The significance of *MDM2* SNP309 and p53 Arg72Pro in young women with breast cancer

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Abstract. The p53 protein and its regulator MDM2 is central to tumorigenesis by directing cells to undergo cell cycle arrest and/or apoptosis in response to DNA damage or other stress signals. The genes encoding these proteins contain nucleotide variation (p53 codon 72, MDM2 SNP309) that influences cellular response. We examined the p53 codon 72 and MDM2 SNP309 to determine their implication with age of disease onset and risk of breast cancer in young women (≤36 years). No risk of breast cancer was observed for the genotypes of p53 and MDM2, however, a tendency (P=0.15) towards increased risk of early onset breast cancer was observed in carriers of two or more Pro and/or G alleles. We further calculated the influence on age at diagnosis. Cases were grouped according to the number of G and Pro alleles (0, 1, 2 or 3-4) and age at diagnosis. A significant trend towards decreased age at diagnosis with increased number of risk alleles was found (P=0.013). Our results suggest that p53 codon 72 and MDM2 SNP309 may be implicated in early onset breast cancer.

Introduction

Early onset breast cancer is a multifactorial disease, and the genesis and progression of most breast cancers are influenced by both environmental and genetic factors. A family history of breast cancer seems to be the greatest risk factor for early onset breast cancer, and the risk is a function of the number of affected relatives, the degree of relationship, and the time of onset (1). The most well-known genetic factors implicated in breast cancer are germ-line mutations in *BRCA1* and *BRCA2* (2,3). However, only a small section of patients have the disease as a consequence of hereditary mutations in one of these genes, and the origin of the vast majority of early onset breast cancers is still unknown. Naturally occurring variations in genes implicated in crucial processes such as apoptosis, DNA repair and proliferation may be modifiers of breast cancer risk.

The p53 protein and its regulator MDM2 are central to tumorigenesis by directing cells to undergo cell cycle arrest and/or apoptosis in response to DNA damage or other stress signals (4-6). The p53 gene harbours a polymorphism (G/C) in codon 72 that results in a substitution of arginine to proline (7) and it has been shown that the arginine allele induces a higher degree of apoptosis compared with the proline allele (8). In addition, the proline allele is more prone to induce increased levels of G1 arrest (9,10). Studies of codon 72 with correlation to breast cancer risk and survival rates have shown contradictory results in different populations (11-15). The MDM2 protein contains a binding domain for p53 and attenuates its growth regulatory functions (16-18). One mechanism by which the MDM2 can impede tumor suppressor activity of p53 involves ubiquitin degradation by the proteasome pathway, resulting from the E3 ligase activity of MDM2 (19-22). In addition, p53 up-regulates the expression of MDM2 mRNA and protein, which subsequently inhibits p53 activity, thus forming a negative feedback loop (18,23). Bond *et al* (24) identified a T→G substitution in the P2 promoter of the *MDM2* gene (SNP309), which is associated with higher affinity for the transcription factor Sp1. This single nucleotide polymorphism leads to increased MDM2 mRNA and protein levels and consequently p53 inhibition. Adding to this, there is a tendency showing earlier cancer onset in certain groups of patients harbouring the G/G or T/G genotype for *MDM2* SNP309 (24,25).

In the present case-control study we investigated polymorphisms in *MDM2* and *p53* and the risk of early onset breast cancer. In addition, since previous studies have shown that younger age groups develop cancer earlier when carrying a homozygote genotype, at least for the *MDM2* SNP309, we also studied the allele influence on age at diagnosis.

Materials and methods

Study populations. The study included 123 young women diagnosed with breast cancer between the years 1980 and 1994 in the South-East Sweden Health Care Region. The patients had a median age of 34 years, raging from 24 to 36

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Table I. Genotype frequencies of the *MDM2* SNP309 and *p53* codon 72 polymorphisms.

	MDM2 SNP309			*p53* codon 72		
	T/T	T/G	G/G	Arg/Arg	Arg/Pro	Pro/Pro
Early onset breast cancer (%)	52 (42.3)	57 (46.3)	14 (11.4)	65 (56)	45 (38.8)	6 (5.2)
Controls (%)	68 (46.6)	60 (41.1)	18 (12.3)	79 (55.6)	58 (40.8)	5 (3.5)

years of age. Tissue samples from archival material were obtained from the pathology departments of hospitals in the South-East Sweden Health Care Region. The control group from the same geographic area consisted of 146 healthy female blood donors between 18-39 years of age with a median age of 30 years.

DNA extraction. DNA from blood donors was isolated using Wizard® SV Genomic DNA Purification System (Promega Corp., Madison, WI, USA). DNA from paraffin-embedded normal lymph node tissues of young breast cancer patients was extracted according to a standard protocol.

Polymerase chain reaction for MDM2 and p53. Amplification of the *MDM2* SNP309 and *p53* codon 72 regions was performed using the following primers; forward, 5'-CGGGAGT TCAGGGTAAAGGT-3'; reverse, 5'-Biotin-TCGGAACGTG TCTGAACTTG-3' for MDM2 and forward, 5'-GAAGACCC AGGTCCAGATGA-3'; reverse, 5'-CTGCCCTGGTAGGTT TTCTG-3' for p53. The PCR reactions for *MDM2* were carried out in a total volume of 30 μl, adding 60 ng of DNA to the reaction mixture. *p53* was amplified using ~40 ng of DNA in a total volume of 20 μl. The PCR reaction mixtures were composed of 1X $MgCl_2$ free PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM each of forward and reverse primer, and 0.5-0.75 units of TaqDNA polymerase (Promega). An initial denaturation at 94˚C for 3 min was followed by 35-40 cycles of denaturation at 94˚C for 45 sec, annealing at 55-60˚C for 45 sec and elongation at 72˚C for 45 sec. An elongation step of 5 min at 72˚C followed the final cycle and the reactions were then kept at 4˚C. All PCR reactions were run on a PTC-200 Peltier Thermal Cycler DNA Engine (MJ Reaserch™ , Inc., Waltham, MA, USA).

Pyrosequencing. The genotypes of SNP309 were verified by pyrosequencing analysis with the sequencing primer 5'-CAG GGTAAAGGTCACG-3' (final concentration 0.3 μM) in an automated PSQ96 MA pyrosequencer instrument (Biotage AB, Uppsala, Sweden). Briefly, 25 μl of the PCR products were immobilized on 3 μl Streptavidin Sepharose™ High Performance beads (Amersham Biosciences AB, Uppsala, Sweden), followed by denaturation, washing, and primer annealing steps, using a vacuum device, according to the manufacturer's instructions (Biotage AB). For all SNP runs, the Pyro Gold reagents kit (Biotage AB) was used and the sequencing was performed as previously described (26). The results were subsequently analysed with the pyrosequencing software PSQ96 MA 2.1 (Biotage AB). However, in 14 samples of the paraffin-embedded normal lymph node tissues of young breast cancer patients no results were obtained. Therefore, purified PCR products of these samples were applied to dideoxy sequencing analysis using isotope ^{33}P, according to the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (USB Corp., Cleveland, USA), and run on a polyacrylamide gel (6% polyacrylamide, 8 M Urea) under denaturating conditions. The gel was then dried and exposed on X-ray film (Kodac Biomax MS using Trans Screen LE intensifying screen) at -70˚C.

Restriction fragment length polymorphism (RFLP). The *p53* codon 72 polymorphism was detected using the restriction enzyme BstUI that recognises a restriction site on the Arg allele. Ten units of BstUI (New England Biolabs) and 1.6 μl NEBuffer 2 (supplied by the manufacturer) were added to 5 μl of PCR products and incubated at 60˚C for 5 h. After digestion, fragments were resolved by electrophoresis on a 3% (w/v) agarose gel containing 1X TBE buffer and ethidium bromide (0.5 μg/μl).

P53 status. Mutations in exon 5-8, loss of heterozygosity as well as protein expression of the p53 gene has been described earlier (27). Briefly, single strand conformational polymorphism (SSCP) was used to screen for mutations in the p53 gene and tumors showing mobility shifts were collected from the gel and sequenced using incorporation of [α-^{32}P] dATP. Loss of heterozygosity was estimated by means of an intragenic microsatellite marker. α-$dATP^{32}$ was incorporated by PCR and amplicons were separated on a denaturating polyacrylamide gel. The signal intensity was then compared between bands of tumor and normal cell origin. For immunohistochemistry, 5 μm tumor sections were deparaffinised and rehydrated before antigen retrieval in microwave oven. Tissue sections were incubated with the monoclonal antibody DO-7 that was coupled with the avidin-biotin-peroxidase complex. Finally, the p53 protein was stained by addition of 3,3'-diaminobenzidine and cells counterstained by Mayer's haematoxylin.

Statistical analysis. Odds ratios (OR) and 95% confidence intervals (CI) were calculated by logistic regression to evaluate the risk of breast cancer for individuals homozygous or heterozygous for the Pro and G alleles in p53 codon 72 and MDM2, respectively. P-values were calculated by χ^2 analysis. One-way analysis of variance (ANOVA) in the Statistical Package for the Social Sciences (SPSS) Advanced Models™ 12.0 software was used for comparing mean age at

Table II. Calculated odds ratios (OR) of *p53* codon 72 and *MDM2* SNP309 for young breast cancer patients and controls.

Genotype	Controls	Cases	OR	95% CI	P-value
MDM2 T/T	68	52			
MDM2 T/G +G/G	78	71	1.19	0.71-1.99	0.48
p53 Arg/Arg	79	65			
p53 Arg/Pro + Pro/Pro	63	51	0.98	0.58-1.66	0.95

Table III. The distribution of p53 status.[a]

	IHC		Mutations		LOH-status	
	+	-	Mutation	wt	LOH	ROH
Codon 72						
Arg/Arg	16	28	9	44	6	9
Arg/Pro	20	15	6	35	8	12
Pro/Pro	2	1	2	2	2	0
P-value	0.23		0.20		0.24	
SNP309						
T/T	17	20	6	38	7	6
T/G	18	25	9	39	6	14
G/G	5	3	4	8	2	5
P-value	0.48		0.29		0.14	

[a]Protein expression estimated by immunohistochemistry, gene mutations and loss of heterozygosity according to *p53* and *MDM2* genotype.

Table IV. Calculated odds ratio of the gene interaction between p53 codon 72 and MDM2 SNP309.

No. of risk alleles	Controls	Cases	OR	95% CI	P-value
0-1	99	71			
≥2	43	24	1.46	0.84-2.53	0.15

Patients were grouped according to the number of risk alleles (i.e., proline in codon 72 and G in SNP309).

diagnosis according to the number of alleles coding for Pro in codon 72 in the *p53* gene and the number of G alleles in SNP309 in the *MDM2* gene.

Results

The genotype frequencies of the *MDM2* and *p53* polymorphisms in the early onset breast cancer cases and controls are presented in Table I. Odds ratios were calculated according to genotypes of p53 and MDM2 but no risk of breast cancer was observed (Table II). The distribution of p53 status, i.e., protein expression estimated by immunohistochemistry, gene mutations and loss of heterozygosity according to genotype is shown in Table III. However, no significant trend was seen. We then grouped the material according to the number of risk alleles (Pro and G) in *p53* codon 72 and *MDM2* SNP309, respectively. Cases and controls with <2 Pro and G alleles were defined as a low risk group and those with ≥2 alleles were grouped together and defined as high risk group. A tendency (P=0.15) towards increased risk of early onset breast cancer was observed among those with ≥2 of the Pro and/or G alleles (Table IV).

We further calculated the influence of p53 and MDM2 on age at diagnosis. Breast cancer cases were grouped according to the number of G and Pro alleles (0, 1, 2 or 3-4) and age at diagnosis. The difference in mean age between groups was tested using one-way analysis of variance (ANOVA). Fig. 1 shows a significant trend towards decreased age at diagnosis with increased number of risk alleles (P=0.013).

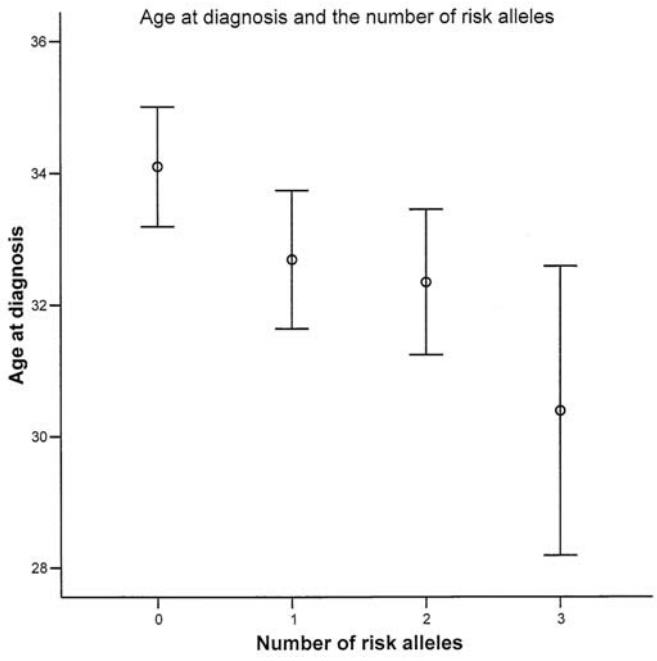

Figure 1. Mean age at breast cancer diagnosis was plotted against the number of risk alleles. A significant trend towards decreased age at diagnosis was found with increased number of risk alleles (P=0.013). Risk alleles are defined as having proline in codon 72 in the *p53* gene or G-allele in SNP309 of the *MDM2* gene. The bars represent standard error of the mean. Difference in mean age according to risk alleles was tested using one-way analysis of variance (ANOVA).

Discussion

In the present study we observed a significant trend towards a decreased age at diagnosis with increasing numbers of *p53* codon 72 Pro and *MDM2* SNP309 G alleles. In addition, when using the control group and calculating the risk of breast cancer we found a weak association between risk and *MDM2* SNP309 and *p53* codon 72.

Previous studies of combined analyses of both *p53* codon 72 and *MDM2* SNP309 and the risk of breast cancer have generally shown lack of association. A report by Cox and collaborators (28), who genotyped participants of a nurse health study, demonstrated no association to breast cancer risk. Similar results were also shown in a pooled series of European cases and controls from 5 studies (29). However, in an Asian population it was reported that carriers of the *MDM2* SNP309 G allele had an increased risk of both post-menopausal and familial breast cancer, although the *p53* codon 72 alleles did not affect breast cancer risk (30). Nevertheless, in their younger age group (≤40 years) results of both *MDM2* SNP309 and *p53* codon 72 polymorphisms were in accordance with our study of early onset cases (30). In investigations of familiar breast cancer, Tommiska *et al* (13) and Wilkening *et al* (31) studied the influence of codon 72 and SNP309, respectively, without finding any relationship to risk of familial breast cancer. On the other hand, Ohayon *et al* (32) showed that arginine homozygosity was associated with increased risk of familial breast cancer in Jewish women without the predominant Jewish mutations in *BRCA1/BRCA2*.

The polymorphisms in *p53* codon 72 and *MDM2* SNP309 have been shown to influence age at onset, especially in hereditary cancers in several reports, but it is less significant in sporadic cancer. In a study of patients with the Li-Fraumeni syndrome, of which the majority depends on germline mutations in *p53*, carriers of SNP309 G and codon 72Arg alleles were diseased significantly earlier (25). Ruijs and colleagues (33) studied the *MDM2* SNP309 in *p53* germline mutation carriers and found in accordance with Bougeard *et al* (25) that the G allele was associated with early onset of tumor formation. High prevalence of somatic *p53* mutations has been reported from head and neck squamous cell carcinoma. Nakashima *et al* (34) investigated this malignancy with respect to the impact of *MDM2* SNP309 and age of onset and the average age at tumor onset was found to be significantly lower in the GG genotype.

In a recent study by Wilkening *et al* (35), including pooled data from 11 reports consisting of both familial and sporadic breast cancer cases, as well as patients with different ethnic origin, no association to earlier tumor formation was found for *MDM2* SNP309. Wilkening and co-workers (31) also studied women with familial breast cancer without *BRCA1* and *BRCA2* mutations and the polymorphism of *MDM2* SNP309 did not seem to modify the age of onset in this population. However, the mutational status of *BRCA1/BRCA2* might influence results since Yarden *et al* (36) found the opposite showing that the GG genotype of SNP309 was significantly associated with earlier diagnosis in Ashkenazi-Jewish with *BRCA1/BRCA2* mutations. In a Chinese breast cancer population, including both sporadic and familial breast cancer cases, Lum *et al* (30) found a significant association between SNP309 T genotype and early age at cancer diagnosis.

The present knowledge of *MDM2* SNP309 and *p53* codon 72 indicates that its implication in carcinogenesis may be dependent on mutational status of genes in the p53 pathway as shown in studies of the Li-Fraumeni syndrome and *BRCAX* mutational carriers. This hypothesis is partly strengthened by the present investigation. *p53* mutation was a minor event in our study population but overexpression of the p53 protein occurred in almost half of cases indicating a possible dys-regulation of the protein. Family history and the status of *BRCA1/BRCA2* genes in the present cohort is unknown since cases were selected from a time period with little focus on hereditary disease, however, a family history of breast cancer and mutations of *BRCA1/BRCA2* is more common in younger patients compared to the elder counterparts. Nevertheless, our results suggest that polymorphisms in *p53* codon 72 and *MDM2* SNP309 may be implicated in breast cancer of young women.

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