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*Original scientific paper*

# Association of *p53Pro72Arg* (rs1042522) and *MDM2*309 (rs2279744) polymorphisms with risk for cervical intraepthelial lesions and cervical cancer development in Macedonian women

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## Abstract

High risk Human Papillomavirus (HPV) is an important etiological factor in initiation of squamous intraepithelial lesions (SIL), but not enough for malignant progression to cervical cancer (CCa). Single nucleotide polymorphisms (SNPs): rs1042522 within the codon 72 of *p53* and rs2279744 within *MDM2* promoter gene are plausible factors for development of SIL or CCa conferring increased attenuation of *p53* pathway. We investigated the association of these SNPs with the HPV positive SIL and CCa among women from the Republic of Macedonia. Using a multiplex PCR SNaPShot analysis we genotyped rs1042522 and rs2279744 in 131 HPV positive women with SIL or CCa and 110 HPV and cytologicaly negative controls subject. No significant difference in either genotype or allelic frequencies for rs1042522 and rs2279744 between cases and control was found. The stratification of patients on the basis of the lesion grade revealed lower frequency of CC genotype and C allele of rs1042522 in HSIL and CCa compared to LSIL [GG vs CC; p=0.001, OR=0.4; CG vs CC; p=0.04, OR=0.03 and CG+ GG vs CC; p=0.004, OR=0.2]. Additionally TT genotype and T allele of *MDM2 309* showed significantly lower frequency in HSIL and CCa group then in LSIL [G vs T p=0.02, OR=0.52; GG vs TT; p=0.04, OR=0.29; TT vs TG+GG; p=0.007, OR=0.34].The Arg variant of rs1042522 and T allele/TT genotype of rs2279744 are associated with progression to LSIL to HSIL or CCa and may be used as prediction markers in CCa management, but the clinical relevant warrants further validation in large and well-designed studies.

**Keywords**: Squamous intraepithelial lesions (SIL), cervical cancer (CCa), Human Papillomavirus (HPV), single nucleotide polymorphism (SNP), cancer and SIL susceptibility

## Introduction

Cervical cancer (CCa) is the fourth most common cancer in woman (Ferlay et al., 2015) and one of the leading causes of morbidity and mortality among women worldwide (Hakama et al., 2008). Human papillomavirus (HPV) is considered the key etiological factor in initiation of squamous intraepithelial lesions (SIL) and cervical cancer (CCa), which are strongly associated with persistentance of this high-risk virus types (Thomison et al., 2008; zur Hausen, 1996). Although the majority infections are spontaneously resolved and do not confer progression to dis-

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ease, evidence confirmed host factors influence in the HPV persistence and progression of cervical lesions to higher grade lesions and CCa.

Many studies have confirmed the association between genetic polymorphisms in cancer susceptibility genes and risk of human malignant tumors (Nunobiki et al., 2011; Tsigris et al., 2007; Ueda et al., 2003). Polymorphisms within the *p53*, damage-response gene and within its main negative regulator, human homolog of mouse double minute 2 (*MDM2*), have been reported as factors that influence cervical carcinogenesis (Wade et al., 2010), but still conclusions remain contradictory.

Storey, et al. (Storey et al., 1998) for the first time reported an association between single nucleotide polymorphisms (SNPs) in codon 72 of *p53* (rs1042522) and persistent HPV infection and cervical cancer. The C to G base change in codon 72 of *p53* replacing proline (Pro) with arginine (Arg) might modify the *p53* susceptibility to ubiquitin dependent degradation lowering its tumor suppression effect (Storey al., 1998). The SNP at position of the intronic promoter of *MDM2* gene (rs2279744) characterized with T to G replacement can also confer susceptibility to SIL and CCa increasing the affinity of the Sp1 transcription factor toward it. This event results with higher level of protein production and attenuation of the p53 pathway (Hun et al., 2007) followed by abrogation of *p53* tumor suppressor activity, higher mutation rate, less efficient DNA repair and reduction of apoptosis all together resulting with faster and more frequent tumor formation (Hu et al., 2010). Elevated MDM2 levels have been detected in different human cancers due to the abnormal expression of the *MDM2* gene (Momand et al., 1998; Oliner et al., 1992) and could be a risk factor for higher susceptibility to various cancers.

Based on this knowledge, we designed a method for simultaneous analysis of both polymorphisms using SNapShot miniseqencing single base extension approach with an aim to find weather there is an association between these SNPs and susceptibility to HPV positive SIL and CCa among Macedonian women. For this purpose we design primers for multiplex reaction and SNP unlabeled primers that anneal one base upstream to the relevant SNP, guided by previous scientific experience (Carvalho and Pena, 2005; Noveski et al., 2014a; Noveski et al., 2014b). Although recent technologies offer sophisticate high-throughput platforms, we used this methodology as a rapid and cost effective one. Genetic characteristic of these SNPs are shown in Table 1.

## Materials and methods

*Study population and clinical characteristic*

The study group included 241 women: 131 HPV positive cases with histologicaly confirmed: low squamous intraepithelial lesions (LSIL) (n=39), high squamous intraepithelial lesions (HSIL) (n=52) and cervical cancer (n=40) and the control group was consisted of 110 HPV and cytologicaly negative women.

For further statistical analysis the study population was stratified in three groups: C1 (all squamous intraepithelial lesions – SILs: LSIL, HSIL and CCa), C2 (HSIL and CCa) and C3 (LSIL). Informed consent was obtained from each participant, before recruitment in the study.

Blood samples and/or cytological swabs from cervical cancer patients were obtained in the period February 2013 to November 2014, from women who underwent cervical cancer screening with HPV testing and/or surgical treatment at the University Clinic for Gynecology and Obstetrics at the Medical Faculty in Skopje, Macedonia. Their cytological/histological status was identified by pathological

Table 1. Characteristic and positions of analyzed SNPs

| dbSNP | Reference sequence | Genome position | transcript | c. DNA | Protein sequence | Amino acid change | Trivial name |
|---|---|---|---|---|---|---|---|
| rs1042522 | NC_000017.10 | g.7579472G>C | NM_000546.5 | c.215C>G | NP_000537.3 | p.Pro72Arg | *p53*Pro72Arg |
| rs2279744 | NC_000012.11 | g.69202580T>G | NM_001145337.2 | c.-291T>G | | | *MDM2* 309T>G |

Table 2. Primers used in multiplex reaction in detection of the SNPs (rs1042522 and rs2279744)

| gene/db SNP ID | primers orientation | 5'>3' | PCR-fragment |
|---|---|---|---|
| *p53* Pro72Argc.215C>G/rs1042522 | F | GAAGACCCAGGTCCAGATGA | 216 |
| | R | ACTGACCGTGCAAGTCACAG | |
| *MDM2*309 c.-291T>G/rs2279744 | F | CGGGAGTTCAGGGTAAAGGT | 194 |
| | R | TCGGAACGTGTCTGAACTTG | |

analysis of cervical swabs or surgical specimens. The cytological diagnosis was performed by cytopathologists using the Bethesda classification system. Histological analyses were performed on specimens collected by a colposcopy-directed biopsy and/or cone specimens collected by the loop excision procedure in patient where it was indicated or on surgical specimens. Median age of the study group was 19-67 (43 ± 7.3). HPV DNA detection and SNP typing were performed in the Laboratory for Virology and Molecular Diagnostics of the Institute of Public Health (IPH) of R. Macedonia and the Research Center for Genetic Engineering and Biotechnology, MASA, R. Macedonia. This study was approved by the local ethics committee of IPH of R. Macedonia.

*DNA isolation and SNP genotyping*

DNA isolation: QIAamp DNA Blood Mini Kit for DNA extraction from peripheral blood and cervical swabs according the manufacture instructions. Samples were obtained considering all bioethical issues and the study protocol was approved by Institute of Public Health of R Macedonia. Informed consent was obtain from the all the participants.

*SNP genotyping by multiplex PCR followed by SNapShot analysis:*

*a). Primer design*

The primers for the first multiplex reaction were designed using primer design software-Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and the reaction resulted amplicons with different fragment sizes (Table 2). Secondary structures and potential primer dimers were predicted using the PriDimerCheck (http://biocompute.bmi.ac.cn/MPprimer/primer_dimer.html) and dimer–dimer exclusion were calculated with OligoCalc (http://biotools.nubic.northwestern.edu/OligoCalc.html; Kibbe 2007). The SNP specific primers were designed to allow 3' attaching to SNP nucleotide and they were 5' tailed with a poly-C sequence to produce extension products 32 to 40 nucleotides long to allow separation by capillary electrophoresis (Table 3).

*b). Multiplex polymerase chain reaction (PCR)*

The multiplex PCR reaction was performed in 25 μL final volume, containing 1x Reaction buffer, 300μM of dNTPs, 2 mM of MgCl2, 2U of AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, USA) and 10 ng of genomic DNA. Cycling conditions were: 10 minutes at 95 °C, followed by 35 cycles of 1 minute at 95 °C, 1 minute and 15 seconds at 59 °C, 2 minutes at 72 °C and a final extension at 72 °C for 10 minutes.

*c). Single-nucleotide primer extension assay*

Before the single base extension reaction, an aliquot of 1 μL of the PCR product was treated with 0.5 μL of ExoSAP-IT (USB Corporation, Clevelend, OH, USA) for 60 minutes at 37 °C to eliminate unincorporated nucleotide triphosphates and excess PCR primers The enzymes were inactivated at 86 °C for 20 minutes. Multiplex single base extension reaction was performed in a 5 μL final volume, combining 2 μL of SNaPshot ready reaction mix (Applied Biosystems), 2 μL of the purified PCR product and 1 μL of the SNPs specific primer cocktail. The cycling conditions were 10 seconds at 96 °C, 10 seconds at 50 °C and 30 seconds at 60 °C, for 25 cycles. To remove unincorporated ddNTPs, the final products were incubated with 1U of shrimp alkaline phosphatase (SAP) (USB Corporation) for 1 hour at 37 °C, followed by 15 minutes at 85 °C to inactivate the enzyme. 1 μl of the SAP-inactivated single-nucleotide extension reaction was diluted with 12 μl HiDi Formamide (Life Technologies) and supplied with 0.5 μl GeneScan 120 LIZ Size Standard (Life Technologies). The mixture was denatured at 95 °C for 5 minutes and loaded onto an ABI PRISM 3010 Genetic Analyzer (Life Technologies). Capillary electrophoresis was performed following manufacturer's instructions. Extension products were visualized and analyzed using GeneScan 4.0 (Life Technologies). Initial validation of the method was performed against DNA sequencing of the amplified region.

Representative results showing the identification of rs1042522 and rs2279744 are shown in Figure 1.

*Statistical analysis*

The difference in genotypic and allelic frequency between patients and controls and their deviation from the

Table 3. Primers for single-nucleotide primer extension reaction

| SNP polymorphism | sequence 5'-> 3' | Base pares | P* | SNP | E** | C(μM) |
|---|---|---|---|---|---|---|
| *p53*Pro72Arg C>G rs1042522 | CTGGTGCAGGGGCCACG | 17+15(C) | 32 | reverse | G/C | 33.3/33.8 |
| *MDM2*309T>G rs 2279744 | TCCGGACCTCCCGCGCCG | 18+22(C) | 40 | reverse | A/C | 39.6/40.1 |

P* -Primer orientation;
E**- Electrophoregram position (referent/variant)

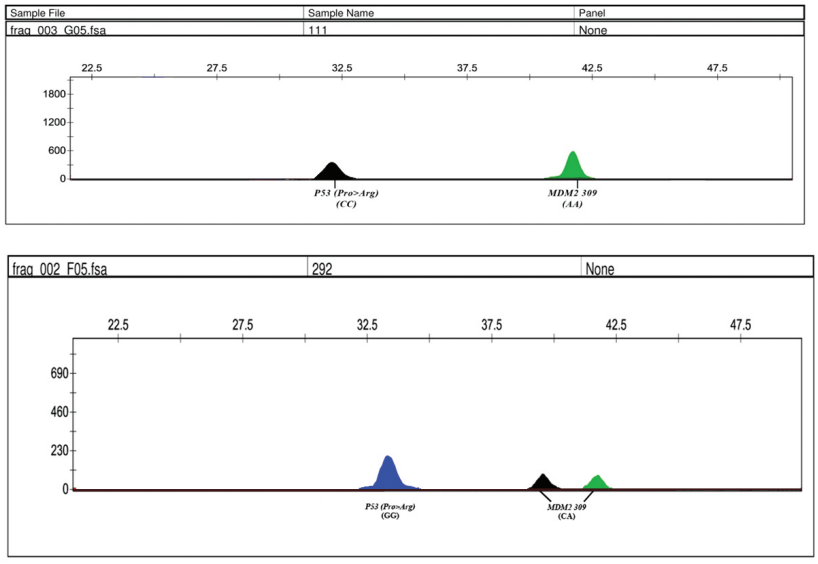


Fig. 1. Results of SNPs after running the multiplex SNP reaction on capillary gel electrophoresis ABI PRISM 3010 Genetic Analyzer (Life Technologies). The electrophoregrams show identification of: A) CC and AA genotypes and B) GG and CA genotype of codon 72 at *p53* and *MDM2 309* polymorphisms respectively.

Hardy-Weinberg (HW) equilibrium were calculated by the Chi-squared test. The significance of Odd Ratio (OR) with their 95% confidence intervals (95% CI) was also assessed by the Chi-square ($\chi^2$) test. In case the latter was not applicable, the Fisher's exact test was used. A *P* value of 0.05 or less is considered statistically significant. All statistical calculations were performed in SPSS 19.0 (SPSS, Chicago, IL, USA) software.

## Results

*Case /Control association analysis*

Descriptive characteristic of both the study and control groups are given in Table 4. Our results reveal an association between the rs1042522, rs2279744 polymorphisms and SIL/CCa among HPV positive cases. Inconclusive results obtained for 9 controls and two cases genotyped for *p53*Pro72Arg were excluded from further analysis. The obtained data from polymorphic variant in control group

Table 4. Characteristics of patients included in the study

| Patients and controls characteristic | Patients | Controls | |
|---|---|---|---|
| age | | | |
| <30 | 30 | 43 | |
| ≥ 30 | 101 | 67 | |
| total | 131 | 110 | |
| Cases Histology | | | |
| LSIL | 39 | | |
| HSIL | 52 | | |
| CCa | 40 | | |
| HPV type | In LSIL (%) | In HSIL and CCa (%) | Total |
| HPV 16 and/or 18 | 6 (15.4) | 56 (60.9) | 62 (47.4) |
| Other High risk type | 23 (59.0) | 20 (21.7) | 43 (32.8) |
| Multiple infections | 10 (25.6) | 16 (17.4) | 26 (19.8) |
| total | 39 (100.0) | 92 (100.0) | 131 (100.0) |

complied with the Hardy-Weinberg equilibrium.

The *p53* Pro72Arg C>G polymorphic variant showed no association with initiation and development of SIL and CCa, but after the stratification of the study group, the GG genotype and the G allele (Arg variant) of *p53* Pro72Arg showed to be associated with progression of LSIL to HSIL and CCa in the presence of HPV. The result showed lower CC genotype frequency in HSIL and CCa (group C2)(6.4%) compared to LSIL (C3group) (25%) [CC vs GG; p=0.001, OR=0.16 (95%CI: 0.04-0.5); CC vs CG; p=0.045, OR=0.3 (95%CI: 0.09-1); GG vs CG+CC; p=0.02, OR=0.4 (95%CI: 0.1-0.8) and [CC vs CG+ GG; p=0.004, OR=0.2 (95%CI: 0.1-0.8)] (Table 5).

The two HPV positive groups: HSIL with CCa and the group of all SIL comparison with control group showed no significant differences in either genotype or allelic frequencies for *MDM2* 309T>G polymorphisms, but when LSIL group was compared with HSIL and CCa, TT genotype and T allele showed significantly lower frequency in the last one (22.8% and 52.7%, respectively) than in LSIL (46.2% and 68.0% respectively) [T vs G p=0.02, OR=0.52 (95%CI: 0.3-0.9; TT vs GG; p=0.04, OR=0.29 (95%CI: 0.08-1.03); TT vs TG+GG; p=0.007, OR=0.34 (95%CI: 0.15-0.75)]. The results of this study suggest that *MDM2* 309T>G might be a potential marker for predicting the progression of LSIL to HSIL and CCa. The results are shown in Table 6.

## Discussion

The results obtained in this study are the first to reveal a significant association between these two polymorphisms and susceptibility to HPV positive SIL and CCa finding

Table 5. Results of allele and genotypic frequencies of rs1805010 in the groups of patients with different cervical lesions and healthy controls rs1042522

| *P53*Pro72Arg rs1042522 | Cases (SIL + CCa) n (%) | HSIL + CCa n (%) | LSIL n (%) | controls n (%) | p1* | p 2* | p 3* | p 4* |
|---|---|---|---|---|---|---|---|---|
| Allele C | 78 (30.2) | 45 (24.4) | 33 (44.6) | 63 (31.2) | 0.8 | 0.15 | 0.04 | 0.001 |
| G | 180 (68.8) | 140 (75.6) | 41(55.4) | 139 (68.8) | | | | |
| Genotype GG | 66 (51.2) | 53 (57.6) | 13 (35.1) | 51 (50.5) | 0.7 | 0.11 | 0.6 | 0.001 |
| GC | 48 (37.2) | 33 (35.9) | 15 40.6) | 37 (36.6) | 0.8 | 0.2 | 0.2 | 0.045 |
| d.CC | 15 (11.6) | 6 (6.5) | 9 (24.3) | 13 (12.9) | Ref | Ref | Ref | 0.02 |
| Recesive model GG/ GC+ CC | | | | | 0.1 | 0.36 | 0.13 | |
| Dominant model GC+GG/CC | | | | | 0.7 | | 0.09 | 0.004 |
| n-total | 129 (100) | 92 (100) | 37 (100) | 101 (100) | - | - | - | - |

(%) Allelic and genotype frequency *p1= cases/controls *p2=/HSIL+CCa/controls; *p3=LSIL/controls; *p4=HSIL+ CCa/LSIL

Table 6. Results of allele and genotypic frequencies of rs1800872 in the group of patients with different cervical lesions and healthy controls

| *MDM2309T>G* rs2279744 | Cases (SIL + CCa) n (%) | HSIL + CCa n (%) | LSIL n (%) | controls n (%) | p1* | p 2* | p 3* | p 4* |
|---|---|---|---|---|---|---|---|---|
| Allele T | 183 (62.0) | 97 (52.7) | 53 (68.0) | 124 (56.4) | 0.21 | 0.4 | 0.07 | 0.02 |
| G | 112 (48.0) | 87 (47.3) | 25 (32.0) | 96 (43.6) | | | | |
| Genotype TT | 39 (29.8) | 21 (22.8) | 18 (46.2) | 30 (27.3) | Ref | ref | ref | ref |
| GT | 72 (55.0) | 55 (59.8) | 17 (43.6) | 64 (58.2) | 0.6 | 0.54 | 0.04 | 0.14 |
| GG | 20 (15.2) | 16 (17.4) | 4 (10.2) | 16 (14.5) | 1 | 0.43 | 0.15 | 0.04 |
| Dominant model GT+GG/TT | | | | | 0.66 | 0.40 | 0.03 | 0.007 |
| Recessive model GG/ TG + TT | - | | | | 0.87 | 0.5 | 0.4 | 0.29 |
| n-total | 131(100) | 92 (100) | 39 (100) | 110 100) | - | - | - | - |

(%) Allelic and genotype frequency *p1= cases/controls *p2=/HSIL+CCa/controls; *p3=LSIL/controls; *p4=HSIL+ CCa/LSIL

a risk of progression of HPV positive LSIL to HSIL or CCa. Genetic analysis of these SNPs was done mostly on Macedonian ethnical origin population and only a small number of them were of Roma and Albanian ethnicity. Codon 72 of the *p53* gene is found to be a site of frequent polymorphism (Matlashewski et al., 1987) and the frequencies of allelic variants at this codon differ among different ethnic groups (Beckman et al., 1994; Sjalander et al., 1996) and it is shown that they are associated with different cancer susceptibility (Birgander et al., 1996; Buller et al., 1997; Kawajiri et al., 1993). The C to G base substitution in codon 72 replacing amino acid Prolin with Arginine is considered to produce a more vulnerable variant of *p53*. Since Storey et al. (Storey et al., 1998) for the first time reported 7 fold higher risk of *p53* Arg homozygotes for HPV associated squamous carcinoma of cervix than heterozygotes, many studies were done in order to confirm this association. Still the results remain contradictory and not conclusive for accepting this SNP as a marker for prediction of HPV associated SIL and CCa susceptibility. Some studies confirm this association with invasive CCa but not with preinvasive lesions (Jee et al., 2004; Koushik et al., 2004).

Recently, a meta-analysis (Habbous et al., 2012) that included 4,292 patients with invasive cervical cancer, 1,519 with high-grade SIL, 810 with low-grade SIL, 648 with SIL of unspecified grade, and 5,326 healthy controls, with known HPV status, showed an increased risk for progression of HPV positive SIL to cervical cancer supporting the HPV biologically modulated mechanism (Habbous et al., 2012). The study did not show association of the Arg variant with initiation and develpompment of cercical cancer nither in HPV positive nor HPV negative patiens. Similar results are obtained in our study showing no association of this SNP with initiation and development of SIL and CCa, but the GG genotype and the G allele (Arg variant) of *p53* Pro72Arg showed to be associated with the progression of LSIL to HSIL and CCa in the presence of HPV.

Previous studies didn't find any association between this polymorphism and CCa in most of the European population with the exception of the United Kingdom and Italy (Sousa et al. 2007). The study of Hu (Hu et al., 2010) shows association of this SNP in Caucasian population particularly in HPV16 and HPV18 positive cases but also suggests that there might be an interaction between this SNP and other environmental factors. The G allele was more frequently inherited in Caucasian population so the frequency in this population is higher (71%) compared to African and American population (65%) what may explain the existence of association of this SNP in the Caucasians.

The opposite conclusion was reached by Zhou e al. (Zhou et al., 2012) in meta study that included 28 case control studies with a total of 3,580 cervical cancer cases and 3,827 healthy controls. The study showed that the Pro/Pro genotype was associated with increased risk of cervical cancer under the heterozygous model among Indian populations, but not among Chinese, Japanese and Korean populations. Nevertheless, our preliminary results are in concordance with studies showing that the (Arg) variant of *p53* Arg72Pro is associated with progression to cervical cancer in the presence of HPV infection.

The inconsistency in the reported results from different studies could be attributed to different material used for analysis, use of insufficient specific molecular technique, or improper population selection. The source of the specimen for analysis is very important step because it might be a source for false results. Use of biopsy specimens can very

Table 7. Results for P value (square test), odd ratio (OR) and 95% Confident interval (95% CI) of statistically significant association between analyzed SNPs and grade of HPV positive CIN or CCa patients

| SNP | Comparation | Model | | p value | OR | 95% CI |
|---|---|---|---|---|---|---|
| *p53Pro72Arg* | HSIL+CCa/LSIL | allelic | G vs C | 0.001 | 0.4 | 0.2-0.7 |
| | | homozygous | GG vs CC | 0.001 | 0.16 | 0.04-0.5 |
| | | heterozygous | GC vs CC | 0.045 | 0.3 | 0.09-1 |
| | | dominant | GC+GG/CC | 0.004 | 0.2 | 0.1-0.8 |
| | | recessive | GG/ C+CC | 0.02 | 0.2 | 0.1-0.8 |
| *MDM2309* | HSIL+CCa/LSIL | allelic | G vs T | 0.02 | 0.5 | 0.3-0.9 |
| | | homozygous | GG vs TT | 0.04 | 0.29 | 0.08-1.03 |
| | | dominant | GT+GG/TT | 0.007 | 0.34 | 0.15-0.75 |
| | LSIL /controls | heterozygous | GT vs TT | 0.04 | 0.44 | 0.2-0.9 |
| | | dominant | GT+GG/TT | 0.03 | 2.28 | 1.1-4.8 |

often yield DNA with poor quality giving poor quality of obtained results.

The results for *MDM2* SNP 309 obtained from 241 samples showed no association between this polymorphism and HPV positive SILs and CCa. Analyses of stratified study group showed higher frequency of TT genotype among HPV positive LSIL cases (46.2%) compared to control group (27.3%) [TT vs TG+ GG, p= 0.03; OR =2.28 (95% CI: 1.1-4.8) under dominant model and under heterozygous model TG vs TT, p=0.04 ; OR=0.44 (95% CI:0.2-0.9)]. But we also found a lower T allele and TT genotype frequencies in HSIL (52.7% and 22.8%) compared to LSIL (68% and 46.2):[(G vs T, p=0.02; OR=0.5 (95% CI:0.3-0.9); homozygous model: GG vs TT, p=0.04; OR=0,29 (95% CI:0.08-1.3) and dominant model: TT vs TG+GG, p=0.007; OR=0.34 (95% CI: 0.15-0.75)].

This significant result indicates an association of this polymorphism with a risk for GG genotype and G allele carrier with progression of LSIL to higher grade lesions and CCa.

Until recently, the nature of the relationship between *MDM2* SNP309 (rs2278744) and cervical cancer was not clear. There have been many studies in this area with contradictory conclusions. The study of Amaral et al. and Meissner (Amaral et al., 2014; Meissner et al., 2007) in Brazilian population did not conclude any association between this SNP and CCa. Contrary to this, Singh et al. (Singh et al., 2009) found an association between higher frequency of G allele and cervical cancer in Indian women infected with HPV. The same conclusion was reached by Nunobiki et al. (Nunobiki et al., 2010), who found the rs2278744 to be associated with cervical carcinogenesis especially in the high-risk HPV positive CIN as well as in a study from 1000 genomic project (www.1000genomes.org) in Chinese and Japan. Recently study of Knappskog (Knappskog and Lonning, 2011a) explained the possible causes of discrepant conclusions about this association between Asian and non-Asian cohorts highlighting the influence of the *MDM2* SNP258 on *MDM2* SNP309 association with CCa. Namely, while 'in vitro' studies have shown SNP309G-allele to elongate a binding site for the transcription factor Sp1, thereby increasing *MDM2* transcription (Bond et al., 2004), new studies find that SNP285C-allele has the opposite effect and this polymorphism significantly reduces Sp1 binding (Knappskog and Lonning, 2011b). Finding that SNP285C/309G haplotype frequency is ~12% in North Western Europeans (Norway, the Netherlands and the UK) (Knappskog and Lonning, 2011a) and a similar frequency was reported in the Caucasian cohorts of the 1000 genomes project (www.1000genomes.org) but it has been notably absent in Asian (Chinese and Japanese) populations (Knappskog and Lonning, 2011b, 2011; www.1000genomes.org,), explain the ethnical influence on different conclusions. Although the presence of the SNP285C variants seems to antagonize the effect of SNP309G there is a limited number of studies that have analyzed this polymorphism with cancer susceptibility and the total sample size was relatively small, which may lead to relatively weak power to detect the real association. The study of Roszak et al. (Roszak et al., 2015) did not find significant association between cervical cancer development or clinic-pathological features and the *MDM2* 309 T>G SNP but they demonstrated that *MDM2* 285G>C polymorphism may protect against SCC development in a sample of the Polish population (Roszak et al., 2015). This study is also the first one that assessed the *MDM2* 285 G>C polymorphism and showed that the 285CC/309GG + 285GC/309GG combined genotype may protect against SCC and 285GG/309GG may increase the risk of SCC in Caucasian populations. But, this study is also characterized by limitation of low statistical power. Several studies about MDM2 SNP285 in different types of cancers reached contradictory results (Bjornslett et al., 2012; Knappskog and Lonning 2011a; Knappskog and Lonning 2011b; Paulin et al., 2008; Piotrowski et al., 2012; Roszak et al., 2015). Our results are in concordance with the Caucasians population studies but lacking results on the MDM2 285G>C polymorphism, it warrants further analysis for obtaining more informative results. Additionally, our results were based on OR without adjustment for individual's age, sex, smoking status, drinking status, environmental factors, and other lifestyles factors that would be taken in account in further studies.

The inconsistent results from the studies could be addressed to the difference in ethnical allele frequency difference, in the source of the extracted DNA for analyses (blood, swabs, cancer tissue) and to the difference in the applied detection methodology. Improving the methodology and using the optimal type of specimens for analysis, could be provide more relevant results. Using of the SNP extension method in our study, we enabled detecting of several SNPs in a single reaction and it has shown to be a fast, accurate and cost effective method.

## Conclusion

According to our knowledge, this study reports for the first time point out the potential of *p53*Pro72Arg rs1042522 and MDM2 rs2279744, as predictive markers for progression of LSIL lesions to HSIL and CCa. The genetic testing for individual susceptibility to HPV infection and CCa that confirm the association between genetic variants at *p53*Arg72Pro and *MDM2* 309 indicated that they could affect cervical carcinogenesis but this finding needs confirmation with further studies performed on a larger cohort and control groups. The results from this study could be used as basic clinical data for recognizing the individual response to the HPV infection but the genetic susceptibility could have comprehensive significance in future if is performing on a sets of genotypes that have been confirmed as a factors that predict the outcome of

cervical changes.

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## References

Amaral, C.M., Cetkovska K., Gurgel, A.P., Cardoso, M.V., Chagas, B.S., Paiva Junior, S.S., de Lima Rde, C., Silva-Neto, J.C., Silva, L.A., Muniz, M.T., Balbino, V.Q., Freitas, A.C., 2014. MDM2 polymorphism associated with the development of cervical lesions in women infected with Human papillomavirus and using of oral contraceptives. Infect. Agent. Cancer 9, 24. DOI: 10.1186/1750-9378-9-24.

Beckman, G., Birgander, R., Sjalander A., Saha, N., Holmberg, P.A., Kivela, A., Beckman, L., 1994. Is *p53* polymorphism maintained by natural selection? Hum. Hered. 44(5), 266-270.

Birgander, R., Sjalander, A., Zhou, Z., Fan, C., Beckman, L., Beckman, G., 1996. *p53* polymorphisms and haplotypes in nasopharyngeal cancer. Hum. Hered. 46(1), 49-54.

Bjornslett, M., Knappskog, S., Lonning, P.E., Dorum, A., 2012. Effect of the MDM2 promoter polymorphisms SNP309T>G and SNP285G>C on the risk of ovarian cancer in BRCA1 mutation carriers. BMC Cancer 12, 454. DOI: 10.1186/1471-2407-12-454.

Bond, G.L., Hu, W., Bond, E.E., Robins, H., Lutzker, S.G., Arva, N.C., Bargonetti, J., Bartel, F., Taubert, H., Wuerl, P., Onel, K., Yip, L., Hwang, S.J., Strong, L.C., Lozano, G., Levine, A.J., 2004. A single nucleotide polymorphism in the MDM2 promoter attenuates the *p53* tumor suppressor pathway and accelerates tumor formation in humans. Cell 119(5), 591-602. DOI: 10.1016/j.cell.2004.11.022.

Buller, R.E., Sood, A., Fullenkamp, C., Sorosky, J., Powills, K., Anderson, B. 1997., The influence of the *p53* codon 72 polymorphism on ovarian carcinogenesis and prognosis. Cancer Gene Ther. 4(4), 239-245.

Carvalho, C.M., Pena, S.D., 2005. Optimization of a multiplex minisequencing protocol for population studies and medical genetics. Genet. Mol. Res. 4(2),115-125.

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D.M, Forman, D., Bray. F., 2015. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int. J cancer 136(5), 359-386.

Habbous, S., Pang, V., Eng, L., Xu, W., Kurtz, G., Liu, F.F., Mackay, H., Amir, E., Liu, G. 2012. *p53* Arg72Pro polymorphism, HPV status and initiation, progression, and development of cervical cancer: a systematic review and meta-analysis. Clin. Cancer Res. 18(23), 6407-6415. DOI: 10.1158/1078-0432.ccr-12-1983.

Hu, X., Zhang, Z., Ma D., Huettner, P.C., Massad, L.S., Nguyen, L., Borecki, I., Rader, J.S., 2010. T*P53*, MDM2, NQO1, and susceptibility to cervical cancer. Cancer Epidemiol. Biomarkers Prev. 19(3), 755-761. DOI: 10.1158/1055-9965.epi-09-0886.

Hu, Z., Jin, G., Wang, L., Chen, F., Wang, X., Shen, H., 2007. MDM2 promoter polymorphism SNP309 contributes to tumor susceptibility: evidence from 21 case-control studies. Cancer Epidemiol. Biomarkers Prev. 16(12), 2717-2723. DOI: 10.1158/1055-9965.epi-07-0634.

Jee, S.H., Won, S.Y., Yun, J.E., Lee, J.E., Park, J.S., Ji, S.S., 2004. Polymorphism *p53* codon-72 and invasive cervical cancer: a meta-analysis. Int. J Gynaecol. Obstet. 85(3), 301-308. DOI: 10.1016/j.ijgo.2003.08.017.

Kawajiri, K., Nakachi, K., Imai, K., Watanabe, J., Hayashi S., 1993. Germ line polymorphisms of *p53* and CYP1A1 genes involved in human lung cancer. Carcinogenesis 14(6),1085-1089.

Knappskog, S., Lonning, P.E. 2011a. MDM2 promoter SNP285 and SNP309; phylogeny and impact on cancer risk. Oncotarget 2(3), 251-258.

Knappskog, S., Lonning, P.E. 2011b. Effects of the MDM2 promoter SNP285 and SNP309 on Sp1 transcription factor binding and cancer risk. Transcription 2(5), 207-210. DOI: 10.4161/trns.2.5.16813.

Knappskog, S., Bjornslett, M., Myklebust, L.M., Huijts, P.E., Vreeswijk, M.P., Edvardsen, H., Guo, Y., Zhang, X., Yang, M., Ylisaukko-Oja, S.K., Alhopuro, P., Arola, J., Tollenaar, R.A., van Asperen, C.J., Seynaeve, C., Staalesen, V., Chrisanthar, R., Lokkevik, E., Salvesen, H.B., Evans, D.G., Newman, W.G., Lin, D., Aaltonen, L.A., Borresen-Dale, A.L., Tell, G.S., Stoltenberg, C., Romundstad, P., Hveem, K., Lillehaug, J.R., Vatten, L., Devilee, P., Dorum, A., Lonning, P.E., 2011. The MDM2 promoter SNP285C/309G haplotype diminishes Sp1 transcription factor binding and reduces risk for breast and ovarian cancer in Caucasians. Cancer Cell 19(2), 273-282. DOI: 10.1016/j.ccr.2010.12.019.

Koushik, A., Platt, R.W., Franco, E.L. 2004. *p53* codon 72 polymorphism and cervical neoplasia: a meta-analysis review. Cancer Epidemiol. Biomarkers Prev 13(1), 11-22.

Matlashewski, G.J., Tuck, S., Pim, D., Lamb, P., Schneider, J., Crawford, L.V., 1987. Primary structure polymorphism at amino acid residue 72 of human *p53*. Mol. Cell Biol. 7(2), 961-963.

Meissner Rde, V., Barbosa, R.N., Fernandes, J.V., Galvao, T.M., Galvao, A.F., Oliveira G.H., 2007. No. association between SNP309 promoter polymorphism in the MDM2 and cervical cancer in a study from northeastern Brazil. Cancer Detect Prev 31(5), 371-374. DOI: 10.1016/j.cdp.2007.09.001.

Momand, J., Jung, D., Wilczynski, S., Niland, J.. 1998. The MDM2 gene amplification database. Nucleic Acids Res. 26(15), 3453-3459.

Noveski, P., Mircevska, M., Plaseski, T., Peterlin, B., Plaseska-Karanfilska D., 2014a. Study of Three Single Nucleotide Polymorphisms in the SLC6A14 Gene in Association with Male Infertility. Balkan J Med. Genet. 17(2), 61-66. DOI: 10.2478/bjmg-2014-2075.

Noveski, P., Madjunkova, S., Mircevska, M., Plaseski, T., Filipovski, V., Plaseska-Karanfilska, D., 2014b. SNaPshot assay for the detection of the most common CFTR mutations in infertile men. PLoS One 9(11), e112498. DOI: 10.1371/journal.pone.0112498.

Nunobiki, O., Ueda, M., Yamamoto, M., Toji, E., Sato, N., Izuma, S., Okamoto, Y., Torii, K., Noda, S., 2010. MDM2 SNP 309 human papillomavirus infection in cervical carcinogenesis. Gynecol. Oncol. 118(3), 258-261. DOI: 10.1016/j.ygyno.2010.05.009.

Nunobiki, O., Ueda, M., Toji, E., Yamamoto, M., Akashi, K., Sato, N., Izuma, S., Torii, K., Tanaka, I., Okamoto, Y., Noda, S., 2011. Genetic Polymorphism of Cancer Susceptibility Genes and HPV Infection in Cervical Carcinogenesis. Patholog. Res. Int. 2011, 364069. DOI: 10.4061/2011/364069.

Oliner, J.D., Kinzler, K.W., Meltzer, P.S., George, D.L., Vogelstein, B., 1992. Amplification of a gene encoding a *p53*-associated protein in human sarcomas. Nature 358(6381), 80-83. DOI: 10.1038/358080a0.

Paulin, F.E., O'Neill, M., McGrego,r G., Cassidy, A., Ashfield A., Ali, C.W., Munro, A.J., Baker, L., Purdie, C.A., Lane, D.P., Thompson, A.M., 2008. MDM2 SNP309 is associated with high grade node positive breast tumours and is in linkage disequilibrium with a novel MDM2 intron 1 polymorphism. BMC Cancer 8, 281. DOI: 10.1186/1471-2407-8-281.

Piotrowski, P., Lianeri,M., Rubis, B., Knula, H., Rybczynska, M., Grodecka-Gazdecka, S., Jagodzinski, P.P., 2012. Murine double minute clone 2,309T/G and 285G/C promoter single nucleotide polymorphism as a risk factor for breast cancer: a Polish experience. Int, J Biol, Markers 27(2), e105-110. DOI: 10.5301/jbm.2012.9140.

Roszak, A., Misztal, M., Sowinska, A., Jagodzinski, P.P., 2015. Murine Double-Minute 2 Homolog Single Nucleotide Polymorphisms 285 and 309 in Cervical Carcinogenesis. Mol, Diagn, Ther, 19(4), 235-244. DOI: 10.1007/s40291-015-0153-4.

Singh, H., Jain, M., Sachan, R., Mittal, B., 2009. Association of TNFA-308G>A and IL-10-819C>T promoter polymorphisms with risk of cervical cancer. Int, J Gynecol, Cancer 19(7), 1190-1194. DOI: 10.1111/IGC.0b013e3181a3a3af.

Sjalander, A., Birgander, R., Saha, N., Beckman, L., Beckman, G., 1996. *p53* polymorphisms and haplotypes show distinct differences between major ethnic groups. Hum, Hered ,46(1):41-48.

Sousa, H., Santos, A.M., Pinto, D., Medeiros, R., 2007. Is the *p53* codon 72 polymorphism a key biomarker for cervical cancer development? A meta-analysis review within European populations. Int. J Mol. Med. 20(5), 731-741.

Storey, A., Thomas, M., Kalita, A., Harwood, C., Gardiol, D., Mantovani, F., Breuer, J., Leigh, I.M., Matlashewski, G., Banks, L., 1998. Role of a *p53* polymorphism in the development of human papillomavirus-associated cancer. Nature 393(6682), 229-234. DOI: 10.1038/30400.

Tsigris, C., Chatzitheofylaktou, A., Xiromeritis, C., Nikiteas, N., Yannopoulos, A., 2007. Genetic association studies in digestive system malignancies. Anticancer Res, 27(5B), 3577-3587.

Ueda, M., Hung, Y.C., Terai, Y., Kanda, K., Takehara, M., Yamashita, H., Yamaguchi, H., Akise, D., Yasuda, M., Nishiyama, K., Ueki, M., 2003. Glutathione S-transferase GSTM1, GSTT1 and *p53* codon 72 polymorphisms in human tumor cells. Hum, Cell 16(4), 241-251.

Wade, M., Wang, Y.V., Wahl, G.M. 2010. The *p53* orchestra: Mdm2 and Mdmx set the tone. Trends Cell Biol, 20(5), 299-309. DOI: 10.1016/j.tcb.2010.01.009.

Zhou, X., Gu, Y., Zhang, S.L., 2012. Association between *p53* codon 72 polymorphism and cervical cancer risk among Asians: a HuGE review and meta-analysis. Asian Pac. J Cancer Prev. 13(10), 4909-4914.

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**Резиме**

# Асоцијација помеѓу *p53Pro72Arg* (rs1042522) и *MDM2*309 (rs2279744) полиморфизмите со ризикот за развој на цервикални интраепителни лезии и цервикален карцином кај македонските жени

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**Клучни зборови**: Сквамозни интраепителни лезии (СИЛ), Цервикален карцином (ЦЦ), Хуман папиломавирус (ХПВ), полиморфизам со еднонуклеотидна замена, подложност на СИЛ и цервикален карцином

Инфекциите со високо-ризичен Хуман папиломавирус (ХПВ) тип се важни етиолошки фактори во иницијација на сквамозните интраепителни лезии (СИЛ), но сепак не се доволни за нивна малигна прогресија до повисок степен на промена или цервикален карцином (ЦЦ). Еднонуклеотидните полиморфизми: rs1042522 во кодонот 72 на *p53* и rs2279744 во 309 промотерниот регион на *MDM2* генот се можни фактори кои дополнително влијаат на развојот на СИЛ и на ЦЦ доведувајќи до зголемена атенуација на *p53* патиштата. Во трудот се испитува асоцијацијата на овие полиморфизми со ХПВ позитивните СИЛ и ЦЦ меѓу жените од Р. Македонија. Користејќи мултиплекс PCR SNaPShot анализи ги генотипизиравме rs1042522 и rs2279744 кај 131 ХПВ позитивна жени со СИЛ или ЦЦ и 110 контролни, со негативен цитолошки и ХПВ наод. Не е утврдена статистики значајна разлика во алелната и генотипската фреквенција кај случаите и контролите. По групирање на пациентките зависно од степенот на лезијата се покажа статистички пониска присутност на ЦЦ генотипот и Ц алелот во групата на пациентки со посисок степен на промени и ЦЦ (ХСИЛ + ЦЦ) во однос на оние со благи лезии (ЛСИЛ) [GG vs CC; p=0,001, OR=0,4; CG vs CC; p=0,04, OR=0,03 and CG+ GG и CC; p=0,004, OR=0,2]. Дополнително ТТ генотипот и Т алелот од *MDM2* 309се значајно поретки кај ХСИЛ и ЦЦ во однос на ЛСИЛ групата [G vs T p=0,02, OR=0,52; GG vs TT; p=0,04, OR=0,29; TT vs TG+GG; p=0,007, OR=0,34]. Според ова заклучуваме дека Арг варијантата на rs1042522 и TT/T -генотип / алелот на rs2279744 се асоцирани со прогресија на ЛСИЛ во ХСИЛ или ЦЦ и може да се користат како маркери за предвидување и менаџирање на ЦЦ, но клиничката значајност бара понатамошна потврда на поголема група испитаници и добро дизајнирана студија.