Mutation analysis of the \textit{MDM4} gene in German breast cancer patients

Scarlett Reincke\textsuperscript{1}, Lina Govbakh\textsuperscript{1}, Bettina Wilhelm\textsuperscript{1}, Haiyan Jin\textsuperscript{1}, Natalia Bogdanova\textsuperscript{1,2}, Michael Bremer\textsuperscript{2}, Johann H Karstens\textsuperscript{2} and Thilo Dörk*\textsuperscript{1}

Address: \textsuperscript{1}Department of Gynaecology and Obstetrics, Hannover Medical School, Carl-Neuberg Str. 1, 30625 Hannover, Germany and \textsuperscript{2}Department of Radiation Oncology, Hannover Medical School, Carl-Neuberg Str. 1, 30625 Hannover, Germany

Email: Scarlett Reincke - doerk.thilo@mh-hannover.de; Lina Govbakh - doerk.thilo@mh-hannover.de; Bettina Wilhelm - doerk.thilo@mh-hannover.de; Haiyan Jin - doerk.thilo@mh-hannover.de; Natalia Bogdanova - doerk.thilo@mh-hannover.de; Michael Bremer - bremer.michael@mh-hannover.de; Johann H Karstens - karstens.jh@mh-hannover.de; Thilo Dörk* - doerk.thilo@mh-hannover.de

* Corresponding author

Abstract

\textbf{Background:} \textit{MDM4} is a negative regulator of p53 and cooperates with \textit{MDM2} in the cellular response to DNA damage. It is unknown, however, whether \textit{MDM4} gene alterations play some role in the inherited component of breast cancer susceptibility.

\textbf{Methods:} We sequenced the whole \textit{MDM4} coding region and flanking untranslated regions in genomic DNA samples obtained from 40 German patients with familial breast cancer. Selected variants were subsequently screened by RFLP-based assays in an extended set of breast cancer cases and controls.

\textbf{Results:} Our resequencing study uncovered two \textit{MDM4} coding variants in 4/40 patients. Three patients carried a silent substitution at codon 74 that was linked with another rare variant in the 5'UTR. No association of this allele with breast cancer was found in a subsequent screening of 133 patients with bilateral breast cancer and 136 controls. The fourth patient was heterozygous for the missense substitution D153G which is located in a less conserved region of the \textit{MDM4} protein but may affect a predicted phosphorylation site. The D153G substitution only partially segregated with breast cancer in the family and was not identified on additional 680 chromosomes screened.

\textbf{Conclusion:} This study did not reveal clearly pathogenic mutations although it uncovered two new unclassified variants at a low frequency. We conclude that there is no evidence for a major role of \textit{MDM4} coding variants in the inherited susceptibility towards breast cancer in German patients.

\textbf{Background}

As part of a genome surveillance network, the tumour suppressor protein p53 becomes stabilized after DNA damage and modulates intracellular responses such as cell cycle arrest, DNA repair, senescence or apoptosis [1-3]. Multiple mechanisms regulate the activity of p53 at the post-
transcriptional level [4,5]. One important antagonist, MDM2, is essential for ubiquitylation and subsequent degradation of p53 to maintain it at low levels in unstressed cells [6]. An MDM2-related protein, MDM4, has more recently emerged as another p53-interacting protein with a central role in the DNA damage response [7-9].

MDM4, also known as MDMX, is a 490 amino acid protein that is structurally related to MDM2 and binds to both, p53 and MDM2 [8]. MDM4 is regarded a negative regulator of p53 and cooperates with MDM2 to antagonize p53 [8-10]. In response to DNA double strand breaks, MDM4 becomes phosphorylated by the ATM and Chk2 kinases in an ATM-dependent manner which leads to a switch from the degradation of p53 to the degradation of MDM4 and consecutive stabilization of p53 [9,11-14].

Disruption of the p53 pathway is a key event in mammary tumorigenesis, and MDM4 is overexpressed in some 19% of breast carcinomas [15]. It is unknown, however, whether the MDM4 gene plays some role in the inherited component of breast cancer susceptibility. In the present study, we investigated the mutational spectrum of the whole MDM4 coding sequence in a group of German patients with familial breast cancer.

Methods

Patients

Our study population consists of a hospital-based series of 1012 unselected breast cancer patients who were treated at the Department of Radiation Oncology at Hannover Medical School from 1996–2001. Median age at onset of breast cancer was 57 years in this patient group, and 157 patients (15.8%) reported at least one first-degree relative with breast cancer. The patient series had been randomly taken from a consecutive series of anonymous female German blood donors recruited in 2005 at the same hospital. Written informed consent was obtained from each patient, and the study was approved by the Ethics commission at Hannover Medical School.

Mutation analyses

Genomic DNA was isolated from peripheral EDTA blood samples using standard phenol-chloroform extraction. All exons of the MDM4 gene were amplified by polymerase chain reaction using primer pairs with sequences flanking the respective exons (Table 1, Genbank NT_004487.18). 35 cycles of PCR were carried out using HotStart Taq DNA Polymerase (Qiagen) with 1 min annealing at the primer specific temperature (Table 1), 1 min extension at 72°C and 1 min denaturation at 94°C. Sequencing reactions were performed using BigDye v1.1 chemistry, and sequences were evaluated on a Genetic Analyzer 3100 Avant (Applied Biosystems). Sequencing primers were the same as the PCR primers (Table 1). The genomic region covering exon 1 of the MDM4 gene was additionally amplified in 133 breast cancer and 136 control samples to allow for a subsequent restriction-enzyme based screening of the c.-103 T/C variant using MboI (New England Biolabs). Reaction products were separated on a 2% agarose gel and were evaluated by staining with GelRed. In the presence of the c.-103T allele, PCR product was cleaved to fragments of 129 bp and 282 bp, whereas product from the C allele remained at 411 bp length. The genomic region covering exon 7 of the MDM4 gene was additionally amplified in 140 breast cancer and 200 control samples to allow for a subsequent restriction-enzyme based screening of the D153G mutation using BbsI (New England BioLabs). Reaction products were separated on a 3% agarose gel and were evaluated by staining with GelRed. In the presence of the D153G mutation, the 334 bp wildtype product was cleaved to fragments of 110 and 224 bp, whereas the mutant product remained uncut. Positive and negative controls were included into each assay and samples that remained uncut were subjected to direct sequencing to avoid false positives.

Results

We established conditions to amplify the eleven exons of the MDM4 gene (Table 1). The whole coding region and flanking sequences were then analysed by direct sequencing in genomic DNA samples from 40 German breast cancer patients with a family history of disease. Eight distinct sequence alterations were identified (Table 2). Six of these were known polymorphisms listed in the NCBI SNP database with four of them located in flanking intron sequences, one in the 5’-UTR and one in the 3’-UTR. Maximum likelihood considerations indicated that the three SNPs in the introns 8, 9 and 10 were in absolute linkage disequilibrium, and that three common haplotypes could be defined on the basis of either the rare allele of rs4252697 in intron 5 (~16%), or the rare alleles of rs4252717-rs2290855-rs2290854 in the intron 8-9-10 block (~26%), or the common alleles at these four loci (~58%). Two less common alterations were identified in the coding region, the silent V74V substitution in 3/40...
patients and the missense substitution D153G in a single case (Table 2).

The V74V substitution is a synonymous transversion c.222A>T that changes a GUA codon to GUU. It is located within a weak potential alternative splice donor site, however maximum entropy calculations predicted that this site is not improved by the substitution [24]. A search for exonic splicing enhancer sequences predicted that the substitution creates an additional binding site for the serine/arginine-rich (SR) protein SC35 which may improve exon recognition [25]. However, preliminary analyses of MDM4 mRNA in lymphoid cells did not reveal evidence for alternative splicing of exon 4 (data not shown), and this exon skipping is not among previously reported alternative splicing events [26,27]. On the basis of the available information, we considered a functional contribution of the V74V substitution unlikely and did not screen further for this substitution. Of some interest, all three patients who were heterozygous for V74V also carried the variant c.-103C>T in the 5’-UTR (rs4252668), suggesting that these two SNPs might be in linkage disequilibrium.

Table 1: Primers and PCR conditions used for MDM4 screening. Summary of primers, annealing temperatures and PCR product sizes for the eleven exons of the MDM4 gene. Forward and reverse primer sequences are listed. Exons 2 – 11 are coding exons. Exon 11 was amplified in two overlapping parts in order to make it more accessible to sequencing analysis.

| Exon | Primer | Annealing (°C) | Product size (bp) |
|------|--------|----------------|------------------|
| 1    | 5'-TCTGATCTCTAATACAGTTGTCGTG-3' 5'-GCCTCCCTACGCTCCCATCCTCAG-3' | 62 | 416 |
| 2    | 5'-CTTGTGGCCCTTCTTGGTGAATG-3' 5'-CTTTGAATCACTGTTAGAGAC-3' | 62 | 296 |
| 3    | 5'-AGAGGTTCTTGGTCCAGAT-3' 5'-GTGGAGATGGCACCACCTCTG-3' | 62 | 253 |
| 4    | 5'-CAAGAATGCAGTCAAGACATG-3' 5'-CAGATCAGTCCATTTCACTCTGAC-3' | 62 | 520 |
| 5    | 5'-CATATTCTCAGTCCCTCATAGG-3' 5'-CTCAAAAGCTGTTATAGCACTG-3' | 62 | 234 |
| 6    | 5'-CAGCCAAATGGAAGAATAC-3' 5'-CAGAGAAGGTTTCACTCTGTC-3' | 62 | 356 |
| 7    | 5'-GTTGGAGCCAGAATGGAACCTG-3' 5'-AAGATGTTAGTAACTGTGGACTG-3' | 62 | 334 |
| 8    | 5'-AGCTTCTGCCACTAGACAG-3' 5'-CAAGAGATACCAAGAATGTTACC-3' | 62 | 389 |
| 9    | 5'-CATGTGTTGACGGACATTGGAG-3' 5'-GATCGAGGAGTAACTGTGGACTG-3' | 62 | 311 |
| 10   | 5'-CTCTCTAGCAATGACAATAGACG-3' 5'-GGATTACATCATCTGAAGATGGG-3' | 62 | 329 |
| 11 (a)| 5'-GCTTATGAGAGGATGATCGAATGC-3' 5'-CTGCTTACCCTTAAATGATC-3' | 59 | 431 |
| 11 (b)| 5'-CTGCTTACCCTTAAATGATC-3' 5'-CTAAAGACACTTCTCTGCAG-3' | 59 | 475 |

Table 2: Genetic alterations of the MDM4 gene in 40 German patients with familial breast cancer. Survey of genetic alterations of the MDM4 gene identified in 40 patients with familial breast cancer. Mutations were designated according to the improved mutation nomenclature recommended by the Human Genome Variation Society [50]. Het, heterozygous; hom, homozygous. Variants c.-103T>C and c.222A>T were identified in the same three individuals. The three SNPs c.672+28C>T, c.823-62T>C and c.903+20G>A were identified in the same 19 individuals, with 17 of them also carrying c.*+32A>C.

| Location | Nucleotide change | Codon | No. of carriers (frequency) | NCBI database |
|----------|------------------|-------|-----------------------------|---------------|
| 5’-UTR   | c.-103T>C       | none  | 3 (.08)                     | 3 (.08)       | rs4252668    |
| Exon 4   | c.222A>T        | Val74Val | 3 (.08)                    | 3 (.08)       | not listed   |
| Intr 5   | c.343+9C>T      | Val74Val | 12 (.30)                   | 1 (.03)       | rs4252697    |
| Exon 7   | c.458A>G        | Asp153Gly | 1 (.03)                    | 1 (.03)       | not listed   |
| Intr 8   | c.672+28C>T     | none  | 19 (.48)                    | 19 (.48)      | rs4252717    |
| Intr 9   | c.823-62T>C     | none  | 19 (.48)                    | 19 (.48)      | rs2290855    |
| Intr 10  | c.903+20G>A     | none  | 19 (.48)                    | 19 (.48)      | rs2290854    |
| 3’-UTR   | c.*+32A>C       | none  | 17 (.43)                    | 17 (.43)      | rs4245739    |
Because SNPs in the 5'-UTR can exert regulatory functions in some genes with implications for breast cancer risk, e.g. in RAD51 [28-31], we assessed the frequency of the rare allele of rs4252668 in an additional set of 133 patients with bilateral breast cancer and 136 population controls. The rare allele was detected in 4/133 cases and 7/136 controls suggesting that it does not confer a significant increase in breast cancer risk (OR 0.6, 95% CI 0.2–2.0, p = 0.38).

The second nucleotide change in the coding region gives rise to a non-conservative amino acid substitution D153G (Figure 1) and was identified in only one patient with familial breast cancer. This patient had been diagnosed with unilateral breast cancer by the age of 24 years. Her mother had unilateral breast cancer by the age of 41 years and basal cell carcinoma by the age of 49 years, and the sister of the mother was diagnosed with unilateral breast cancer by the age of 44 years. We confirmed the D153G mutation in separate amplification reactions and also in the patient’s mother but not in the maternal aunt, indicating an incomplete segregation pattern. The D153G substitution is embedded within a region of unknown structure and with no apparent homology to MDM2. A screen for evolutionary conservation with SIFT v.2.0 predicted this substitution to be tolerated [32]. A search for exonic splicing enhancers revealed that the D153G substitution is predicted to destroy a binding site for the SF2/ASF splicing factor [25]. However, only one of two overlapping binding sites is affected by the substitution suggesting that there might be no gross change in exon recognition. A PROSITE search for protein binding and phosphorylation sites revealed one possible motif, 150-TTED-153, which is a predicted casein kinase II (CK2) phosphorylation site [33]. We screened another 140 cases with bilateral breast cancer and 200 random female control individuals for the presence of the D153G mutation using a restriction enzyme-based assay. No further carrier was detected indicating that this missense substitution is very rare, at least in the German population.

**Discussion**

Dysregulation of the p53 network is pivotal to mammary carcinogenesis, and germline alterations in the TP53 and MDM2 genes have been found to modulate the inherited risk of breast cancer [34-39]. The MDM4 protein associates with and regulates both proteins, p53 and Mdm2, in the DNA damage response pathway. It was therefore tempting to investigate whether activating germ-line mutations exist in the MDM4 coding sequence that could also contribute to breast cancer risk. We thus sequenced all exons and flanking non-coding sequences of MDM4 in 40 German patients with familial breast cancer. It is the first study, to our knowledge, that assesses the frequency distribution of MDM4 germ-line alterations in familial breast cancer.

Outside of the coding region, we confirmed common single nucleotide polymorphisms (SNPs) in the introns 5, 8, 9 and 10 as well as in the 5' and 3' untranslated regions (UTR). Although we cannot assign any function to these SNPs, the SNPs in the untranslated regions may be useful to measure allelic imbalances at the mRNA level. We also noticed that there appeared to be a slight underrepresentation of rare homozygotes for the coupled intronic variants in IVS8, 9 and 10 as one may have expected 2–3
All patients were scored for the presence of breast cancer, and no patients with familial breast cancer were identified. There were only partial segregation data available for these families, and it is therefore possible that rare mutations exist which may be more likely to harbour polygenic susceptibility alleles rather than highly penetrant mutations. It is also possible that more families with the D153G mutation may be detected in an extended case-control series. However, several thousands of samples would have to be screened to confirm, for instance, a 2–3 fold risk for a variant with a carrier frequency of less than 1%. The paucity of mutations in our study population seems to be consistent with the lack of evidence from breast cancer linkage studies for linkage to the MDM4 locus, and so highly penetrant founder mutations in the MDM4 gene might at best account for only a small proportion of German breast cancer patients.

Conclusion
In summary, a resequencing study of the MDM4 gene in 40 German breast cancer patients selected for family history did not reveal clearly pathogenic mutations although it uncovered two new unclassified variants at a low frequency. We conclude that there is no evidence for a major role of MDM4 coding alterations in the inherited susceptibility towards breast cancer in German patients.

Competing interests
The author(s) declare that they have no competing interests.
Authors’ contributions
SR established PCR and sequencing conditions to analyse the MDM4 coding region. SR, LG and BW performed the sequencing analyses. HJ and NB performed the case-control screening studies. MB and JHK provided blood samples and clinical records from the patients of the Department of Radiation Oncology. TD initiated and coordinated the study and drafted the manuscript. Authors took part in the critical discussion and proofreading of the manuscript.

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