Published in final edited form as:

Breast Cancer Res Treat. 2010 June ; 121(2): 453–460. doi:10.1007/s10549-009-0587-7.

Alcohol consumption and breast tumor mitochondrial DNA mutations

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Abstract

Mitochondrial DNA (mtDNA) mutations are frequent in breast tumors, but the etiology of these mutations is unknown. We hypothesized that these mutations are associated with exposures that affect oxidative stress such as alcohol metabolism. Using archived tumor blocks from incident breast cancer cases in a case control study, the Western New York Exposures and Breast Cancer (WEB) study, analysis of mtDNA mutations was conducted on 128 breast cancer cases selected based on extremes of alcohol intake. Temporal temperature gradient gel electrophoresis (TTGE) was used to screen the entire mtDNA genome and sequencing was completed for all TTGE positive samples. Case–case comparisons were completed using unconditional logistic regression to determine the relative prevalence of the mutations by exposures including alcohol consumption, *manganese superoxide dismutase* (*MnSOD*) genotype, nutrient intake related to oxidative stress and established breast cancer risk factors. Somatic mtDNA mutations were found in 60 of the 128 tumors examined. There were no differences in the prevalence of mtDNA mutations by alcohol consumption, *MnSOD* genotype or dietary intake. The likelihood of mtDNA mutations was reduced among those with a positive family history for breast cancer (OR = 0.33, CI = 0.12–0.92), among postmenopausal women who used hormone replacement therapy (OR = 0.46, CI = 0.19–1.08, $P = 0.08$) and was increased for ER negative tumors (OR = 2.05, CI = 0.95–4.43, $P = 0.07$). Consistent with previous studies, we found that mtDNA mutations are a frequent occurrence in breast tumors. An understanding of the etiology of mtDNA mutations may provide insight into breast carcinogenesis.

Keywords

Breast cancer epidemiology; Mitochondrial DNA mutations; Oxidative stress; Alcohol consumption

Introduction

Mitochondria are organelles that perform essential functions related to cellular metabolism and apoptosis. They are an important source of superoxide anions (O_2^-) and are limited in DNA repair capacity. Furthermore, mitochondrial DNA (mtDNA) lack histones and other

structural features making them very susceptible to damage by these anions [1]. mtDNA mutations have been identified in human malignancies including breast tumors. Tan et al. observed somatic mutations in the mtDNA of 74% of a series of breast tumors [2]. Others have identified mtDNA mutations in fine needle aspirates of primary breast tumors [3, 4]. The etiology of mtDNA mutations may prove to be important in understanding breast carcinogenesis.

Oxidative stress, an excess of oxidants over antioxidants, has been shown in animal models and in female breast tissue, to contribute to DNA damage and tumor formation [5, 6]. Several recent studies have demonstrated that higher levels of oxidative DNA damage and/or lipid peroxidation products are found in human breast cancer cases than in controls [7–10]. Ethanol metabolism generates reactive oxygen species as well as lipid peroxidation products including malondialdehyde [11].

One of the first line cellular defense mechanisms against oxidative stress within the mitochondria is the antioxidant enzyme, manganese superoxide dismutase (MnSOD). There is a polymorphism in the mitochondrial targeting sequence of the *MnSOD* gene (*rs1799725* currently combined into *rs4880*) that alters the transport of the enzyme into the inner matrix of the mitochondria [12]. A recent meta-analysis from 13 published case–control studies showed no overall association of this polymorphism with risk for breast cancer [13]. To our knowledge, there have been no prior studies examining *MnSOD* genotype and mtDNA mutations among women with breast cancer.

While mtDNA mutations have been examined to a large extent in the context of rare genetic diseases, there has been limited examination of these mutations in cancer [14]. Examinations of how exposures affect the prevalence of mtDNA mutations in breast tumors may provide insight into their etiology and breast carcinogenesis. Using tumor blocks from women in a population-based case–control study; we examined the likelihood of mtDNA mutations by alcohol intake, by *MnSOD* genotype, by intake of nutrients related to oxidative stress and by established breast cancer risk factors.

Materials and methods

Study population

Data were collected as part of a case–control study of breast cancer focused on alcohol consumption, the Western New York Exposures and Breast Cancer (WEB) Study described in detail elsewhere [15]. Subjects were accrued between 1996 and 2001. Briefly, cases were residents of Erie and Niagara counties (35–79 years old) with incident, primary, histologically confirmed breast cancer ($n = 1170$; 72% participation rate). Controls ($n = 2115$; 63% participation rate) were frequency matched to cases on age and race. Controls under the age of 65 were randomly selected from the Department of Motor Vehicle driver's license lists and women age 65 and over were randomly selected from the Health Care Financing Administration. Both cases and controls were limited to women who had no previous history of cancer other than non-melanoma skin cancer. Data on demographics, past medical history and other study variables were collected via questionnaires by trained interviewers or by self-administered questionnaires.

Diet and alcohol intake

Recall of dietary intake 12–24 months prior to the interview was collected using a self-administered modified version of the Health Habits and History food frequency questionnaire (FFQ) [16]. Nutrient intakes were calculated using the DietSys nutrient analysis software (version 3.7) developed specifically for the FFQ. Dietary intake was adjusted for total energy [17]. Data on alcohol consumption was collected by trained interviewers during in-person computer assisted interviews. Participants were asked about recent alcohol consumption (12–24 months prior to the interview for controls or diagnosis for cases), as well as detailed information regarding alcohol consumption throughout their lifetime (up to 2 years prior to interview or diagnosis). Intensity of alcohol consumption, the number of drinks per drinking day, for these same time periods, was also determined.

Immunohistochemistry and genotyping

Immunohistochemical staining for estrogen receptor (ER) status was performed in sections of paraffin-embedded breast tumor blocks. The Allred score was used to evaluate staining for ER status [18]; all determinations were made by a single pathologist. A fasting blood sample was collected from all participants who agreed to a blood draw (78% of cases and 88% of controls). For some of the participants who did not agree or were unable to provide a blood draw, an oral rinse sample using the method of Lumand LeMarchand [19] was collected (approximately 17% of cases and 8% of controls). DNA extraction from blood or oral cells was done with the DNAQuik™ (BioServe, Beltsville, MD) extraction kit according to the manufacturer's instructions.

Polymerase chain reaction (PCR) for *MnSOD* genotype (*rs1799725* currently combined into *rs4880*) was conducted using primers 5'-ACGTTGGATGCTGTGCTTTCTCGTCTTCAG-3' and 5'-ACGTTGGATGTTCTGCCTGGAGCCCAGATA-3'. The Homogenous Mass Extend reaction was used to prepare the PCR products for matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Quality control procedures were followed for all laboratory assays and included positive and negative controls in all runs and samples analyzed as blind duplicates (20%). The genotype assays were validated for confirming polymorphic Mendelian inheritance patterns in seven human family cell lines, each encompassing at least three generations. All genotyping included cases and controls together in the runs; laboratory personnel were blinded to case–control status. Ninety percent of the cases selected for this study were successfully genotyped for the *MnSOD* polymorphism.

Selection of cases for examination of mtDNA mutations

Examination of mtDNA mutations were completed for 128 pairs of breast tumor and matched normal breast tissue from archived tumor blocks. These pairs were selected from a list of 922 participating cases from the parent case control study who had tumor samples and were classified as being at either extreme of alcohol intake. Lifetime nondrinkers ($n = 67$) were randomly selected from among women who reported less than 12 drinks in any 1 year throughout their lifetime. Lifetime drinkers ($n = 61$) were selected randomly from among women with the highest alcohol consumption; those whose alcohol intakes were in the top

20% of reports for between 2 and 10 years prior to diagnosis of breast cancer and in the top 30% of the reports of lifetime consumption.

mtDNA mutation screening

Temporal temperature gradient gel electrophoresis (TTGE) was used to screen the entire mtDNA. Comprehensive mutation analysis of the entire mitochondrial genome by TTGE has been described previously [2, 20]. A total of 32 pairs of overlapping primers were used to PCR amplify the entire 16.6 kb mitochondrial genome [20]. PCR products were denatured at 95°C for 30 s and slowly cooled to 45°C for a period of 45 min at a rate of 1.1°C/min. TTGE was performed on a Bio-Rad D-Code mutation detection apparatus, using two back-to-back 20 cm × 20 cm × 1 mm 4.5–6% polyacrylamide (acrylamide:Bis = 37.5:1) gels in 1.25× TAE buffer containing 6 mol/l urea. The electrophoresis was carried out at 145 V for 4–5 h at a constant temperature increment of 1–2°C/h. The temperature range (50–60°C) was determined by computer simulation (MacMelt software, Bio-Rad Laboratories).

Any DNA fragments showing differences in banding patterns between the control (normal tissue) and samples (tumor tissue) were sequenced to identify the exact mutations and variation. DNA sequencing was performed by using DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences) and a MegaBACE 1000 DNA analysis system (Amersham Life Sciences) automated sequencer.

The results of DNA sequence analysis were compared with the published Human Mitochondrial DNA Revised Cambridge Reference Sequence (Mitomap Database: <http://www.mitomap.org>) using Vector NTI 9.0 software [21]. Those not recorded in the database were categorized as novel mtDNA mutations, and those appearing in the database were reported polymorphisms or mutations.

Statistical analysis

Statistical analyses were done using Statistical Package for the Social Sciences (Version 15, SPSS Inc. Headquarters, Chicago, IL). Breast cancer risk factors, dietary intake related to antioxidant status, alcohol intake, and *MnSOD* genotype were compared for cases with and without mutations. The alcohol variables were categorized into three levels: lifetime nondrinkers, low drinkers, and high drinkers. Low and high drinker categories were based on the median intake in the distribution of lifetime drinkers chosen for this study. Nutrient variables were categorized into high and low variables based on the median intake of the total population ($n = 128$). Because of the small sample size, the Fisher Exact Test was used to examine categorical variables and the Mann–Whitney U was used to examine continuous variables.

Hardy–Weinberg equilibrium (HWE) of genotype frequency was tested with the Pearson goodness-of-fit statistic among cases and controls and the statistic conformed to Hardy–Weinberg proportions [22].

Case–case comparisons of mtDNA positive and negative cases to determine the relative prevalence of the mutations by exposure were conducted using unconditional logistic regression. Unadjusted and adjusted odds ratios (OR) were calculated and 95% confidence

intervals (CI) estimated. We examined established risk factors for breast cancer, estrogen receptor status of the tumor, lifetime alcohol consumption, *MnSOD* genotype, and dietary factors including intake of fruits and vegetables, citrus, vitamin C, carotenoids, and iron.

Results

Of the 128 tumors examined, 60 (46.9%) exhibited somatic mtDNA mutations in comparison to the control germline DNA. There were a total of 119 mutations identified. The range of mutation number found per case was 1–5 mutations. Twenty-nine tumors (48.3%) had a single mutation and 31 tumors (51.7%) had multiple mutations. Most of the mutations (78%) were in the hypervariable D-loop region. The majority of the total somatic mutations were point mutations (89.9%). Nine mutations that were not previously identified were discovered.

Descriptive information relating to breast cancer risk factors, *MnSOD* genotype and drinker status for the population is shown in Table 1. Breast cancer risk factors that were different or had borderline statistically significant differences between cases with and without mutations included family history of breast cancer, ER status of the tumor and use of hormone replacement therapy (HRT) among postmenopausal women. The distribution of *MnSOD* genotype or drinker status was not different between cases with mutations and those without mutations. Other characteristics that did not differ between the two groups included, age, education and race. Additionally, among drinkers, there was no difference for categories of drinking for those with mutations or without mtDNA mutations. There were also no differences in intake of nutrients related to oxidative stress (Table 2).

Results for case–case comparisons are shown in Table 3. As the results were similar for pre- and postmenopausal women (data not shown), analyses shown were not stratified on menopausal status. There was decreased likelihood of having a mtDNA mutation for those with a family history of breast cancer compared to those without a family history (OR = 0.33, CI = 0.12–0.92). Compared to cases with ER positive tumors, there was an indication of an increased likelihood of having a mutation if the tumor was ER negative (OR = 2.05, 95% CI = 0.95–4.43), although the difference did not reach formal statistical significance. When selecting for only mutations in the D-Loop region, there was no difference in mutation status between those who were ER positive or ER negative. Among postmenopausal women who used HRT, the likelihood of having a mutation was decreased (OR = 0.46, 95% CI = 0.19–1.08), although not statistically significantly.

The *MnSOD* variant genotype was not associated with the likelihood of a mtDNA mutation although there was a suggestion of decreased likelihood of mutation with the *TT* genotype for higher alcohol consumption when genotype was stratified by drinking status.

Contrary to our hypothesis, there were no differences in likelihood of mutation by drinker status or categories for alcohol consumption (data not shown for categories of alcohol consumption). Additionally, there were no differences in likelihood of mutation by intake of dietary factors related to oxidative stress (data not shown).

Discussion

For more than 70 years, mitochondrial defects including mtDNA mutations have been suspected to be a part of carcinogenesis [1]. Somatic mutations in the mtDNA have been found in breast tumors and in fine needle aspirates of primary breast tumors [2–4]. mtDNA is highly susceptible to damage by oxidative stress products [1] and increased levels of oxidative damage have been demonstrated in human breast cancer cases [7–10].

We had hypothesized that an increase in oxidative damage that is produced from alcohol metabolites and the possible resulting production of mtDNA mutations may explain, at least in part, the association between alcohol and breast cancer risk. The metabolism of ethanol generates cytosolic nicotinamide adenine dinucleotide (NADH) which is then oxidized indirectly by mitochondrial electron transport depending on hydrogen shuttling mechanisms that involve metabolite carriers in the inner membrane [23, 24]. Chronic ethanol treatment has been shown to affect mitochondrial oxidative phosphorylation by suppressing synthesis of the main respiratory complexes (Complex I, III, IV, and V) of the electron transport system encoded on mtDNA [23, 25]. The resulting inefficient oxidative phosphorylation promotes mitochondrial formation of the superoxide anion [23]. A cellular defense mechanism against oxidative stress within the mitochondria involves the antioxidant enzyme *MnSOD*. There is a polymorphism in the mitochondrial targeting sequence of the *MnSOD* gene (*rs1799725* currently combined into *rs4880*) which may alter the level of oxidative stress within the mitochondrion and potentially have an effect on the formation of mtDNA mutations.

The literature is limited in investigations of mtDNA mutations and breast cancer and there has not been an examination of how exposures including established breast cancer risk factors and exposures related to oxidative stress such as alcohol consumption, affect the prevalence of mtDNA mutations in breast tumors. We hypothesized that there would be more mtDNA mutations in breast tumors from women with increased oxidative stress and examined alcohol consumption as a promoter of oxidative stress, the *MnSOD* genotype and investigated if differences in established breast cancer risk factors would result in differences in the number of mtDNA mutations.

We found somatic mtDNA mutations in 60 tumors (47%). Previous studies with sample sizes of approximately 15–20, have reported mutation frequencies of 61–93% [2–4]. Similar to Zhu et al. [4], we found mtDNA mutations both inside and outside of the (D) loop region; however, most of the mutations were in the (D) loop region similar to findings from Tan and Parrella [2, 3]. The (D) loop region of mtDNA is a non-coding region which acts as a promoter for both heavy and light strands of mtDNA and contains essential transcription and replication elements [26]. Since mtDNA is a circle double strand, the light strands refer to the inner loop which encodes approximately seven genes. The heavy strands refer to the outer loop where most of the mtDNA genes are encoded [1]. This (D) loop is considered a “hot” spot for mtDNA mutations [1].

There were no differences in prevalence of mutations by alcohol consumption, dietary antioxidant intake or by *MnSOD* genotype. We did find an indication that among drinkers,

MnSOD genotype may be associated with risk, with lower risks for those with the *TT* genotype; however confidence intervals were wide and included one. The *MnSOD* genotype has not been examined with mtDNA mutations previously. Other epidemiologic studies examining the risk of breast cancer have shown decreased risk of breast cancer associated with the *TT* genotype [27–30]. In one study, there was a report of an increased risk of breast cancer with having a *C* allele among those with higher alcohol consumption [31]. In a recent meta-analysis, however, based on 13 published case control studies, *MnSOD* genotype was not associated with breast cancer risk [13].

Exposures that showed either a statistically significant or borderline change in the likelihood of mtDNA mutations were family history of breast cancer, use of hormone replacement therapy among postmenopausal women and ER negative tumors. For family history and HRT, there was a decreased likelihood of mtDNA mutations among cases. It may be that for women with a family history or postmenopausal women who use HRT, that their cancers were related to alternate causal pathways that do not include mtDNA mutations. Similar to the findings of Tseng et al., we found that ER negative tumors may have a higher likelihood of mtDNA mutations. Unlike Tseng et al., we did not find a higher prevalence of (D) loop mutations for ER negative tumors [14].

While this was the largest study examining mtDNA mutations in breast tumors to date and the only one examining the association of these mutations with exposures, we were still hindered by small sample size. There is a possibility of measurement error, particularly for measurement of lifetime alcohol. Nondifferential error in measurement of intake would likely bias results to the null. Despite this potential for error, our methods for measuring alcohol consumption were more comprehensive and detailed than previous assessments of alcohol intake [32].

As has been shown previously, we found that mtDNA mutations are frequent in breast tumors [2–4]. Our results suggest among women with a family history of breast cancer and among postmenopausal women who used HRT, that mtDNA mutations may not be a common causal pathway. For women with ER negative tumors however, it would appear that mtDNA mutations are involved in tumorigenesis. Ours is the second report showing increased likelihood of mtDNA mutations among women with ER negative tumors. Further examinations of exposures and mtDNA mutations in breast tumors may provide information concerning the etiology of these mutations and ultimately, provide insight into breast carcinogenesis.

Acknowledgments

This work was funded in part by the Department of Defense grants number DAMD 170310446 and 170010417 and the National Institutes of Health grants number RO1CA092040, P50AA09802 and R25CA114101.

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Table 1

Characteristics of cases by mtDNA mutation status: the WEB study

Characteristics	Subjects		<i>P</i> value ^a
	No mutation <i>n</i> = 68 (53.1%) Median	Mutation <i>n</i> = 60 (46.9%) Median	
Age (years)	61.50	59	0.48
Education (years)	13	13	0.35
Age at first birth (years)	22	23	0.26
Age at menarche (years)	12	13	0.48
Age at menopause (years) (postmenopausal only)	<i>n</i> = 52 50	<i>n</i> = 43 50	0.98
Parity	2	2	0.73
BMI (postmenopausal only)	<i>n</i> = 52 26.56	<i>n</i> = 43 29.53	0.19
			<i>P</i> value ^b
	No mutation <i>n</i> = 68 (53.1%) Frequency (%)	Mutation <i>n</i> = 60 (46.9%) Frequency (%)	
Menopausal status			
Premenopausal	16 (23.5)	17 (28.3)	0.55
Postmenopausal	52 (76.5)	43 (71.7)	
Race			
White	60 (88.2)	53 (88.3)	1.00
Non White	8 (11.8)	7 (11.7)	
Ever use HRT (postmenopausal)	<i>n</i> = 52	<i>n</i> = 43	0.06
Yes	33 (63.5)	18 (42.9)	
No	19 (36.5)	25 (58.1)	
Have/Had Benign breast disease	<i>n</i> = 65	<i>n</i> = 58	0.85
Yes	23 (35.4)	22 (37.9)	
No	42 (64.6)	36 (62.1)	
Family history of breast cancer	<i>n</i> = 62	<i>n</i> = 53	0.04
Yes	17 (27.4)	6 (11.3)	
No	45 (72.6)	47 (88.7)	
ER status	<i>n</i> = 66	<i>n</i> = 58	0.06
Negative	17 (25.8)	25 (43.1)	
Positive	49 (74.2)	33 (56.9)	
MnSod polymorphism	<i>n</i> = 61	<i>n</i> = 54	0.48
CC	10 (16.4)	12 (22.2)	
CT/TT	51 (83.6)	42 (77.8)	
Drinker status			
Nondrinkers	36 (52.9)	31 (51.7)	0.51
Drinkers	32 (47.1)	29 (48.3)	

^aMann–Whitney *U* was used to examine differences for continuous variables

^b Fisher Exact Analysis was used to examine differences for categorical variables

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Table 2

Alcohol and dietary intake of cases by mtDNA mutation status: the WEB study

Characteristics	Subjects		<i>P</i> value *
	No mutation <i>N</i> = 32 (47.1%) Median	Mutation <i>N</i> = 29 (48.3%) Median	
Alcohol consumption among drinkers			
Lifetime total oz. of Alcohol	8494.20	7674.42	0.52
Total oz. of Alcohol, 2–10 ^a	2637.48	2382.02	0.25
Total oz. of Alcohol, 10–20 ^a	3156.51	2552.16	0.13
Lifetime # of usual drinks per usual drinking day	2.40	2.45	0.80
# of usual drinks per usual drinking day, 2–10a	2.00	2.00	0.61
# of usual drinks per usual drinking day, 10–20a	2.00	2.00	0.78
Dietary intake	No mutation <i>n</i> = 68 (53.1%) Median	Mutation <i>n</i> = 60 (46.9%) Median	<i>P</i> value *
Daily fruit and vegetables (servings)	3.60	3.50	0.80
Daily citrus (servings)	0.50	0.70	0.39
Daily iron (mg) ^b	10.14	10.40	0.95
Daily vitamin C (mg) ^b	99.95	115.23	0.29
Daily carotenoids (mcg) ^b	2688.65	2750.94	0.75

* Mann–Whitney *U*^a Years prior to diagnosis^b Energy adjusted using the residual method [17]

Table 3

Case–case comparisons of mtDNA mutations: the WEB study

Characteristic	Number of cases yes/no mutation	OR (95% CI)*	P value
Family history			
No (ref)	47/45	1.00	
Yes	6/17	0.33 (0.12–0.92)	0.03
ER status			
ER (+) (ref)	33/49	1.00	
ER (–)	25/17	2.05 (0.95–4.43)	0.07
ER status among premenopausal			
ER (+) (ref)	6/10	1.00	
ER (–)	10/5	3.52 (0.78–15.96)	0.10
ER status among postmenopausal			
ER (+) (ref)	27/39	1.00	
ER (–)	15/12	1.70 (0.67–4.28)	0.26
Post menopausal women only			
Ever use HRT never (ref)	24/19	1.00	
Ever	18/33	0.46 (0.19–1.08)	0.08
<i>MnSOD</i> polymorphism			
<i>CC</i> (ref)	12/10	1.00	
<i>CT</i>	24/22	0.97 (0.35–2.71)	0.95
<i>TT</i>	18/29	0.54 (0.19–1.50)	0.24
<i>MnSOD</i> polymorphism among nondrinkers			
<i>CC</i> (ref)	5/6	1.00	
<i>CT</i>	14/12	1.32 (0.32–5.53)	0.70
<i>TT</i>	10/13	0.93 (0.21–4.08)	0.93
<i>MnSOD</i> polymorphism among drinkers			
<i>CC</i> (ref)	7/4	1.00	
<i>CT</i>	10/10	0.68 (0.14–3.22)	0.62
<i>TT</i>	8/16	0.30 (0.06–1.41)	0.13
Drinker status			
Nondrinkers (ref)	31/36	1.00	
Drinkers	29/32	1.07 (0.53–2.16)	0.86

* Adjusted for age and education