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# Genetic polymorphisms of glutathione S transferase and cervical intraepithelial neoplasia

## Polimorfismele genice ale glutathione S-transferazei și neoplazia intraepitelială de col uterin

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### Abstract

**Aim:** The present study aim to analyze the relationship between GST M/T genotypes of glutathione S-transferases and cervical intraepithelial neoplasia. **Materials and Methods:** A prospective case-control study has been designed including 69 cases with different degrees of cervical dysplasia and 107 controls. All patients had been examined colposcopically. For every patient both cervical and blood specimen have been obtained. The peripheral blood was used for GST M/T genotyping. The statistical analysis was performed using OR and chi-square at a level of significance inferior to 0.05.

**Results:** No statistically significant differences had been found between cases and controls for GST T/M- genotype ( $T/M-$ ,  $\chi^2=0.03$ ,  $p=0.8610$ ) and  $T+/M+$   $\chi^2=0.65$ ,  $p=0.4197$ . Patients with in situ carcinoma had significant GST genotype association for  $T/M+$  genotype ( $OR=4.66$ ,  $CI\ 95\% [0.6528, 24.9725]$ ,  $\chi^2=4.6$ ,  $p=0.0314$ ) and for  $T+/M-$  genotype ( $OR=0.12$ ,  $CI\ 95\% [0.0027, 0.9465]$ ,  $\chi^2=0.05$ ,  $p=0.0219$ ). **Conclusion:** The combination of GST genotypes can be included in a predictive score for patients with cervical carcinoma.

**Keywords:** cervical intraepithelial neoplasia, HPV, SNP, GST.

### Rezumat

**Obiectiv:** Cercetarea prezintă și propune să analizeze relația dintre genotipurile GST M/T ale glutathione S-transferazei și neoplazia intraepitelială intracervicală. **Material și metode:** A fost realizat un studiu prospectiv de tip caz-martor, ce a inclus 69 pacienți cu diferite grade de neoplazie intraepitelială cervicală și 107 controale negative pentru leziuni intraepiteliale. Fiecare pacientă a fost examinată colposcopic obținându-se o probă cervicală. Probele de sânge

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obținute au fost utilizate pentru genotiparea GST M/T. Analiza statistică a fost efectuată utilizând testele OR și Hi-patrat la un prag de semnificație statistică inferior 0.05. **Rezultate:** La comparația globală caz-martor nu au fost detectate diferențe semnificative statistic pentru genotipul GST T-/M- ( $T-/M-$ ,  $\chi^2=0.03$ ,  $p=0.8610$ ) și T+/M+ ( $\chi^2=0.65$ ,  $p=0.4197$ ). În schimb, în cazul pacienților cu carcinom in situ asocierile au fost semnificative: risc crescut genotipul T-/M+ (OR=4.66, CI 95% [0.6528,24.9725],  $\chi^2=4.6$ ,  $p=0.0314$ ) și respectiv efect protector în cazul genotipului T+/M- (OR=0.12, CI 95% [0.0027,0.9465],  $\chi^2=0.05$ ,  $p=0.0219$ ). **Concluzie:** Genotipurile GST pot fi incluse într-un sistem de scor pentru carcinomul cervical.

**Cuvinte cheie:** neoplazia cervicală intraepitelială, HPV, SNP, GST.

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## Introduction

Cervical neoplasia is the second most prevalent cancer in women after lung cancer, representing one of the major morbidity causes, especially in young women (1, 2).

Scientific medical research over the past two decades has brought conclusive and unquestionable evidence that cervical neoplasia results from persistent chronic infections with oncogenic types of the human papilloma virus (HPV) known as high-risk HPV (hrHPV). Virtually, hrHPV DNA is found in all (99%) of invasive cervical tumors of the affected women (3, 4).

Although the viral etiology of cervical cancer is incontestable, only a part of the HPV infected women develop cancer, so the pathogenesis of this neoplastic event involves potential immune and genetic individual characteristics. The molecular study of HPV carcinogenesis has been a major research topic trying to identify the genetic predisposing factors involved in the etiopathogenesis of cervical cancer (5). There are a number of studies demonstrating that personal genetic particularities are important when defining higher susceptibility to HPV infections and cervical neoplasia, particularly the effect of the viral oncoproteins E6 and E7, the loss of heterozygosity in some chromosomal region, and, last but not least, different polymorphisms of the MHC, p53, immune response and detoxifying genes (6, 7). Most of the existing data related to cervical cancer have mainly focused on oncogen-

ic HPV strains and other environmental factors, therefore, the role of host genetic susceptibility to cervical carcinogenesis is still largely unknown (8). Previous research has suggested the potential implication of genetic polymorphisms in cancer susceptibility (8, 9). Ongoing research related to genetic predisposition upon viral induced malignancy is expected to have a potential impact on prevention strategy and future prognosis (9, 10).

The glutathione S-transferases (GSTs) represent an important class of detoxifying enzymes belonging to phase II detoxification enzymes superfamily, which protect cells against endogenous and exogenous molecular injury inducing compounds including mutagens, carcinogens and other toxic chemicals, thus playing a crucial role in the detoxification process (11). The genes for both GSTM1 and GSTT1 isoforms could exhibit an insertion/deletion polymorphism null alleles/genotypes resulting in loss of enzymatic activity which was associated with a number of malignancies including cervical neoplasia (12-14). Previous reports have associated GST M1 and T1 null genotype with an enhanced risk of precancerous lesions or cervical cancer (15, 16), but still, other studies have not confirmed this correlation (12). Considering that null alleles of GSTM1 and GSTT1 genes could be used as potential molecular markers in cervical cancer prevention, further large studies and adequate meta-analyses are needed to confirm and validate this hypothesis.

The present research aims to analyze the potential link between GSTM1 and GSTT1 null

variants and cervical intraepithelial neoplasia in a group of women from Transylvania, Romania.

## Materials and Methods

### *Cases and controls*

The present research received the approval of the Ethics Committee of the directing institution (University of Medicine and Pharmacy "Iuliu Hatieganu", Cluj-Napoca, Romania) and was conducted according to the Helsinki Declaration. All subjects included in the current study agreed and signed the written informed consent. The study was cross-sectional.

The study included a total of 176 female subjects (women of Romanian and Hungarian ethnicity, all Caucasians), of whom 69 cases and 107 healthy volunteers, and was performed in the 1<sup>st</sup> Obstetrics and Gynecology Clinic of Cluj-Napoca, Romania between 1<sup>st</sup> of January 2014-30<sup>th</sup> of June 2014.

Every patient was scheduled for two visits. During the first visit, a physical examination was performed, followed by cervical probe collection. Cervical probes were used for cervical cytology classification according to the Bethesda system (17) and HPV testing. The HPV testing was performed in a qualitative manner using an immunohistological test that could detect the presence of HPV strains (Viroaktiv from Virofem®). During the second visit, colposcopy was performed. At the end of the visit 2 mL of peripheral blood were drawn and stored at 4°C for subsequent DNA extraction.

Controls were represented by cytological negative for intraepithelial neoplasia (NILM) patients according to the Bethesda terminology, negative for HPV testing and also with a normal colposcopy.

The group of cases was represented by patients with abnormal Pap smear results and positive for hrHPV. The colposcopy was performed according to 2011 colposcopic terminology of

the International Federation for Cervical Pathology and Colposcopy (18). Colposcopy was used to choose the most appropriate site of biopsy. The results of the biopsy allowed the classification of cases into the following histological categories: in situ carcinoma (CIS), high grade intraepithelial lesions (HSIL), and low grade intraepithelial lesion (LSIL). No case of invasive carcinoma was recorded in the study group. The histological classification of cervical dysplasia was based on ASCCP 2012 terminology (American Society for Colposcopy and Cervical Pathology - ASCCP) (19). Patients with abnormal Pap smears results but negative for hrHPV were not included in the study.

### *Molecular analysis of null alleles of GSTM1 and GSTT1 genes*

Genotyping for M1 and T1 alleles of GST gene was performed by Multiplex PCR using a protocol previously described by Bid HK (20). Although the current protocol does not allow discriminating between GSTM1 and GSTT1 homozygous and heterozygous genotypes, 4 different genotypes were identified and used for further statistical analysis: GSTM1 wild type or positive normal M1 allele (M1+), GSTT1 wild type or positive T1 allele (T+), GSTM1 variant or null allele (M1-), GSTT1 variant or null allele (T1-).

### *Statistical analysis*

GSTM1 and GSTT1 polymorphism genotype distribution between different cytological groups was compared using Fisher's exact test. For estimation of the relationship between the odds ratio (OR) of different cytological classes was used at 95% confidence interval (CI). The risk associated with each genotype was assessed by logistic regression analysis. P-value under 0.05 was assumed statistically significant. STATA Intercooled 10 (College Station, Texas, USA) and SPSS 18.0 for Windows were used for statistical calculations (SPSS, Inc, Chicago, IL., USA).

## Results

The average mean age of patients was 39.30 years, 42.49 years in the control group, respectively, with no statistical significant differences ( $p=0.258$ ). Enrolled patients were classified in

specific subgroups according to 2012 ASCCP Consensus Guidelines (19). The largest category was represented by high grade disease and in situ carcinoma 43.48%.

While comparing cases versus controls, no link was found between cervical intraepitheli-

**Table 1. GST M1/T1 genotype analysis**

Comparison	OR	OR 95% CI	Chi-squared	p
<b>T-/M-</b>				
cases/controls	1.0846	[0.3836,2.9348]	0.03	0.8610
LSIL/controls	-	-	-	-
HSIL/controls	0.8034	[0.814,4.0543]	0.07	0.7845
LSIL+HSIL+CIS/controls	0.6573	[0.1134,2.6138]	0.39	0.5298
CIS/controls	0.8034	[0.0170,6.7155]	0.04	0.8413
HSIL+CIS/controls	0.7747	[0.1325,3.1138]	0.14	0.7050
<b>T-/M+</b>				
cases/controls	1.166	[0.1902,5.0958]	0.05	0.8256
LSIL/controls	7.2592	[0.5234,70.2258]	5.38	0.0203
HSIL/controls	0	[0,2.1848]	1.81	0.1784
CIS/controls	4.6666	[0.6528,24.9725]	4.63	0.0314
LSIL+HSIL+CIS/controls	1.7562	[0.4274,6.3411]	0.92	0.3387
HSIL+CIS/controls	1.1666	[0.1902,5.0958]	0.05	0.8256
<b>T+/M-</b>				
cases/controls	1.2982	[0.6533,2.5654]	0.65	0.4197
LSIL/controls	-	-	-	-
HSIL/controls	1.0980	[0.3755,3.2072]	0.04	0.8478
CIS/controls	0.1220	[0.0027,0.9465]	5.25	<b>0.0219</b>
LSIL+HSIL+CIS/controls	0.4831	[0.8222,4.4447]	2.80	0.0942
HSIL+CIS/controls	0.6039	[0.2376,1.4801]	1.44	0.2300
<b>T+/M+</b>				
cases/controls	1.2982	[0.6533,4.0543]	0.07	0.7845
LSIL/controls	3.2205	[0.3480,39.76]	1.72	0.1897
HSIL/controls	1.4313	[0.4606,4.2162]	0.51	0.4730
CIS/controls	2.1470	[0.4576,9.9428]	1.37	0.2424
LSIL+HSIL+CIS/controls	1.9221	[0.8222,4.4447]	2.80	0.0942
HSIL+CIS/controls	1.7681	[0.7138,4.3082]	1.90	0.1682

al neoplasia and GST M1 and GST T1 genetic polymorphisms (T-/M-,  $\chi^2=0.03$ ,  $p=0.8610$ ; T-/M+,  $\chi^2=0.15$ ,  $p=0.6961$ ; T+/M-,  $\chi^2=1.24$ ,  $p=0.2659$ ; T+/M+  $\chi^2=0.65$ ,  $p=0.4197$ ). The complete analysis of GST M1 and T1 genotypes is presented in table I. Instead of a more subtle analysis on subgroup of cervical intraepithelial neoplasia (CIN), significant findings were noted.

The combination of GST T-/M+ genotype represents a significant risk factor for LSIL and CIS (OR=7.2592,  $p=0.0203$ , and OR=4.6666,  $p=0.0314$ , respectively). On the other hand, the T+/M- genotype combination seems to exert a protective effect in patients with CIS (OR=0.1220,  $p=0.0219$ ). The effect of genotypes was confirmed by the logistic regression analysis,

where each cytological class was considered as dependent variable and the two genotypes as independent variables (table II). The results of logistic regression were concordant with previous analysis: significant increased risk for GST T-/M+ for LSIL (OR=7.259, [1.069; 49.268],  $p=0.042$ ) and CIS (OR=4.667, [1.026-21.231,  $p=0.046$ ], and significant decreased risk for developing CIS in case of GST T+/M- (OR=0.122, [0.015; 0.997],  $p=0.05$ ).

## Discussion

Worldwide, cervical cancer remains the second most common cause of neoplasia related deaths in women. Persistent hrHPV cervical infections represent the most important contributing factor

**Table 2. Effect genotypes by logistic regression analysis**

	Genotype	OR	95% CI	p
<b>LSIL</b>				
LSIL#	GST T-/M-	-		
LSIL	GST T-/M+	7.259	1.069-49.268	0.042
LSIL#	GST T+/M-	-		
LSIL	GST T+/M+	3.220	0.514-20.175	0.212
<b>HSIL</b>				
HSIL	GST T-/M-	0.803	0.167-3.868	0.785
HSIL#	GST T-/M+	-		
HSIL	GST T+/M-	1.098	0.422-2.853	0.848
HSIL	GST T+/M+	1.431	0.536-3.824	0.474
<b>CIS</b>				
CIS	GST T-/M-	0.803	0.0940-6.869	0.842
CIS	GST T-/M+	4.667	1.026-21.231	0.046
CIS	GST T+/M-	0.122	0.015-0.997	0.050
CIS	GST T+/M+	2.147	0.582-7.915	0.251
<b>LSIL+HSIL+CIS</b>				
LSIL+HSIL+CIS	GST T-/M-	0.678	0.181-2.532	0.563
LSIL+HSIL+CIS	GST T-/M+	1.814	0.565-5.831	0.317
LSIL+HSIL+CIS	GST T+/M-	0.503	0.224-1.129	0.096
LSIL+HSIL+CIS	GST T+/M+	1.808	0.829-3.943	0.137

# - insufficient number of cases in this category for statistical analysis.

in its etiology and progression (21, 22). Infections with hrHPV could evolve in inducing or transforming cervical intraepithelial neoplasia lesions (23). In patients with HSIL lesions, hrHPV are found integrated in the cell nucleus in so-called transformed infections (23, 24). In high grade lesions with persistent oncogenic HPV, molecular anomalies in host cell genome accumulate gradually, underlying the prior characteristic progression to cancer (24). Although hrHPV infections represent undoubtedly the major risk factor for cervical cancer, a broad array of individual genetic variants that accompany HPV infections in different stages of preneoplastic events and promotion to cervical cancer have been identified in recent decades. These data are strongly supported by epidemiological studies (25, 26).

Different genetic polymorphisms might be related to HPV persistence and progression to cancer, some of them related to cervical cancer (25, 26). Among these, genetic polymorphisms of GST genes were intensely studied in relationship to chronic hrHPV infections and cervical cancer, but results are still inconclusive even if large populations with different ethnicities were included (27, 28). GST polymorphisms have been previously analyzed in study lots from Transylvania in relationship with other pathologies such as glaucoma and lung cancer (29, 30).

The persistent HPV HR infections are asymptomatic, but can determine low grade, high grade lesions, in situ carcinoma, or cervical cancer. Since it is very difficult to realize a longitudinal study that might include patients without any cytological anomalies for decades in order to see the relationship between GST M1 and T1 genetic polymorphisms and HPV HR infections, the present study has included HPV-HR positive patients with LSIL, HSIL or in situ carcinoma, which, accordingly to the known HPV natural history (1, 2, 4, 5, 22, 23), are due to persistent

HPV-HR that cause significant squamous cell modifications.

A meta-analysis, including a total of 15 case-control studies comprising 1825 cases and 2104 controls, provides well-founded evidence that the GSTM1 null genotype is correlated with the development of cervical neoplasia, especially in Indian and Chinese populations. In the same meta-analysis, smoking is linked between GSTM1 null genotype and cervical cancer (27). In contrast, other studies show that polymorphisms in GSTM1 and GSTT1 genes are not associated with a higher risk for cervical cancer in HPV infected women (16, 28). In our study, the GST M1 null genotype was a significant protecting factor for CIS ( $OR=0.1220$ ,  $\chi^2=5.25$ ,  $p=0.0219$ ), while GST T-/M+ genotype was associated with a significantly increased risk for developing LSIL and CIS ( $OR=7.2592$ ,  $p=0.0203$ , and  $OR=4.6666$ ,  $p=0.0314$ , respectively).

As most of the current data refer to Asian populations, previous studies focused on Caucasian populations in order to identify possible ethnic differences for GST polymorphism distribution and their relationship with cervical cancer. The results of a study that included 253 women with a persistent high-risk HPV positivity showed a significant association of GSTM1 and GSTT1 null genotypes with cervical dysplasia in Caucasians (31). Hence, null GSTM1 and GSTT1 genotypes should both be counted in the risk assessment algorithms of Caucasian individuals as well in Asian ones (31).

To the best of our knowledge, the current comparative analysis of GSTM1 and GSTT1 variants in patients diagnosed with cervical intraepithelial neoplasia is the first one in Eastern Europe, and therefore, also in Romania. All subjects enrolled in the study were Caucasians. The study group, from a strictly statistical point of view, cannot be considered population representative, the estimation of the exact number of

cases being difficult due to the unknown prevalence of cervical dysplasia in Transylvania. The relatively small number of enrolled patients is a limitation of the present study, but this research is conclusive from a statistical point of view and the results encourage more complex future studies. Overall, null genotypes of GSTM1 and GSTT1 genes are not associated with cervical cancer and precancerous lesions. The results are concordant with other previous studies (32, 33).

In contrast, T-/M+ genotype was associated with a significant higher risk for LSIL (OR=7.25, chi-square=5.38,  $p=0.0203$ ) and CIS (OR=4.66, chi-square=4.63,  $p=0.0314$ ). Also, a significant association has been found between T+/M- genotype and CIS (OR=0.1220, chi-square=5.25,  $p=0.0219$ ). The results are conflicting at first sight, but we have to bear in mind that GSTs play an important role in cell detoxification and that it is impossible to assess all the potential co-carcinogens. Among them, tobacco smoking represents a well-known cofactor for cervical cancer; the risk of developing a CIN2+ lesion is higher among women who smoke with abnormal Pap smears (34). Moreover, GSTs are involved in major detoxification pathways (35).

One of the limitations of the present study is that it does not evaluate GST polymorphisms in relationship with other associated risk factors involved in cervical neoplasia. All meta-analyses and multicentric reports highlight that smoking is an important environmental carcinogenic cofactor for cervical neoplasia (36-39). A study taking into account 150 smoking women diagnosed with cervical neoplasia showed significant association with an increased risk of 4.19-folds of developing cervical cancer in association of GSTM1 null genotypes (40). Other studies confirm that there is a positive association between active or passive smoking, GST null genotypes and the risk of developing cervical cancer (27, 28).

A seven-year follow-up study of patients with persistent high-risk HPV infections shows that they are more likely to develop a high grade disease in the presence of GSTM1 and GSTT1 null genotype (40).

It is known that GSTT- allele alone, or the combined GSTT-/GSTM- genotype is considered a risk factor for cancer in general and also for cervical cancer (41). In the present study, the double null genotype was not significantly associated with any category of cervical dysplasia.

Although the enzymatic deficiency due to GSTM null genotype is considered a risk factor in cervical neoplasia, the T+/M- genotype seems to exert an inverse statistical relationship, if not a protective effect, in a patient with CIS in our study (OR=0.1220, chi-square=5.25,  $p=0.0219$ ).

The continuous and particular interest in the molecular influences of GST detoxifying enzymes and their impact on the onset and progression of cancer could explain the pathogeny of the disorder, on the one hand, and could influence the response to therapy on the other. As only a part of the HPV infected women develop precancerous cervical lesions or cancer, prophylactic evaluation of GST family enzymes could play a major role in identifying high risk individuals for an efficient therapeutic interference, here including HPV vaccination (42, 43).

Our data support the inclusion of GST M/T genotypes in a potential predictive score system for cervical intraepithelial neoplasia.

## Abbreviations

bp	= base pairs
CI	= confidence interval
CIS	= in situ carcinoma
GSTs	= Glutathione S-transferases
HPV	= human papillomavirus
hrHPV	= high risk HPV
HSIL	= high grade intraepithelial lesion

LSIL = Low grade intraepithelial lesion  
 MHC = major histocompatibility complex  
 M- = null homozygous GST-M1  
       (variant) genotype  
 M+ = heterozygous or homozygous  
       GST-M1 wild genotype  
 T- = null homozygous GST T1 (variant)  
       genotype  
 T+ = heterozygous or homozygous  
       GST-T1 wild genotype

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