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Aberrant methylation in promoter region induced silencing of $P^{16/NK4a}$ in colorectal cancer in Iranian patients

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Abstract

Aberrant methylation in promoter regions of genes might lead to change gene functions and result in cancer. Hence, biomarker identification for aberrant methylated genes would be very useful for early diagnosis, prognosis, and therapeutic treatment of colorectal cancer (CRC). The aim of the present study was to detection correlation between methylation status in promoter region of P16/NK4a with P16/NK4a expression level, CRC occurence and with demographic and clinocopathological characteristics of CRC. Methylation status in promoter region of P16/NK4a was assayed by methylation-specific polymerase chain reaction (MS-PCR) and P16/NK4a gene expression was performed by real time quantitative PCR (qPCR). RNA from embedded paraffin sections of colorectal tissue (in 70 sporadic colorectal tumors as well as adjoining and normal tissue specimens) was reverse transcribed, quantified and analyzed by Q-PCR. Aberrant promoter methylation of p16 gene was detected in 27 (38.6%) tumor samples and in 8 (11.4%) adjacent normal tissues. Thus, aberrant promoter methylation of p16 is significantly correlated with CRC occurance. Aberrant promoter methylation was found significantly associated with tumor stage II (P=0.000), but not with other clinocopathological and demographic characteristics. P16/INK4a expression level in tumor tissues was 8.6-fold more than normal adjacent tissues. In conclusion, this study has identified aberrant promoter methylation of P16/INK4a was significantly correlated with CRC, because aberrant promoter methylation effects on P16/INK4a expression. Our approaches revealed P16/INK4a can be as a potential biomarker for CRC as diagnostic, prognostic and therapeutic targets in the future.

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Introduction

Colorectal cancer (CRC) is generally the third leading cause of cancer-related mortality. CRC incidence and mortality rates vary over 10-fold worldwide. Lowest incidence rates are observed in Africa and highest ones are found in Western societies. Although incidence rates in developed countries are stabilizing, they are severely increasing in both developing countries and several areas historically at low risk (Jemal *et al.*, 2011). Also in USA, data from migration population studies revealed that some ethnic groups are showing increased CRC incidence rate while they are migrating from low-risk to high-risk areas (Grulich *et al.*, 1995; Lee *et al.*, 2007; Flood *et al.*, 2000). Despite genetic variation, these epidemiological data strongly suggest a role of environmental and lifestyle factors deeply contributing to the etiology of CRC. Significant lifestyle risk factors are represented by sedentarity and changes in dietary habits, from a moderate to a Western like enriched diet associated with high consumption of unsaturated fats and red meat, high intake of alcohol, and smoking.

It is well accepted that environmental and dietary factors greatly influence epigenetic events including hypermetylation. Hypermethylation or aberrant methylation in tumor suppressor genes has been reported in various diseases including cancers. The promoter hypermethylation is one of the mechanisms leading to gene silencing by either physically inhibiting the binding of transcription factors, or by recruiting proteins that have transcription repressive properties in the cancer pathogenesis (Miyamoto *et al.*, 2005; Baylin, 2005; Radhakrishnan *et al.*, 2011). Thus, this epigenetic process may act as a native to genetic alterations involving DNA mutations or chromosomal aberrations that disrupt functions of the oncogenes or tumour suppressor genes (Khor *et al.*, 2011; Baylin *et al.*, 2006; Khor *et al.*, 2009). Many investigations conducted on cancer-associated genes including cyclin-dependent kinase inhibitor 2A (*P16*), E-cadherin (*CDH1*), O^6-methyl guanine methyl transferase (*MGMT*) and Estrogen Receptor-α (*ESR-α*) genes, have been found to harbor hypermethylated regulatory sequences that lead to gene silencing in CRC (Xing *et al.*, 2013; Yu *et al.*, 2005; Wani *et al.*, 2013) and other cancers (Jundong *et al.*, 2013; Changmei *et al.*, 2013; Rasti *et al.*, 2009; Ovchinnikov *et al.*, 2012; Mulero-Navarro and Esteller, 2008). However, numbers of studies to evaluate methylation profile of P16 in promoter region is low in CRC and further investigation is necessary. The identification of biomarkers for early diagnosis or as therapeutic target in CRC treatment is urgently needed.

The inhibitor of cyclin-dependent kinases $P^{16/INK4a}$ inhibits CDK43 and CDK6, which are key regulators of the progression of eukaryotic cells through the G1phase of the cell cycle (Serrano *et al.*, 1993). The *P16/CDKN2* gene resides on chromosome band 9p2l, a region frequently altered in diverse tumor types (Fountain *et al.*, 1992; van der Riet *et al.*, 1993). This gene is an excellent candidate for a tumor suppressor, and silencing of *P16* by aberrant methylation can be a reason for CRC. Thus, *P16/INK4a* was selected and studied in this investigation.

Here we used methylation-specific polymerase chain reaction (PCR) (MS PCR) to investigate *P16/INK4a* methylation to distinguish methylated from unmethylated cytosines. MS PCR is very sensitive to detect one methylated cytosine in 1000, and the primers used are highly specific and cost effective. Also we used real -time quantitative PCR, that it is a very precision method, to assay *P16* mRNA expression. In fact, the aim of this study was to evaluate the effect of methylation status on *P16* mRNA expression and correlation between these cases and CRC risk.

Materials and methods

Patient population and tissue samples

Seventy patients with histologically confirmed colorectal cancer (primary sporadic CRC) were examined in the present study. All patients underwent colorectal resection primarily at Neyshabur Medical Science Faculty Hospital (Neyshabur, Iran) from January 2008 to February

2013. No patient had received preoperative treatment in the form of radiation or chemotherapy. Patients who had other malignant disease except CRC were excluded. This study was approved by the IAU-Neyshabur and Neyshabur Medical science Faculty. All the tissue samples were collected within 10 minutes of resection. Tumor samples were divided into two parts; one part was sent for histopathological diagnosis and staging and the other half was stored as formalin fixed paraffin embedded (FFPE) as well as adjacent normal samples (normal non tumorous healthy tissue at >2 cm distance from the tumor) (Li *et al.*, 2012). Histopathological grades and clinical staging were evaluated according to the standard criteria (Edge *et al.*, 2010). Only histopathologically confirmed cases were included for further molecular analysis.

DNA and RNA extraction from tissue samples

Genomic DNA was extracted 3 times from 70 paired normal background and tumor FFPE tissue specimens with the AccuPrep Genomic DNA Extraction Kit (BioNEER, Cat.NO.: K-3032, Korea).

The most important consideration in generating useful data with real-time q-PCR is the quality of the isolated RNA. RNA isolated 5 times with the Jena Bioscience kit (Cat.-No. PP-210xs, Germany).

Before all extractions FFPE tissue specimens was deparaffinized by xylem. Quantity/quality was checked spectrophotometrically /1% agarose gel electrophoresis and stored for further use at -20°C.

Bisulfite modification and methylation- specific polymerase chain reaction (MS PCR)

Aberrant DNA methylation in the CpG islands in the promoter region of the *p16* in 70 patients of CRC was determined by chemical modification of genomic DNA with sodium bisulfite followed by MS PCR. Genomic DNA was treated with sodium bisulfate as reported by Herman *et al.,* (1996). Briefly, 2 µg of genomic DNA was denatured in 50 µl NaOH (final concentration, 0.2 M) for 10-15 min at 50 °C. Thirty microliters of 10 mM hydroquinone (Fluka) and 520 µl 3 M sodium bisulfite (Fluka) at pH 5, freshly both prepared, were added and mixed, and the samples were incubated at 50 °C for 16 h. The unmethylated cytosine was converted to thymine, whereas methylated cytosine remains unchanged.

The primer sequence of p16 was based on the previous report and is listed in Table 1. Bisulfite-modified DNA was denatured in a total volume of 25 µl containing 1 µl of each primer, 2 µl of deoxynucleotide triphosphate, 2.5 µl of 10× PCR buffer, and 0.4 µl of hot-taq polymerase at 95^{oC} for 10 minutes. This was followed by 40 cycles of 95^{oC} for 30 seconds, incubation at the primer specific annealing temperature for 45 seconds, and 72^{oC} for 45 seconds. Samples were finally incubated at 72^{oC} for 10 minutes. Annealing temperature was 63.3^{oC}. Blood Sample of normal human DNA was used as the control for unmethylated alleles. In vitro methylated DNA from placenta with SssI methyltransferase was used as a positive control for methylated reaction. Distilled water was used as a negative control.

Table 1. Primer sequences used in methylation-specific PCR (An *et al.,* 2005).

Gene	Profile	Sequence
P16/NK4a	Unmetylated	5'-TTATTAGAGGGTGGGGTGGATTGT-3' (sense)
		5'-CAACCCCAAACCACAACCATAA-3' (antisense)
	Metylated	5'-TTATTAGAGGGTGGGGCGGATCGC-3' (sense)
		5'-GACCCCGAACCGCGACCGTAA-3' (antisense)

PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. Samples were scored as methylated when there was a clearly visible band on the gel with the methylation-

specific primers. The sizes of PCR product in this study were 150 bp for methylation and 151 bp for unmethylation respectively. All samples were examined by one experimenter who was unaware of the patient's clinical features.

Real-time quantitative PCR (qPCR)

Two-step procedure was conducted in this study. cDNA was prepared according to manufacturer's instructions of kit (AccuPower RoketScript RT PerMix) from BioNEER company of korea. q PCR was performed in triplicates with the Applied Biosystems step one Real-Time PCR System, using SYBR® Green PCR Master Mix (Part number 4309155) in comparative Ct method {ΔCt = Ct(main target cDNA) – Ct (refrence cDNA) and ΔΔCt = ΔCt (test cDNA) - ΔCt (calibrator cDNA) }. The primers were used for $P^{16/NK4a}$ and GAPDH are shown in table 2 based on references 80. qPCR was performed in a total reaction volume of 25 µl containing 12.5 µl, 1.25 µl (30 pmol) primermix and 11.25 µl cDNA, or water as control. Thermal cycling conditions included 2 min at 50°C to allow for cleavage of cDNA double-strands and 10 min at 95°C to activate the Taq polymerase, followed by 45 cycles at 95°C for 15 sec and 60°C for 1 minute. Relative mRNA expression was calculated with the Relative Quantification Software using comparative Ct. GAPDH gene is a housekeeping gene and this gene has previously been shown to be appropriate for normalization in human tumor and normal colorectal tissue (Caradec *et al*., 2010).

Table 2. Primer sequences used in qPCR (Ivanova *et al.,* 2007).

Gene	Sequence	PCR product size (bp)
$P^{16/NK4a}$	5′-GTGGACCTGGCTGAGGAG -3′ (sense)	132
	5′-CTTTCAATCGGGGATGTCTG-3′ (antisense)	
GAPDH	5′-GCTCTCTCCTCCTGTTCG-3′ (sense)	115
	5′-ACGACCAAATCCGTTGACTC-3′ (antisense)	

Statistical analysis

Statistical analyses were performed with SPSS statistical software, version 20.0 for Windows (SPSS IBM, IL, USA). Pearson chi-square test was performed to analyze the distribution of hypermethylation in test groups compared with control. Statistical significance was defined as $P < 0.05$.

Results

Patients and tumor characteristics

A total of 140 consecutive surgically resected FFPE tissue specimens comprising of 70 tumor tissues and 70 adjacent normal control regions of primary sporadic CRC were collected for analysis in the present study. Their ages ranged from 35 to 87 years (median, 66 years). Patients included 47 men (67.1%) and 23 women (32.9%). Of the 70 cases analyzed, tumor was located in the colon, rectum and rectosigmoid regions in 55.7%, 31.4% and 12.9% cases, respectively. Histological examination was done in all of the cases to evaluate tumor histotype. Details of each patient related to their demographic profile, clinical characteristics and methylation status of p^{16} in tissues have been shown in table 2.

Methylation status of $P^{16/INK4a}$

Methylation in p^{16} promoter was detected in 38.6% of tumor tissues and %11.4 in adjacent normal control regions. Then aberrant methylation status significantly correlates with occurrence of CRC (p-value 0.000).

A significant number of cases with methylated $P^{16/INK4a}$ gene, 47.1%, had tumor stage II (p-value 0.024), but not significant cases had moderately differentiated grade (p-value 0.747). As it is shown in table 3, the correlation of methylation status with

gender, age and tumor location is also not significant (p-values 0.0102, 0.367 and 0.309, respectively).

Expression analysis of ^{p16}/ ^{INK4a} by qPCR

Table 3. Demographic and clinical characteristics of 70 CRC tissues.

Patient characteristic	No. of samples (%)	$P^{16/INK4a}$ methylatation status (tumor tissue)		P value Pearson Chi - Square
		No. of M (%)	No. of UM (%)	
Total	70 (100)	27(38.6)	43 (61.4)	
Gender				
Male	47 (67.1)	15	32	0.102
Female	23 (32.9)	12	11	
Age				
≤50	28 (40)	9	12	0.367
50 <	42 (60)	16	24	
Tumor location				
Colon	39 (55.7)	12	27	0.309
Rectum	22 (31.4)	11	11	
Rectosigmoid	9 (12.9)	4	5	
Tumor Stage				
I	15 (21.4)	4	11	0.024
II	33 (47.1)	13	20	
III	17 (24.3)	5	12	
IV	5 (7.1)	5	0	
Tumor grade#				
WD	20 (28.6)	9	11	0.747
MD	33 (47.1)	13	20	
PD	12 (17.1)	4	8	
U	5 (7.1)	1	4	

UM, unmethylated; M, methylated; #WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; U, undifferentiated. p value <0.05 was taken as significant

We performed a quantitative -PCR assay to analyze the mRNA expression of *P16* in FFPE tissue samples of colorectal cancer and normal adjacent tissue. We could detect *P16* mRNA expression in normal tissue than tumor is 8.7- fold. Therefore, *P16* expression was significantly reduced in colorectal cancer compared to normal adjacent tissue. As expected, expression of *P16* mRNA was clearly decreased in colorectal cancer stage II compared to the expression in normal adjacent tissue. The amount of P^{16} mRNA did not differ significantly between tumor grades.

Discussion

As it is mentioned above, colorectal cancer (CRC) is one of the most important problems for human and there has been a dramatic increase in the incidence of

CRC in developing countries, but its main cause is not known. Thus, the present study was performed to investigate the molecular genetic alterations responsible for the development of CRC in the Iranian population. p^{16} protein is a cell cycle regulator and inactivation of this gene leads to uncontrolled cell proliferation and growth. The inactivation of P^{16} is mainly associated with aberrant promoter methylation (Nakamura *et al*., 1999; Matsuda *et al*., 1999; Zöchbauer-Müller *et al*., 2001; Esteller *et al*., 2001; Nakahara *et al*., 2001; Nielsen *et al*., 2001; Tannapfel *et al*., 2000; Trzeciak *et al*., 2001). Therefore in the present study the authors analyzed the promoter methylation and expression of the *p16* gene in colorectal cancer in Iranian patients.

In the present study MS PCR was used to assay methyaltion status of P^{16}. The prevalence hypermethylation of P^{16} in promoter region in colorectal tumors, reported in some studies. In our study 40% of cases had aberrant methylated in promoter of P^{16}, in European it ranged from 32%-37% (Esteller *et al*., 2001; Nakahara *et al*., 2001; Nielsen *et al*., 2001; Tannapfel *et al*., 2000; Trzeciak *et al*., 2001; Muto *et al*., 2000; Sanchez-Cespedes *et al*., 2000; Guo *et al*., 2000), 19%-36% in US (Wiencke *et al*., 1999; Ashktorab *et al*., 2003; Van Rijnsoever *et al*., 2003) and 29%-42% in Asian population (Liang *et al*., 1999; Yi *et al*., 2001. The percentage of *p16* methylation in our study located within the range of those mentioned in the literature. Similar results were also reported by other investigators (Wiencke *et al*., 1999). This genetic aberration was found to be significantly correlated with the stage of the tumor but not with the other clinicopathological parameters examined.

In this study for first time, expression of P^{16} in CRC was investigated by q-PCR, but other studies by immunohistochemistry method have investigated it (Tada *et al*., 2003; Zhao *et al*., 2003). The results of present study have shown a significant correlation between aberrant methylation and reduced expression of p^{16}. p^{16} methylated tumors showed reduced expression. Most studies have suggested that detectable p^{16} gene methylation is necessarily linked to the inactivation of p^{16} protein or transcriptional silencing of p^{16} gene (El-Naggar *et al*., 1997; Shim *et al*., 2000). Coexistence of p^{16} gene methylation and p^{16} expression in the same specimen has also been frequently described (El-Naggar *et al*., 1997) and this might reflect the cell heterogeneity, in which some cells contained showed p^{16} gene methylation and loss of p16 expression whereas others expressed or even overexpressed p^{16} protein. In one study (Ohhara *et al*., 1996) has proposed that activation but not inactivation of the p^{16} gene was associated with primary CRC. But as mentioned above in the present study, we found that the methylation of the p^{16} gene results in reduced expression of p16 mRNA. The reduced expression of p^{16} in some tumors lacking methylation suggested that not only the methylation but also genetic alterations are responsible. This other genetic alteration could possibility be mutation or polymorphism in the promoter or coding region of p^{16} gene which could reduce the expression of p^{16} mRNA in tumors lacking methylation. The overexpression of p16 protein in some of the tumors lacking methylation indicated that the activation, but not the inactivation, of the gene was associated with the overexpression and tumor progression. Taken together these results suggest that p^{16} hypermethylation may contribute to reduced expression of p^{16}. The illustration of the relationship between p^{16} expression and *p16* gene methylation in primary tumors requires further studies on a large number of samples and this may certainly help us to better understand the role of methylation of tumor suppressor genes in carcinogenesis.

Some investigators have shown that hypermethylation of the p^{16} gene was associated with advanced tumor stage (Liang *et al*., 1999; Yi *et al*., 2001). In our study also it established that there is this relationship.

The present study determined that there was aberrant methylation in the adjoining tissues, too, and it

suggests that normal tissues may contaminant with tumor tissue. This is the first study in the Iranian population in which p^{16} gene has been analyzed at both epigenetic and expression level in CRC in relation to clinicopathological features and prognosis and confined to North East Iran. We need more data from the other parts of the country to validate our findings.

In conclusion, our study demonstrated that aberrant methylation P^{16} can reduce its expression among tumor tissues in CRC, and may contribute to tumor stage progression.

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References

An C, Choi IS, Yao JC, Worah S, Xie K, Mansfield PF. 2005. Prognostic significance of CpG island methylator phenotype and microsatellite instability in gastric carcinoma. Clinical Cancer Research **11,** 656-663.

Ashktorab H, Smoot DT, Carethers JM, Rahmanian M, Kittles R, Vosganian G, Doura M, Nidhiry E, Naab T, Momen B. 2003. Highmincidence of microsatellite instability in colorectal cancer from African Americans. Clinical Cancer Research **9,** 1112-1117.

Baylin SB. 2005. DNA methylation and gene silencing in cancer. Nature Clinical Practice Oncology **2,** 104-111.

Baylin SB, Ohm JE. 2006. Epigenetic gene silencing in cancer – a mechanism for early oncogenic pathway addiction? Nature Reviews Cancer **6,** 107-116.

Caradec J, Sirab N, Revaud D, Keumeugni C, Loric S. 2010. Is *GAPDH* a relevant housekeeping gene for normalization in colorectal cancer experiments? British Journal of Cancer **103,** 1475 – 1476.

Changmei G, Jiachun L, Tianpen C, Cheng L, Hao S, Wenmao X, Xueli Y, Xiaobo Y, Yangxin H, Meixia L. 2013. Association between *MGMT* Promoter Methylation and Non-Small Cell Lung Cancer: A Meta-Analysis. PLOS ONE **8 (9),** 72-633.

Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL. 2010. Cancer Staging Handbook: From the AJCC Cancer Staging Manual.: Springer Publishing Company, 173–206

El-Naggar AK, Lai S, Clayman G, Lee JK, Luna MA, Goepfert H, Batsakis JG. 1997. Methylation, a major mechanism of *p16/CDKN2* gene inactivation in head and neck squamous carcinoma. American Journal of Pathology **151,** 1767-1774.

Esteller M, González S, Risques RA, Marcuello E, Mangues R, Germà JR, Herman JG, Capellà G, Peinado MA. 2001. K-ras and p16 aberrations confer poor prognosis in human colorectal cancer. Journal of Clinical Oncology **19,** 299-304.

Flood DM, Weiss NS, Cook LS. 2000. Colorectal cancer incidence in Asian migrants to the United States and their descendants. Cancer Causes Control **11(5),** 403–411.

Fountain JW, Karayiogou M, Ernstoff MS, Kirkwood JM, Viock DR,Titus-Emstoff,L, Bouchard B, Vijayasaradh S, Houghton AN, Lahti,J. 1992. Homozygous deletions within human chromosome band 9p21 in melanoma. of the National Academy Sciences USA **89,** 1055-10561.

Grulich AE, McCredie M, Coates M. 1995. Cancer incidence in Asian migrants to New South

Wales. Australia. British Journal of Cancer **71(2),** 400–408.

Jemal A, Bray F, Center MM. 2011. Global cancer statistics. Cancer Journal for Clinicians **61(2),** 69–90.

Herman JG, Graff JR, Myohanen S. 1996. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proceedings of the National Academy Sciences USA **93,** 98-216.

Ivanova TA, Golovina DA, Zavalishina LE, Volgareva GM, Katargin AL, Andreeva YY, Frank GA, Kisseljov FL, Kisseljova NP. 2007. Up-regulation of expression and lack of 5' CpG island hypermethylation of *p16 INK4a* in HPV-positive cervical carcinomas. BMC Cancer **7,** 47.

Jundong G, Yanjun W, Siwei Z. Feng H, Hui Z, Hongrui X, Jiacong Y,Linlin S, Weiqiang W, Jun C, Qinghua Z. 2013. Association between P16INK4a Promoter Methylation and Non-Small Cell Lung Cancer: A Meta-Analysis. PLOS ONE **4,** 60-107.

Khor GH. 2009. Chromosome 17 Aberration of Oral Squamous Cell Carcinoma in Malaysia. Global Journal of Health Science **1,** 150-156.

Khor GH, Hassan MI, Siar CH. 2011. p53 Expression as a marker of microinvasion in oral squamous cell carcinoma. Asian Pacific Journal of Cancer Prevention **12,** 1017-1022.

Lee J, Demissie K, Lu SE, Rhoads GG. 2007. Cancer incidence among Korean-American immigrants in the United States and native Koreans in South Korea. Cancer Control **14(1),** 78–85.

Li Z, Fang ZY, Ding Y, Yao WT, Yang Y. 2012. Amplifications of NCOA3 gene in colorectal cancers in a Chinese population. World Journal of Gastroenterology **18,** 855–860.

Liang JT, Chang KJ, Chen JC, Lee CC, Cheng YM, Hsu HC, Wu MS, Wang SM, Lin JT, Cheng AL. 1999. Hypermethylation of the p16 gene in sporadic T3N0M0 stage colorectal cancers: association with DNA replication error and shorter survival. Oncology **57,** 149-156.

Matsuda Y, Ichida T, Matsuzawa J, Sugimura K, Asakura H. 1999. p16(INK4) is inactivated by extensive CpG methylation in human hepatocellular carcinoma. Gastroenterology **116,** 394-400.

Miyamoto K, Ushijima T. 2005. Diagnostic and Therapeutic Applications of Epigenetics. Japanese Journal of Clinical Oncology **35,** 293-301.

Radhakrishnan R, Kabekkodu S, Satyamoorthy K. 2011. DNA hypermethylation as an epigenetic mark for oral cancer diagnosis. Journal of Oral Pathology & Medicine **40,** 665-676.

Mulero-Navarro S, Esteller M. 2008. Epigenetic biomarkers for human cancer: The time is now. Critical Reviews in Oncology/Hematology **68,** 1-11.

Nakahara Y, Shintani S, Mihara M, Ueyama Y, Matsumura T. 2001. High frequency of homozygous deletion and methylation of p16(INK4A) gene in oral squamous cell carcinomas. Cancer Letter **163,** 221-228.

Nakamura M, Sugita K, Inukai T, Goi K, Iijima K, Tezuka T, Kojika S, Shiraishi K, Miyamoto N, Karakida N. 1999. p16/MTS1/INK4A gene is frequently inactivated by hypermethylation in childhood acute lymphoblastic leukemia with 11q23 translocation. Leukemia **13,** 884-890.

Nielsen NH, Roos G, Emdin SO, Landberg G. 2001. Methylation of the p16(Ink4a) tumor suppressor gene 5'-CpG island in breast cancer. Cancer Letter **163,** 59-69.

Ohhara M, Esumi M, Kurosu Y. 1996. Activation but not inactivation of the MTS1 gene is associated with primary colorectal carcinomas. Biochemical and Biophysical Research Communication **226,** 791-795.

Ovchinnikov DA, Cooper MA, Pandit P. 2011. Tumor-suppressor Gene Promoter Hypermethylation in Saliva of Head and Neck Cancer Patients. Translational oncology **5,** 321-326.

Rasti M, Entezam M, Monabati A. 2009. Hypermethylation of E-Cadherin and Estrogen Receptor-a Gene Promoter and Its Association with Clinicopathological Features of Breast Cancer in Iranian Patients. *International Journal of Molecular Sciences* **34(3),** 50-56.

Serrano M., Hannon G J, Beach D. 1993. A new regulatory motif in cell-cycle control causing specific inhibitionof cyclin D/CDK4. Nature (Land.) **366,** 704-707.

Shim YH, Kang GH, Ro JY. 2000. Correlation of p16 hypermethylation with p16 protein loss in sporadic gastric carcinomas. Laboratory Investigation **80,** 689-695.

Tada T, Watanabe T, Kazama S, Kanazawa T, Hata K, Komuro Y, Nagawa H. 2003. Reduced p16 expression correlates with lymphatic invasion in colorectal cancers. Hepatogastroenterology **50,** 1756-1760.

Tannapfel A, Benicke M, Katalinic A, Uhlmann D, Köckerling F, Hauss J, Wittekind C. 2001. Frequency of p16(INK4A) alterations and K-ras mutations in intrahepatic cholangiocarcinoma of the liver. Gut **47,** 721-727.

Trzeciak L, Hennig E, Kolodziejski J, Nowacki M, Ostrowski J. 2001. Mutations, methylation and expression of CDKN2a/p16 gene in colorectal cancer and normal colonic mucosa. Cancer Letter **163,** 17-23.

van der Riet P, Nawroz H, Hruban R H, Corio R, Tokino K, Koch w, Sidransky D. 1994. Frequent loss of chromosome 9p2l-22 early in head and neck cancer progression. Cancer Research **54,** 1156-1158.

Van Rijnsoever M, Elsaleh H, Joseph D, McCaul K, Iacopetta B. 2003. CpG island methylator phenotype is an independent predictor of survival benefit from 5-fluorouracil in stage III colorectal cancer. Clinical Cancer Res **9,** 2898-2903.

Wani HA, Beigh MA, Amin S, Bhat AA, Bhat S, Khan H, Mattoo AA, Showkat M, Masood A, Majid S. 2013. Methylation profile of promoter region of p16 gene in colorectal cancer patients of Kashmir valley. Journal of Biological Regulation Homeostais Agents **27(2),** 297-307.

Wiencke JK, Zheng S, Lafuente A, Lafuente MJ, Grudzen C, Wrensch MR, Miike R, Ballesta A, Trias M. 1999. Aberrant methylation of $p^{16INK4a}$ in anatomic and gender-specific subtypes of sporadic colorectal cancer. Cancer Epidemiological Biomarkers Prevention **8,** 501-506.

Xing XB, Cai WB, Luo L, Liu LS, Shi HJ, Chen MH. 2013. The Prognostic Value of p16 Hypermethylation in Cancer: A Meta-Analysis. PLoS One **8(6),** e66587.

Yi J, Wang ZW, Cang H, Chen YY, Zhao R, Yu BM, Tang XM. 2001. p16 gene methylation in colorectal cancers associated with Duke's staging. World Journal of Gastroenterology **7,** 722-725.

Yu J, Zhang HY, Sun MH, Gu J, Du X, Shi DR, Wang P, Yang ZH, Zhu JD. 2004. Methylation profile of the promoter CpG islands of 31 genes that may contribute to colorectal carcinogenesis. World Journal of Gastroenterology **10(23),** 3441-54**.**

Zhao P, Hu YC, Talbot IC. 2003. Expressing patterns of p16 and CDK4 correlated to prognosis in

colorectal carcinoma. World Journal of Gastroenterology **9,** 2202-2206.

Zöchbauer-Müller S, Fong KM, Virmani AK, Geradts J, Gazdar AF, Minna JD. 2001. Aberrant promoter methylation of multiple genes in non-small cell lung cancers. Cancer Research **61,** 249-255.