Methylenetetrahydrofolate reductase (MTHFR) polymorphisms and promoter methylation in cervical oncogenic lesions and cancer

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Abstract

The aim of this study was to investigate the role of methylenetetrahydrofolate reductase (MTHFR) polymorphisms and MTHFR methylation pattern in cervical lesions development among women from Romania, a country with high prevalence of human papillomavirus (HPV) cervical infections. To achieve this goal, blood samples and cervical cytology specimens (n = 77)/tumour tissue specimens (n = 23) were investigated. As control, blood and negative cytological smears (n = 50) were used. A statistically significant association was found between T allele of C677T polymorphism and cervical lesions, heterozygote women presenting a threefold increased risk (normal/cervical lesions and tumours: wild homozygote 34/41 (0.68/0.41), heterozygote 14/51 (0.28/0.51), mutant homozygote 2/8 (0.04/0.08); OR = 3.081, P = 0.0035). Using χ² square test for the control group, the HPV-negative and HPV-positive patients with cervix lesions, a significant correlation between viral infection and T allele of C677T polymorphism (P = 0.0287) was found. The MTHFR promoter was methylated in all HGSIL and tumour samples, significant differences being noted between HPV-positive samples, control group and cases of cervical dysplastic lesions without HPV DNA (P < 0.0001) and between samples from patients with high-risk (hr)HPV versus low-risk (lr)HPV (P = 0.0026). No correlations between polymorphisms and methylation were observed. In Romania, individuals carrying T allele are susceptible for cervical lesions. MTHFR promoter methylation is associated with cervical severity lesions and with hrHPV.

Keywords: MTHFR polymorphism • hrHPV • cervical cancer • DNA methylation

Introduction

Although cervical cancer is a model for early cancer detection, it remains one of the most important causes of mortality affecting women worldwide. Molecular and epidemiological data proved that human papillomavirus (HPV) is the aetiological agent of cervical cancer [1]. Many women are infected with HPV, but only a minority develop cervical cancer; therefore other factors like HPV genotypes, multiple sexual partners, early start of sexual life, multiple pregnancies, diet, smoking and oral contraceptives [2–5] are linked with cervical oncogenesis. Among host factors, immunomodulatory and metabolic pathways might be determinants for the susceptibility to the development of cervical neoplasia or cancer.

In this context, some investigations have been focused on the role of folate in carcinogenesis, including cervical one [6]. Folate is essential for the synthesis of nucleotides, its deficit inducing double-strand breaks and increased cancer risk. Low levels of folate correlate with alterations in cell replication, DNA excision and repair and DNA methylation pattern [7]. The metabolism of folate is regulated by methylenetetrahydrofolate reductase (MTHFR), an enzyme that catalyses the reduction of 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methylTHF, the methyl donor for methionine synthesis from homocysteine [8]. DNA methylation also depends on methyl-group donor S-adenosylmethionine (SAM). The methyl group used by SAM for methylation reactions is mainly derived from folate metabolism [9].

Although multiple polymorphisms in MTHFR gene were described, epidemiologic studies have shown that only two of them
affect the enzymatic activity and are related to different disorders including malignancies [10–12]. These polymorphisms are C677T (which results in an alanine-to-valine substitution) and A1298C (leading to the replacement of glutamine by alanine). A1298C polymorphism has a lower effect in reducing enzyme activity, compared with the C677T mutation, which determined different enzyme thermostability degree (assessed as residual activity after heat inactivation). The enzyme residual activity is almost 18–22% in 677TT individuals compared with heterozygous 677CT (56%) and normal 677CC (66–67%) [13, 14].

Whereas 677CT heterozygote patients were reported to have mild elevation levels of plasma homocysteine and non-methylated folate in red blood cells (both conditions involving impairment in 5-methylTHF synthesis) [14], 677TT genotype was frequently associated with different types of cancer (colorectal cancer, hepatocellular carcinoma) and cardiovascular diseases [14–16]. On the other hand, A1298C polymorphism correlates with ovarian cancer and lymphoblastic acute leukemia [17–19].

It is already known that MTHFR polymorphisms can modulate DNA methylation status and the alteration of DNA methylation pattern is associated with carcinogenesis [20]. Maintaining the DNA methylation pattern is a major feature of epigenetic control on genome stability and gene transcription regulation [21]. In addition, the MTHFR gene may be subjected to promoter CpG island methylation. To our knowledge, little is known regarding the involvement of MTHFR gene promoter methylation in cancer. The MTHFR promoter was found to be hypermethylated in renal tumours [22] and in male infertility [23]. Sun et al. performed an integrated analysis of gene expression related to CpG islands methylation. They concluded that differentially expressed genes which exhibited differential methylation pattern of one or more CpG islands within the region promoter of the gene, presented an inversely correlation between mRNA abundance and CpG methylation status [24]. On the other hand, reduced expression of metabolic enzyme genes, including MTHFR, was significantly associated with oesophageal squamous cell carcinoma [25].

This study aimed to investigate MTHFR polymorphisms and the methylation pattern of MTHFR promoter in relationship with susceptibility to cervical lesions and cervix papillomaviruses infection in Romania, which has one of the highest rates of cervical cancer in Europe (incidence 33.9/100,000; mortality 16.9/100,000 – Romanian Statistic Annual 2004).

Materials and methods

Sample collection

Investigated patients were selected based on suggestive cervical pathology for human papillomavirus infection. Cervical cytology specimens (n = 77) from women with dysplastic cervical lesions were collected by a gynaecologist, and conventional cytological screening or histological exams of the samples were performed by a trained pathologist. According to cytological investigation, the cervical samples were divided as: ASCUS (atypical squamous cells of undetermined significance) (21/77, age range: 18–58 years, median: 30), LGSIL (low-grade squamous intraepithelial lesion) (27/77, age range: 18–63 years, median: 35) and HSIL (high-grade squamous intraepithelial lesion) (26/77, age range: 25–60 years, median: 36). Tissue specimens (n = 23) were obtained from patients with squamous cervical carcinoma (SCC) who underwent surgery for cervix tumour mass removal (age range: 22–69 years, median: 36). Tumours were immediately stored at −80°C.

Control group consisted of women (n = 50) with negative cytological smears attending gynaecological clinic for regular investigations (age range: 20–53 years, median: 35). For nucleic acids preservation all samples were collected in sterile tubes with ESwab Collection and Transport System medium (Copan, Brescia, Italy). Blood samples used for the detection of MTHFR polymorphisms were collected on EDTA from all the women enrolled in this study. No age or ethnicity exclusion criteria were used.

DNA isolation

DNA isolation from blood and cervical samples (smears and tumours) was performed with High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Mannheim, Germany), according to manufacturer’s recommendations. Isolated DNAs were subsequently stored at −20°C. The concentration and purity of each DNA sample was evaluated with NanoDrop spectrophotometer (NanoDropTechnologies, Montchanin, DE, USA).

HPV genotyping

Human papillomavirus genotyping was performed with Linear Array HPV Genotyping Test (Roche Molecular Biochemicals, Mannheim, Germany), according to manufacturer’s instructions. A pool of biotinylated primers amplify near 450 base pairs from L1 gene of 37 HPV genotypes including 13 high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). An additional primer pair targets the human β-globin gene to provide a control for cell adequacy, extraction and amplification.

Bisulphite treatment

Bisulphite treatment for unmethylated C residues conversion was realized with EpiTect Bisulfite kit (Qiagen, Valencia, California, USA). Seven-hundred nanogram of each isolated DNA (from cervical swabs and tumours) in a maximum volume of 20 μl was bisulphite treated along with positive (CpGenome Universal Methylated DNA) and negative (CpGenome Universal Unmethylated DNA) controls (Millipore, Billerica, MA, USA).

Primers

Primers to distinguish methylated and unmethylated status of MTHFR following bisulphite treatments were designed using Methprimer (Li and Dahiya, 2002) and were synthesized by Invitrogen Corporation (Carlsbad, CA, USA):

- MTHFR methylated FW 5′-TAGATTAGGTAGTTAGTAGGTAAGAC-3′;
- MTHFR methylated R 5′-GAAACACTATATAAAACCGAGCA-3′.
MTHFR unmethylated FW 5′-TTAGGTATGTAAGTAGGTTAGTGT-3′.
MTHFR unmethylated R 5′-CAAAAAACTAA TAAAAACCAACAA-3′.

Methylation-Specific PCR (MS-PCR)

Methylation-specific PCR was performed in a final volume of 25 μl using Platinum Taq DNA Polymerase (1 U), 1× enzyme buffer, 1.5 mM MgCl₂, 200 μM of each dNTP, 0.3 μM of each specific primers, 5 μl target DNA. The PCR conditions were as follows: 95°C – 5 min., 35 cycles of 95°C – 30 sec., 66°C – 30 sec., 72°C -30 sec. and a final elongation of 72°C – 7 min. The methylated/unmethylated status of the target gene was estimated in 2% agarose gel electrophoresis.

Direct Q-MSP of Genomic DNA

Direct Q-MSP of genomic DNA was used for evaluating the degree of sample methylation. Standard curves were designed using serial dilution (10 pg, 100 pg, 1 ng and 10 ng) of positive (DNA fully methylated) and negative controls (DNA fully unmethylated), according to Applied Biosystem Guide (Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System, Foster City, CA, USA). The quantity of unmethylated and methylated DNA for each patient sample was extrapolated using the standard curves.

Direct Q-MSP was performed in 50 μl final volume comprising 50 ng/μl DNA, 25 μl FastStart Universal SYBR Green Master (ROX; Roche Molecular Biochemicals, Mannheim, Germany) and 0.3 μM primers. PCR parameters we used were as follows: 95°C/10 min. (1 cycle), 40 cycles at 95°C/15 sec. and 60 sec./specific annealing temperature.

qPCR experiments were performed in duplicate and mean values were used for calculations. To control the quality of DNA samples we used ACTB gene as reference [26].

Methylation percentage

Methylation percentage was calculated according to method described by Fackler et al. (% M = 100× [ng methylated gene A/(ng methylated gene A+ unmethylated gene A)]) [26].

MTHFR polymorphisms (C677T and A1298C)

Methylenetetrahydrofolate reductase polymorphisms (C677T and A1298C) were performed in PCR – RFLP technique, according to Frost et al. [14]. The primers’ sequences for C677T mutation were as follows: FW 5′-TGAAGGAAGTTGCTGGGGA-3′ and R 5′-AGGCCGCT GCCTGAGATG-3′ whereas for A1298C polymorphism we used: FW 5′-GGGAGGTGACAGCAGCAGCAG-3′ and R 5′-GGCGGTCAGGCA GGGGCG -3′. The PCR conditions were as follows: for C677T polymorphism, 30 cycles of 94°C/45 sec., 55°C/45 sec., 72°C/45 sec.; for A1298C polymorphism, 35 cycles of 94°C/45 sec., 65°C/45 sec., 72°C/45 sec. 198 bp amplicons for C677T polymorphism and 138 bp amplicons for A1298C polymorphism were subjected to enzymatic restriction with HinfI and Fnu4HI, respectively, according to manufacturer’s instructions (New England Biolabs). Following digestion, the samples were evaluated in 3% agarose gel electrophoresis. Heterozygote (CT) presented three lanes of 198 pb, 175 bp and 23 bp whereas heterozygote (AC) displayed 138, 119 and 19 bp lanes.

Statistical analysis

Statistical analysis was performed with GraphPadInstat3. The association between MTHFR polymorphisms and Pap test was analysed using Fisher’s exact test, wherever appropriate. The confidence interval was established at 95%. ANOVA (Kruskal–Wallis and Mann–Whitney tests) was used to compare the variance between the investigated groups. The biological samples were provided with patient’s agreement and the study protocol was approved by the Ethics Committee.

Results

HPV testing

Viral test results for each category of investigated patients are presented in Table 1. In the study group 75/100 patients were HPV positive. The overall prevalence (single and co-infections) of hrHPV genotypes was as follows: 32% HPV16 (16.7% in ASCUS, 7.4% in LSIL, 42.3% in HSIL and 65.2% in SCC), 11.8% HPV 31; 9.2% HPV 18; 9.2% HPV 66; 3.9% HPV 33 and 3.9% HPV 51.

MTHFR polymorphisms and their impact on cervical tumourigenesis

The analysis of MTHFR C677T polymorphism in all patients included into the study revealed that 75 cases were homozygote for normal allele (CC), 65 were heterozygote (CT) and 10 were mutant homozygote (TT). The calculated frequency of C allele in population was 0.72, whereas the frequency of T allele was 0.28. The χ² test was performed with 1 degree of freedom and 5% level of significance (3.84). The value of χ² test was 0.674 (below 3.8), the population being in Hardy–Weinberg equilibrium. The calculated frequency of A allele for 1298 polymorphism in our patients was 0.75, whereas the frequency of C allele was 0.25. Eighty-one of the investigated patients were normal homozygous (AA), 63 were heterozygous (AC) and 6 were mutant

| HPV (+) (%) | HPV single infection/total HPV-positive | HPV co-infections/total HPV-positive |
|------------|---------------------------------------|-------------------------------------|
| ASCUS      | (62.5)  15/24                         | 8/15                                |
| LGSIL      | (73)  19/26                           | 11/19                               |
| HSIL       | (77.8) 21/27                          | 15/21                               |
| SCC        | (87)  20/23                           | 13/20                               |
homzygous (CC). The population was in Hardy–Weinberg equilibrium, the value of $\chi^2$ test being 2.16.

When comparing patients with cervical lesions (including cervical cancer) with the normal group, a statistically significant association was found between $T$ allele of $C677T$ polymorphism and cervical lesions, heterozygote women presenting a threefold increased risk (normal/cervical lesions and tumours: wild homozygote 34/41 (0.68/0.41), heterozygote 14/51 (0.28/0.51), mutant homozygote 2/8 (0.04/0.08); OR = 3.081, $P = 0.0035$). By contrast, the MTHFR polymorphism $A1298C$ was not associated with cervical disease development (normal/cervical lesions and tumours: wild homozygote 30/51 (0.60/0.44), heterozygote 19/44 (0.41/0.51), mutant homozygote 1/5 (0.02/0.06); OR = 1.441, $P = 0.2278$).

Furthermore, we evaluated a possible link between MTHFR polymorphisms and HPV infection. Using chi-square test for control group, HPV-negative and HPV-positive patients with cervix lesions, we found no correlation ($\chi^2$ test to compare the MTHFR polymorphisms and HPV type morphisms in the investigated groups are presented in Table 2: using chi-square test for control group and in human papillomavirus (HPV)-positive and -negative patients with cervix lesions, heterozygote women presenting a threefold increased risk of cervical lesions without HPV DNA ($P < 0.0001$). Moreover, statistic differences ($P = 0.0026$) between samples from patients with hrHPV infection versus lrHPV were obtained (Table 4).

We have to mention that some samples presented % M levels below the cut-off. Using Kruskal–Wallis for MTHFR promoter methylation we noted highly significant differences in median data between different cytological groups (Table 3).

Table 2 Contingency table of the methylenetetrahydrofolate reductase (MTHFR) polymorphisms in control group and in human papillomavirus (HPV)-positive and -negative patients

| MTHFR $C677T$ | $\chi^2$ | $P$ value | MTHFR $A1298C$ | $\chi^2$ | $P$ value |
|---------------|----------|-----------|----------------|----------|-----------|
| Control group | CC CC CC CC | 10.82 | 0.0287 | AA AA AA AA | 7.18 | 0.1267 |
| HPV– cervical lesions | CT CT CT CT | | | AC AC AC AC | | |
| HPV+ cervical lesions | TT TT TT TT | | | CC CC CC CC | | |

Table 3 Methylenetetrahydrofolate reductase (MTHFR) methylation values in the investigated groups

| Range | Median | Mean ± SE | Lower 95% confidence limit | Upper 95% confidence limit | $P$ value |
|-------|--------|-----------|---------------------------|---------------------------|-----------|
| Normal | 0–10 0 | 0.8716 ± 0.4685 | −0.095 | 1.839 | <0.0001 |
| ASCUS | 0–100 | 36.07 | 38.62 ± 6.666 | 24.83 | 52.41 | <0.0001 |
| LGSIL | 0–65.38 | 15.11 | 22.08 ± 3.966 | 13.91 | 30.25 | <0.0002 |
| HGSIL | 22.98–100 | 56.15 | 57.13 ± 4.700 | 47.47 | 66.79 | <0.0001 |
| SSC | 34.55–100 | 75.01 | 73.75 ± 4.034 | 65.39 | 82.12 | <0.0001 |

The methylation percentage (% M) value for negative standard was approximately 0 and no amplification of methylated standard with unmethylated primers was obtained. We validated our data regarding quantitative methylation analysis based on the similar differences between $\Delta Ct$ values ($\Delta Ct = CtM – CtU$) for all dilutions ($\Delta Ct \approx 0.726$). On the other hand the slope value for all the standard curves were around the −3.16 value that means a twofold increase in PCR product per cycle during the linear phase of real-time PCR. The average of calculated efficiencies was around 107.3. The standard curves showed a correlation coefficient ($R^2$) around 0.999 providing evidence of linearity over the entire range of template concentration.

To eliminate false-positive % M values in cervical samples due to blood cell infiltration/contamination, a cut-off value of 1.569 was established. The cut-off was calculated as the 95th percentile from blood % M values of MTHFR promoter in the blood samples of the patients. We have to mention that some samples presented % M levels below the cut-off. The methylation percentage (% M) value for negative standard was approximately 0 and no amplification of methylated standard with unmethylated primers was obtained. We validated our data regarding quantitative methylation analysis based on the similar differences between $\Delta Ct$ values ($\Delta Ct = CtM – CtU$) for all dilutions ($\Delta Ct \approx 0.726$). On the other hand the slope value for all the standard curves were around the −3.16 value that means a twofold increase in PCR product per cycle during the linear phase of real-time PCR. The average of calculated efficiencies was around 107.3. The standard curves showed a correlation coefficient ($R^2$) around 0.999 providing evidence of linearity over the entire range of template concentration.

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Considering positive for methylation all the cervical samples over cut-off, the methylated cases were as follows: 2/25 of control group, 18/24 ASCUS patients and 18/26 LGSIL patients. All HGSIL and tumour samples presented MTHFR promoter methylation, 14/27 HSIL samples and 18/23 tumours displaying more than >60% methylation percentage.

Significant differences in methylation were found between HPV-positive samples, control group and cases of cervical dysplastic lesions without HPV DNA ($P < 0.0001$). Moreover, statistic differences ($P = 0.0026$) between samples from patients with hrHPV infection versus lrHPV were obtained (Table 4).

Although, no significant association between MTHFR methylation and MTHFR polymorphisms was noted, we have to mention that CC...
genotype of A1298C polymorphism presented the highest median value (Table 5).

**Discussion**

The MTHFR gene encodes for a protein with important enzymatic functions in methyl-group metabolism. Numerous studies have addressed the correlation of MTHFR polymorphisms and risk of cancers. On the other hand, the role of persistent hrHPV infections in the development of cervical precursor lesions and cancer is unequivocally demonstrated [27].

The impairment of MTHFR enzymatic activity through C677T polymorphism was frequently associated with different type of malignancy [28–30], but its link to cervical cancer is still under debate, the results being controversial. Whereas some current meta-analysis suggested that C677T MTHFR polymorphism may not be associated with cervical cancer [31], other studies found a correlation (without statistically robustness) between mutant C677T in white women and low risk of cervical cancer [32]. Further meta-analysis highlights the association between C677T MTHFR polymorphism and cervical cancer, emphasizing that this association may be race specific [33]. Taking into account the geographical variability in the MTHFR polymorphisms we found a prevalence of TT homozygosity (C677T polymorphism) in our samples of around 6.67%, which is in agreement with data published by Osian et al. [34]. Still, for CT heterozygosity, we found a higher prevalence (43.33%) in our group. The analysis of C677T polymorphism in correlation with the severity of cervix lesion associate the presence of T allele with cervical cancer susceptibility \( (P = 0.0035) \). Our results are similar with the study of Goodman et al. [35], but are in contrast with those of Lambropoulos et al. [36]. Goodman et al. reported that women with CT genotype had twice the risk of cervical LGSILs, whereas TT genotype women had almost three times the risk of LGSILs compared with women with the homozygous MTHFR CC genotype [35].

We investigated patients with different cytology, including cancer. Overall, for both CT and TT genotypes we found a threefold increased risk for cervical lesion development. These observations are in contrast with Agodi et al. [37], who reported that the C677T allele of MTHFR is a protective factor against cervical carcinogenesis. Moreover, the results from our investigation support the hypothesis that individuals carrying the T allele are susceptible for acquiring HPV cervix infection \( (P < 0.001, OR=14.22) \). The susceptibility of C677T mutation carriers individuals to HPV infection is due to genomic instability [21] created by decreased enzyme activity. This is an important finding which suggests that MTHFR polymorphism do not cause only cancer susceptibility but also for acquiring viral infection.

In our study, HPV-positive women who had at least one variant of T allele (C677T polymorphism) had a 14-fold increased risk \( (P < 0.001, OR=14.22) \) for cervical dysplasia compared with HPV-negative women with the CC genotype. On the other hand, the T allele is almost sixfold more frequent in patients with tumours than in control group \( (P = 0.0084, OR = 5.667) \). No association between HPV types (high or low risk) and MTHFR polymorphisms was found. Mei et al. highlighted that the association between C677T MTHFR...
polymorphism and cervical cancer could be affected by HPV status. C677T polymorphism might lead to cervical cancer by impairing DNA methylation in both host and viral genomes [33].

On the other hand, our findings concerning the Romanian women assert the results of Kang et al. [38], who did not found any association between MTHFR A1298C polymorphism and cervical cancer. Gathering these findings, we support the idea that MTHFR polymorphism presence could represent a risk factor for cervical HPV acquisition and persistence. Furthermore, it is assumed that MTHFR plays an important role in DNA methylation, an important epigenetic mechanism which modulates gene expression. Differences in genome-wide methylation are linked with pathological conditions, including cervical neoplasia [39]. As aberrant methylation of tumour-related genes has been recognized as an important mechanism associated with the development and progression of cancer [40], methylation of CpG islands from MTHFR gene promoter is also a result of the oncogenesis process. Thus, the MTHFR methylation analysis can be included in a panel of hypermethylated genes, which may be useful for a sensitive and specific diagnosis of cervical lesions.

We found that MTHFR hypermethylation is rare in LGSIL lesion, but more frequent in HGSIL and cancer lesions. Significant differences were observed between HPV-positive samples, control group and HPV-negative dysplastic lesions ($P < 0.0001$). These findings support the involvement of papillomaviruses in oncogenesis, a process associated with aberrant methylation. No association between MTHFR genotype and MTHFR methylation was found. Therefore, we concluded that MTHFR polymorphism is a factor for promoting cervical dysplasia, but promoter methylation might result from cervical lesion severity linked to viral infection. This scenario is supported by the fact that the methylation level of MTHFR seems to vary between high-risk and low-risk HPV types. Moreover, the most powerful methylation HPV type is HPV16, in single- and co-infections. To our knowledge, these data has not been reported before.

**Conclusion**

Our study shows that the C677T and not the A1298C MTHFR polymorphism represents a risk factor of cervical dysplasia. MTHFR promoter methylation varies between high-risk and low-risk HPV types, with HPV 16 being linked to most methylation changes in the promoter. No link between MTHFR polymorphism at C677T and MTHFR promoter methylation was observed. Given that in Romania a high prevalence of HPV infection is found, our patient cohort might prove valuable for studies of the dynamics of MTHFR promoter methylation.

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**Conflict of interest**

The authors declare no conflicts of interest.

**References**

1. zur Hausen H. Human papilloma viruses and their possible role in squamous cell carcinomas. *Curr Top Microbiol Immunol*. 1977; 78: 1–30.
2. Chan K, Monk BJ, Brewer C, et al. HPV infection and number of lifetime sexual partners are strong predictors for natural regression of CIN 2 and 3. *Br J Cancer*. 2003; 89: 1062–6.
3. Kjellberg L, Wang Z, Wiklund F, et al. Sexual behaviour and papillomavirus exposure in cervical intraepithelial neoplasia: a population-based case-control study. *J Gen Virol*. 1999; 80: 391–8.
4. Slattery ML, Robison LM, Schuman KL, et al. Cigarette smoking and exposure to passive smoke are risk factors for cervical cancer. *JAMA*. 1989; 261: 1593–8.
5. Brinton LA, Reeves WC, Brenes MM, et al. Oral contraceptive use and risk of invasive cervical cancer. *Int J Epidemiol*. 1990; 19: 4–11.
6. Alberg AJ, Selhub J, Shah KV, et al. The risk of cervical cancer in relation to serum concentrations of folate, vitamin B12, and homocysteine. *Cancer Epidemiol Biomarkers Prev*. 2000; 9: 761–4.
7. Fowler BM, Giuliano AR, Piyathilake C, et al. Hypomethylation in cervical tissue: is there a correlation with folate status? *Cancer Epidem Biol Markers*. 1998; 7: 901–6.
8. Bailey LB, Gregory JF 3rd. Polymorphisms of methylenetetrahydrofolate reductase and other enzymes: metabolic significance, risks and impact on folate requirement. *J Nutr*. 1999; 29: 919–22.
9. Friso S, Chol SW, Girelli D, et al. A common mutation in the 5,10 - methylentetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. *Proc Natl Acad Sci USA*. 2002; 99: 5606–11.
10. Li XL, Xu JH. MTHFR polymorphism and the risk of prostate cancer: a meta-analysis of case-control studies, prostate cancer and prostatic diseases. *Neuromolecular Med*. 2012; 14: 84–90.
11. Chiouso P, Reddicento G, Farina G, et al. MTHFR polymorphisms’ influence on outcome and toxicity in acute lymphoblastic leukemia patients. *Leuk Res*. 2007; 31: 1669–74.
12. Martin DN, Boersma BJ, Howe TM, et al. Association of MTHFR gene polymorphisms with breast cancer survival. *BMC Cancer*. 2006; 6: 257–67.
13. Palomino-Morales R, Gonzalez-Juanatey C, Vazquez-Rodriguez TR, et al. A1298C polymorphism in the MTHFR gene predisposes to cardiovascular risk in rheumatoid arthritis. *Arthritis Res Ther*. 2010; 12: R71.
14. Frostell P, Blom HG, Milos R, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet*. 1995; 10: 11–3.
15. Mu LN, Cao W, Zhang ZF, et al. Methylene-tetrahydrofolate reductase (MTHFR) C677T and A1298C polymorphisms and the risk of primary hepatocellular carcinoma (HCC) in a Chinese population. *Cancer Causes Control*. 2007; 18: 665–75.
16. Lewis SJ, Ebrahim S, Smith GD. Meta-analysis of MTHFR 677C→T polymorphism and coronary heart disease: does totality of
evidence support causal role for homocysteine and preventive potential of folate? BMJ. 2005; 331: 1053.
17. Gershoni-Baruch R, Dagan E, Israel D, et al. Association of the C677T polymorphism in the MTHFR gene with breast and/or ovarian cancer risk in Jewish women. *Eur J Cancer*. 2000; 36: 2313–6.
18. Wiemels J, Smith RN, Taylor GM, et al. Methylene-tetrahydrofolate reductase (MTHFR) polymorphisms and risk of molecularly defined subtypes of childhood acute leukemia. *Proc Natl Acad Sci USA*. 2001; 98: 4004–9.
19. Schnakenberg E, Mehlies A, Carlo G, et al. Polymorphisms of methylenetetrahydrofolate reductase (MTHFR) and susceptibility to pediatric acute lymphoblastic leukemia in a German study population. *BMC Med Genet*. 2005; 6: 23–7.
20. Feinberg AP, Tycko B. The history of cancer epigenetics. *Nat Rev Cancer*. 2004; 4: 143–53.
21. Wolff AP, Matzke MA. Epigenetics: regulation through repression. *Science*. 1999; 286: 481–6.
22. Costa VL, Henrique R, Ribeiro FR, et al. Quantitative promoter methylation analysis of multiple cancer-related genes in renal cell tumors. *BMC Cancer*. 2007; 7: 133.
23. Khazamipour N, Noruzinia M, Fatehmanesh P, et al. MTHFR promoter hypermethylation in testicular biopsies of patients with non-obstructive azospermia: the role of epigenetics in male infertility. *Hum Reprod*. 2009; 24: 2361–4.
24. Sun Z, Asmann YW, Kalari KR, et al. Integrated analysis of gene expression, CpG Island methylation, and gene copy number in breast cancer cells by deep sequencing. *PLoS ONE*. 2011; 6: e17490.
25. Liu R, Yin L, Pu Y. Association between gene expression of metabolizing enzymes and esophageal squamous cell carcinomas in China. *Genet Test Mol Biomarkers*. 2012; 16: 1211–7.
26. Fackler MJ, McVeigh M, Mehrotra J, et al. Quantitative multiplex methylation-specific PCR assay for the detection of promoter hypermethylation in multiple genes in breast cancer. *Cancer Res*. 2004; 64: 4442–52.
27. Schiffman M, Castle PE, Jereimono J, et al. Human papillomavirus and cervical cancer. *Lancet*. 2007; 370: 890–907.
28. Chen J, Giovannucci E, Kelsey K, et al. A methylene-tetrahydrofolate reductase polymorphism and the risk of colorectal cancer. *Cancer Res*. 1996; 56: 4862–4.
29. Kimura F, Franke KH, Steinhoff C, et al. The association between MTHFR 677C>T polymorphism and cervical cancer. *Cancer Res*. 2003; 63: 907.
30. Shen H, Xu Y, Zheng Y, et al. Polymorphisms of 5,10-methylenetetrahydrofolate reductase and risk of gastric cancer in a Chinese population: a case-control study. *Int J Cancer*. 2001; 95: 322–6.
31. Xu XJ, Zou LW, Wang DB. Cervical cancer with polymorphism in MTHFR C677T gene: a systematic review and meta-analysis. *Mol Biol Rep*. 2013; 40: 245–62.
32. Luo YL, Ye P, Zhang OH, et al. Methylenetetrahydrofolate reductase C677T polymorphism and susceptibility to cervical cancer and cervical intraepithelial neoplasia: a meta-analysis. *PLoS ONE*. 2012; 7: e46272.
33. Mei Q, Zhou D, Gao J, et al. The association between MTHFR 677C>T polymorphism and cervical cancer: evidence from a meta-analysis. *BMC Cancer*. 2012; 12: 467.
34. Osian G, Procopciuc L, Vlad L. MTHFR polymorphisms as prognostic factors in sporadic colorectal cancer. *Gastrointestin Liver Dis*. 2007; 16: 251–6.
35. Goodman MT, McDuffie K, Hernandez B, et al. Association of methylenetetrahydrofolate reductase polymorphism C677T and dietary folate with the risk of cervical dysplasia. *Cancer Epidemiol Biomarkers Prev*. 2001; 10: 1275–80.
36. Lambropoulos AF, Agorastos T, Foka ZJ, et al. Methylene-tetrahydrofolate reductase polymorphism C677T is not associated to the risk of cervical dysplasia. *Cancer Lett*. 2003; 191: 187–91.
37. Agodi A, Barchitta M, Cipresso R, et al. Distribution of p53, GST, and MTHFR polymorphisms and risk of cervical intraepithelial lesions in Sicily. *Int J Gynecol Cancer*. 2010; 20: 141–6.
38. Kang S, Kim JW, Kang GH, et al. Polymorphism in folate- and methionine-metabolizing enzyme and aberrant CpG island hypermethylation in uterine cervical cancer. *Gynecol Oncol*. 2005; 96: 173–80.
39. Lai HC, Lin YW, Huang TH, et al. Identification of novel DNA methylation markers in cervical cancer. *Int J Cancer*. 2008; 123: 161–7.
40. Calvisi DF, Simile MM, Luddo S, et al. Altered methionine metabolism and global DNA methylation in liver cancer: relationship with genomic instability and prognosis. *Int J Cancer*. 2007; 121: 2410–20.