV 18 is more common in adenocarcinoma (Das et al. 1993, Iwasawa et al. 1996). Although infection with HPV is important, but not sufficient alone, for the development and progression of cervical cancer this indicates a role for host genetic factors. Therefore, it is worthwhile investigating the various host markers in different clinical stages/histological grades of cervical tumours in order to understand the aetiology of the disease, which may provide a very effective tool for the better management of this dreaded disease.

Cervical cancer is a suitable tumour model for the study of genetic and epigenetic changes because of the incidence of multiple aetiologies as well as prolonged well-differentiated intraepithelial lesions, a key feature of cervical carcinogenesis. But, still the role of epigenetic factors involved in the development of cervical carcinogenesis is poorly understood and only a few reports are available showing promoter methylation of specific tumour suppressor genes (Dong et al. 2001, Virmani et al. 2001). There are

only a few studies reporting hypermethylation of both *FHIT* and *RASSF1A* genes together in cancers, including cervical cancer (Tomizawa et al. 2004, Chang et al. 2005, Choi et al. 2007). Therefore, in the present study we have analysed the aberrant methylation pattern of the promoter region of these two genes in cervical carcinoma patients in an Indian population using MSP.

Several studies showed separately that methylation of CpG islands of *FHIT* and *RASSFIA* genes has a significant role in the development of cervical cancer (Kuzmin et al. 2003, Yu et al. 2003, Maliukova et al. 2004, Shi et al. 2005, Ren et al. 2006), but only in one study in a Korean population have the two genes been considered simultaneously (Choi et al. 2007). Ren et al. (2006) showed that in a Chinese population, methylation of the *FHIT* gene occurred in 53.33% of cervical cancer tissue samples but in the Caucasian population, the incidence was barely more than 20% (Virmani et al. 2001). In the study by Choi et al. in a Korean population it was shown to be 24% (Choi et al. 2007) which supports our data (28.3%). Interestingly, we found a trend for methylation in the *FHIT* gene in association with clinical stage, i.e. from 20% in stage Ib to 75% in stage IVa, except for stage IIIB (21%), and despite a smaller sample size for stage IVa cases. In agreement with this study, Shi et al. showed that the incidence of methylation increases from stage I to stage II of cancer but they could not find a relationship in later stages (Shi et al. 2005). When we compared histological grades, there was certainly a trend of increase in frequency of methylation with disease severity. Thus, our data strongly support the theory that methylation of the *FHIT* gene in cervical cancer cases in the Indian population is a late event which may be associated with carcinogenesis. Our data show that the aberrant promoter hypermethylation of *FHIT* gene was highly significant ($p < 0.01$) for the cervical cancer cases compared with control samples. Also, promoter methylation of *FHIT* was found to be significantly ($p < 0.01$) associated with cervical cancer compared with unmethylated and total (methylated + unmethylated), thereby strengthening the relation between cervical cancer and methylation.

Various reports suggested that epigenetic event of *RASSFIA* gene may play a key role in the development of cervical cancer (Cohen et al. 2003, Maliukova et al. 2004). Our study showed that, promoter methylation of the *RASSF1A* gene was found in 35.0% of cases compared with the control, similar to SCC of uterine cervical cancer cases in a Chinese population (Yu et al. 2003). In contrast, Kuzmin et al. (2003) showed that promoter hypermethylation of *RASSF1A* occurred in only 10% of SCC cases in the Caucasian population; however, no methylation was detected in SCC cases in a Korean population (Choi et al. 2007). In another study, it was reported that hypermethylation of the *RASSF1A* promoter region is a common phenomenon in adenocarcinoma but a rare event in SCC of the uterine cervix (Cohen et al. 2003). Although in our study there was no significant difference in methylation pattern among the various clinical stages and histological grades, hypermethylation of the *RASSF1A* gene was found to be an early event. Similar to the *FHIT* gene, the promoter methylation state of the *RASSF1A* gene was significantly ($p < 0.01$) linked with cervical cancer cases with respect to unmethylated and with total (i.e. methylated + unmethylated).

The *RASSF1A* gene was observed to be commonly methylated in comparison with the *FHIT* gene in the studied cohort (Table I). Overall, aberrant promoter methylation in at least one of these genes was detected in 63.33% of the total cervical cancer cases, which was found to be statistically significant ($p < 0.01$). By studying the methylation

profile of the *FHIT* and *RASSF1A* genes for different stages/grades of SCC, we found that the frequency of methylation of the *RASSF1A* gene is significantly higher ($p < 0.01$) compared with the *FHIT* gene in cervical cancer cases, although both genes were methylated in only 13.3% cases (Table I). Both these genes are located on chromosome 3p (*FHIT* at 3p14.2 and *RASSF1A* at 3p21.31) but the pattern of methylation of these two genes was independent of location, indicating that aberrant methylation of promoter regions is gene specific rather than associated with the location of the gene on the chromosome.

In conclusion, to our knowledge, this is the first study indicating a correlation between the results of MSP analysis of the *FHIT* and *RASSF1A* genes with clinical staging and histological grading of cervical carcinoma in Indian women. Studies are underway in our laboratory to examine the practical implications of the methylation profile of these two tumour suppressor genes in HPV-mediated cervical carcinogenesis.

Acknowledgements

The authors thank Dr Rajyashri Sharma, Department of Obstetrics and Gynecology, JNMC, Aligarh, for providing clinical samples. The authors also thank Dr Saima Wajid, National Institute of Immunology, New Delhi for critical evaluation of the manuscript.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Bosch FX. 2003. Epidemiology of human papillomavirus infections: new options for cervical cancer prevention. Salud Publica de Mexico 45(Suppl. 3):S326–S339.

Chang Q, Pang JC, Li KK, Poon WS, Zhou L, Ng HK. 2005. Promoter hypermethylation profile of RASSF1A, FHIT, and sFRP1 in intracranial primitive neuroectodermal tumors. Human Pathology 36:1265–1272.

Choi CH, Lee KM, Choi JJ, Kim TJ, Kim WY, Lee JW, Lee SJ, Lee JH, Bae DS, Kim BG. 2007. Hypermethylation and loss of heterozygosity of tumor suppressor genes on chromosome 3p in cervical cancer. Cancer Letters 255:26–33.

Cohen Y, Singer G, Lavie O, Dong SM, Beller U, Sidransky D. 2003. The RASSF1A tumor suppressor gene is commonly inactivated in adenocarcinoma of uterine cervix. Clinical Cancer Research 9:2981–2984.

Costello JF, Fruhwald MC, Smiraglia DJ, Rush LJ, Robertson GP, Gao X, Wright FA, Feramisco JD, Peltomäki P, Lang JC, Schuller DE, Yu L, Bloomfield CD, Caligiuri MA, Yates A, Nishikawa R, Su Huang H, Petrelli NJ, Zhang X, O'Dorisio MS, Held WA, Cavenee WK, Plass C. 2000. Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. Nature Genetics 24:132–138.

Das BC, Gopalkrishna V, Hedau S, Katiyar S. 2000. Cancer of the uterine cervix and human papillomaviruses infection. Current Science 78:52–63.

Das BC, Showket H, Vilas N, Bharadwaj M. 2008. Prospects and prejudices of human papillomavirus vaccines in India. Vaccine (In press).

Das BC, Sharma JK, Gopalkrishna V, Das DK, Singh V, Gissman L, et al. 1992. A high frequency of human papillomavirus DNA sequences in cervical carcinomas of Indian women as revealed by Southern blot hybridization and polymerase chain reaction. Journal of Medical Virology 36:239–245.

Das BC, Gopalkrishna V, Das DK, Sharma JK, Singh V, Luthra UK. 1993. Human papillomavirus DNA sequences in adenocarcinoma of the uterine cervix in Indian women. Cancer 72:147–153.

Dong SM, Kim HS, Rha SH, Sidransky D. 2001. Promoter hypermethylation of multiple genes in carcinoma of the uterine cervix. Clinical Cancer Research 7:1982–1986.

Durst M, Gissmann L, Ikenberg H, zur Hausen H. 1983. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proceedings of National Academy of Sciences USA 80:3812–3815.

Esteller M, Corn PG, Baylin SB, Herman JG. 2001. A gene hypermethylation profile of human cancer. Cancer Research 6:3225–3229.

Franceschi S, Rajkumar T, Vaccarella S, Gajalakshmi V, Sharmila A, Snijders PJ et al. 2003. Human papillomavirus and risk factors for cervical cancer in Chennai, in India: a case control study. International Journal of Cancer 107:127–133.

Han SY, Iliopoulos D, Druck T, Guler G, Grubbs CJ, Pereira M, Zhang Z, You M, Lubet RA, Fong LYY, Huebner K. 2004. CpG methylation in the Fhit regulatory region: relation to Fhit expression in murine tumors. Oncogene 23:3990–3998.

Hanada M, Delia D, Aiello A, Stadtmauer E, Reed JC. 1993. Bcl-2 gene hypomethylation and high-level expression in B-cell chronic lymphocytic leukemia. Blood 82:1820–1828.

Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. 1996. Methylation-specific PCR a novel PCR assay for methylation status of CpG islands. Proceedings of the National Academy of Sciences USA 93:9821–9826.

Iwasawa A, Nieminen P, Lehtinen M, Paavonen J. 1996. Human papillomavirus DNA in uterine cervix squamous cell carcinoma and adenocarcinoma detected by polymerase chain reaction. Cancer 77:2275–2279.

Jemal A, Thomas A, Murray T, Thun M. 2002. Cancer statistics, 2002. CA. A Cancer Journal for Clinicians 52:23–47.

Jones PA, Baylin SB. 2002. The fundamental role of epigenetic events in cancer. Nature Review Genetics 3:415–428.

Kailash U, Soundararajan CC, Lakshmy R, Arora R, Vivekanandhan S, et al. 2006. Telomerase activity as an adjunct to high-risk human papillomavirus types 16 and 18 and cytology screening in cervical cancer. British Journal of Cancer 95:1250–1257.

Kohaar I, Thakur N, Salhan S, Batra S, Singh V, Sharma A, Sodhani P, Das BC, Sarkar DP, Bharadwaj M. 2007. TNFα-308 G/A polymorphism as a risk factor for HPV associated cervical cancer in Indian population. Cellular Oncology 29:249–256.

Kuzmin I, Gillespie JW, Protopopov A, Geil L, Dreijerink K, Yang Y, Vocke CD, Duh FM, Zabarovsky E, Minna JD, Rhim JS, Emmert-Buck MR, Linehan WM, Lermam MI. 2002. The RASSF1A Tumor suppressor gene is inactivated in prostate tumors and suppresses growth of prostate carcinoma cells. Cancer Research 62:3498–3502.

Kuzmin I, Liu L, Dammann R, Geil L, Stanbridge EJ, Wilczynski SP, Lerman MI, Pfeifer GP. 2003. Inactivation of RAS association domain family 1A gene in cervical carcinomas and the role of human papillomavirus infection. Cancer Research 63:1888–1893.

Lea JS, Ashfaq R, Muneer S, Burbee DG, Miller DS, Minna JD, Muller CY. 2004. Understanding the mechanisms of FHIT inactivation in cervical cancer for biomarker development. Journal of the Society for Gynecologic Investigation 11:329–337.

Maliukova AV, Loginov VI, Khodyrev DS, Kadyrova EL, Pronina IV, Ivanova TA, Kiselev FL, Zabarovskii NP, Braga EA. 2004. Methylation of the putative tumor suppressor gene, RASSF1A, in primary cervical tumors. Molekuliarnaia Biologiia (Mosk) 38:1005–1013.

Manos MM, Ting Y, Wright DK, Lewis AJ, Broker TR, Wolinski SM. 1989. Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. Cancer Cells 7:209–214.

Maruyama R, Toyooka S, Toyooka KO, Virmani AK, Zöchbauer-Müller S, Farinas AJ, Minna JD, McConnell J, Frenkel EP, Gazdar AF. 2002. Aberrant promoter methylation profile of prostate cancers and its relationship to clinicopathological features. Clinical Cancer Research 8:514–519.

Parkin DM, Bray F, Ferlay J, Pisani P. 2005. Global cancer statistics, 2000. CA. A Cancer Journal for Clinicians 55:74–108.

Ren CC, Miao XH, Yang B, Zhao L, Sun R, Song WQ. 2006. Methylation status of the 5′ CpG islands in FHIT gene in the plasma and tissues of cervical cancer patients. Yi Chuan 28:1061–1066.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA. 1988. Primer-directed amplification of DNA with a thermostable DNA Polymerase. Science 239:487–491.

Sambrook J, Fritsh EF, Maniatis T. 1989. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. p. 9.16–9.19.

Shi HR, Wu QH, Suo ZH, Nesland JM. 2005. Correlation between methylation of 5′-CpG islands and inactivation of FHIT gene in cervical cancer. Ai Zheng 24:7–11.

Sowjanya AP, Jain M, Poli UR. 2005. Prevalence and distribution of high-risk human papillomavirus (HPV) types in invasive squamous cell carcinoma of the cervix and in normal women in Andhra Pradesh, India. BMC Infectious Diseases 5:116–122.

Tomizawa Y, Iijima H, Nomoto T, Iwasaki Y, Otani Y, Tsuchiya S, Saito R, Dobashi K, Nakajima T, Mori M. 2004. Clinicopathological significance of aberrant methylation of RARbeta2 at 3p24, RASSF1A at 3p21.3, and FHIT at 3p14.2 in patients with non-small cell lung cancer. Lung Cancer 46:305–312.

Virmani AK, Muller C, Rathi A, Zoechbauer-Mueller S, Mathis M, Gazdar AF. 2001. Aberrant methylation during cervical carcinogenesis. Clinical Cancer Research 7:584–589.

Vizcaino AP, Moreno V, Bosch FX, Muñoz N, Barros-Dios XM, Borras J, Parkin DM. 2000. International trends in incidence of cervical cancer: II. Squamous-cell carcinoma. International Journal of Cancer 86:429–435.

Yang Q, Nakamura M, Nakamura Y. 2002. Two-hit inactivation of FHIT by loss of heterozygosity and hypermethylation in breast cancer. Clinical Cancer Research 8:2890–2893.

Yu MY, Tong JH, Chan PK, Lee TL, Chan MW, Chan AW, Lo KW, To KF. 2003. Hypermethylation of the tumor suppressor gene RASSFIA and frequent concomitant loss of heterozygosity at 3p21 in cervical cancers. International Journal of Cancer 105:204–209.

zur Hausen H, Rosl F. 1994. Pathogenesis of cancer of the cervix Cold Spring Harbor Symposia on Quantitative Biology, vol. LIX. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. p. 623–628.