Genetic polymorphisms in one-carbon metabolism increased the risk of persistence of pre-neoplastic cervical lesions

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Abstract

Background: Cervical cancer is caused by high-risk Human Papillomavirus (hr-HPV) infection associated with cofactors that has been analyzed as predictors of cytological abnormalities remission or persistence. These cofactors may be classified as environmental, epigenetic or genetic. Polymorphism in genes of enzymes that act on one-carbon metabolism alter their activity and may be associated with cervical carcinogenesis because they affect DNA synthesis and repair, and gene expression. Therefore, the objective of this study was to analyze the risk of persistence of pre-neoplastic cervical lesions according to genetic polymorphisms involved in one-carbon metabolism. Sample group was divided in Remission (n=60) - presence of pre-neoplastic lesion at first meeting (T1), and normal cytology after six months of follow-up (T2), and Persistence (n=46) - presence of pre-neoplastic lesion at T1 and T2. Cervical samples were obtained for cytological analysis (T1 and T2), HPV detection (T1), and evaluation of polymorphism C667T of Methylenetetrahydrofolate Reductase (MTHFR C677T), A2756G of Methionine Synthase (MS A2756G), A66G of Methionine Synthase Reductase (MTRR A66G), double or triple 28 bp tandem repeat in 5'-untranslated enhanced region of Thymidylate Synthase (TSER), and 6 bp deletion at nucleotide1494 in TS 3'-untranslated region (TS3'UTR). Genetic Risk Score (GRS) was calculated for analyze all genetic polymorphism simultaneously.

Results: No differences were observed between Remission and Persistence groups of GRS, or genotypic and allelic distribution of MTHFR C677T and MS A2756G polymorphisms. However, higher risk of persistence was observed among women presenting heterozygote genotype - ins/del [OR (IC95%): 3.22 (1.19 – 8.69), p=0.021], or polymorphic genotype – del/del [OR (IC95%): 6.50 (1.71 – 24.70), p=0.006] of TS3'UTR.

Conclusions: Presence of TS3'UTR polymorphism increased risk of persistence of cervical abnormalities. This genetic variant could be considered as potential marker of cervical carcinogenesis, assisting follow-up of women with persistent pre-neoplastic cervical lesions.

1) Background

Persistent high-risk Human Papillomavirus (hr-HPV) infection is the main cause of cervical cancer. However, only 10% of women with viral infection will develop pre-neoplastic lesions, and less than 1% will progress to cervical cancer. Furthermore, only approximately 30% of high-grade cervical lesions progresses to cancer in uterine cervix, and spontaneous regression occurs in 20–40% of cases (1–4). Thus, risk of cervical carcinogenesis depends on hr-HPV infection and host-dependent features (5, 6). Environmental, genetic and epigenetic cofactors of cervical carcinogenesis has been analyzed as markers for diagnosis, prognosis, and for auxiliary in treatment of pre-neoplastic cervical lesions, since they could be predictors of cytological abnormalities remission or persistence (7–10).

Several genetic alterations characterize cervical cancer and they may have a substantial impact on risk of cervical carcinogenesis, as genomic instability, chromosomal aberration, and integration of HPV DNA into

host genome (4, 7). Polymorphism in genes of enzymes that act on one-carbon metabolism, such as Methylenetetrahydrofolate Reductase (MTHFR), Methionine Synthase (MS), Methionine Synthase Reductase (MTRR), and Thymidylate Synthase (TS), alter their activity and may be associated with cervical carcinogenesis (11–13).

MTHFR enzyme, whose gene is located on chromosome 1p36.3, is a flavoprotein that acts on folate metabolism, being essential for DNA integrity (14). MTHFR C677T polymorphism consists of cytosine (C) exchange for thymine (T) at nucleotide 677, and results in substitution of Alanine for Valine, leading to decrease in MTHFR activity (14, 15). This Single Nucleotide Polymorphism (SNP) may modify the susceptibility to carcinogenesis by modulating the availability of 5,10-methyleneTHF at different points in folate metabolism (16).

MS is a vitamin B12-dependent enzyme, essential to intracellular folate levels maintenance, and catalyzes the methylation of homocysteine to methionine (17, 18). MS A2756G polymorphism is caused by an exchange of adenine (A) for guanine (G) at nucleotide 2756, resulting in the substitution of Aspartic Acid for Glycine, close to the binding domain of vitamin B12 (19, 20). Van Der Put et al. (1997) suggested that this SNP affects the secondary structure of MS, and has functional consequences. An association between the polymorphic allele (G) of MS and reduction of the number of hypermethylated CpG islands in tumor suppressor genes has been demonstrated (21). Thus, it is possible that the presence of MS A2756G alters the activity of tumor suppressor genes, explaining its association with the development of several types of tumor (22).

MTRR catalyzes the methylation of vitamin B12, that is a cofactor of MS enzyme (23). A66G polymorphism of MTRR enzyme (MTRR A66G) leads to exchange of A by G in nucleotide 66, and to substitution of Isoleucine by Methionine, which results in decrease of MTRR affinity by MS (24). Thus, polymorphic genotype was negatively associated with homocysteinemia, which alters DNA methylation and, consequently, gene expression (23, 25).

TS enzyme catalyzes the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), the only de novo source of thymidine for DNA synthesis and repair. TS binds to RNA for repression of translation of its own messenger RNA (mRNA) or other proteins, and can regulate cell cycle progression (26–28). Moreover, TS expression is an index of cell proliferation and biological malignancy of cancer (29). The polymorphisms most frequently studied are double or triple 28 bp tandem repeat in 5'-untranslated enhanced region (TSER), and 6 bp deletion/insertion at nucleotide 1494 in TS 3'-untranslated region (TS3'UTR). These genetic variations may influence the TS gene expression and the stability of its mRNA, respectively (28).

When the one-carbon metabolism is altered, the integrity of genetic material is compromised due to changes in nucleotide pool and uracil incorporation, leading to DNA instability. In addition, global hypomethylation and site-specific hypermethylation are observed, which lead to activation of proto-oncogenes and silencing of tumor suppressor genes (10, 30, 31).

Therefore, this study evaluated the risk of persistence of pre-neoplastic cervical lesions according to genetic polymorphisms involved in one-carbon metabolism.

2) Results

Mean age of participants was 39.7 ± 11.4 years, ranging from 19 to 71, and 32.1% (n=34) were between 35 and 44 years old. Most of women resided in urban area (n=97, 91.5%), had family financial income <US\$250/month (n=70, 74.5%), high school education (n=46, 47.9%), was not smoker (n=81, 84.4%), and ingested alcoholic beverages (n=57, 59.4%). Moreover, a higher percentage of participants were married or had a fixed partner (n=77, 80.2%), reported having had the first sexual intercourse with at least 18 years old (n=53, 56.4%), and three or more sexual partners (n=55, 57.3%). In addition, most of participants was not using hormonal contraceptives (n=61, 63.5%), and have had pregnancies (n=81, 84.4%) (Table 1).

Similar frequencies of these characteristics and no significant association ($p<0.05$) were observed between Remission and Persistence groups (Table 1).

In relation to HPV, 50.9% (n=54) presented viral infection and significate higher frequency were observed among women from Persistence group (n=31, 67.4%) if compared with Remission (n=23, 38.3%) ($p=0.003$). Similar results were obtained analyzing only HPV-AR infection ($p=0.000$) (data not showed).

TABLE 1: Sociodemographic and behavioral characteristics

Characteristics	Total n (%)	Cytological abnormality		p
		Remission n (%)	Persistence n (%)	
Age (years)				
<25	12 (11.3)	7 (11.7)	5 (10.9)	0.302
25-34	21 (19.8)	9 (15.0)	12 (26.1)	
35-44	34 (32.1)	18 (30.0)	16 (34.8)	
45-54	28 (26.4)	17 (28.3)	11 (23.9)	
≥55	11 (10.4)	9 (15.0)	2 (4.3)	
Area				
Urban	97 (91.5)	54 (90.0)	43 (93.5)	0.524
Countryside	9 (8.5)	6 (10.0)	3 (6.5)	
Income per person (US\$/month)¹				
<250	70 (74.5)	40 (72.7)	30 (76.9)	0.333
250-500	21 (22.3)	12 (21.8)	9 (23.1)	
≥500	3 (3.2)	3 (5.5)	0	
Education²				
University	6 (6.3)	5 (9.1)	1 (2.4)	0.341
High school	46 (47.9)	27 (49.1)	19 (46.3)	
Elementary school/Illiterate	44 (45.8)	23 (41.8)	21 (51.2)	
Smoker²				
No	81 (84.4)	48 (87.3)	33 (80.5)	0.365
Yes ^a	15 (15.6)	7 (12.7)	8 (19.5)	
Use of alcoholic beverage²				
No	39 (40.6)	22 (40.0)	17 (41.5)	0.885
Yes ^a	57 (59.4)	33 (60.0)	24 (58.5)	
Marital status²				
Married/Fixed Partner	77 (80.2)	40 (72.7)	37 (90.2)	0.073

Single	9 (9.4)	8 (14.5)	1 (2.4)	
Widow/Divorced	10 (10.4)	7 (12.7)	3 (7.3)	
Age at first vaginal intercourse (years) ¹				
≥18	53 (56.4)	30 (54.5)	23 (59.0)	0.670
<18	41 (43.6)	25 (45.5)	16 (41.0)	
Lifetime sexual partners ²				
1	23 (23.9)	13 (23.6)	10 (24.4)	0.936
2	18 (18.8)	11 (20.0)	7 (17.1)	
≥3	55 (57.3)	31 (56.4)	24 (58.5)	
Use of hormonal contraceptive ²				
No	61 (63.5)	39 (70.9)	22 (53.7)	0.082
Yes	35 (36.5)	16 (29.1)	19 (46.3)	
Pregnancies ²				
0	15 (15.6)	9 (16.4)	6 (14.6)	0.130
1	19 (19.8)	14 (25.5)	5 (12.2)	
2	26 (27.1)	10 (18.2)	16 (39.0)	
3	22 (22.9)	12 (21.8)	10 (24.4)	
≥4	14 (14.6)	10 (18.2)	4 (9.8)	
HPV infection		52 (49.1)		
Negative	54 (50.9)	37 (61.7)	15 (32.6)	0.003
Positive		23 (38.3)	31 (67.4)	

Participants excluded due to absence of information: ¹Twelve; ²Ten. ^aAmount or frequency not determined. Remission: presence of pre-neoplastic lesion at T₁, and normal cytology at T₂; Persistence: pre-neoplastic lesion detected at T₁ and T₂.

MTRR A66G genotypic frequencies were 10.4% (n=11), 76.4% (n=81), and 13.2% (n=14) of AA, AG, and GG, respectively. Genotypic frequency of TSER was 35.8% (n=38) of 2R/2R, 31.1% (n=33) of 2R/3R,

and 33.0% (n=35) of 3R/3R. However, distributions of genotypes of MTRR A66G and TSER of Remission group were not found under Hardy-Weinberg equilibrium (p=0.000). Thus, these polymorphisms were excluded from further analyses of this study.

MTHFR C677T genotypic frequencies were 50.0% (n=53) of CC, 45.3% (n=48) of CT, and 4.7% (n=5) of TT, and T allelic frequency was 27.4%. MS A2756G polymorphic genotype was detected in 3.8% (n=4) of samples, and G allele in 19.8%. On the other hand, higher frequency of women presented polymorphic genotype for TS3'UTR genetic variation (16.0%, n=17), and del allelic frequency of 41.5% was observed (Table 2).

TABLE 2: Genotypic and allelic frequencies, and GRS, according Remission or Persistence of pre-neoplastic cervical lesions.

Polymorphisms		Cytological abnormality			OR (IC95%) ^a	P
		Total	Remission (n=60)	Persistence (n=46)		
MTHFR C677T ¹	Genotype n (%)					
	CC	53 (50.0)	28 (46.7)	25 (54.3)	1.0	
	CT	48 (45.3)	28 (46.7)	20 (43.5)	0.93 (0.40 – 2.12)	0.856
	TT	5 (2.2)	4 (6.6)	1 (2.2)	0.25 (0.02 – 2.48)	0.245
	Allele %					
	C	72.6	70.0	76.1	1.0	
	T	27.4	30.0	23.9	0.71 (0.34 – 2.11)	0.842
MS A2756G ²	Genotype n (%)					
	AA	69 (65.4)	42 (70.0)	27 (58.7)	1.0	
	AG	33 (31.1)	17 (28.3)	16 (34.8)	1.20 (0.50 – 2.89)	0.690
	GG	4 (3.8)	1 (1.7)	3 (6.5)	4.99 (0.46 – 54.57)	0.188
	Allele %					
	A	80.2	83.3	76.1	1.0	
	G	19.8	16.7	23.9	1.63 (0.59 – 4.53)	0.349
TS3'UTR ³	Genotype n (%)					
	ins/ins	34 (32.1)	26 (43.3)	8 (17.4)	1.0	
	ins/del	55 (51.9)	28 (46.7)	27 (58.7)	3.22 (1.19 – 8.69)	0.021
	del/del	17 (16.0)	6 (10.0)	11 (23.9)	6.50 (1.71 – 24,70)	0.006
	Allele %					
	ins	58.5	66.7	47.8	1.0	
	del	41.5	33.3	52.2	2.28 (1.00 – 5.22)	0.051
GRS n(%)	≤2	77 (72.6)	48 (80.0)	29 (63.0)	1.00	
	≥3	29 (27.4)	12 (20.0)	17 (37.0)	2.21 (0.89 – 5.48)	0.086

Hardy-Weinberg Equilibrium (HWE): ¹p=0.389; ²p=0.625; ³p=0.699. ^aAdjusted for HPV infection. Remission: presence of pre-neoplastic lesion at T₁, and normal cytology at T₂; Persistence: pre-neoplastic lesion detected at T₁ and T₂.

To evaluate the association between MTHFR C677T, MS A2756G, and TS3'UTR polymorphisms according to the course of cytological abnormalities, we compared genotype distribution of Remission and Persistence groups (Table 2).

No differences were observed between distribution of MTHFR C677T and MS A2756G, and course of cytological abnormalities (Table 2). On the other hand, higher heterozygote and polymorphic genotypic frequencies of TS3'UTR was observed among women presenting persistent lesions if compared with Remission group. Furthermore, ins/del and del/del genotypes increased the risk of persistence at least three times [OR (IC95%): 3.13 (1.21 – 8.12), $p=0.019$; OR (IC95%): 5.96 (1.67 – 21.25), $p=0.006$ - respectively] (Table 2).

To evaluate the presence of MTHFR C677T, MS A2756G, and TS3'UTR simultaneously, the Genetic Risk Score (GRS) were determined, and women with $GRS \geq 3$ was classified as presenting high number of genetic variants. Higher frequency of women with $GRS \geq 3$ was observed in Persistence group ($n=17$, 37.0%) if compared with Remission group ($n=12$, 20.0%), and women with high number of genetic variants presented higher risk of persistent lesions [OR (IC95%): 2.21 (0.89 – 5.48), $p=0.086$] (Table 2). However, when adjusted for TS3'UTR, the risk of persistence according GRS was modified [OR (IC 95%): 1.26 (0.44-3.61), $p=0.669$], evidencing that between three polymorphism analyzed, only TS3'UTR was associated with course of cytological abnormality.

3) Discussion

Although persistent hr-HPV infection is the main cause of cervical cancer development, genetic alterations may alter the risk of this neoplasia. Thus, genetic markers may be useful in the screening of pre-neoplastic and neoplastic cervical lesions, especially in cases of persistence of HPV infections or recurrent cytological abnormalities (7, 32). Moreover, the conventional methods used for screening of cervical cancer are not able to differentiate pre-neoplastic cervical lesions that will regress, or persist and progress. Therefore, the prognosis of individual pre-neoplastic lesions should be predictable, with the purpose of selecting women with higher risk of persistence and progression, which could decrease the number or unnecessarily treatment of lesions (4).

In this study, five genetic polymorphisms in enzymes that act on one-carbon metabolism were evaluated: MTHFR C677T, MS A2756G, MTRR A66G, TSER, and TS3'UTR. However, distribution of MTRR A66G and TSER were not under Hardy-Weinberg Equilibrium (HWE), which led to their exclusion from further analyzes. Some studies with Brazilian population also did not present genotypic distribution of this polymorphisms attending HWE (33, 34).

Many polymorphisms were identified in TS gene, which is located on chromosome 18p11.32. One of most frequently studied is TS3'UTR, that was related to increased in vitro degradation of mRNA, leading to decrease of expression protein (35). We observed that the presence of polymorphic allele (del) of TS3'UTR increased twice the risk of persistence of cytological abnormalities. Probably it occurred due to decreased synthesis of thymidylate, catalyzed by TS enzyme whose activity is decreased by TS3'UTR polymorphism, leading to DNA uracil incorporation. It results in DNA instability and chromosome damage, crucial events for carcinogenesis (26, 35, 36).

On the other hands, although MTHFR C677T and MS A2756G polymorphisms have already been associated with the presence of pre-neoplastic and neoplastic lesions in uterine cervix, we did not observe any association of these SNPs with the persistence of cytological abnormalities (11, 13).

This was the first study in which the risk of persistence of pre-neoplastic cervical lesions was evaluated in relation to the presence of genetic polymorphisms in enzymes that act on folate metabolism. Evaluation of TS3'UTR polymorphism as a possible marker of cervical carcinogenesis has shown promising results, and further study should be performed. Some authors have shown an association between this polymorphism and esophageal, gastric and breast cancer, although the results were controversial and varied with ethnicity (37–41).

However, this research had some limitations, as the small sample size, the losses of participants during the study, and the short follow-up time. Thus, it is necessary to carry out larger studies, for a longer period and with different population groups to better understand the role of genetic cofactors on cervical carcinogenesis. Besides, considering the different grades of cytological abnormalities separately may be important. In this study, 69.7% (n = 32) presented same cervical lesion at T₁ and T₂, while 23.9% (n = 11) presented LSIL at T₁ and ASC-US at T₂, and 6.4% (n = 3) presented LSIL or ASC-US at T₁ and ASC-H at T₂. However, no differences of characteristics analyzed were observed among these participants (data not showed).

4) Conclusions

Presence of TS3'UTR polymorphism increased risk of cervical abnormalities persistence. Thus, this genetic variant could be considered as potential marker of cervical carcinogenesis, assisting follow-up of women with persistent pre-neoplastic cervical lesions.

5) Methods

5.1) Study design

From October 2016 to September 2018, 280 women living in Minas Gerais State and attended at Basic Health Units of Ouro Preto, and State Specialized Care Center of Itabirito were selected for this study.

Inclusion criterion was age ≥ 18 years old. Exclusion criteria were pregnancy in a period < 6 months, history of neoplasia, and presence of cervical atypia in glandular cells.

At the first meeting (T_1), an interview was performed for information about sociodemographic and behavioral characteristics, and cervical sample was collected for cytological analysis, genetic polymorphism evaluation, and HPV detection.

After six months of follow-up (T_2), 164 women performed the second cytological analysis. From this group, 50 participants presenting normal cytology at T_1 and T_2 , and eight women with normal cytology at T_1 and presence of pre-neoplastic lesion at T_2 were excluded (Figure 1). Thus, sample group ($n=106$) was divided into:

- Remission ($n=60$): presence of pre-neoplastic lesion at T_1 , and normal cytology at T_2 ;
- Persistence ($n=46$): pre-neoplastic lesion detected at T_1 and T_2 .

Presence of pre-neoplastic lesion was considered when Atypical Squamous Cells of Undetermined Significance (ASC-US), Low Grade Squamous Intraepithelial Lesion (LSIL), High Grade Squamous Intraepithelial Lesion (HSIL), or Atypical Squamous Cell – cannot exclude HSIL (ASC-H) were detected at two times.

Research Ethics Committee of Federal University of Ouro Preto approved this study (CAAE 57187316.7.0000.5150, and CAAE 88479718.0.0000.5150).

5.2) Sample collection

Cervical samples were obtained through conventional double collection by health care professionals, using Ayre spatulas for ectocervical sample, and cylindrical brushes for endocervical sample. After confection of cervical smear for cytological analysis, brush was conditioned in Phosphate-Buffered Saline (PBS) pH 7.2, and stored at -80°C for genetic polymorphisms evaluation.

5.3) Cytological analysis

Cervical smears were stained according to Papanicolaou method, and samples were evaluated based on cytomorphological criteria described in Bethesda System 2014 for reporting cervical cytological

diagnoses (Nayar and Wilbur, 2015). All samples were evaluated by two cytopathologists. In case of disagreement between results, a third professional evaluated the sample. The analyses were performed in Laboratório de Análises Clínicas (LAPAC) from Federal University of Ouro Preto.

5.4) DNA extraction

DNA extraction from cervical samples was performed with illustra blood genomicPrep Mini Spin™ Kit (GE Healthcare, Chicago, Illinois, USA). Evaluation of quality and integrity of DNA was performed by amplification of *β-actin* gene (42).

5.5) HPV detection

HPV detection was performed by conventional Polymerase Chain Reaction (PCR) with MY09/MY11 primers, as described by Miranda *et al.* (2013). For positive samples, HPV genotype was analyzed by Restriction Fragment Length Polymorphism (RFLP) (42). HPV negative samples were also analyzed by conventional PCR with GP5+/GP6+ primers (43).

5.6) Genetic polymorphisms

MTHFR C677T (rs1801133), MS A2756G (rs1805087), MTRR A66G (rs1801394), and TS3'UTR (rs151264360) polymorphisms were evaluated by PCR-RFLP (14, 35, 44, 45). TSER (rs34743033) was evaluated by PCR (46).

Sequences of primers, restriction enzymes, and PCR protocols were presented in *Supplementary Tables 1, 2, and 3*.

Table 3 shows the size of DNA fragments that characterize the genotypes of polymorphisms analyzed.

TABLE 3: Size of DNA fragments for genetic polymorphisms analysis

Polymorphisms	Genotypes		
	No polymorphic	Heterozygote	Polymorphic
MTHFR C677T	198 bp	23 bp, 175 bp and 198 bp	175 bp and 198 bp
MS A2756G	211bp	80 bp, 131 bp and 211 bp	80 bp and 131bp
MTRR A66G	22 bp and 44 bp	22 bp, 44 bp and 66 bp	66 bp
TS3'UTR	70 bp and 88 bp	70 bp, 88 bp and 152 bp	152 bp
TSER	220 bp	220 bp and 248 bp	248 bp

GRS was calculated to evaluate the presence of all polymorphisms simultaneously, as described by Tomita *et al.* (2013). Presence of heterozygosity or polymorphic homozygotes received one or two points, respectively. Non-polymorphic homozygotes were not scored (zero) (13). Thus, the higher the GRS, the higher the frequency of genetic polymorphisms.

5.7) Statistical analysis

Data were tabulated by Microsoft Office Excel™ (Microsoft, Redmond, Washington, USA), and analyzed by Statistical Package for the Social Sciences™ 17.0 (International Business Machines, New York, USA).

Descriptive statistics were performed to evaluate the frequency of genotypes. Allelic frequency was calculated by Genepop software (47). HWE of genotypic frequencies was calculated by HWE calculator including analysis for ascertainment bias (48).

Chi-square was used for comparison between groups. Binary logistic regression was used to calculate the relative risk (Odds Ratio), with a 95% confidence interval.

p values <0.05 were considered as evidence of statistically significant association.

6) List Of Abbreviations

A: Adenine;

ASC-H: Atypical Squamous Cell – cannot exclude High Grade Squamous Intraepithelial Lesion;

ASC-US: Atypical Squamous Cells of Undetermined Significance;

C: Cytosine;

dTMP: deoxythymidine monophosphate;

dUMP: deoxyuridine monophosphate;

G: Guanine;

GRS: Genetic Risk Score;

HPV: Human Papillomavirus;

hr-HPV: high-risk Human Papillomavirus;

HSIL: High Grade Squamous Intraepithelial Lesion;

HWE: Hardy-Weinberg Equilibrium;

LAPAC: Laboratório de Análises Clínicas;

LSIL: Low Grade Squamous Intraepithelial Lesion;

mRNA: messenger RNA;

MS: Methionine Synthase;

MS A2756G: polymorphism A2756G of Methionine Synthase;

MTHFR: Methylenetetrahydrofolate Reductase;

MTHFR C677T: polymorphism C667T of Methylenetetrahydrofolate Reductase;

MTRR: Methionine Synthase Reductase;

MTRR A66G: polymorphism A66G of Methionine Synthase Reductase;

PBS: Phosphate-Buffered Saline;

PCR: Polymerase Chain Reaction;

RFLP: Restriction Fragment Length Polymorphism;

SNP: Single Nucleotide Polymorphism;

T: Thymine;

T₁: First meeting;

T₂: Second meeting, after six months of follow-up;

TS: Thymidylate Synthase;

TS3'UTR: 6 bp deletion at nucleotide1494 in TS 3'-untranslated region;

TSER: Double or triple 28 bp tandem repeat in 5'-untranslated enhanced region of Thymidylate Synthase.

7) Declarations

Ethics approval and consent to participate: Research Ethics Committee of Federal University of Ouro Preto approved this study (CAAE 57187316.7.0000.5150, and CAAE 88479718.0.0000.5150). All women invited and who agreed to participate in this study signed the consent form to participate.

Consent for publication: Not applicable.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: There are no competing interests.

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Authors' contributions: NNTS, ACSS, CMC, and AAL analyzed and interpreted the patient data regarding the cytological analyses, HPV infection, and genetic polymorphisms. VMN assisted in selection of participants, and collection of cervical samples. All authors read and approved the final manuscript.

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9) Supplementary Material

TABLE 1: Sequences of primers, and restrictions enzymes used for polymorphisms analyses.

Polymorphism	Nucleotide Sequences	Restriction Enzyme	Reference
MTHFR	5' TGAAGGAGAAGGTGTCTGCGGGA 3'	<i>HinfI</i>	(14)
C677T	5' AGGACGGTGCGGTGAGAGTG 3'		
MS A2756G	5' TGTTCCAGACAGTTAGATGAAAATC3' 5' GATCCAAAGCCTTTTACACTCCTC3'	<i>HaeIII</i>	(44)
MTRR A66G	5'GCAAAGGCCATCGCAGAAGACAT3' 5'GTGAAGATCTGCAGAAAATCCATGTA3'	<i>NdeI</i>	(45)
TS3'UTR	5'CAAATCTGAGGGAGCTGAGT3' 5'CAGATAAGTGGCAGTACAGA3'	<i>DraI</i>	(35)
TSER	5'GTGGCTCCTGCGTTTCCCCC3' 5'CCAAGCTTCGCTCCGAGCCGCCACAGGCATGGCGCGG3'	NA ¹	(46)

¹NA: Not applicable.

TABLE 2: PCR protocol used for polymorphisms analyses.

Polymorphism	Number of cycles	Temperature (°C)	Time (minutes)	Stage
MTFHR C677T	1	94	1	Denaturation
		94	1	Denaturation
	35	59	0.5	Annealing
		72	0.5	Extension
	1	72	5	Final Extension
MS A2756G	1	95	2	Denaturation
		95	1	Denaturation
	35	48	1	Annealing
		72	1	Extension
	1	72	7	Final Extension
MTRR A66G	1	94	4	Denaturation
		94	0.5	Denaturation
	35	53	0.5	Annealing
		72	0.5	Extension
	1	72	5	Final Extension
TSER	1	94	4	Denaturation
	35	94	1	Denaturation
		60,6	1	Annealing
		72	1	Extension
	1	72	7	Final Extension
TS3'UTR	1	94	5	Denaturation
	30	94	0.5	Denaturation
		53	0.75	Annealing
		72	0.75	Extension
	1	72	5	Final Extension

TABLE 3: Reagents for polymorphisms analysis by PCR

Reagents	Polymorphisms				
	MTHFR C677T	MS A2756G	MTRR A66G	TSER	TS3'UTR
PCR Master Mix ^{™1}	12.5 µl	12.5 µl	12.5 µl	12.5 µl	12.5 µl
Primers ² (10 pmol/µl)	0.25 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl
DNA (15 ng/µl)	1.0 µl	1.0 µl	2.5 µl	5.0 µl	2.5 µl
Water ³	11.0 µl	9.5µl	8.0 µl	5.5 µl	8.0 µl

¹0.2mM each deoxyribonucleotide (dNTP), 1.5 mM MgCl₂ and 1.0 unit of Taq DNA (Promega, Madison, Wisconsin, USA); ²IDT (Coralville, Iowa, USA); ³Nuclease Free-Water (Promega, Madison, Wisconsin, USA).

Figures

Figure 1

Study flow diagram.

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