

**Investigation of allelic polymorphisms
influencing the development and
prognosis of cervical tumors and
preblastomas**

Submitted for the degree of Doctor of Philosophy (PhD)

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The significance of tumor diseases

In developed countries, death caused by tumors stand second place after cardiovascular diseases. Most women with tumors suffer from breast cancer in the world. Instead of raw numbers, calculating age standardized rates for the whole population is more informative, and using these we can see that cervical tumor is one of the most common tumors causing death among women, has advanced from third to first place (Figures 1. and 2.).

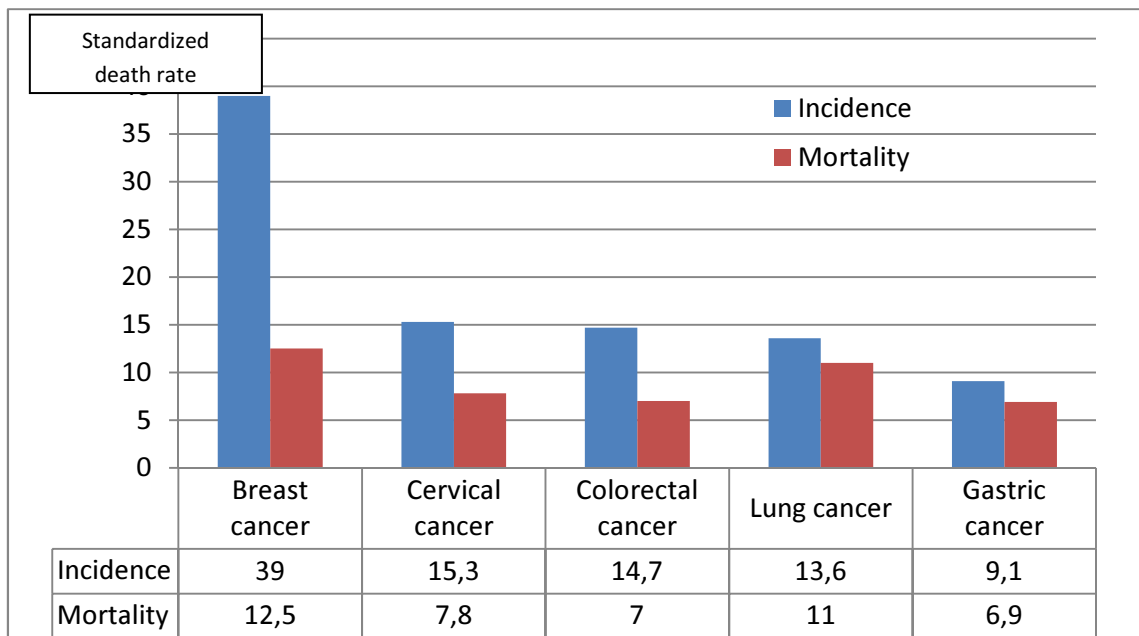


Figure 1. Age specific incidence and mortality rates, standardised to the female population of the world. (<http://globocan.iarc.fr/>).

Epidemiology of cervical cancer

In 2008, almost 300 000 women died of cervical cancer in the world. The number of new diseases was half a million, despite the introduction of the screening of healthy women for cervical cancer due to which there was a significant improvement in the morbidity of the disease. In Hungary, the number of new cases approached a thousand in 2010, and the number of fatalities was almost 400. It is a significant problem that the disease starts at an

increasingly earlier age, manifestation of the illness is continually increasing among women aged 35-40.

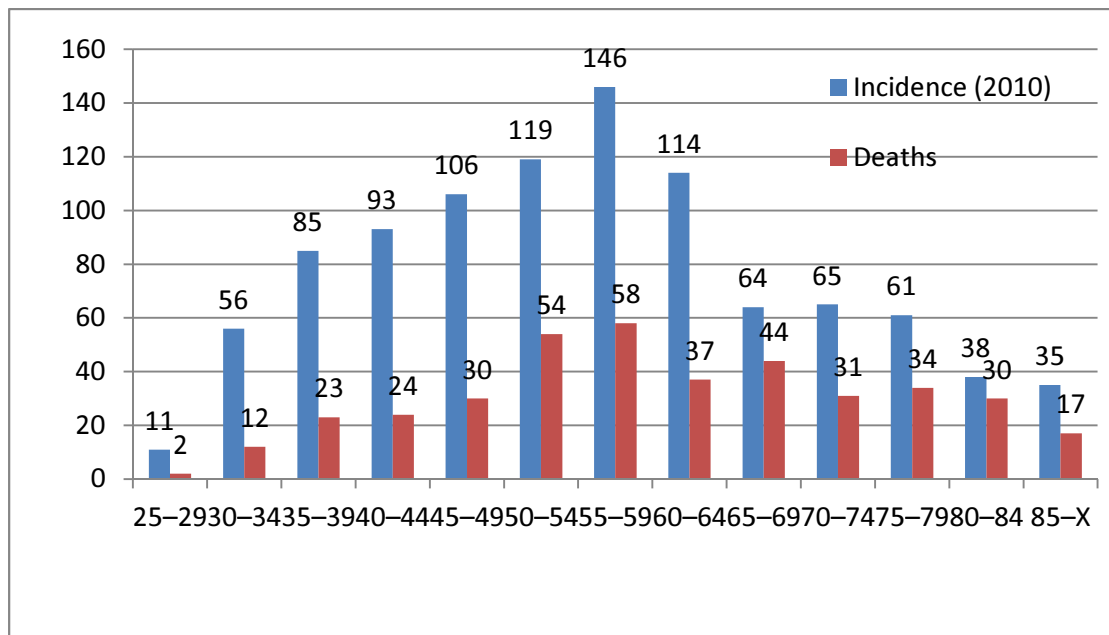


Figure 2. Cervical tumors and associated deaths by age groups, Hungary, 2010. (KSH, Demographic Yearbook, Cancer registry).

Major risk factors of cervical cancer

Human papillomavirus

The main risk factor for the disease is the Human Papillomavirus (HPV), the presence of which can be detected in 90% of cervical and genital tumors. The virus invades the basal epithelium via micro- and macrodamage during sexual intercourse, where it attacks the vulnerable immature squamous epithelial cells. Afterwards it resides in the cervical mucosa for years in an inactive state, preying on the host system. In case of a persistent infection that lasts for years, the two oncoproteins of the virus cause changes on the cervix that result in intraepithelial dysplasia of different severity. Without treatment, this precancerous state leads to cervical tumor in the upcoming years or decades. HPV infection can be detected in more than 95% of cervical cancers, 75-95% of CIN II/III stages and 25-40% of CIN I stages.

Table I. presents the summary of further cofactors influencing the development of the disease.

- Age
- Race
- Number of childbirths
- Number of abortions
- Young age at first delivery
- Oral anticoncipients
- Number of sexual partners
- Smoking
- Immune suppression
- Chlamydia infection
- Obesity
- Family history of cervical cancer
- Low socioeconomic status
- Diet factors (e.g.: low fruit and vegetable consumption)

Table 1.: Risk factors for cervical cancer.

Personality as a risk factor

Personality has an important role in the development and experience of illnesses. It has been shown in numerous cases in the last decades, that environmental stress and psychological factors influence the risk of illnesses.

Dopaminergic systems, dopamine receptors, DRD2

Dopamine is an important neurotransmitter synthesized in the central nervous system and adrenal glands. Dopamine receptors can be found both on pre- and postsynaptic neurons which denotes its multifunctional role. During the last few years one of the most intensively examined gene has been the one that of dopamine receptor D2 (DRD2) in connection with psychological processes and addictive behavior. DRD2 gene plays a key role in stress management and motivation, therefore A1/A2 polymorphism has been investigated in numerous neuropsychiatric and mental diseases and in case of the effect of environmental stress as well. Since DRD2 gene affects numerous factors that influence the risk of cervical cancer (e.g. it has an effect on sexual activity, the development of early menarche and smoking habits, and it also influences alcohol and drug cravings), we felt it was feasible to assess the allele prevalence of the dopamine receptor gene D2 as well in the population we investigated.

Metabolic enzymes

Numerous procarcinogenic compounds get into the human system during environmental exposition. These compounds go through metabolic transformation in the body, in which metabolic enzymes have a significant role. The activity of metabolic enzymes can depend on age, sex, stress, burden, time of the day or certain medications. However, the genotypes of these enzymes have outstanding significance as well, since the majority of metabolic enzymes are genetically polymorph, meaning they have different types of allele variants. These, often only one base differences could be enough to slightly modify the function and activity of the protein that is translated from the gene.

GSTM1 and GSTT1 enzymes have a prominent role in the formation of the defense system of the human body, since they participate in the transformation of the potential carcinogens that are produced due to diet, transport and smoking habits. The most significant polymorphism of GSTM1 and GSTT1 enzymes is the so-called 0/+ polymorphism. The + genotype denotes the functional protein, while in the opposite case a section of the gene is deleted and the protein translated from the gene is dysfunctional, namely it is not able to conjugate. In case of homozygotic deletion (called null genotype), thus the individual does not have the functional GSTM1 enzyme that participates in the biotransformation and elimination of active carcinogenic metabolites.

II. AIMS AND OBJECTIVES

- Is there a difference between the distribution of DRD2/ANKK1 A1/A2 alleles in case of the existence of persistent HPV 16 or 18 infection in women with high-grade cervical dysplasia and healthy women or women with low-grade dysplasia (in other

words, does DRD2/ANKK1 A1/A2 allele polymorphism have an effect on the development of high-grade cervical dysplasia)?

- Is there a difference between the distribution of DRD2/ANKK1 A1/A2 alleles in patients with cervical cancer or preblastoma with bad or good prognosis (in other words, does DRD2/ANKK1 A1/A2 allele polymorphism influence the prognosis of cervical cancer and preblastoma)?
- Is there a difference between the distribution of GSTM1 és GSTT1 0/+ genotypes in women with high-grade cervical dysplasia and healthy controls or women with low-grade dysplasia, in case of persistent HPV 16 or 18 infection (in other words, does GSTM1 and/or GSTT 0/+ polymorphism influence the development of cervical dysplasia)?
- Do the coexistence of the supposedly high-risk GSTM1 and GSTT1 genotypes show interaction (and if yes, to what extent) in the development of cervical preblastoma?

MATERIALS AND METHODS

In the investigation regarding the risk of cervical dysplasia, 214 women participated in the experiment in which we investigated DRD2 polymorphism, and 253 women participated in the one in which we examined GST polymorphism, all had persistent HPV infection (type 16 or 18). Women were recruited from the Obstetrics and Gynaecology Departments of Szent György Hospital of Fejér county and Diósgyőr Hospital. The investigation was carried out in possession of ethical approval and all women participated voluntarily. Confirmation of HPV infection was carried out from archived biopsy material and samples taken for cervical smear test, retrospectively in some of the cases. The relevant demographic parameters of the investigated participants (age, age at the time of menarche, age at first sexual intercourse, number of children and number of abortions) were recorded. Information regarding diagnosis, treatment history and the outcome of the illness was acquired from hospital patient records.

We registered the development of cervical dysplasias and cancers for at least 7 years from the time of the confirmation of HPV infection. Women were regarded as 'positives' if cytology (cervical smear test) showed HSIL, or biopsy confirmed CIN II or CIN II (high-grade dysplasia group), whereas members of the control group either showed no cervical changes or had LSIL or CIN I diagnosis at the most. In order to establish whether certain GST and DRD2 alleles influence the development of high-grade cervical dysplasia, we compared allele prevalences in the high-grade dysplasia group and control group which showed no such change.

Regarding DRD2 allele polymorphism, we investigated the employability of polymorphism as a prognostic marker as well. In this case there were 239 participants, with a diagnosis of CIN III or I cervical cancer. We have also confirmed the presence of type 16 or 18 HPV infection. The patients received treatment appropriate for tumors or precancerous conditions, completely independent of the present study. Every participant was grouped into one of the following two groups at the end of the 5 year period after diagnosis: 1) Progression, incidental death, presence of residual tumor or need for treatment at the end of the observation period. 2) Tumor free state without any signs of residual tumor, recurrence or metastasis. After DRD2 genotyping, allele frequency was compared between groups of patients that showed good or bad prognosis.

HPV assay

The of verification of HPV types 16 and 18 was carried out with nested PCR. **External primers:** Forward: ACCGAAAACGGTTGAACCGAAAACGGT, reverse: AATAATGTCTATATTTCACTAATT. **Internal, type-specific primers:** HPV16 forward: ATGTITCAGGACCCACAGGA, reverse: CCTCACGTGCGAGTAACTGT, HPV18 forward: ATGGCGCGCTITGAGGATCC, reverse: GCATGCGGTATACTGTCTCT. (124 bp and 188 bp fragments).

Examination of allele polymorphisms

GSTM1 and GSTT1 genotyping

After DNA isolation with phenol-chloroform extraction, GSTM1 and GSTT1 genotyping was carried out simultaneously with polymerase chain reaction (PCR). Amplification was

carried out with internal control in all cases. The reaction mixture, in 15 µl in total volume contained 0.5 U Taq DNA polymerase (Go Taq, Promega), 1x buffer (Promega), 2 µl DNA template, 200 µM dNTP and 1.5 mM MgCl₂, 30-30 pmol GSTT1-F and GSTT1-R primers, 50-50 pmol GSTM1-F and GSTM1-R primers, 20-20 pmol β-globin-F and β-globin-R primers.

After amplification the sample was electrophoresed in agarose gel containing 2% ethidium bromide. As a result of amplification there were two fragments visible under UV light in addition to the control: a 215 bp long band in case of the GSTM1 + genotype and a 480 bp long band in case of the GSTT1 + genotype.

DRD2/ANKK1 genotyping:

The PCR reaction mixture in 15 µl total volume contained 0.5-0.5 µM primer (5'CACGGCTGGCCAAGTTGTCTA3', 5'CACCTTCTGAGTGTCATCAA3'), 0,5 U Taq DNA polymerase (Go Taq, Promega), 1X buffer (Promega), 2 µl DNS template, 200 µM dNTP and 2,5 mM MgCl₂.

After amplification the PCR product was digested with TaqI restriction endonuclease, and the the DNA fragments were separated with electrophoresis in 1.4% agarose gel. In case of A1 allele, one single 300 bp fragment indicated lack of digestion, whereas in case of the presence of A2 allele, 125 and 175 bp fragments were visible in the ethidium bromide agarose gel.

Statistical analysis

Demographic parameters of the groups were compared with Student t-test in case of continuous variables, and Pearson chi-squared test was employed in case of prevalences. In case of the distribution of genotypes (both DRD2 and GST genes) the comparison of the groups was carried out with logistic regression analysis both in case of risk analysis and the investigation of effect on survival. A1/A1 and A1/A2 genotypes were treated as a common group in DRD2 polymorphism analysis, due to the rare prevalence of A1 homozygote genotype. During logistic regression analysis, the effect of genotype was examined in a controlled manner, standardised to demographic variables (age, age at first period, age at first sexual intercourse, number of childbirths and number of abortions).

In case of the table like presentation of the results, odds ratios (OR) and their 95% confidence intervals and p-values were given. All statistical analyses presented in this work were carried out with IBM SPSS Statistics 19 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Investigation of the effect of DRD2/ANKK1 polymorphism. The effect on the risk of dysplasia.

	Stages HSIL, CIN II/III	Control	OR (95% CI)	p-value
A2/A2	56 (54,9%)	78 (69,6%)	1,00 (reference)	-
A1/A2	39 (38,2%)	30 (26,8%)	1,87 (1,05-3,33)	p=0,034
A1/A1	7 (6,9%)	4 (3,6%)		
Összesen	102 (100,0%)	112 (100,0%)		

Table II.: DRD2 *TaqI* value prevalences in different groups of women infected with HPV.

The effect of DRD2/ANKK1 polymorphism on prognosis.

	Worse prognosis	Better prognosis	OR (95% CI)	p-value
A2/A2	27 (47,4%)	118 (64,8%)	1,00 (reference)	-
A1/A2	25 (43,8%)	55 (30,2%)	2,00 (1,07-3,74)	p=0,030
A1/A1	5 (8,8%)	9 (5,0%)		
Összesen	57 (100%)	182 (100%)		

Table III.: DRD2 *TaqI* allele prevalences in case of patients with cervical cancer or preblastoma with good and bad prognosis.

The effect of *GSTM1* and *GSTT1* allele polymorphisms on the development of cervical dysplasia.

Genotype	Stages HSIL, CIN II/III	Control	OR (95% CI)	p-value
<i>GSTM1</i> +	54 (46,2%)	83 (61,0%)	1,00 (reference)	-
0	63 (53,8%)	53 (39,0%)	1,78 (1,06-2,97)	0,028
<i>GSTT1</i> +	70 (59,8%)	101 (74,3 %)	1,00 (reference)	-
0	47 (40,2%)	35 (25,7%)	1,89 (1,10-3,26)	0,022
<i>GSTM1</i> és/vagy <i>GSTT1</i> +	90 (76,9%)	121 (89,0 %)	1,00 (reference)	-
Mindkettő 0	27 (23,1%)	15 (11,0%)	2,35 (1,17-4,73)	0,017

Table IV.: Distribution of *GSTM1* and *GSTT1* genotypes in infected women with persistent high-risk HPV.

DISCUSSION

One of the key factors in the relationship between HPV infection and cervical cancer is the type of the virus. Infection with the high-risk type significantly increases the risk of developing cervical dysplasia and the consequent cervical cancer. Within this group, HPV types 16 and 18 receive the most attention and it is nowadays clear that due to its carcinogenic effect and widespread distribution, HPV type 16 is considered to be the most dangerous. Altogether 70% of cervical cancers are caused by these two types of viruses in the world.

From the point of view of the dissertation it is a crucial question what further factors influence the risk of cervical cancer and the preceding dysplasia apart from HPV infection. Numerous factors have been proven to have a connection with the likelihood of developing cervical cancer and preblastoma. A proportion of these factors have an effect by increasing the risk of infection, therefore they are not considered to be risk modifying factors regarding the question we investigated. Other risk factors in contrast influence risk via HPV independent mechanisms, these are for example the number of successful pregnancies, contraceptives or smoking.

We have long known the influence of metabolic enzymes on carcinogenesis. The amount of active carcinogens is determined by - beyond carcinogenic exposure - the activity of phase I and II metabolic enzymes. The GST superfamily belongs to the group of phase II detoxifying enzymes. The members of the enzyme superfamily we have investigated (GSTM1 and GSTT1) show insertion-deletion polymorphism, consequently the given enzymes are not present in a functional form in the homozygotic 0 allele carriers. This does not cause a great deal of a problem due to the substrate overlap of the metabolic enzymes, but results in some decrease of the detoxifying capacity. According to the above mentioned, numerous investigations have found a relationship between the 0 genotype and the increased risk of numerous tumors. Certain investigations have shown a similar relationship regarding cervical cancer and preblastomas while others have not confirmed it.

In our investigation we attempted to rule out interfering factors as much as possible. Hence we decided to include a uniform population of HPV infected cases with long-term persistence in our study, and employed HSIL and CIN II/III stages as an end point. This approach is relevant from a practical aspect as well, since these changes necessitate medical intervention.

Our investigation has confirmed that GSTM1 and GSTT1 0 genotypes increase the risk of developing cervical preblastomas when persistent HPV infection is present. Women with dual 0 genotypes have shown further increased risk, which denotes an interaction between the two factors. According to our results, GSTM1 and GSTT1 allele polymorphisms increase

the risk of developing cervical dysplasia in the caucasian population as well, and the relationship between the them is not only present in the Asian population.

On the contrary to the above mentioned, there could be a completely different, indirect relationship between DRD2 gene allele polymorphism and the risk of cervical cancer. The mechanisms that could play a role could have an impact through psychological factors, for example certain addictive risks (smoking, alcoholism and drugs), that could enhance exposition with chemical or biological agents. Certain DRD2 associated behaviors (multiple sexual partners) could also increase the risk of infection, but naturally we could not measure this in our experiments, since the participants were solely HPV positive women. We assume that DRD2/ANKK1 allele polymorphism has its main risk altering effect by influencing stress management.

In terms of mental stress factors we have to distinguish external and internal components. The external component is stress itself, namely the environmental psychological strain. Internal factors, on the other hand are the management and processing of stress. It has been first proven in case of cardiovascular risk assessment, that it is not only -or mainly- the 'amount' of stress, but rather the personality, stress management and its processing is important in terms of the risk of the illness. The development of personality traits -the quality of stress management amongst others - can be influenced by certain genetic factors. Since dopaminerg systems can play an important role in the central regulation of certain mental functions and processes (motivation, pleasure-seeking behavior), DRD2 allele polymorphisms could also play a role in the formation of personality and our psychological character.

According to our knowledge, a publication that investigates the role of DRD2 A1/A2 allele polymorphism in increasing the risk of cervical cancer has not been published yet. The risk increasing effect of DRD2/ANKK1 *TaqIa* allele polymorphism supposedly has an effect through numerous indirect mechanisms and interactions. These addictive behaviors and mental diseases could influence further known cancer risk factors. It is a very difficult task to measure these factors in isolation and is not necessarily the appropriate solution. According to epidemiological methodology as well, the appropriate method leaves out intermediate variables from the analysis and analyses the relationship of the risk factor with the end point. We have followed this strategy in our analysis as well.

The prognostic effect can be explained in view of the above as well, moreover, to a certain extent even more easily, since psychological factors and the behavior of the patient are proven to influence the prognosis of several illnesses. Our observation confirms this, we have proven that DRD2 A1 allele carriers have worse prognosis.

OWN RESULTS

- The distribution of DRD2/ANKK1 A1/A2 alleles showed a statistically significant difference between women who had high-grade cervical dysplasia and healthy women or women with low-grade dysplasia (OR: 1,87, 95% CI: 1,05-3,33; p=0,034). Therefore, DRD2/ANKK1 A1/A2 allele polymorphism influenced the development of high-grade cervical dysplasia, namely A1 allele has been proven to be a risk factor.
- The distribution of DRD2/ANKK1 A1/A2 alleles showed a statistically significant difference between patients with cervical cancer or preblastoma that had good or bad prognosis (OR: 2,00, 95% CI: 1,07-3,74; p=0,030). DRD2/ANKK1 A1/A2 allele polymorphism therefore has proven to be an influencing factor in the development of cervical cancer and preblastoma, and the carriers of A1 allele had worse prognosis.
- Both GSTM1 (OR: 1,78, 95% CI: 1,06-2,97; p=0,028) and GSTT1 0 (OR=1,89, 95% CI=1,10-3.26; p=0,022) genotypes were significantly more frequent in women with high-grade cervical dysplasia as compared to healthy women or women with low-grade dysplasia. GSTM1 és a GSTT1 0 genotypes therefore increased the risk of developing cervical dysplasia.
- The coexistence of GSTM1 and GSTT1 0 genotypes increased the risk of developing cervical preblastoma to a greater extent (OR=2.35, 95% CI=1.17-4.73; p=0.017), compared to the presence of GSTM1 or GSTT1 0 on their own. This confirms the presence of an interaction between the two high-risk genotypes.

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