A polymorphism C3435T of the MDR-1 gene associated with smoking or high body mass index increases the risk of sporadic breast cancer in women

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Abstract. The human multidrug resistance gene 1 (MDR-1) encodes a plasma membrane P-glycoprotein (P-gp) that functions as the transmembrane efflux pump for various structurally unrelated anticancer agents and toxins. Polymorphisms in the MDR-1 gene may have an impact on the expression and function of P-gp, thereby influencing the susceptibility to various diseases, including cancer. We investigated the incidence of C3435T polymorphisms at exon 26 in the MDR-1 gene in 92 women with breast cancer and potential association of altered genotypes with smoking and high body mass index in cancer development among patients. The MDR-1^{C3435T} allelotype and genotype analysis revealed a high incidence (75.0%) of polymorph alteration in the MDR-1 gene. The frequencies of homozygous T/T, heterozygous C/T and homozygous C/C genotypes were 25.0, 50.0 and 25.0%, respectively. The risk of breast carcinoma in patients with MDR-1 polymorphism was significantly associated with the higher body mass index, where women with BMI >30 kg/m^2 and C allele in genotype had a higher risk of disease compared to patients with lower amounts of body fat tissue (p=0.0439). The risk was highest for the homozygous carriers of C allele with BMI >30 kg/m^2 compared to patients with BMI 25.1-30 or ≤25 kg/m^2 (OR 3.65, 95% CI 0.94-14.20; or OR 2.50, 95% CI 0.55-11.41), respectively. Consistent with the results of genotyping and BMI analyses, smoking patients harboring the C/T or C/C genotype had an increased risk of cancer (OR 1.28, 95% CI 0.23-7.17; OR 1.58, 95% CI 0.28-10.44, respectively) when exposed to carcinogens in tobacco smoke, although it was not statistically significant. Our findings suggest that the MDR-1^{C3435T} polymorphism occurs in high incidence among women with breast carcinoma where C allele carriers have increased risk of developing cancer when exposed to toxic substances. Our observations are the first that indicate this polymorphism as a modulator of health to be associated with an increased risk of breast cancer.

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Introduction

Breast cancer is one of the major causes of cancer-related death in women with different survival and mortality rates worldwide (1). The incidence rises steadily, however it may vary by age, race, socioeconomic status and stage (2). Susceptibility to breast cancer represents a complex interplay between exposure to potential toxins, carcinogens and the genes involved in the detoxification pathways. Additionally, p53-specific disruption of the cell cycle as well as the disruption of DNA structure cannot be omitted (3,4). The genetic predisposition or disturbance of these genes in association with exogenous carcinogens (i.e., benzopyrene in tobacco smoke, polychlorinated biphenyls, organochlorine pesticides in air, food and water), high-protein or fatty diet may enhance the proliferative effects of carcinogenic compounds and might contribute to tumor promotion in estrogen-sensitive tissues (5).

It is generally accepted that tobacco smoke is carcinogenic for not only the respiratory tract, but for many tumors of a non-respiratory nature, including breast tissue, as well (6). The polycyclic aromatic hydrocarbons including benzopyrene in tobacco smoke are mutagenic for the p53 suppressor gene in humans, and 7,12-dimethylbenzanthracene is an inductor of mammary tumors in animals (7). Previous studies showed increased risk of breast cancer for active or passive smokers (8,9). Most of the 30 carcinogenic substances, which are present in tobacco smoke are fat-soluble, resistant to metabolism and, may be due to their lipophilic properties, stored in breast adipose tissue (10,11). Otherwise, obesity alone is a known risk factor for breast cancer, especially in postmenopausal women (12-15), and evidence for a causal link between obesity and breast cancer risk, particularly the effects of metabolic syndrome, insulin resistance, peripheral estrogen aromatization

in adipose tissue, and direct effect of adipokines on molecular basis was established (16). These factors are potentially oncogenic via the shared mitogen-activated protein kinase (MAPK), mitogen/extracellular signal-regulated kinase (MEK) and extracellular signal-regulated kinase (ERK) cellular pathways (17). Obese breast cancer patients appear to have a higher risk of lymph node metastases, large tumors and death when compared with non-obese breast cancer patients (18).

From this view, it may be inferred that transporter molecules involved in distribution, delivery and penetration of environmental (xenobiotic) agents into cellular and subcellular compartments or expulsion of reactive free radicals, toxic endogenous substances and metabolites out of the cell may be involved in cancer development, including breast carcinoma. One such transporter with cell protective effects is P-glycoprotein, an energy dependent efflux pump, transporting a wide variety of divergent lipophilic compounds from the intracellular to the extracellular compartment (19,20). This protein is encoded by the MDR-1 gene located on human chromosome 7 at q21.1 (21) and belongs to a large group of transport proteins, known as the ATP-binding cassette (ABC) superfamily (22). P-glycoprotein is highly expressed in apical membranes of organs with excretory function, such as liver, small intestine or kidney (23-25), where it mediates the excretion of xenobiotics into the bile, urine and gut lumen. In addition, high expression was found in endothelial cells of the central nervous system (26) and breast carcinoma (27). In addition to the known association between increased expression of this glycoprotein in tumor cells and the phenomenon of multidrug resistance against antineoplastic agents (28) are reports on the relationship between MDR-1 genotype and susceptibility to cancer (29-31), based on the fact that some of the polymorphic variants of the MDR-1 gene, including C3435T at exon 26, have been shown to affect the expression and function of P-glycoprotein (32,33). Of great importance is also the finding that polymorphism C3435T of the MDR-1 gene can predict response to preoperative chemotherapy in locally advanced breast cancer (34). Considering all these observations together we hypothesize that C3435T polymorphism in the MDR-1 gene may limit the local detoxification activity in breast tissue and be a risk factor for cancer development and behavior. Although several studies have focused on the association between polymorphism of xenobiotic metabolizing enzymes, smoking and breast cancer risk (35,36), not one focused on MDR-1^{C3435T} polymorphism. We present the first report on the link between the disease risk, smoking, high body mass index and this genetic alteration in women.

Materials and methods

Patients. Between September 2004 and June 2006 a total of 92 patients with breast cancer surgically treated at our Department (Department of Obstetrics and Gynecology, Jessenius Medical Faculty, Comenius University, Martin, Slovakia) were enrolled in this study. All patients underwent quadrantectomy or modified radical mastectomy with axillary dissection and diagnosis of the disease was confirmed by histological examinations. All participants were of Slavic origin (Caucasian race). Only patients in clinical stage I-II with no history of any other malignant or serious systemic disease were included in the study. Women with a history of hormone replacement therapy and oral contraceptives were excluded. All women in this trial filled out a structured questionnaire including patient history and risk factors in relation to breast cancer development. The questionnaire concentrated on demographic and lifestyle characteristics, reproductive and menstrual history, physical activities, estrogen exposure, anthropometrical variables and family history. However, in this study we used only data related to smoking habits and anthropometrical variables expressed by body mass index (BMI kg/m^2). For final analyses of disease risk only active smokers (≥5 cigarettes daily for more than one year) were used for cancer risk expression. Occasional (e.g., 1-2 cigarettes weekly) or passive smoking in non-smokers was not considered as sufficient risk of toxicity exposure. The ethics committee of the Jessenius Faculty of Medicine approved the study protocol, and biologic samples were obtained after written informed consent from the patient.

Patients, who fulfilled inclusion criteria, filled out an entrance questionnaire and subsequently underwent a breast operation and DNA-testing using PCR-assay. Based on the results from genotyping they were divided into three subgroups reflecting the character of alteration in the MDR-1 gene (homozygote for T, heterozygote or homozygote for the C allele). Obtained data were used in statistical analysis.

Genotyping. Genomic DNA was extracted from lymphocytes in peripheral venous blood (10 ml) by proteinase K digestion and standard phenol/chloroform extraction. The MDR-1^{C3435T} genotypes were determined by the use of PCR-RLFP assay as described previously (30). PCR was performed in a 20-μl-reaction volume containing 100 ng of genomic DNA. PCR reaction mix containing reaction buffer, $MgCl_2$, dNTPs, primers and *Taq* polymerase (Promega, Madison, WI, USA). Each PCR amplification was after initial denaturation for 2 min at 94˚C carried out in 30 cycles of denaturation for 90 sec at 94˚C, annealing at 54˚C for 60 sec and extension for 90 sec at 72˚C with terminal extension at 72˚C for 7 min. We used a forward primer 5'-TTG ATG GCA AAG AAA TAA AGC-3' and reverse primer 5'-CTT ACA TTA GGC AGT GAC TCG-3' for amplification of the 206-bp PCR products, which were digested by restriction enzyme *Mbo*I (New England Biolabs, USA) for 16 h at 37˚C evaluated on 3% agarose gel. Restriction fragments were visualized after ethidium bromide staining with the use of an ultraviolet transilluminator (Fig. 1). Electrophoretic pattern showed two bands (148 and 59 bp) for homozygous C allele and three bands (207, 148 and 59 bp) for heterozygous C/T genotype and one band (207 bp) for homozygous T allele.

Histological data of the specimens. All bioptic material was embedded in paraffin-coated blocks, and managed with standard histological methods. Tumor size, grade and histological classification were examined according to the criteria of the WHO (37).

Statistics. A χ^2 test for trend was used to evaluate the association between clinical variables and MDR-1 genotypes, as well as for determination of the deviation from the Hardy-Weinberg equilibrium. Odds ratio (OR) and 95% confidence intervals (95% CIs), obtained from unconditional logistic

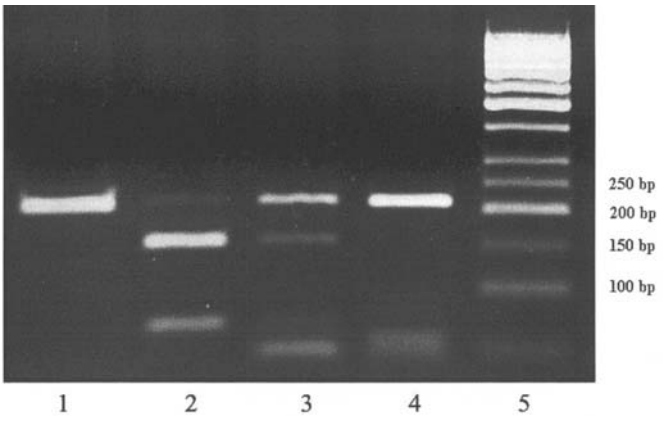

Figure 1. Agarose gel electrophoresis of MDR-1C3435T PCR products digested with restriction enzymes. Lane 1, amplified PCR product of exon 26 in the MDR-1 gene (207); lane 2, homozygote for C allele (148 and 59 bp); lane 3, heterozygote for C and T allele (207, 148 and 59 bp); lane 4, homozygote for T allele (207); and lane 5, marker of molecular size standards (50 bp).

regression, were used to set the association between the MDR-1 polymorphism and cancer risk. The statistical level of significance was set to two-sided $p \leq 0.05$. All statistical calculations were performed using the statistical package MedCalc 8.1.1 (MedCalc Inc.®, Belgium).

Results

The study population consisted of 92 women with histologically confirmed breast carcinoma with an age range from 34 to 82 years (mean 57.8 years) at the time of diagnosis. The majority of women were postmenopausal (77.17%) with middle degree of education (61.96%). Invasive ductal carcinoma of higher tumor grade and size over 2 cm was the dominant type of disease in patients. The detailed clinical and histopathologic characteristics of the patients are summarized in Table I.

The frequencies of the MDR-1^{C3435T} allelotype and genotype analysis revealed a high incidence, up to 75.0% of polymorph alteration in the MDR-1 gene. The frequencies of homozygous T/T, heterozygous C/T and homozygous C/C genotype forms were 25.0, 50.0 and 25.0%, respectively. The genotype distributions among patients were in Hardy-Weinberg equilibrium (p=NS). No significant difference was observed in the genotype distribution of MDR-1^{C3435T} polymorphism in the patients when stratified by age (<50 versus ≥50 years), Table II.

Subsequently, when we analyzed obtained data concerning cancer risk expression in association with smoking and body mass index, we found an increased risk of breast cancer in smoking carriers with C allele in genotype. The risk for smoking heterozygous and homozygous carriers was increased by an OR of 1.28 (95% CI 0.23-7.17), and 1.58 (95% CI 0.28-10.44), respectively. If expressed for both genotypes (C/T + C/C) the risk was increased by an OR=1.36, however this increase was not significant. Despite this, a significant association was revealed in patients with high body mass index, where women with BMI >30 kg/m^2 and C allele in genotype had a higher risk of disease compared to breast cancer patients with lower amounts of body fat tissue (p=0.0439). The risk was highest for the homozygous carriers of C allele with BMI >30 kg/m^2 compared to patients with BMI 25.1-30 or ≤25 kg/m^2 (OR=3.65, 95% CI 0.94-14.20 or OR=2.50, 95% CI 0.55-11.41), respectively. Frequency and statistical data of these analyses are given in Table III and genotype distribution in patients with different BMI in Table IV.

Table I. The clinical and histopathologic characteristics of patients.

Variable	Value (%)
Age at diagnosis (years)	
Mean	57.8
Range, ±SD	34-82, ±10.8
Education	
Elementary	29 (31.52)
Middle	57 (61.96)
University	6 (6.52)
Menstrual status	
Premenopause	12 (13.05)
Perimenopause	9 (9.78)
Postmenopause	71 (77.17)
Tumor size (cm)	
≤1	25 (27.17)
1-2	31 (33.70)
>2	36 (39.13)
Tumor type	
Invasive ductal	74 (80.44)
Invasive lobular	13 (14.13)
Other	5 (5.43)
Tumor grade	
G1	24 (26.09)
G2	30 (32.61)
G3	38 (41.30)
Lymph node status	
Positive	49 (53.26)
Negative	43 (46.74)

Discussion

The MDR-1 gene is highly polymorphic with differing frequencies of allelic variants in different ethnic groups, e.g., African, Caucasian or Asian populations (38,39). To date, at least 40 SNPs have been found in this gene (33). Most of the detected polymorphisms are intronic or silent. These naturally occurring genetic variants of MDR-1 can not only affect interindividual variability in the pharmacokinetics and pharmacodynamics of many drugs and account for differences in the oral availability of various P-gp substrates or multidrug

Table II. MDR-1 allele and genotype frequency distribution in patients.

	Total		<50 (years)		≥50 (years)	
	N	(%)	N	(%)	N	(%)
Genotype						
T/T	23	25.0	8	36.4	15	21.4
C/T	46	50.0	7	31.8	39	55.7
C/C	23	25.0	7	31.8	16	22.9
C/T + C/C	69	75.0	14	63.6	55	78.6
Allele						
T	92	50.0	23	52.3	66	48.2
C	92	50.0	21	47.7	71	51.8

Hardy-Weinberg equilibrium, P-value = NS.

Table III. Association between MDR-1 genotype and breast cancer risk expressed for body weight and smoking.

Genotype/ factor	Smokers[a]	Non-smokers	OR	95% CI
T/T	2	21	1.0	(ref.)
C/T	5	41	1.28	(0.23-7.17)
C/C	3	20	1.58	(0.28-10.44)
C/T + C/C	8	62	1.36	(0.27-6.89)
	>30 BMI[b]	25.1-30 BMI		
T/T	4	19	1.0	(ref.)
C/T	10	36	1.32	(0.37-4.77)
C/C	10	13	3.65	(0.94-14.20)
C/T + C/C	20	49	1.94	(0.59-6.42)
	>30 BMI[a]	≤25 BMI		
T/T	4	8	1.0	(ref.)
C/T	10	16	1.25	(0.30-5.26)
C/C	10	8	2.50	(0.55-11.41)
C/T + C/C	20	24	1.67	(0.44-6.36)

[a]p=NS, [b]p=0.0439, P-value two sided, from χ^2 test for trend. BMI, kg/m^2.

Table IV. MDR-1 genotype distribution in patients with different BMI.

	BMI (kg/m^2)		
Genotype	≤25 (n/%)	25.1-30 (n/%)	>30 (n/%)
T/T	8/34.8	11/47.8	4/17.4
C/T	16/34.8	20/43.5	10/21.7
C/C	8/34.8	5/21.7	10/43.5

resistance in chemotherapy of some types of cancer, but also modify the cell detoxification activity.

In recent years the relationship between the MDR-1^{C3435T} polymorphism and susceptibility or progression to some diseases, such as gastrointestinal tract infections (40), Parkinson's disease (41), childhood acute lymphoblastic leukemia (30), renal epithelial tumors (29), colon cancer (31) or adult glioma (42) has been studied. These studies more or less revealed a positive association with this genetic alteration, however the results varied in level of statistical significance and role of genotype consequence. Whereas the T/T form was in majority associated with higher risk of disease origin, the carriers of C/C genotype were supposed to have a worse prognosis (30,31,34).

In this study we tested the hypothesis that the functional polymorphism C3435T in the MDR-1 gene may alter the detoxification protective function of P-glycoprotein in breast tissue cells exposed to environmental carcinogens in tobacco smoke and its possible association with higher local fat compound. Primarily, we found a high, up to 75.0% occurrence of polymorphic 3435>C variants in the MDR-1 gene (homozygous C/C form in 25.0% and heterozygous C/T form in 50.0%). To the best of our knowledge, there is only one study to compare the results in breast cancer patients, published by Kafka *et al* (34) who after genotyping 68 patients revealed the C/T genotype in 57% and C/C form in 21% of the subjects. From both studies it is obvious that approximately 2/3 of all patients with breast cancer are carriers of aberrant C allele in genotype, however some difference may be present in genotype forms. These findings represent the first reports on high incidence of MDR-1^{C3435T} polymorphism in breast cancer patients.

The revealed high prevalence of C3435T polymorphism in our subjects and the roles of MDR-1 in detoxification systems of human tissues have led us to suggest the possible link between altered ability of P-glycoprotein to facilitate the efflux of carcinogens included in tobacco smoke and this polymorphism under the possible impact of increased breast tissue adiposity. We found a non-significant increased risk of disease in smoking carriers of C allele in genotype, where the risk for heterozygous and homozygous carriers was 1.28 (95% CI 0.23-7.17), and 1.58 (95% CI 0.28-10.44), respectively. Our data are in concordance with studies presenting smoking as a risk for breast cancer in women (8,9), however due to the different methods used in the studies it is difficult to tell whether the risk is independent of MDR-1 polymorphism or augmented by it. As our study primarily deals with MDR-1^{C3435T} alteration, smoking and breast cancer, we can conclude only that non-smokers with breast carcinoma and C allele in genotype have a lower risk of disease compared to smoking women with breast carcinoma. The altered, probably lower, P-glycoprotein activity in carriers of C allele results in higher intracellular concentrations of mutagens, which can damage the DNA, and eventually transform the cell to a cancer cell. Especially, some studies (43,44) showed a possible role of MDR-1 in regulating cell differentiation, proliferation,

immunology, survival and apoptosis, where cells are protected against caspase-dependent apoptosis induced by cytotoxic drugs, Fas ligation, tumor necrosis factor, and ultraviolet radiation. In this way the polymorphism of the MDR-1 gene might be a risk factor for any disease, especially in cases where MDR-1 etiology is demonstrated.

Additionally, the risk of carcinogen exposure is increased when lipophilic substances of tobacco smoke accumulate in adipose tissue. Therefore, obesity (including increase of breast adipose tissue) should serve as an augmentation factor for breast cancer. Considering the low number of smoking patients in our study we did not provide multifactorial analysis (smoking + high BMI) for risk assessment in carriers of C allele in genotype to avoid doubtful results (unpublished observations). Therefore, only the influence of BMI in unifactorial analysis was evaluated. Subsequently, we have found a significant association between high BMI (>30 kg/m^2) and increased cancer risk in patients with C allele in genotype and this susceptibility was the highest for homozygous carriers. Our results are in line with results from other studies (12,13) of breast cancer risk and high BMI, where obese women (BMI >30 kg/m^2) had a 31% excess risk compared to women with BMI ≤25 kg/m^2 (12). Since in our study the risk for breast cancer patients bearing MDR-1 polymorphism in their genotype is demonstated, it is not proper to compare these situations.

Several hypotheses have been proposed to explain the association of obesity and breast cancer. One places adipocytes and their autocrine, paracrine, and endocrine functions at center stage. This hypothesis proposes that obesity should be considered as an endocrine tumor (45) and adipocyte as an endocrine cell (46). In such a hypothesis the following pathways can be included in obesity conditioned breast cancer among women: a) increased local estrogen biosynthesis in breast via aromatase activity increasing with age and BMI (47) and modulated by tumor necrosis factor α or interleukin 6 (48); b) insulin resistance and increased insulin concentrations (49) together with overexpression of IGF-I (50); c) decreased concentrations of sex-hormone-binding globulin (SHBG) due to hyperinsulinemia that leads to an increase in the bioavaliable fraction of circulating estradiol (16,51); d) adipokines secreted by adipocytes (52); e) leptin resistance in which the membrane leptin receptor and the JAK-STAT pathway are blocked with results of increased intracellular concentrations of lipid metabolites, increased non-oxidative metabolism by adipocytes, and stimulation of the cell estrogen cycle [under these conditions the oncogenic property is activated via the shared mitogen-activated protein kinase (MAPK), mitogen/extracellular signal-regulated kinase (MEK) and extracellular signal-regulated kinase (ERK) cellular pathways (17,53)]; and f) a novel complement-related hormone secreted exclusively by adipocytes, adiponectin (16).

In addition, oxidative catabolism of estrogen, mediated by various cytochrome P450 enzymes, generates reactive free radicals that can cause oxidative damage. The same enzymes of estrogenic metabolic pathways catalyze biological activation of several environmental (xenobiotic) chemicals. These mutagens may exert their pathological effects through generation of reactive free radicals. The DNA-reactive metabolites of different xenobiotic compounds have been detected in breast tissue and many of the xenobiotic metabolizing enzymes are expressed in both normal and cancerous breast tissues. These enzymes play a significant role in the activation/detoxification of xenobiotic, carcinogen and endogenous compounds including estrogens (11). Also the lipotoxicity itself after overnutrition is considered risky. Slawik and Vidal-Puig have found that excess lipid intake in the cell may exceed the limited triacylglycerol buffer capacity and under these conditions alternative non-oxidative pathways with production of toxic reactive lipid species occur (54).

In conclusion, this study investigated the allelic frequency distribution of C3435T polymorphism in breast cancer patients and for the first time also the risk of disease in association with active smoking and higher body mass index. We found that patients with breast carcinoma had a high incidence of MDR-1^{C3435T} polymorphism, where C allele carriers have increased risk of disease when active smoking or high body mass index was present. These observations may indicate that this polymorphism as a modulator of health is associated with an increased risk of breast cancer by virtue of lower protection against specific P-glycoprotein-dependent carcinogens. Furthermore, our results should serve as a basis for larger studies focused on cancer prevention and together with results from Kafka *et al* (34) can be used for individualized chemotherapy in locally advanced breast cancer.

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