Associations of MDM2 and MDM4 Polymorphisms with Early-Stage Breast Cancer

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Abstract: Breast cancer is one of the most common cancers worldwide. Single nucleotide polymorphisms (SNPs) in MDM2 and MDM4 have been associated with various cancers. However, the influence on clinical characteristics of breast cancer has not been sufficiently investigated yet. Thus, this study aimed to investigate the relationship between SNPs in MDM2 (rs2279744, rs937283, rs937282) and MDM4 (rs1380576, rs4245739) and I–II stage breast cancer. For analysis, the genomic DNA was extracted from 100 unrelated women peripheral blood. Polymorphisms were analyzed with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The study showed that MDM2 rs937283 and rs937282 were significantly associated with estrogen receptor status and human epidermal growth factor receptor 2 (HER2) status. SNPs rs1380576 and rs4245739, located in MDM4, were significantly associated with status of estrogen and progesterone receptors. Our findings suggest that rs937283 AG, rs937282 CG, rs1380576 CC, and rs4245739 AA genotypes were linked to hormonal receptor positive breast cancer and may be useful genetic markers for disease assessment.

Keywords: breast cancer; SNP; MDM2; MDM4; associations

1. Introduction
Breast cancer is one of the most common cancers among women [1,2]. Today many studies claim that breast cancer is a multifactorial disease, and the etiology of cancer is often unknown [3]. However, it has been shown that the major cause is the combination of genetic and environmental factors [1,4–6]. It has been demonstrated that overexpression or amplification of MDM2 and MDM4 genes are common in many malignancies, including breast cancer [7–10]. MDM2, which is mapped to chromosome 12q14.3–q15, and MDM4, which is located on chromosome 1 region q32, encode MDM2 and MDM4 proteins, respectively [11]. The evidence suggests that MDM2 and MDM4 may play significant roles in breast cancer formation, progression, prognosis, and protection from cancer [7,8,12–14]. The cellular processes could be related with other important protein p53, product of TP53 gene, which is a key regulator in genomic stability, cell cycle, autophagy, apoptosis, and necrosis [4,10,15]. It is known that MDM2 and MDM4 proteins perform distinct but cooperative functions in regulation of cellular p53 activity through a combination of p53 degradation and direct transcriptional squelching [16–19]. TP53 is the most common inactivated tumor suppressor gene in various human cancer types (including breast cancer). In spite of this fact, protein p53 encoded by wild-type TP53 can be functionally inactivated.
by abnormal structure or elevated levels of MDM2 and MDM4 [9,20–23]. Consequently, blocking MDM2 and MDM4 has been proposed as a cancer treatment strategy [24–30].

Several studies observed the associations between single nucleotide polymorphisms (SNPs) in MDM2 and MDM4 genes and various cancers [9,16,23,24,31–36]. In spite of this, the influence on breast cancer has not been sufficiently investigated yet. There is the lack of studies investigating the MDM2 and MDM4 associations with the clinical and morphological characteristics such as tumor size, nodal status, histologic grade, estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) status, proliferation rate, etc. This information might assist as prognostic factor and/or predictive marker for response to treatment of breast cancer. The prognostic and predictive strength of various characteristics is different. However, it was found that women with ER-positive breast cancer, compared to ER-negative, have a better prognosis and the treatment for positive status tumor is more efficacious. Low histologic grade as well as PR-positive and HER2-negative status is also usually associated with better breast cancer prognosis. Meanwhile, high histologic grade, high proliferation rate, and triple negative breast cancer subtype are characterized by unfavorable prognosis [37–40].

In the present study we determined the relationship between MDM2 and MDM4 SNPs and characteristics of breast tumor. The information may improve the assessment of prognostic and/or predictive value of MDM2 and MDM4 genotypes in breast cancer patients.

2. Materials and Methods

2.1. Patients

The homogeneous study group consisted of 100 unrelated Lithuanian women with a diagnosis of primary breast cancer. All women were treated in the Hospital of Lithuanian University of Health Sciences Kaunas Clinics. The age between 30 and 50 years at the time of diagnosis, early stage (I–II) of the disease and premenopausal status were preferred. All clinical and tumor pathomorphological data of the patients were obtained from the medical records with the help of the oncologists. The exclusion criteria were other malignancies, significant comorbidities, and incomplete medical documentation. Study data included the age at diagnosis, pathological tumor size (pT), status of pathological lymph node involvement (N), status of estrogen (ER) and progesterone (PR) receptors, human epidermal growth factor receptor 2 (HER2) status, tumor grade (G1 and G2, G3), progress, metastasis, and death.

The study was performed at the Oncology Research Laboratory (Oncology Institute, Lithuanian University of Health Sciences). The study was approved by Kaunas Regional Biomedical Research Ethical Committee (protocols Nr. BE-2-10 and Nr. P1-BE-2-10/2014). A written informed consent was obtained from all the participants.

2.2. DNA Isolation and Genotyping

The blood samples were collected in EDTA-containing tubes from all included subjects in 2014–2017. For SNP analysis genomic DNA was extracted from peripheral blood leukocytes with a commercially available DNA extraction kit (Thermo Fisher Scientific Baltics, Lithuania). The SNPs in MDM2 and MDM4 genes were analyzed with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay according to a self-made protocol. PCR reactions were carried out in a total volume of 25 µL containing distilled water (dH₂O), 1x DreamTaq buffer, 0.2 mM of each dNTP, DMSO, 0.24 pmol/µL of forward and reverse primers, 0.02 U DreamTaq polymerase and template DNA. Only the PCR mix for rs937283 was different and composed of distilled water (dH₂O), 1x Taq buffer (with NH₄), 0.2 mM of each dNTP, MgCl₂, 0.4 pmol/µL of forward and reverse primers, 0.025 U Taq polymerase and template DNA. The negative control was included in each experiment to ensure the accuracy of the amplification. The primer sequences were described previously [4,5,41–43]. The thermal conditions and primer sequences are shown in Table 1.
Table 1. The SNP and thermal conditions for analysis.

| Gene, SNP     | Primer Sequence F-Forward, R-Reverse | Thermal Conditions                                                                 | Fragments, bp |
|---------------|-------------------------------------|------------------------------------------------------------------------------------|---------------|
| MDM2 rs2279744 | F 5'-CGCGGGAGTTCAGGGTAAAG-3'        | 5 min of denaturation at 95 °C, then 35 cycles of 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s. The final cycle had a 7 min extension at 72 °C | 157           |
|               | R 5'-CTGAGTCAACCTGCCCACCCTG-3'     |                                                                                     |               |
| MDM2 rs937283  | F 5'-TGACGGAGATCCTGCTGCTTTC-3'      | 5 min of denaturation at 95 °C, then 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The final cycle had a 7 min extension at 72 °C | 617           |
|               | R 5'-TGAGTCAACCTGCCCACTGAAC-3'     |                                                                                     |               |
| MDM2 rs937282  | F 5'-GGTAACACGGCAGACCGGAGAT-3'     | 5 min of denaturation at 95 °C, then 35 cycles of 95 °C for 30 s, 57.5 °C for 30 s, and 72 °C for 30 s. The final cycle had a 7 min extension at 72 °C | 231           |
|               | R 5'-CTCCGGGATGATGGAGTG-3'         |                                                                                     |               |
| MDM4 rs1380576 | F 5'-GAAGAGGTGACATTTACCTGGAAC-3'   | 5 min of denaturation at 94 °C, then 35 cycles of 94 °C for 30 s, 56.6 °C for 45 s, and 72 °C for 30 s. The final cycle had a 10 min extension at 72 °C | 195           |
|               | R 5'-GTGGTCTATCCCCCTCAAGACATTTCCA-3' |                                                                                   |               |
| MDM4 rs4245739 | F 5'-AAGACTAAAGAAGGCTGGGG-3'       | 3 min of denaturation at 94 °C, then 35 cycles of 94 °C for 30 s, 51 °C for 30 s, and 72 °C for 30 s. The final cycle had a 10 min extension at 72 °C | 134           |
|               | R 5'-TTCAAAATAATGTGTAAGTGAGCC-3'   |                                                                                     |               |

Nucleotide base in underlined letter is mismatched base added in the primer sequence to create restriction enzyme site.

Table 2. The SNP and restriction conditions for analysis.

| Gene and SNP     | Restriction Endonuclease | Restriction Conditions | Fragments, bp |
|------------------|--------------------------|------------------------|---------------|
| MDM2 rs2279744   | MspA11                    | 37 °C for 1 h          | T allele 157; G allele 109 + 48 |
| MDM2 rs937283    | MboI                     | 37 °C for 1–16 h       | A allele 437 + 87 + 86 + 7; G allele 524 + 86 + 7 |
| MDM2 rs937282    | Hpy188I                  | 37 °C for 1 h          | C allele 231; G allele 182 + 49 |
| MDM4 rs1380576   | BseNI (BsrI)             | 65 °C for 1–16 h       | C allele 172 + 23; C allele 110 + 24; A allele 134 |
| MDM4 rs4245739   | MspI (HpaII)             | 37 °C for 1–16 h       | C allele 110 + 24; A allele 134 |

2.3. Statistical Analysis

Statistical analysis was performed by using SPSS (Statistical Package for the Social Sciences) version 20.0 statistical software (SPSS Inc., Chicago, IL, USA). The deviation from Hardy–Weinberg equilibrium was tested (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Pearson’s Chi-square or Monte Carlo tests were used to determine the statistical significance of the association between categorical values for each genotype group (genotype
model). The odds ratios (ORs) and 95% confidence intervals (CIs) were obtained from univariate logistic regression analyses to evaluate associations between SNPs and cancer characteristics. Additionally, multivariate logistic regression analyses were performed to estimate the adjusted ORs. The multivariate analyses were conducted in three models (No. 1, No. 2 and No. 3). Model No. 1 included combination of SNPs in MDM2 or MDM4 as potential covariates. In model No. 2, the receptors as additional confounding variables were included. In model No. 3, SNPs and all analyzed breast cancer characteristics (age, status of receptors, pT, N, and G) were considered as potential covariates. The overall survival was measured from the date of diagnosis till the event—last follow-up or death. Survival plots were generated using the Kaplan–Meier method. The differences between genotypes were assessed using the log-rank test. \( p < 0.05 \) was determined as criterion for statistical significance for all executed statistical tests.

3. Results

3.1. The Distribution of the Breast Tumor Features, Alleles and Genotypes

Our analysis included 100 Lithuanian breast cancer patients and 65 of them were diagnosed over 40 years. 68% of patients had \( >1 \) mm but \( \leq 5 \) mm tumors and 45% of the subjects had tumors spread to the lymph nodes. 57% of patients had positive ER expression, 48% had positive PR expression, and 22% had increased HER2 expression. In 71% of the subjects, the tumor was well or moderately differentiated (grade G1 and G2). In 31 of 100 cases, cancer had progressed, and 22% women died of breast cancer (Table 3).

Table 3. The clinicopathological characteristics of the study group.

| Characteristics                         | Frequencies (%) |
|----------------------------------------|-----------------|
| Age                                    |                 |
| 30–40 years                            | 35              |
| 41–50 years                            | 65              |
| Pathological tumor size (pT)           |                 |
| T1a                                    | 68              |
| T1b                                    | 32              |
| Pathological lymph node involvement (N)|                 |
| negative (N0)                          | 55              |
| positive (N1)                          | 45              |
| Estrogen receptor (ER)                 |                 |
| negative                               | 43              |
| positive                               | 57              |
| Progesterone receptor (PR)             |                 |
| negative                               | 52              |
| positive                               | 48              |
| Human epidermal growth factor receptor 2 (HER2) |        |
| negative                               | 78              |
| positive                               | 22              |
| Tumor grade (G)                        |                 |
| G1 and G2                              | 71              |
| G3                                     | 29              |
| Progress                               |                 |
| absent                                 | 69              |
| present                                | 31              |
| Metastasis                             |                 |
| absent                                 | 74              |
| present                                | 26              |
| Death                                  |                 |
| absent                                 | 78              |
| present                                | 22              |

T1a—\( >1 \) mm but \( \leq 5 \) mm, T1b—\( >5 \) mm but \( \leq 10 \) mm across, G1—well differentiated (low grade), G2—moderately differentiated (intermediate grade), G3—poorly differentiated.
Five SNPs across MDM2 (rs2279744, rs937283, rs937282) and MDM4 (rs1380576, rs4245739) genes were evaluated for associations with breast cancer phenotype. All observed genotype distributions, excluding MDM4 rs1380576 \((p = 0.02)\), were in agreement with the Hardy–Weinberg equilibrium \((p > 0.05)\). The allele and genotype distributions of the studied SNPs are summarized in Table 4.

Table 4. Allele and genotype distribution of MDM2 and MDM4 polymorphisms in the study group.

| Polymorphism | Allele Frequency | Genotype Frequency |
|--------------|-----------------|-------------------|
| MDM2 rs2279744 | T-0.68 (0.65) | TT-0.49 |
| MDM2 rs937283 | A-0.47 (0.61) | AG-0.46 |
| MDM2 rs937283 | G-0.53 (0.39) | GG-0.13 |
| MDM2 rs937283 | C-0.46 (0.51) | CC-0.23 |
| MDM2 rs937282 | G-0.54 (0.49) | GG-0.30 |
| MDM2 rs937282 | C-0.76 (0.69) | GC-0.28 |
| MDM4 rs1380576 | G-0.24 (0.31) | GG-0.62 |
| MDM4 rs4245739 | C-0.80 (0.26) | CC-0.07 |
| MDM4 rs4245739 | A-0.74 (0.74) | AC-0.26 |

3.2. The Associations of MDM2 Polymorphisms with Breast Cancer Characteristics

The results of the analysis showed some significant associations between the MDM2 polymorphisms and tumor features. The SNPs rs937283 and rs937282 were significantly associated with ER \((p = 0.023\) and \(p = 0.021\), respectively) and HER2 \((p = 0.010\) and \(p = 0.033\), respectively) status. Meanwhile, no significant differences were observed in the association analysis between the rs2279744 genotypes and tumor features \((p > 0.05)\). Results are summarized in Supplementary Material Table S1. Furthermore, the effect of SNPs in MDM2 on the overall survival was analyzed but no significant associations were detected (Supplementary Material Figure S1).

As shown in Table 5, after identifying MDM2 rs937283, it was found that patients carrying the AG genotype were predisposed to higher rates of ER-positive disease \((\text{OR} = 2.538, 95\%\ CI 1.336–4.823, p = 0.004)\). When compared with the AA genotype, the AG was also significantly associated with decreased chances of HER2-positive breast cancer \((\text{OR} = 0.231, 95\%\ CI 0.060–0.894, p = 0.034)\). In addition, the logistic regression analysis revealed a significant association \((\text{OR} = 2.538, 95\%\ CI 1.366–4.823, p = 0.004)\) between rs937282 CG genotype and ER-positive status (Table 5.)

Table 5. Odds ratio for associations of rs937283 A > G and rs937282 C > G with tumor characteristics.

| SNP             | Genotype | Positive ER \(95\%\ CI\) | \(p\) | Positive HER2 \(95\%\ CI\) | \(p\) |
|-----------------|----------|--------------------------|------|---------------------------|------|
| MDM2 rs937283   | AA       | 1.00 (ref.)              | -    | 1.00 (ref.)               | -    |
|                 | AG       | 2.538 1.336–4.823        | 0.004| 0.231 0.060–0.894         | 0.034|
|                 | GG       | 0.765 0.371–1.574        | 0.467| 1.406 0.444–4.448         | 0.562|
| MDM2 rs937282   | CC       | 1.00 (ref.)              | -    | 1.00 (ref.)               | -    |
|                 | CG       | 2.538 1.366–4.823        | 0.004| 0.346 0.093–1.287         | 0.113|
|                 | GG       | 0.722 0.354–1.474        | 0.371| 1.558 0.476–5.104         | 0.464|

We also performed a combined evaluation of rs937283 and rs937282 with status of ER and HER2 (Supplementary Material Table S2). However, the analysis of a possible joint effect did not reveal significant results. Following the adjustment for more confounding variables (models No. 2 and No. 3), the associations also remained non-significant.
3.3. The Associations of MDM4 Polymorphisms with Breast Cancer Characteristics

Both SNPs, located in MDM4, rs1380576 and rs4245739, showed statistically significant associations with ER ($p = 0.005$ and $p = 0.010$, respectively) and PR ($p = 0.010$ and $p = 0.016$, respectively) status. Results are summarized in Supplementary Material Table S1. Results also showed that SNPs in MDM4 were not statistically associated with overall survival (Supplementary Material Figure S1).

Individuals having the rs1380576 CC genotype had an OR of 2.263 (95% CI 1.319–3.883, $p = 0.003$) of an ER-positive status compared to individual having the GG genotype. Moreover, CC genotype was significantly associated with the PR-positive disease (OR = 12.462, 95% CI 1.486–104.514, $p = 0.020$) (Table 6).

Table 6. Odds ratio for associations of rs1380576 G>C and rs4245739 C>A with tumor characteristics.

| SNP        | Genotype | Positive ER OR   | 95% CI       | $p$  | Positive PR OR  | 95% CI       | $p$  |
|------------|----------|------------------|--------------|------|-----------------|--------------|------|
| MDM4 rs1380576 | GG       | 1.00 (ref.)      | -            |      | 1.00 (ref.)     | -            |      |
|            | CG       | 0.647            | 0.303–1.381  | 0.261| 5.824           | 0.645–52.599 | 0.117|
|            | CC       | 2.263            | 1.319–3.883  | 0.003| 12.462          | 1.486–104.514| 0.020|
| MDM4 rs4245739 | CC       | 1.00 (ref.)      | -            |      | 1.00 (ref.)     | -            |      |
|            | AC       | 0.857            | 0.396–1.853  | 0.695| 0.733           | 0.337–1.597  | 0.435|
|            | AA       | 1.913            | 1.155–3.168  | 0.012| 1.233           | 0.762–1.996  | 0.393|

As indicated in Table 6, we found that rs4245739 AA genotype was significantly associated with ER-positive breast cancer in comparison with CC genotype (OR = 1.913, 95% CI 1.155–3.168, $p = 0.012$). After logistic regression analysis, no reliable differences were found between rs4245739 polymorphism genotype and PR-positive status ($p > 0.05$).

However, according to results of multivariate logistic regression analysis, all investigated associations resulted in loss of significance (Supplementary Material Table S2). The results suggested that in this case other factors were more important.

4. Discussion

Breast cancer is one of the most common cancers affecting females worldwide [2]. The etiology and pathogenesis of breast cancer is complicated [6]. Various risk factors, including genetic predisposition, have been characterized, but the accurate molecular mechanisms of breast cancer cause and characteristics are still unclear [23]. Additionally, some studies predicate that overexpression/amplification of MDM2 and MDM4 are common in many malignancies [7–9,17]. Genetic variations (for example, SNPs) in the MDM2 and MDM4 genes may also be associated with breast cancer. Moreover, it is established that TP53 pathway is one of the central pathways involved in various human cancers, including breast cancer [23,44,45]. Many studies have shown that MDM2 and MDM4 proteins are also involved in TP53 pathway by performing cooperative functions in negatively regulating p53 protein, which is the key regulator in essential cellular processes [16,17,19].

In our study we investigated the MDM2 (rs2279744, rs937283, rs937282) and MDM4 (rs1380576, rs4245739) single nucleotide polymorphisms in 100 Lithuanian breast cancer patients. We investigated the relationship of MDM2 and MDM4 polymorphisms genotype with the clinicopathological features, including the patient’s age at diagnosis, pathological tumor size (pT), pathological lymph node involvement (N), status of estrogen (ER), progesterone (PR) receptors and human epidermal growth factor receptor 2 (HER2), tumor grade (G1 and G2, G3), progress, metastasis, and death. For comparisons of all genotype and associated features, logistic regression analysis was used to determine odds ratios and $p$ values. Our results revealed that some SNPs of MDM2 and MDM4 might be significantly associated with the breast cancer characteristics. To the best of our knowledge, this is the first study to examine the role of MDM2 and MDM4 polymorphisms in breast cancer patients in Lithuania.
As the data of our study shows, associations for MDM2 and MDM4 SNPs with age at breast cancer diagnosis, pathological tumor size, lymph node involvement status, histological grade, progression, metastasis, and death were evaluated but no significant associations were found. In addition, no significant associations between SNPs and overall survival were detected.

MDM2 rs2279744 polymorphism is located in the promoter region. It has been shown to increase the affinity of the stimulatory protein 1 (Sp1, transcriptional activator), resulting in higher levels of MDM2 mRNA and protein [46]. In our study, rs2279744 polymorphism did not show any significant association with analyzed breast cancer characteristics. Like our study, Miedl et al. [47] have reported no significant associations of rs2279744 with breast cancer features (age, menopausal status, ER, PR, and HER2 status, stage, grade, and tumor size). Similarly, Yilmaz et al. [48] did not find any relationships among tumor grade, tumor size, lymph node involvement, metastasis status, and this polymorphism. In contrast, Yadav with colleagues [23] have found a significant correlation between rs2279744 and HER2/neu-positive status and distant metastasis (\( p = 0.003 \) and \( p = 0.04 \), respectively) in breast cancer. Furthermore, analyzing control and breast cancer cohorts, Paulin et al. [36] indicated that rs2279744 was associated with tumor grade and nodal involvement. Their study revealed that GG genotype was associated with high grade tumors (OR = 1.64, 95% CI = 1.06–2.53, \( p = 0.025 \)) and greater nodal involvement (OR = 2.51, 95% CI = 1.26–4.98, \( p = 0.009 \)). Moreover, in concordance with our findings, they report that rs2279744 was not associated with age at diagnosis of breast cancer, tumor ER, PR, HER2 status, and menopausal status (\( p > 0.05 \)).

rs937283 is located in the 5' untranslated region (UTR). This genetic variant enhances the transcription activity of the MDM2 gene thereby increases the mRNA and protein expression levels of MDM2 [35]. Meanwhile, rs937282 is located in the promoter region of MDM2. Similarly to rs2279744, rs937282 can affect the affinity of CAAT/enhancer binding protein \( \alpha \) (C/EBP \( \alpha \)), resulting in higher expression of MDM2 [33]. In the present study, we found the relationships between these two SNPs of MDM2 (rs937283 and rs937282) and ER, HER2 status. We observed that heterozygous genotypes were significantly associated with ER-positive breast cancer. The analysis also revealed a statistically significant association between the rs937283 AG genotype and decreased chances of HER2-positive breast cancer. It is known that ER receptor positive breast cancer is normally associated with much better prognosis. Consequently, our results suggest that heterozygous genotypes of rs937283 and rs937282 polymorphisms could indicate better prognosis for breast cancer patients. Literature sources presented only the data of these SNPs and their influence on the risk of various cancers, but there was no evidence that rs937283 and rs937282 had been analyzed with breast cancer characteristics. Some correlations with other types of tumors have been recently identified. For example, Jiao et al. [35] studied association of rs937283 with clinicopathological characteristics (gender, age at diagnosis, family history, invasion, aggression, and lag-time) in retinoblastoma patients, and significant associations with tumor invasion and high aggression were identified.

MDM4 rs1380576 is the intronic variant and its exact functional changes remain unknown. We have observed that rs1380576 polymorphism was significantly associated with ER and PR status. In the present study, we found that patients with CC genotype were linked to positive status of ER and PR. Conversely, Hashemi et al. [5] did not show significant associations between rs1380576 and status of ER and PR (\( p > 0.05 \)). In agreement with our study, they have also reported no significant relationship between rs1380576 and other clinical breast cancer characteristics such as age, tumor size, grade, stage, histological type, HER2 status. There is the lack of studies investigating the rs1380576 associations with the clinical and morphological characteristics of breast cancer. Consequently, further studies concerning the role of this SNP may be considered.

rs4245739 polymorphism is located in the 3' untranslated region (UTR) of the MDM4 gene, affects mRNA stability and proteins level [31]. In this study, we confirmed the impact of MDM4 rs4245739 on ER status, and we consider that AA genotype could indicate better
breast cancer prognosis. Our findings for rs4245739 and ER status concur with those of Bauer et al. [17]. They analyzed rs4245739 in 815 breast cancer patients and results showed that AA genotype was associated with a 1.8-fold increased probability to develop an ER-positive tumor ($p = 0.042$). We discovered AA genotype to be associated with ER-positive status with OR = 1.913. What is more, according to Purrington et al. [49], rs4245739 C allele was related to ER-negative status (OR = 1.14, 95% CI 1.10–1.18, $p = 2.1 \times 10^{-12}$). Meanwhile, Milne et al. [50] in their study claimed that rs4245739 was not associated with ER-positive disease. Moreover, Pedram et al. [51] (Azerbaijan population) and Hashemi et al. [5] (Southeast Iranian population) found that MDM4 rs4245739 had no significant association with breast cancer clinicopathological factors, such as age, involved breast side, tumor size and type, grade of tumor, count of involved lymph nodes and stage of cancer, status of receptors. Liu with colleagues [4] reported no associations with age, age at menarche, menstrual history, or family history of breast cancer.

We have to admit that this is the first analysis of polymorphisms in the MDM2 and MDM4 genes in relation to the breast cancer clinical characteristics in Lithuania; consequently, a much larger cohort of breast cancer patients would be required to verify our findings.

5. Conclusions

This study provides basic information about the genotype frequency distributions of MDM2 rs2279744, rs937283, rs937282 and MDM4 rs1380576, rs4245739 polymorphisms in Lithuanian population. Our results suggest that heterozygous genotypes of rs937283 and rs937282, as well as rs1380576 CC and rs4245739 AA genotypes, are associated with early-stage hormonal receptor positive breast cancer and may be useful genetic markers for disease assessment. This information may improve patient stratification in the future; however, further large and functional studies are needed to assess the validity of these associations.

Supplementary Materials: The following are available online at https://www.mdpi.com/2077-0383/10/4/866/s1, Table S1: Associations between genotype of each polymorphism with clinicopathological features of breast cancer. Table S2: Multivariate logistic regression analysis of the combined SNPs in MDM2 and MDM4 genes. Figure S1. Kaplan–Meier estimates of overall survival: (a) rs2279744, (b) rs937283, (c) rs937282, (d) rs1380576, (e) rs4245739.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Kaunas Regional Biomedical Research Ethical Committee (protocol codes BE-2-10 and P1-BE-2-10/2014).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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References

1. Dai, X.; Li, T.; Bai, Z.; Yang, Y.; Liu, X.; Zhan, J.; Shi, B. Breast cancer intrinsic subtype classification, clinical use and future trends. *Am. J. Cancer Res.* 2015, 5, 2929–2943. [PubMed]

2. Coughlin, S.S.; Ekwueme, D.U. Breast cancer as a global health concern. *Cancer Epidemiol.* 2009, 33, 315–318. [CrossRef]

3. Golubnitschaja, O.; Debald, M.; Yeghiazaryan, K.; Kuhn, W.; Pešta, M.; Costiglola, V.; Grech, G. Breast cancer epidemic in the early twenty-first century: Evaluation of risk factors, cumulative questionnaires and recommendations for preventive measures. *Tumor Biol.* 2016, 37, 12941–12957. [CrossRef]

4. Liu, J.; Tang, X.; Li, M.; Lu, C.; Shi, J.; Zhou, L.; Yuan, Q.; Yang, M. Functional *MDM4* rs4245739 genetic variant, alone and in combination with *P53* Arg72Pro polymorphism, contributes to breast cancer susceptibility. *Breast Cancer Res. Treat.* 2013, 140, 151–157. [CrossRef]

5. Hashemi, M.; Sanaei, S.; Hashemi, S.M.; Eskandari, E.; Bahari, G. Association of Single Nucleotide Polymorphisms of the *MDM4* Gene with the Susceptibility to Breast Cancer in a Southeast Iranian Population Sample. *Clin. Breast Cancer* 2018, 18, e883–e891. [CrossRef]

6. Rudolph, A.; Chang-Claude, J.; Schmidt, M.K. Gene–environment interaction and risk of breast cancer. *Br. J. Cancer* 2016, 114, 125–133. [CrossRef]

7. Yu, Q.; Li, Y.; Mu, K.; Li, Z.; Meng, Q.; Wu, X.; Wang, Y.; Li, L. Amplification of Mdmx and overexpression of *MDM2* contribute to mammary carcinogenesis by substituting for *p53* mutations. *Diagn. Pathol.* 2014, 9, 71. [PubMed]

8. Haupt, S.; Vijayakumaran, R.; Miranda, P.J.; Burgess, A.; Lim, E.; Haupt, Y. The role of *MDM2* and *MDM4* in breast cancer development and prevention. *J. Mol. Cell Biol.* 2017, 9, 53–61. [CrossRef]

9. Karni-Schmidt, O.; Lokshin, M.; Prives, C. The roles of *MDM2* and *MDMX* in cancer. *Annu. Rev. Pathol.* 2016, 11, 617–644. [CrossRef] [PubMed]

10. Yu, H.; Wang, L.-E.; Liu, Z.; Wei, S.; Li, G.; Sturgis, E.M.; Wei, Q. Polymorphisms of *MDM4* and risk of squamous cell carcinoma of the head and neck. *Pharm. Genom.* 2011, 21, 388–396. [CrossRef]

11. Mendola, M.; Mandani, G.; Momand, J. The *MDM2* gene family. *Biomol. Concepts* 2014, 5, 9–19. [CrossRef]

12. Gao, C.; Xiao, G.; Piersigilli, A.; Gou, J.; Ogunwobi, O.; Bargonetti, J. Context-dependent roles of *MDMX* (*MDM4*) and *MDM2* in breast cancer proliferation and circulating tumor cells. *Breast Cancer Res.* 2019, 21, 5. [CrossRef]

13. Li, Q.; Lozano, G. Molecular Pathways: Targeting Mdm2 and Mdm4 in Cancer Therapy. *Clin. Cancer Res.* 2013, 19, 34–41. [CrossRef] [PubMed]

14. Swetzig, W.M.; Wang, J.; Das, G.M. Estrogen receptor alpha (ERα/ESR1) mediates the p53-independent overexpression of *MDM4*/*MDMX* and *MDM2* in human breast cancer. *Oncotarget* 2016, 7, 16049–16069. [CrossRef]

15. Silwal-Pandit, L.; Vollan, H.K.M.; Chin, S.-F.; Rueda, O.M.; McKinney, S.; Osako, T.; Quigley, D.A.; Kristensen, V.N.; Aparicio, S.; Barresen-Dale, A.-L.; et al. TP53 Mutation Spectrum in Breast Cancer Is Subtype Specific and Has Distinct Prognostic Relevance. *Clin. Cancer Res.* 2014, 20, 3569–3580. [CrossRef]

16. Wang, M.-J.; Luo, Y.-J.; Shi, Z.-Y.; Xu, X.-L.; Yao, G.-L.; Liu, R.-P.; Zhao, H. The associations between *MDM4* gene polymorphisms and cancer risk. *Oncotarget* 2016, 7, 55611–55623. [CrossRef]

17. Bauer, M.; Kantelhardt, E.J.; Siwiec, T.; Nist, A.; Mernberger, M.; Politt, K.; Hanf, V.; Lantzsch, T.; Uleer, C.; Peschel, S.; et al. Specific allelic variants of SNPs in the *MDM2* and *MDMX* genes are associated with earlier tumor onset and progression in Caucasian breast cancer patients. *Oncotarget* 2019, 10, 1975–1992. [CrossRef]

18. Junttila, M.R.; Evan, G.I. *p53*—A Jack of all trades but master of none. *Nat. Rev. Cancer* 2009, 9, 821–829. [CrossRef]

19. Haupt, S.; Buckley, D.L.; Pang, J.-M.B.; Panimaya, J.; Paul, P.J.; Gamell, C.; Takano, E.A.; Lee, Y.Y.; Hiddingh, S.; Rogers, T.-M.; et al. Targeting Mdm2 to treat breast cancers with wild-type p53. *Cell Death Dis.* 2015, 6, e1821. [CrossRef] [PubMed]

20. Wade, M.; Li, Y.C.; Wahl, G.M. *MDM2*, *MDMX* and *p53* in oncogenesis and cancer therapy. *Nat. Rev. Cancer* 2013, 13, 83–96. [CrossRef]

21. Marine, J.-C. *MDM2* and *MDMX in Cancer and Development*; Elsevier: Amsterdam, The Netherlands, 2011; Volume 94, pp. 45–75.

22. Gansmo, L.B.; Bjørnslett, M.; Halle, M.K.; Salvesen, H.B.; Dørum, A.; Birkeland, E.; Hveem, K.; Romundstad, P.; Vatten, L.; Lenning, P.E.; et al. The *MDM4* SNP rs34091 (rs4245739) C-allele is associated with increased risk of ovarian but not endometrial cancer. *Tumor Biol.* 2016, 37, 10697–10702. [CrossRef]

23. Yadav, P.; Masoor, M.; Tanwer, K.; Mir, R.; Javid, J.; Ahmad, I.; Zuberi, M.; Kaza, R.C.M.; Jain, S.K.; Khurana, N.; et al. Clinical significance of TP53 (R72P) and MDM2 (T309G) polymorphisms in breast cancer patients. *Clin. Transl. Oncol.* 2015, 18, 728–734. [CrossRef] [PubMed]

24. Haupt, S.; Mejía-Hernández, J.O.; Vijayakumaran, R.; Keam, S.P.; Haupt, Y. The long and the short of it: The *MDM4* tail so far. *J. Mol. Cell Biol.* 2019, 11, 231–244. [CrossRef] [PubMed]

25. Miranda, P.J.; Buckley, D.; Raghu, D.; Pang, J.-M.B.; Takano, E.A.; Vijayakumaran, R.; Teunisse, A.F.; Posner, A.; Procter, T.; Herold, M.J.; et al. *MDM4* is a rational target for treating breast cancers with mutant p53. *J. Pathol.* 2017, 241, 661–670. [CrossRef]

26. Xiong, S.; Pant, V.; Zhang, Y.; Aryal, N.K.; You, M.J.; Kusewitt, D.; Lozano, G. The p53 inhibitor Mdm4 cooperates with multiple genetic lesions in tumourigenesis. *J. Pathol.* 2017, 244, 501–510. [CrossRef]

27. Burgess, A.; Chia, K.M.; Haupt, S.; Thomas, D.; Haupt, Y.; Lim, E. Clinical Overview of MDM2/X-Targeted Therapies. *Front. Oncol.* 2016, 6, 7. [CrossRef] [PubMed]
28. Portman, N.; Milioli, H.H.; Alexandrou, S.; Coulson, R.; Yong, A.; Fernandez, K.J.; Chia, K.M.; Halilovic, E.; Segara, D.; Parker, A.; et al. MDM2 inhibition in combination with endocrine therapy and CDK4/6 inhibition for the treatment of ER-positive breast cancer. Breast Cancer Res. 2020, 22, 1–17. [CrossRef] [PubMed]

29. Gupta, A.; Shah, K.; Oza, M.J.; Behl, T. Reactivation of p53 gene by MDM2 inhibitors: A novel therapy for cancer treatment. Biomed. Pharmacother. 2019, 109, 484–492. [CrossRef] [PubMed]

30. Liu, Y.; Wang, X.; Wang, G.; Yang, Y.; Yuan, Y.; Ouyang, L. The past, present and future of potential small-molecule drugs targeting p53-MDM2/MDMX for cancer therapy. Eur. J. Med. Chem. 2019, 176, 92–104. [CrossRef]

31. Xu, C.; Zhu, J.; Fu, W.; Liang, Z.; Song, S.; Zhao, Y.; Lyu, L.; Zhang, A.; He, J.; Duan, P. MDM4 rs4245739 A > C polymorphism correlates with reduced overall cancer risk in a meta-analysis of 69477 subjects. Oncotarget 2016, 7, 71718–71726. [CrossRef]

32. Wynendaele, J.; Böhnke, A.; Leucci, E.; Nielsen, S.J.; Lambertz, I.; Hammer, S.; Sbrzesny, N.; Kubitz, D.; Wolf, A.; Gradhand, E.; et al. An illegitimate microRNA Target Site within the 3’ UTR of MDM4 Affects Ovarian Cancer Progression and Chemosensitivity. Cancer Res. 2010, 70, 9641–9649. [CrossRef]

33. Wang, M.; Zhang, Z.; Zhu, H.; Fu, G.; Wang, S.; Wu, D.; Zhou, J.; Wei, Q.; Zhang, A. A Novel Functional Polymorphism C1797G in the MDM2 Promoter Is Associated with Risk of Bladder Cancer in a Chinese Population. Clin. Cancer Res. 2008, 14, 3633–3640. [CrossRef] [PubMed]

34. Chen, B.; Wang, J.; Zhang, J.; Gu, X.; Feng, X. The Study of MDM2 rs937283 Variant and Cancer Susceptibility in a Central Chinese Population. Technol. Cancer Res. Treat. 2018, 17, 1–7. [CrossRef] [PubMed]

35. Jiao, Y.; Jiang, Z.; Wu, Y.; Chen, X.; Xiao, X.; Yu, H. A Functional Polymorphism (rs937283) in the MDM2 Promoter Region is Associated with Poor Prognosis of Retinoblastoma in Chinese Han Population. Sci. Rep. 2016, 6, 1–11. [CrossRef] [PubMed]

36. Paulin, F.E.M.; O’Neill, M.; McGregor, G.; Cassidy, A.; Ashfield, A.; Ali, C.W.; Munro, A.J.; Baker, L.; Purdie, C.; Lane, D.P.; et al. The MDM2 T309G polymorphism and gastric cancer. Biomed. Rep. 2015, 3, 717–726. [CrossRef] [PubMed]

37. North, A.; Atabey, M.; Caglayan, G.; Bostanci, M.E.; Bolukbasi, S.S.; Topcu, A026302. [CrossRef]

38. Bykov, V.J.N.; Eriksson, S.E.; Bianchi, J.; Wiman, K.G. Targeting mutant p53 for efficient cancer therapy. Nat. Rev. Cancer 2018, 18, 89–92. [CrossRef] [PubMed]

39. Basu, S.; Murphy, M.E. Genetic Modifiers of the p53 Pathway. Cold Spring Harb. Perspect. Med. 2016, 6, a026302. [CrossRef]

40. Møller, H.; Henson, K.; Lüchtenborg, M.; Broggio, J.; Charman, J.; Coupland, V.H.; Davies, E.; Jack, R.H.; Sullivan, R.; Vedsted, P.; et al. Short-term breast cancer survival in relation to ethnicity, stage, grade and receptor status: National cohort study in England. Br. J. Cancer 2016, 115, 1408–1415. [CrossRef]

41. Xu, B.; Xu, Z.; Cheng, G.; Min, Z.-C.; Mi, Y.; Zhang, Z.-Z.; Tao, J.; Li, P.-C.; Wang, M.-L.; Tang, J.-L.; et al. Association between polymorphisms of TP53 and MDM2 and prostate cancer risk in southern Chinese. Cancer Genet. Cytogenet. 2010, 202, 76–81. [CrossRef] [PubMed]

42. Jin, L.; Xu, L.; Song, X.; Wei, Q.; Sturgis, E.M.; Li, G. Genetic Variation in MDM2 and p14ARF and Susceptibility to Salivary Gland Carcinoma. PLoS ONE 2012, 7, e49361. [CrossRef]

43. Basu, S.; Murphy, M.E. Genetic Modifiers of the p53 Pathway. Cold Spring Harb. Perspect. Med. 2016, 6, a026302. [CrossRef]

44. Lalonde, M.-E.; Ouimet, M.; Larivière, M.; A Kritikou, E.; Sinnett, D. Identification of functional DNA variants in the constitutive promoter region of MDM2. Hum. Genom. 2012, 6, 15. [CrossRef] [PubMed]

45. Miedl, H.; Lebhard, J.; Ehart, L.; Schreiber, M. Association of the MDM2 SNP285 and SNP309 genetic variants with the risk, age at onset and prognosis of breast cancer in central European women: A hospital-based case-control study. Int. J. Mol. Sci. 2019, 20, 509. [CrossRef]

46. Albright, E.; A Kritikou, E.; Sinnett, D. Identification of functional DNA variants in the constitutive promoter region of MDM2. Hum. Genom. 2012, 6, 15. [CrossRef] [PubMed]

47. Miedl, H.; Lebhard, J.; Ehart, L.; Schreiber, M. Association of the MDM2 SNP285 and SNP309 genetic variants with the risk, age at onset and prognosis of breast cancer in central European women: A hospital-based case-control study. Int. J. Mol. Sci. 2019, 20, 509. [CrossRef]

48. Yilmaz, M.; Tas, A.; Donmez, G.; Kacan, T.; Silig, Y. Significant association of the MDM2 SNP285 and SNP309 genetic variants with the risk, age at onset and prognosis of breast cancer in a Turkish population. Asian Pac. J. Cancer Prev. 2018, 19, 1059–1062. [CrossRef] [PubMed]

49. Purrington, K.S.; Slager, S.; Eccles, D.; Yannoukakos, D.; Fasching, P.A.; Miron, P.; Carpenter, J.; Chang-Claude, J.; Martin, N.G.; Montgomery, G.W.; et al. Genome-wide association study identifies 25 known breast cancer susceptibility loci as risk factors for triple-negative breast cancer. Carcinogenesis 2014, 35, 1012–1019. [CrossRef]
50. Milne, R.L.; Kuchenbaecker, K.B.; Michailidou, K.; Beesley, J.; Kar, S.; Lindström, S.; Hui, S.; Lemaçon, A.; Soucy, P.; Lemaçon, A.; et al. Identification of ten variants associated with risk of estrogen-receptor-negative breast cancer. *Nat. Genet.* 2017, 49, 1767–1778. [CrossRef]

51. Pedram, N.; Pouladi, N.; Hosseinpour Feizi, M.A.; Montazeri, V.; Sakhinia, E.; Estiar, M.A. Analysis of the Association between MDM4 rs4245739 Single Nucleotide Polymorphism and Breast Cancer Susceptibility. *Clin. Lab.* 2016, 59, 1303–1308. [CrossRef]