GSTM1, GSTT1 and GSTP1 in Patients with Multiple Breast Cancers and Breast Cancer in Association with another Type of Cancer

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Rezumat

Glutation S-transferazele M1, T1 și P1 la pacientele cu cancere mamare multiple și asocierea cancerului mamar cu un alt tip de cancer

Introducere: Cancerul mamar prezintă cea mai ridicată incidenţă la femei. Glutation S-transferazele reprezintă un grup important de enzime care participă în metabolismul xenobioticelor. Membrii acestei superfamilii de gene sunt implicaţi în dezvoltarea a multiple cancere.
Obiective: am căutat să vedem dacă polimorfismul genelor GSTM1, GSTT1 şi GSTP1 reprezintă factori de risc pentru pacientele diagnosticate cu tumori maligne multiple, dintre care cel puţin una este localizată la nivelul sânului.
Material şi metodă: în perioada 2005-2012 din rândul a 520 paciente diagnosticate cu cancer mamar, 69 au avut tumori maligne primitive multiple, dintre care cel puţin una a fost localizată mamar. Cercetarea genotipurilor GSTM1, GSTT1 şi GSTP1 a cuprins 59 de paciente diagnosticate cu cancere mamare multiple sau asocierea unui cancer mamar cu un alt cancer, comparativ cu un lot de martori sănătoşi.
Rezultate: în sublotul de paciente cu asociere de cancer mamar cu alt cancer genotipul nul GSTM1 a fost găsit la 61,2% dintre paciente, comparativ cu 29% dintre martori; în sublotul de cancere mamare metacrone prezenţa oricărui genotip nul GSTM1 sau GSTT1 a fost semnificativ diferită statistic faţă de martori (65,2% faţă de 28,5%); în sublotul cu cancere sincrone genotipul nul GSTM1 a fost constatat la 66,6% dintre paciente comparativ cu 9% în cazul martorilor, iar prezenţa oricărui genotip nul (GSTM1 sau GSTT1) a fost tot semnificativă statistic în lotul studiat.
Concluzii: Genotipul nul GSTM1 este un factor de risc pentru cancerele mamare sincrone şi pentru asocierile de cancer mamar cu unul extramamar; prezenţa genotipului nul (GSTM1 sau GSTT1) reprezintă un factor de risc pentru cancerul mamar multiplu (bilateral sau sincron); genotipul nul GSTT1 şi genotipurile variante heterozigot Ile105Val şi homozigot Val105Val al GSTP1 nu sunt factori de risc pentru cazurile luate în studiu.

Cuvinte cheie: cancere multiple mamare, asociere de cancere diferite cu cancer mamar, GSTM1, GSTT1 şi GSTP1

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Abstract

Introduction: breast cancer has the highest incidence in women. Glutathione S-transferases (GSTs) are a large group of enzymes involved in the metabolism of xenobiotics. The members of this gene superfamily are involved in the development of multiple cancers.
Objectives: the aim of the study was to see whether the GSTM1, GSTT1 and GSTP1 genetic polymorphisms are risk factors for patients diagnosed with multiple malignancies, of

which at least one is located in the breast.

Materials and Methods: in the period between 2005 and 2012, of the 520 patients diagnosed with breast cancer, 69 had multiple primitive malignant tumors, of which at least one was localized in the breast. The research on GSTM1, GSTT1 and GSTP1 genotypes consisted of 59 patients diagnosed with multiple breast cancers or with breast cancer in association with another type of cancer, compared with a group of healthy controls.

Results: in the subgroup of patients with breast cancer in association with another type of cancer, the GSTM1 null genotype was present in 61.2% of patients, compared to 29% of controls; the subgroup of metachronous breast cancers, the presence of any of the GSTT1 or GSTM1 null genotypes was statistically significantly different from that of controls (65.2% vs. 28.5%); in the subgroup with synchronous cancers, the GSTM1 null genotype was found in 66.6% of patients compared to 9% for the controls, and the presence of any null genotype (GSTM1 and GSTT1) was also statistically significant in the case group.

Conclusions: the GSTM1 null genotype is a risk factor for synchronous breast cancers and for breast cancer associated with extramammary cancer; the presence of null genotypes (GSTM1 or GSTT1) is a risk factor for multiple breast cancer (bilateral or synchronous); the GSTT1 null genotype and the heterozygous variant allele (Ile105Val) and homozygous variant allele (Val105Val) of GSTP1 are not risk factors for the cases studied.

Key words: breast multiple cancers, association of different cancers with breast cancer, GSTM1, GSTT1 and GSTP1

Introduction

Numerous advances in imaging methods employed for the diagnosis of malignant diseases, as well as the widespread use of minimally invasive diagnostic and treatment methods, of effective adjuvant and neoadjuvant therapies in recent years, have led to an improved prognosis in different cancers, resulting in more and more cured patients of those with neoplastic disease. The increase in life expectancy of cancer patients has led to an increase in the possibility of developing other primitive malignancies, of which many can be cured. Breast cancer in women is on the first place among malignant tumors and mortality rates in this type of cancer are also the highest. For both men and women, breast cancer ranks second after lung cancer. The GST superfamily has a protective role against DNA damage caused by exogenous and endogenous oxidative agents (1) via the conjugation of xenobiotic compounds (herbicides, insecticides, environmental carcinogens, alkylating agents, and platinum) with glutathione, promoting their excretion in the urine. The role of these genes in breast cancer has provided mixed results in various studies.

Objectives

This research aims to find an answer to whether GST (GSTM1, GSTT1 and GSTP1) gene polymorphisms are linked to an increased risk of developing breast cancer or breast cancer in association with other cancers.

Materials and Methods

Between 2005 and 2012, 521 patients with breast cancer were admitted to Cluj-Napoca Municipal Clinical Hospital, representing 10.41% of 5,003 patients (2) diagnosed with malignant tumors. Of these, 69 patients (13.47%) also had another type of cancer: 40 patients (57.97%) had multiple primitive malignant mammary tumors, 13 patients (18.84%) had breast cancer in association with another type of cancer (3 cases of cervical cancer, 2 cases of endometrial cancer, 2 cases of ovarian cancer and 2 cases of skin basal cell carcinomas, 1 case of rectal cancer, 1 case of gastric cancer, 1 case of melanoma skin cancer, and 1 case of lymphoma), 16 patients (23.18%) had a history of breast cancer associated with bladder (4), kidney (2), colorectal (2) and ovarian (2) cancer, 3 patients had a retroperitoneal malignant tumor (kidney, spleen, colon), 1 thyroid cancer, 1 cervical cancer, 1 vaginal cancer, 1 endometrial cancer, and 1 stomach cancer. The age of these patients ranged between 34 and 83 years. Ten patients developed a third cancer: ovarian, endometrial, thyroid, retrobulbar, kidney, lymphoma, and breast cancer. One patient developed a fourth cancer (gastric cancer).

The genetic study included 59 patients diagnosed with multiple breast cancers or breast cancer associated with other cancers. Only patients who signed their informed consent for participation in the study were included. The study was approved by the Ethics Committee of Cluj-Napoca Medical Civil Society. Blood was harvested from admitted patients to determine the genetic profile for three GSTs, which is known to be possibly involved in the development of breast cancer.

Breast cancer was associated with another type of cancer, most commonly metachronous cancer: 3 cases of association with cervical cancer, 3 with bladder cancer, 2 patients with endometrial cancer, 2 ovary, 2 kidney cancers and 2 basal cell carcinomas, 1 patient with rectal cancer, 1 gastric, 1 melanoma skin cancer and 1 lymphoma. In 9 cases, breast cancer was the second malignant tumor, occurring at a mean interval of 13.42 years after the index tumor (ranging between 5 and 34 years). In 6 cases, breast cancer was the index malignancy, followed by a second malignant tumor after a period of 8 years (range 7 to 10 years). There were also 3 cases of synchronous malignant tumors.

The DNA samples were obtained from 2 ml of peripheral blood on EDTA and from paraffin-embedded tumor. We used a multiplex PCR protocol (3) to determine simultaneously the presence or absence of the GSTT1 or GSTM1 genes; we were able to identify the null genotypes, but could not distinguish between heterozygous and homozygous subjects. The primers for GSTM1, GSTT1 used for amplification of 215 bp in case of GSTM1 allele and 480 bp for GSTT1 allele were: FwM1 5'-

GAACTC CCTGAAAAGCTAAAGC-3'; RevM1 5'-GTTGGGCTCAAATATAGGGTGG- 3'and FwT1 5'-TTC-CTTACT GGTCCTCACATCTC-3'; RevT1 5'- TCACCG-GATCATGGC CAGCA-3'. As an internal amplification control we used the primer pair for a co-amplification of 268 bp of β Globin gene. A gradient thermocycler (Mastercycler Gradient, Eppendorf®, Germany) was used for PCR reactions: 94°C for 5 minutes and then 35 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min and a final polymerization step at 72°C for 10 min. A total amount of approximately 100 ng of genomic DNA was obtained, and it was amplified in a total volume of 25 μl reaction mixture containing 12.5 μl 2xPCR Master Mix (Fermentas MBI, Lituania®), 1 μl BSA (Bovine Serum Albumine, Fermentas MBI, Lituania®) solution 5 mg/ml, 8 pM of each primer, forward and reverse (Eurogentec, Belgium®) and water free of nucleases to complete the 25 μl reaction volume. The electrophoresis in MetaPhor agarose gel (Lonza®, Basel, Switzerland) was used to analyse the amplification products; the null genotypes were considered in the absence of amplification products (215 or 480 bp).

For GSTP1 polymorphism we applied a Wizard Genomic DNA Purification Kit (Promega®, MA, USA) in order to extract genomic DNA from 300 μl of blood (leucocytes). The Ile105Val polymorphism of GSTP1 gene was analysed by the PCR–RFLP technique (polymerase chain reaction-restriction fragment length polymorphism), modifying a protocol described by Harries et al, 1997 (4). The amplification of the DNA was made with the primers pair 105F (5'-ACC-CCAGGGCTCTATGGGAA-3') and 105R (5'-TGAGGGCA-CAAGAAGCCCCT-3'), 12.5 μl 2xPCR Master Mix (Fermentas MBI, Lituania®), 1 μl BSA (Bovine Serum Albumine, Fermentas MBI, Lituania®) solution 2 mg/ml, 8 pM of each primer, forward and reverse (Eurogentec, Belgium®) and water free of nucleases to complete the 25 μl volume. A gradient thermocycler (MastercyclerGradient, Eppendorf®) was used for the PCR reactions: denaturation for 5 minutes at 95°C, then in 30 seconds another denaturation at 94°C (30 cycles), primer annealing for 30 seconds at 55°C, 30 seconds of polymerization at 72°C and an elongation of 5 minutes at 72°C. The PCR product was an amplified fragment of 176 bp, digested with 5 U BsmAI (Fermentas MBI, Lituania®), and then the fragments were separated on a 3.0% Metaphor® agarose gel (Lonza®, Basel, Switzerland), and visualized in a UV transilluminator (VilberLourmat Imaging System®, Marne-la-Vallée, France) after staining with ethidium bromide. We obtained: three fragments of 176, 91 and 85 bp, which belong to the Ile/Val genotype; two fragments of 91 and 85 bp represented a Val/Val homozygous genotype; an undigested product of 176 bp corresponding with the absence of restriction site, corresponding with an Ile/Ile genotype (5).

We extracted the DNA from paraffin embedded samples through a multiple steps procedure: multiple sections of 5 μm thickness cut from the samples were put in microcentrifuge tubes at 4°C; a QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) was used for extraction of genetic material, using the protocol provided by the manufacturer; the paraffin was removed with xylene; the samples were rehydrated with ethanol and after 12 hours of incubation at 56 degrees C, in the presence of proteinase K, the DNA was recovered and purified on QIAamp MinElute column and eluted in nuclease free water, measuring the concentrations with a Nanodrop spectrophotometer (NanoDrop Technologies; Wilmington, DE).

Results

The study evaluated 59 patients with at least two cancers, of which at least one was breast cancer, and 39 healthy individuals who were close to patient age (±2 years). The average age of the first cancer diagnosis in the case group was 53.32 ± 12.43 years (ranging between 28 and 84 years). The average age of second cancer diagnosis in the case group was 63.22 ± 9.95 years (ranging between 45 and 84 years). The average age in the witness group was 64.56 ± 10.39 years (ranging between 44 and 86 years). The ages of patients and witnesses had a normal distribution (Kolomorov-Smirnov test) and did not reveal statistically significant differences between average patient age and average witness age during study enrolment (t test, p=0.5).

There were 22 female cancer patients (36%) and 12 female controls (30%) who were smokers. The percentage of smokers was not significantly different between the case group and the controls (chi-square test, p=0.7). Breast cancer was: invasive ductal carcinoma (57.45%), invasive lobular carcinoma (31.91%) and other types of carcinoma (mucinous, squamous and papillary) representing 10.64%. The GSTM1 null genotype was present in 35 patients in the case group (59.3%) and in 17 women in the witness group (43.5%), without any statistically significant differences in its frequency between the two groups (chi-square test, p=0.1). The GSTT1 null genotype was found in 14 patients in the group with cancer (23.7%) and in 4 women (10.2%) in the witness group, and there were no statistically significant differences in its frequency between the two groups (chi-square test, p=0.1). The GSTP1 Ile105Ile (common) genotype was present in 29 patients in the group with cancer (49.1%). In this group, the heterozygous variant Ile105Val was found in 26 patients (44%) and the homozygous variant Val105Val was identified in 3 patients (5%). In the control group, the GSTP1 Ile105Ile genotype was present in 21 women (53.8 %), the Ile105Val genotype in 17 women (43.5%), and the Val105Val genotype in 1 woman (2.5%). Alleles were in Hardy-Weinberg equilibrium (chi-square test = 2, p=0.1), without any statistically significant differences in genotype frequency between the patient group and the controls (chi-square test, p=0.8). There were no statistically significant differences in the frequency of null genotype between different locations of the first or second cancer for either GSTM1 (chi-square test, p=0.8, p=0.5) or GSTT1 (chi-square test, p=09, p=0.8). The frequency of the GSTP1 genetic polymorphisms at position 105 did not differ between the various locations of the first or second cancer (chi-square test, p=0.5, p=0.2). The histological types of breast cancer have not been associated with the presence of the GSTM1 null genotype (chi-square test, p=0.5), of the GSTT1 null genotype

(chi-square test, p=0.4) or of a particular GSTP1 genotype (chi-square test, p=0.6). Both null genotypes (GSTM1 and GSTT1) were found in 8 patients (13.5%) in the case group and in 2 women (5.1%) in the witness group, the difference in percentage not being statistically significant (chi-square test, p=0.3). There were 41 patients (69.4%) in the case group and 19 women (48.7%) in the witness group with one of the two types of null genotypes (GSTM1 and GSTT1), the difference in percentage not being statistically significant (chi-square test; p=0.06).

We analysed a subgroup of 31 patients who had breast cancer associated with a second cancer with another location than the breast, and compared with a control group consisting of 31 women, close in age (±2 years). For the case group, the average age during the diagnosis of the first cancer was 53.97 ± 11.78 years (ranging between 31 and 84 years), while the average age during the diagnosis of the second cancer was 64 ± 11.3 years (ranging between 50 and 84 years). For the control group, the average age was 63.58 ± 10.01 years (ranging between 50 and 85 years). Patient and witness ages had normal distribution (Kolomorov-Smirnov test). There were no statistically significant differences between the average age of patients during study enrolment and that of the controls (t test, p=0.8). Ten of the patients in the cancer group were smokers (32.2%), whereas 12 women in the control group (38.7%) were smokers, the percentage of smokers not being significantly different (chi-square test, p=0.7). GSTM1 null genotype was present in 19 patients (61.2%) and 9 healthy women (29%), with statistically significant differences in the frequency of the null genotype between the patient group and controls (chi-square test, p=0.02). GSTT1 null genotype was detected in 5 patients (16.1%) and 2 women (6.4%) in the witness group, with no statistically significant differences in its frequency (chi-square test, p=0.4). In the cancer group, the GSTP1 Ile/Ile genetic polymorphism at position 105 was found in 16 patients (51.6%), Ile/Val genotype in 14 patients (45.1%), and Val/Val genotype in 1 patient (3.2%). In the control group, GSTP1 Ile/Ile genotype was identified in 15 women (48.3%), Ile/Val genotype in 15 women (48.3 %), and Val/Val genotype in 1 woman (3.2%). Alleles were in Hardy-Weinberg equilibrium (chi-square test = 2.4, p=0.1). There were no statistically significant differences in genotype frequency between the patient group and the witness group (chi-square test, p=0.9). In the case group, 3 patients (9.6 %) indicated the presence of both null genotypes (GSTM1 and GSTT1). The percentage difference was not statistically significant (chi-square test, p=0.2). Either of the two null genotypes (GSTM1 and GSTT1) were present in 21 of the patients (67%) and 11 women (35.4%) in the witnesses group, the percentage difference being statistically significant (chi-square test, p=0.02). We calculated a mean difference of 10.6 ± 8.3 years between the occurrence of the first cancer and the diagnosis of the second neoplasm. Although patients with GSTM1 null genotype developed a second cancer much faster (9.9 ± 6.8 years) than those with at least one allele (12 ± 11.1 years), there was no statistically significant difference between the years-difference average (t test, p=0.6).

Another subgroup of 23 patients had metachronous contralateral breast cancer (we excluded synchronous breast cancers) as the second neoplasm, and was compared with a control group made of 21 women close in age (±2 years). The average age during the diagnosis of the first cancer in the case group was 51.2 ± 12.5 years (ranging between 28 and 77 years). The average age during the diagnosis of the second neoplasm in the case group was 60.8 ± 10.2 years (ranging between 50 and 80 years). The average age for the witness group was 61.3 ± 14 years (ranging between 40 and 82 years). The ages of patients and witnesses had a normal distribution (Kolomorov-Smirnov test). There were no statistically significant differences between the average age of patients from study enrolment and that of controls (t test, p=0.8). There were 14 patients (46.6%) and 9 healthy women (31%) who were smokers. The percentage of smokers was not significantly different (chi-square test, p=0.3). GSTM1 null genotype was found in 11 patients (47.8%) and in 5 controls (23%), without any statistically significant differences in its frequency (chi-square test, p=0.1). GSTT1 null genotype was present in 6 patients (26%) and 3 controls (9.5%), with no statistically significant differences (chi-square test, p=0.2). In the group with cancers, GSTP1 Ile105Ile genotype was found in 10 patients (43.4%), heterozygous variant Ile105Val in 12 patients (52.1%) and homozygous variant Val105Val in 1 patient (4.3%). In the control group, GSTP1 Ile105Ile genotype was present in 11 women (52.3%), Ile105Val genotype in 9 women (42.5%) and Val105Val genotype in 1 woman (4.7%). Alleles were in Hardy-Weinberg equilibrium (chi-square test = 1.05, p=0.3). There were no statistically significant differences in the frequency of GSTP1 genotypes between the patient group and the witness group (chi-square test, p=0.8). Both null genotypes (GSTM1 and GSTT1) were identified in two patients (8.9%) and one healthy woman (4.7%), the percentage difference not being statistically significant (chi-square test, p=1). Fifteen patients (65.2%) and 6 female controls (28.5%) carried either of the two null genotypes (GSTM1 and GSTT1), the percentage difference not being statistically significant (chi-square test, p=0.03).

Another subgroup of 12 patients with synchronous neoplasms was compared with a control group of 11 women close in age (±2 years). The average age during the diagnosis of synchronous neoplasms for the case group was 59.1 ± 13.4 years (ranging between 43 and 84 years). The average age during the diagnosis of another cancer in the case group was 62.67 ± 11.2 years (ranging between 45 and 84 years). The average age for the witness group was 62.82 ± 13.8 years (ranging between 44 and 86 years). The ages of patients and witnesses had a normal distribution (Kolomorov-Smirnov test). There were no statistically significant differences between the average age of patients from study enrolment and the controls (t test, p=0.9). Five patients (33.3%) and 9 female controls (28.5%) were smokers, and the percentage was not significantly different (chi-square test, p=1). GSTM1 null genotype was found in 8 patients (66.6%) and 1 control (9%), with statistically significant differences (chi-squared χ^2 test, p=0.01). GSTT1 null genotype was found in 4 patients (33.3%) and no

witness, but the difference was not statistically significant (chi-square test, p=0.1). In the cancer group, GSTP1 Ile105Ile genotype was identified in 6 patients (50%), Ile105Val genotype in 6 patients (50%) and Val105Val genotype was not identified in any patient. In the control group, GSTP1 Ile105Ile genotype was identified in 4 women (36.6%), Ile105Val genotype in 6 women (54.4%) and Val105Val genotype in 1 female (9%). Alleles were in Hardy-Weinberg equilibrium (chi-square test = 2.4, p=0.1). There were no statistically significant differences in the frequency of GSTP1 genotypes between the patient group and the controls (chi-square test, p=0.4). Nine patients (75%) and one healthy woman (9%) carried at least one null genotype (GSTM1 and GSTT1), the percentage difference being statistically significant (chi-square test, p=0.03). The estimation of the relative risk of the presence of GSTM1 null genotype for our studied cases with breast cancer and another associated cancer resulted in an OR value of 1.9 (CI95% 1.1, 3.2). The same risk is present for any of the null genotypes, either GSTT1 or GSTM1 (CI95% 1.1, 3.4). For bilateral breast cancers, OR value is 2 (CI95% 1.1, 3.8) associated with any of the two null alleles (GSTM1 and GSTT1), while for synchronous cancers, OR value is 3.1 (CI95% 1.3, 7 3) associated with the GSTM1 null genotype, and 3.9 (CI95% 1.4, 10.7) associated with any of the null alleles (GSTM1 and GSTT1).

Discussion

In developed countries, breast cancer is among the first primitive malignant tumors in women, accounting for 23 % of all cancers in women, followed by colorectal, lung, ovarian and endometrial cancers. In 2008, there were 1.38 million new cases diagnosed with breast cancer, which thus ranked II (10.9 % of all cancers diagnosed), the most common in both developed and developing regions [GLOBOCAN project 2008 (6)]. Mortality rates were lower (6-19/100,000), ranking breast cancer fifth in top cancer deaths (458,000 deaths), still representing the most frequent cause of cancer death in women. Romania had an incidence rate of 45.4/100,000 (7929 cases), with a mortality rate of 15.6/100,000 (3,101 deaths). In our study, the patients diagnosed with breast cancer represented 10.41 % of all cancer patients admitted to our hospital during the period under study.

In 1921, Kilgore (7) quotes Willard Parker, who in 1855 described the first 14 patients with bilateral breast cancer selected from a group of 397 women diagnosed with breast cancer, suggesting, like other authors (8,9), that the emergence of these multiple bilateral primitive tumors may indicate a high susceptibility to developing breast cancer. Breast cancers are among the most common synchronous cancers, their diagnosis currently being achieved by mammography screening. We consider two malignant mammary tumors primitive if they fulfil the following criteria: the presence of carcinoma in situ in the 2nd malignancy or in both cancers; if the 2 tumor is of different histopathological type or has a lower degree of differentiation than the first, or if it has an in situ component; if the first two criteria are not met and if there is no clear evidence of local, regional or distant metastasis from the first tumor (10). Bilateral tumors are often individual primitive tumors, whereas the multitude of synchronous malignant tumors located in a single breast are generally similar from the point of view of the genome. However, the possibility of developing two synchronous independent unilateral tumors has also been confirmed, as well as the fact that a breast tumor can trigger contralateral metastasis (11). Metastasis is supported by the presence of bilateral positive lymph nodes, the same degree of histological differentiation and the type of ductal carcinoma (12,13). Multiple synchronous tumors in the same breast are either multifocal (in the same quadrant) or multicentric (in different quadrants), considering that they develop more frequently [25-50% of patients may have an outbreak of a different carcinoma in the same breast (14,15)] than bilateral synchronous tumors. The indicators of the independent character of a breast tumor are: the presence of healthy tissue that separates the lesions, the location of tumor foci in different quadrants of the breast (16). The following techniques have been used in order to differentiate between primitive and secondary tumors: X-chromosome inactivation analysis, the comparison of allelic imbalance patterns (17-19), or the distribution of p53 protein mutations (20,21), but the correlation with the histological analysis still triggers unsatisfactory results. In the literature, the incidence of synchronous breast tumors is variable (0.3%-9%) and can be even 5 times higher when performing screening mammography (22,23).

Metachronous malignant tumors are more common (24), their incidence ranging between 1% and 12%, which is highlighted in patients with multiple breast cancers diagnosed and operated in our hospital. The authors assess different diagnostic range indicating synchronous breast tumors, most of them accepting the 6 month range.

There have been indications of the occurrence of secondary cancers following the first breast cancer, mainly contralateral breast cancers, as well as endometrial, ovarian, gastric, thyroid, kidney, colorectal cancers, leukemia. In addition, 27 patients diagnosed with multiple malignant tumors have developed breast cancer at an age of ≤ 50 years, knowing that the diagnosis of breast cancer under the age of 50 years is a risk factor for other malignant tumors. The age below 50 years is also important because patients could be considered premenopausal, knowing that menopause is a period of hormonal balance, which may influence the development of malignant tumors. GSTs also detoxify endogenous electrophilic molecules generated in lipid metabolism and products of oxidative stress, oxidative metabolites of estrogen (25,26), reducing the concentration of estrogen quinones and the possibility of these oxidative metabolites to cause DNA damage. It is assumed that GSTs act by means of an increase in the frequency of tumor suppressor gene mutations, such as p53 (27). An increased risk of developing breast cancer has been achieved for the GSTM1 null genotype in some of the studies performed (28). GSTT1 null genotype is related to an increased susceptibility to developing breast cancer (29-33). This has not been achieved in other studies (34-35). Some studies (36-40) have reported the existence of a not very high risk for

developing breast cancer in the case of combined changes in GSTs, and other studies have indicated no causal relationship (41,42). In breast cancer, GSTT1, GSTM1, GSTP1 genetic polymorphisms may be involved in modifying the response to neoadjuvant chemotherapy, given that null alleles or the changes in the genes involved lead to enzyme deficiency, which is linked to the inability of the chemo-therapy drug to export cells (43-45). GSTP1 is found in healthy and carcinoma breast tissue (46) in the liver, in the erythrocytes and it is polymorphic. GSTP1 Val (105) genotype has twice lower catalytic activity than the normal variant, so the survival of breast cancer patients will be better due to the low catalytic activity of GSTP1 in thiotepa-based chemo-therapy (47). A meta-analysis (48) revealed a significantly increased risk for breast cancer for GSTM1 null genotype (1.10) in the Caucasian (1.05) and Asian (1.21) population, especially in postmenopausal women (1.11). A 2-fold increase in the risk for breast cancer is found for the combination between GSTT1 and GSTM1 null genotypes. A 1.7-increase was found for the GSTM1 null genotype, 1.3 for the GSTT1 null genotype and 4.1 for the association of the two null genotypes with catechol-O-methyl transferase genotype (49). Our research has revealed an association between the GSTM1 null genotype and synchronous breast cancers, as well as the presence of at least one null genotype (either GSTM1 or GSTT1) associated with multiple breast cancers, and breast cancer associated with another type of cancer. There has been no statistically significant association with the GSTT1 null genotype or with the heterozygous variant Ile105Val, or the homozygous variant Val105Val of the GSTP1 genotype, even if more patients were identified with the GSTT1 null genotype than those in the witness group. We note that most of the white population carried the GSTM1 and GSTT1 null genotypes, indicating that 45.28% of the women selected as controls carried the GSTM1 null genotype, 36.41% the GSTT1 null genotype, 18.86% carried both null genotypes, and 47.16% carried at least one altered allele for the GSTP1 polymorphism at position 105. Even if there were no statistically significant differences, we want to point out that patients with the GSTM1 null genotype have developed a second cancer about two years faster than those who carried at least one allele. The statistical significance was very close to the threshold (p=0.06) for the presence of any of the null genotypes (GSTM1 and GSTT1) in patients with two primitive malignant tumors, of which at least one was breast cancer. Of course, our study has limitations: the relatively small number of patients, the short period considered for the study, the study was carried out in a single hospital unit, the retrospective analysis, worldwide comparative data on incidence and mortality rates for various cancers only for 2008 (the latest data is only available for the United States), the refusal to participate in the study expressed by some of the patients, the death of some patients, the inability to conduct genetic research by analysing DNA from some parts embedded in paraffin. These polymorphisms have also been studied for other multiple malignancies (50).

Conclusions

In our study, the GSTM1 null genotype is a risk factor for synchronous breast cancers and for breast cancer associated with one extramammary cancer. The presence of null genotypes (GSTM1 and GSTT1) is a risk factor for multiple breast cancer (bilateral or synchronous). The GSTT1 null genotype and the heterozygous variant Ile105Val and homozygous variant Val105Val of GSTP1 genotype are risk factors for the cases studied.

Conflict of interest

We declare that there is no conflict of interest for this manuscript, and manuscript is approved by all authors for publication. The work described is original and has not been published previously, and not under consideration for publication elsewhere.

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