A polymorphic variant in human MDM4 associates with accelerated age of onset of estrogen receptor negative breast cancer

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Abstract

Mouse knockout studies have shed significant light on the dynamics of the p53–MDM2–MDM4 interaction and have highlighted the biological role of these molecules during embryonic development as well as oncogenesis. Whereas the p53 null mouse is viable and prone to oncogenesis (15), the Mdm2 single knockout exhibits a p53 dependent early embryonic lethal phenotype as evidenced by excessive apoptosis, which in turn is rescued with p53 knockout (16,17). Similarly, the Mdm4 knockout in mice results in p53-dependent embryonic lethality with defects in proliferation and not apoptosis but also becomes viable in a p53-null background (18–20). The distinct phenotypes seen in Mdm2- and Mdm4-null mice suggest that MDM2 and MDM4 have non-overlapping roles in p53 regulation (20). This is further supported by the fact that deletion of Bax, a proapoptotic gene, marginally delays the embryonic lethality of Mdm2-null mice (21,22), whereas the lethality in Mdm4-null mice is partially rescued by deletion of p21 (23). However, recent evidence suggests that the MDM4 deficiency can be completely rescued by Mdm2 transgene revealing functional overlap of MDM2 and MDM4 during development (24). Lastly, mice lacking p53, Mdm2 and Mdm4 are viable but are susceptible to tumorigenesis similar to that of p53–/– mice (25).

It is a well-established fact that the human MDM2 gene is amplified to high copy numbers or overexpressed in a wide variety of human cancers (26–28). The majority of these malignancies possess wild-type p53, with functional inactivation of p53 due to overexpression of MDM2. The human MDM4 gene is also amplified or overexpressed in a subset of malignant gliomas, soft tissue sarcomas and retinoblastomas (29–31). Tumors showing absence of p53 mutations or MDM2 amplification often display MDM4 overexpression as an alternative and independent molecular mechanism for tumorigenesis. The overall data support the hypothesis that deregulated expression of MDM2 or MDM4 leads to functional inactivation of wild-type p53 and carcinogenesis.

Earlier age onset breast cancer is more probably to have genetic associations, yet most women with breast cancer do not have mutations in known breast cancer susceptibility genes (32,33). Our laboratory has previously reported a single-nucleotide polymorphism (SNP) located in the promoter region of human MDM2, known as SNP309, associating with higher protein expression, but only in the presence of active estrogen signaling (34–36). Furthermore, the presence of the variant associates with early onset of estrogen receptor (ER) positive, but not ER-negative breast cancers (34). A recent report described the haplotype structure and correlation between SNPs in MDM4 with risk of breast and ovarian cancers (37). In this study, the association of a SNP in MDM4 with clinical phenotypes of breast cancer, in particular age of onset of breast cancer based on tumor subtype, was evaluated.

Materials and methods

Subjects

Cases consisted of two cohorts. Cohort 1 was derived from consecutive patients evaluated at The Cancer Institute of New Jersey who were invited to participate in this prospective study from 2004 to 2009. Greater than 95% of eligible individuals gave consent for participation. Eligibility included a history of biopsy-proven breast cancer verified by pathology records and confirmed on review by our institutional breast pathologist. In <5% of cases, slides were not available for review and pathological features were based on available pathology reports from other institutions. Clinical information was abstracted through chart review. ER staining <10% was considered negative. BRCA1/2 testing was performed where clinically indicated. Patients with known BRCA1/2 mutations were excluded from age association analyses due to potential confounding bias. Investigations were performed with approval by the University of Medicine and Dentistry of New Jersey/Robert Wood Johnson Medical School Institutional Review Board.

Abbreviations:
ER, estrogen receptor; MDM2, murine double minute 2; MDM4, murine double minute 4; SNP, single-nucleotide polymorphism.
Cohort 2, an independent group of cases, consisted of individuals evaluated and enrolled consecutively through an institutional review board-approved protocol at Yale Medical School from 1996 to 2007. This cohort included 148 patients treated with conservative surgery and radiation therapy to the intact breast who were \( \leq 52 \) years at the time of diagnosis. All patients in this cohort had lumpectomy followed by radiation therapy to the intact breast for early stage I or II breast cancer. Clinical information was obtained from clinical records, de-identified and stored in a clinical database.

Genotyping

Genomic DNA was extracted from 1 ml of peripheral blood, obtained through venipuncture, using a spin column-based method according to manufacturer’s protocol (QiAGEN, Valencia, CA). Genotyping for the human MDM4 SNP (rs1563828) was performed using an Applied Biosystems TaqMan assay on the ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Briefly, reactions were performed with 5–10 ng genomic DNA in a 20 μl vol. PCR cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min. The assay failed in <1% of cases. The alleles at the locus are C and T where T is defined as the ancestral allele.

Statistical analysis

A permutation test (108 permutations) was performed to determine the statistical significance of differences in mean age at diagnosis between different genotype groups (e.g. CC or CT versus TT homozygous). This test was chosen because it is non-parametric, making no assumptions about the age of diagnosis. The statistical significance in categorical values was determined using Fischer’s exact test. The associated odds ratio and 95% confidence interval were computed using a Bayesian estimate for the odds ratio posterior distribution.

Results

The polymorphic locus in human MDM4 tags a haplotype under recent natural selection

A recent report indicates that a major haplotype of the human MDM4 gene has undergone recent selection in the Caucasian population (37). This haplotype is tagged by several SNPs across the MDM4 gene that are in high linkage disequilibrium (37), including the polymorphic locus rs1563828 located within intron 10. To confirm this, the haplotype structure of the human MDM4 gene in the Caucasian population was analyzed. To this end, genotypes from the HapMap project [http://www.hapmap.org] were used, specifically for the HapMap CEU samples (Centre d’Etude du Polymorphisme Humain) representing 90 Utah residents with ancestry from northern and western Europe. Fifteen SNPs were found to be in linkage disequilibrium with rs1563828 (normalized mutual information above 0.7). These linked SNPs include 13 within the MDM4 gene and two downstream (Figure 1) with two major haplotypes accounting for 95.5% of the genotypes observed in the CEU samples. More importantly, these two haplotypes are tagged by the genotype at rs1563828: the C allele and T allele containing haplotypes being 70 and 25.5%, respectively. In practice, this means that, with 95.5% confidence, whenever the C or T alleles are observed, the associated haplotype is actually being observed. Furthermore, any association found between the genotype at rs1563828 and clinical phenotypes is in fact an association between the haplotype and the clinical variable. Based on these findings, the SNP locus was selected for genotyping in the two breast cancer cohorts.

Patient characteristics and human MDM4 genotypes in the two study cohorts

The demographics of both cohorts are depicted in Table I. The data demonstrate that the majority of women were Caucasian and the majority of cases were of ductal origin in both groups. Overall in cohort 1, the average age at diagnosis was 51.9 years where half of women were diagnosed <51 of age; the average age of menopause was 38.8 years. While nearly 75% of breast cancers in cohort 1 were positive for ER, cohort 2 had nearly equal representation of ER-positive and negative breast cancers. The latter group reflects the higher propensity of early onset breast cancer to be ER negative.

As distribution of genotype frequencies may be population specific, MDM4 genotype frequencies were analyzed by race (Table II). Consistent with the ancestral allele information (dbSNP: [http://www.ncbi.nlm.nih.gov/sites/entrez]), the T allele was more prevalent in African-American women in both cohorts. The major allele in Caucasians was the C allele. Although MDM4 genotypes had population-specific frequencies, these frequencies did not deviate from Hardy–Weinberg Equilibrium except in African-Americans in both the cohorts.

Because of the age association with hormone receptor status (39), the frequency of MDM4 genotypes in ER-negative and ER-positive breast cancers was evaluated. The distribution of C- and T-allele frequencies for ER-positive and ER-negative breast cancers was not statistically significant in both the cohorts (data not shown). Because breast cancer represents a very heterogenous disease, the distribution of genotype frequencies among the different cancer subtypes, such as ductal carcinoma in situ, invasive ductal carcinomas, invasive lobular carcinomas and other subtypes, was examined as well. There was no significant allele enrichment by breast cancer subtype (data not shown).

![Fig. 1. The haplotype structure of human MDM4 gene. The introns and exons in human MDM4 are denoted in gray and black, respectively. The SNP rs1563828 present in intron 10 is marked by an arrow and is in linkage disequilibrium with 15 other SNP loci, 13 within the MDM4 gene and two downstream. The possible haplotypes and their frequencies in HapMap CEU population are shown. Two major haplotypes (70 and 25.5%) are observed in Caucasians, each tagged by the two alleles (C or T) of this SNP.](https://academic.oup.com/carcin/article-abstract/30/11/1910/2629508)
The TT genotype associates with an earlier age at diagnosis of ER-negative breast cancers

Age of onset analyses in cohort 1 revealed that African-American and Hispanic women were diagnosed at an earlier age than Caucasian and Asian women (Table III; \( P = 0.0045 \)). In contrast, Caucasian women were diagnosed earlier than African-American women in the second cohort (Table III; \( P = 3.59 \times 10^{-6} \)). Overall and as expected, ER-negative breast cancers were diagnosed at an earlier age as compared with ER-positive breast cancers regardless of MDM4 genotype in cohort 1. Of note, African-Americans were diagnosed with ER-positive breast cancers at an age earlier than those with ER-negative disease in this cohort. This difference was not significant and probably reflects one outlier diagnosed with an ER-negative breast cancer in her 70s. The average at diagnosis was similar in cohort 2 irrespective of hormone receptor status (Table III). The vast majority of ER-negative breast cancers were of ductal origin and invasive in both the cohorts (data not shown).

The mean age at diagnosis by genotype without grouping by ER status demonstrated no significant difference in both the cohorts (Table III). However, subgroup analysis of age at diagnosis by genotype and ER status revealed distinct differences with women homozygous for the T allele demonstrating an earlier mean age at diagnosis of ER-negative breast cancer compared with women homozygous for the C allele (Table III). The mean age of onset for TC genotype carriers was not significantly different from CC carriers in both the cohorts. The most significant increase occurred between the TT and CC+TC groups, with a difference in mean age at diagnosis of 5.0 years (\( P = 0.018 \)) in cohort 1 and a difference of 3.8 years (\( P = 0.006 \)) in cohort 2. Finally, when comparing alleles, it was observed that the T allele associates with a mean age at diagnosis 2.0 years earlier than C allele (\( P = 0.053 \)) in cohort 1 and 2.8 years (\( P = 0.002 \)) earlier in cohort 2. In contrast, no genotype-specific difference in age at diagnosis was observed for ER-positive breast cancer in either cohort (Table III; \( P > 0.05 \)).

As a consequence of population-specific genotype frequencies, potential differences due to genetic background and breast cancer characteristics, any analysis made pooling together all samples may be misleading. One would conclude that the TT genotype associates with an earlier age at diagnosis in ER-negative breast cancers independent of ethnicity. However, this may be a consequence of the fact that the majority of cases are of Caucasian origin. To reduce the heterogeneity in the study population and many age-specific differences due to breast cancer subtypes and/or ethnicity, samples were stratified into groups with the same cancer subtype and ethnic background. However, only the group of ductal carcinomas (patients with ductal carcinoma in situ and invasive ductal carcinoma) in Caucasian women was represented by a significant number of samples in both the cohorts (\( n = 476 \) in cohort 1 and \( n = 90 \) in cohort 2). Thus, the association between the genotype and the age at diagnosis of ductal carcinomas was evaluated in Caucasian women. For ER-positive ductal carcinomas in Caucasian females, there was no statistically significant association between any of the MDM4 genotypes and the age of diagnosis (Figure 2A and B). For the homozygous wild-type (CC), the heterozygote (CT) and the homozygous variant (TT), the mean age at diagnosis were 52.7, 53.5 and 53.8 years, respectively, in cohort 1 (\( P > 0.3 \)) and 38.1, 36.0 and 36.2 years, respectively, in cohort 2 (\( P = 0.17 \)). However, there was a left shift in the cumulative incidence curve for ER-negative ductal cancers corresponding to a 7.5 year earlier onset in women homozygous for TT (41.7 years) as compared with the CC homozygote (49.2 years; \( P = 0.031 \)) in cohort 1 (Figure 2C). Similarly, there was a 6.2 year earlier onset in TT carriers (33.2 years) as compared with CC carriers (39.4 years; \( P = 7 \times 10^{-5} \)) in the second cohort (Figure 2D). Finally, when comparing alleles, the T allele associates with a mean age of diagnosis 2.9 years earlier than C allele (\( P = 0.017 \)) in cohort 1 and 4.0 years earlier than C allele (\( P = 1 \times 10^{-6} \)) in cohort 2.
The MDM4 TT genotype shows enrichment in ER-negative breast cancers developing at a younger age

Because the risk for breast cancer is related to age at menopause, the data from cohort 1 were also analyzed to evaluate the genotype-specific risk of developing ER-negative breast cancer before 51 years, the average age of menopause in USA (38). Thus, women diagnosed with cancer at ≥51 years were used as a comparison group for the cases diagnosed at an earlier age (Figure 3). The genotype frequencies for the <51-year-old cohort were 29% CC, 49% TC and 21% TT. For those ≥51 years at diagnosis, that distribution was 44, 48 and 8%, respectively. This indicates that there was enrichment of the TT genotype in the younger age group as compared with the older age group (P = 0.021, one-tailed Fischer’s exact test). The odds ratio of 4.6 and 95% confidence interval [0.94–10.42] demonstrates an increased risk for developing ER-negative breast cancer under age 51 years for TT over CC genotypes. Furthermore, while 51 and 63% of Caucasian women carrying the CC or TC genotypes, respectively, were diagnosed with ER-negative ductal breast cancers by the age of 51, 87% carrying the TT genotype were diagnosed by age 51 years (P = 0.026, one-tailed Fischer’s exact test). This corresponded to an odds ratio = 12.5, 95% confidence interval (1.14–28.6) where the TT genotype associated with a higher risk of developing ER-negative ductal breast cancers in Caucasian women before age 51.

The second cohort, which is particularly enriched in women diagnosed with breast cancer before the age of 51 years, also demonstrated a similar trend in age at diagnosis when this cohort was stratified into women diagnosed with ER-negative breast cancer before the age of 40 years or ≥40 years. The cut off of 40 years was chosen since that was the median age at diagnosis in cohort 2 by when ~50% women were diagnosed with breast cancer. The genotype frequencies for the <40-year-old cohort were 17% CC, 25% TC and 58% TT and those for ≥40 years at diagnosis were 38% CC, 36% TC and 26% TT, respectively, confirming the enrichment of TT genotype in younger patients (P = 0.0039). Similarly, 40 and 50% Caucasian women carrying CC or TC genotypes were diagnosed with ER-negative ductal cancers by the age of 40 years, by which time 92% of the TT carriers had the disease (P = 0.0056). Taken together, the data suggest that women carrying the T allele or homozygous for this variant are at increased risk of developing ER-negative breast cancer at a younger age. There were no differences noted in the development of ER-positive breast cancer, even when looking at ductal and lobular breast cancers separately (data not shown).

Fig. 2. The TT genotype of human MDM4 associates with an accelerated age of onset of ER-negative but not ER-positive ductal breast cancers in Caucasian women. The cumulative incidence of cancer for individuals with TT genotype (black triangles), TC (dark gray squares) or CC genotype (light gray diamonds) is plotted as a function of age at diagnosis. The analysis was limited to a sample size of 476 patients (cohort 1) and 90 patients (cohort 2) consisting of Caucasian women with ductal breast cancers. Panels (A and B) depict ER positive and panels (C and D) represent ER-negative ductal breast cancers from cohorts 1 and 2, respectively.

Fig. 3. The TT genotype of human MDM4 is enriched in ER-negative breast cancers developing at a younger age. Genotype frequencies were calculated for women developing ER-negative breast cancers before age 51 and at age 51 and older including all ethnicities and cancer subtypes from cohort 1. CC represents the wild-type and TT the homozygous variant.
Discussion

In this descriptive, case-only study design of breast cancer patients, a SNP located in an intronic region of MDM4 (rs1563828), was found to be associated with earlier age at diagnosis of ER-negative breast cancers, but not ER-positive breast cancers. To our knowledge, this is the first study describing such an association. Although these studies identify an association with a single SNP, by virtue of being in possible linkage disequilibrium with a different locus, an alternative locus may represent the true functional polymorphism. The haplotype structure including other SNP loci in MDM4 that are linked to this polymorphism supports this hypothesis (37). MDM4 represents an attractive candidate for study of potentially functional polymorphisms due to its role in the p53 pathway. Our laboratory had similarly evaluated a SNP in the human MDM2 gene known as SNP309 that associated with increased levels of MDM2 and increased risk of tumorigenesis in familial as well as sporadic breast cancers (34,35).

It was further demonstrated that this polymorphism also associated with earlier age of onset of ER-positive but not ER-negative breast cancers as well as other tumors, e.g. diffuse large B cell lymphoma and soft tissue sarcoma, in a gender-specific manner (34). The presence of this polymorphic locus within an Sp1 transcription factor-binding site and existence of an estrogen-response element within 10 base pairs of this locus led to the hypothesis that both ER and Sp1 increase MDM2 levels through synergistic activation of the MDM2 promoter (34,35,40). This was further substantiated when estrogen was shown to selectively increase MDM2 levels in cells containing the G allele of SNP309 (36).

Unlike SNP309 in MDM2, the MDM4 SNP appears to associate with earlier age at diagnosis of ER-negative breast cancer. However, the SNP is not associated with an increased risk of developing this subtype of breast cancer over ER-positive disease. A growing body of literature supports the hypothesis that ER-positive and ER-negative breast cancers derive from different progenitor or breast stem cells (41,42). Altered expression of MDM4 may more effectively disrupt normal homeostasis in a cell type-dependent manner, e.g. mammary stem cell/progenitor or ER-negative duct epithelial cell, or in a developmental time point-specific manner.

In general, ER-negative breast cancers are thought to have higher genomic instability (43). DNA repair occurs when cells are in cell cycle arrest mediated by p53. If MDM4 directly binds p21 and disrupts p21 (44) in addition to p53, this may help explain the propensity of this MDM4 genotype to display a phenotype in ER-negative breast cancers. In presence of DNA damage, MDM4 interacts with isoforms of 14-3-3, which is achieved through phosphorylation of MDM4 at residue S367 by CHK2 kinase (45). The binding with 14-3-3 in turn promotes nuclear import of MDM4 and its subsequent MDM2-dependent ubiquitination and degradation, resulting in p53 stabilization.

MDM4 is also known to have shorter isoforms, one arising from a short internal deletion of 68 base pairs and giving rise to a truncated protein known as MDM4-S, and is better at binding and inhibiting p53-induced transactivation than full-length MDM4 (46). There is evidence for human MDM4 gene amplification or overexpression of human MDM4-S messenger RNA splice variant in soft tissue sarcomas, malignant gliomas and retinoblastomas with the expression of the splice variant being associated with worse prognosis (29–31). Another isoform of MDM4 (MDMX), the MDMX211 splice variant stabilizes MDM2 (47). It is possible that the SNP in MDM4 associates with expression of one of the shorter isoforms of MDM4. The effect on splicing could be a direct effect of this SNP or an indirect effect mediated through one of the linked SNPs within the haplotype. If this SNP indeed promotes the expression of a shorter MDM4 isoform with more efficient binding to p53 or MDM2 and less efficient binding to 14-3-3, this would result in less degradation of MDM4 and enhanced p53 inactivation even in the presence of DNA damage.

Both MDM2 and MDM4 play distinct but coordinated roles in p53 regulation. Unlike MDM2, MDM4 is not known to be hormonally regulated (Entrez Gene) despite some computational predictions for an estrogen-response element in the promoter and first intron of MDM4 (A. Vazquez, personal communication). We hypothesize that while SNP309 of MDM2 is functionally active in the presence of active estrogen signaling, the negative effects of MDM4 become more dominant in the absence of that active hormone signaling and the ratio of MDM4 to MDM2 increases. In an in vitro model system of point mutations in the C-terminus of MDM2, MDM4 was shown to contribute to the E3 function of MDM2 (14), evidence pointing toward a cooperative action between MDM2 and MDM4. Furthermore, there is also functional overlap between MDM2 and MDM4 as evidenced by complete rescue of Mdm4-null phenotype in mice by Mdm2 transgene (24). Thus, it can be hypothesized that in ER-negative breast cancers where MDM2 expression is lower, MDM4 acts both cooperatively with MDM2 and independently from MDM2 in exerting its effects.

Strengths of this study include the availability of information for all study participants from pathology reports and that the association analysis incorporated disease subtype. The latter is especially important given the heterogeneity of breast cancer as a disease. Although the effect of carrying the TT genotype in MDM4 on ER-negative breast cancers may represent a true biologic effect, the shift to earlier age at diagnosis may represent a bias in detecting more rapidly growing breast cancers that are detected earlier. This is particularly important as breast cancers are thought to be present for several years before they reach a threshold for detection. That being said, the distribution in stage at diagnosis using the TNM (Tumor Node Metastasis) classification was not significantly different between MDM4 genotypes.

The association in this study was strongest for ductal carcinomas but may not be representative of all breast cancer types, e.g. lobular, metaplastic, colloid, tubular breast cancers. In fact, lobular and tubular breast cancers would be predicted to have no association given their strongly positive ER status. Lastly, one would also expect that younger women might suffer from delay in diagnosis due to dense breast tissue relative to the sensitivity of mammography and lower suspicion for diagnosis of breast cancer in a young woman. This effect would tend to decrease the differences in age at diagnosis.

The specific ER-dependent associations observed in this descriptive study are hypothesis generating for the role of human MDM4 in hormone receptor-negative disease that will subsequently need to be tested in additional patient populations. Genetic association studies are often criticized for lack of reproducibility (48). However, in this study and in spite of its small size, cohort 2 provides reproducibility in the observed association with the MDM4 SNP locus. Differences in the genotype frequencies among ethnicities between the two cohorts can be attributed to factors such as smaller sample size in cohort 2 and admixture in different regions of the country. Due to the small size of cohort 2 with limited statistical power, a larger comprehensive study should be performed for confirmation of these associations. This would be particularly true since the smaller groups resulting from subgroup analysis are more probably to lead to Type 1 errors. Analyses using larger patient populations would help refute this type of error. As this study is a case-only design, it does not examine the overall risk of developing breast cancer. However, Atwal et al. (37) demonstrated risk associated with this SNP locus in human MDM4 in both breast and ovarian cancers. That being said, defining the molecular mechanism of SNP functionality, or that of a closely linked SNP, would even further support the observed positive associations. Because of the known role of MDM2 in breast cancer, the role of MDM4 in regulating the tumor suppressor p53, and known interactions between MDM2 and MDM4, there is biological plausibility for this association in breast cancer. Future studies may also benefit from controlling for potential confounders such as gynecological factors, e.g. age at menarche, age at first live birth, number of pregnancies.

In summary, we found that SNP rs1563828 located in intron 10 of human MDM4 associated with earlier age at diagnosis of ER-negative breast cancer but not ER-positive breast cancer. These findings were confirmed in a second cohort of breast cancer cases. However, further studies are needed to confirm our findings and molecular studies to identify functionality of this polymorphism are underway.
MDM4 polymorphism in ER-negative breast cancer

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References

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