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Genetic variation in adiponectin, leptin and leptin receptors with reference to risk of breast cancer in Northeast obese women in India

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ABSTRACT

Adiponectin and leptin are adipose tissue derived hormones, adiponectin properties are anti-inflammatory, anti-atherogenic, anti-diabetic, and plays an important role in the development and progression of various cancers, especially obesity-related cancers. Studies show the SNPs in adiponectin is cause of SNPs in insulin resistance, type2 diabetes and breast cancer. Leptin is an obesity mediator; leptin and its receptor are overexpressed in breast cancer, especially in high grade tumors. The aim of this study is to estimate the allele polymorphisms of adiponectin, leptin, leptin receptor in a population of northeast India and investigate the role of those polymorphisms that increase risk of breast cancer. The study type is a case-control. 205 patients with positive breast cancer as cases group and 205 people as controls group were determined genotyping by using PCR-RFLP. Comparing cases and controls, there is positive association between adiponectin SNPs (276G/T, 45T/G) and leptin SNP and Breast cancer (P = 0.04, OR = 1.64, 95% Cl = 1.00 – 2.62), (P = 0.05, OR = 1.77, 95% CI = 0.96 – 3.17) & (P = 0.04, OR = 1.44, 95% Cl = 0.98 – 2.13). But there is no evidence for a strong association between leptin receptor SNPs (109A/G, 223A/G) and breast cancer (P = 0.87, OR = 1.05, 95% Cl = 0.43 – 2.91), (P = 0.31, OR = 0.56, 95% Cl = 0.17 – 1.83). The results of this study indicate that the polymorphisms in the adiponectin and leptin may be a risk factor for breast cancer.

KEY WORDS: POLYMORPHISM, ADIPONECTIN, LEPTIN, LEPTIN RECEPTOR, OBESITY, BREAST CANCER

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INTRODUCTION

Obesity is a risk factor for decreased survival in breast cancer patients (Reeves *et al.*, 2007; Schapira *et al.,* 1991). Adipocytokines are hormones produced by adipose tissue, and its circulating levels are correlated with obesity, they show an effect on cell proliferation, migration and invasion, they are found to be expressed in breast tissues (Fredriksson *et al.*, 2006; Rose *et al.*, 2004; Schaffler *et al.*, 2007; Vona-Davis *et al.*, 2007). Adiponectin has potent effects for insulin sensitizing, increases nitric oxide levels and decreases the rate of apoptosis and oxidative stress (Cheng *et al.*, 2007; Kobayashi *et al.*, 2004; Tschritter *et al.*, 2003). In vitro studies, shows adiponectin is associated with growth inhibition and apoptosis induction in cancer cell lines (Byeon *et al.*, 2010). In vivo studies in mice showed the developments of more intestinal tumors are associated with the disruptions in serum adiponectin (Mutoh *et al.*, 2011). Many studies have also shown that the low levels of adiponectin are associated with increased body fat resulting in obesity (Maeda *et al.*, 1996). Insulin is an important biomolecules, which plays an important role in the progression of metastatic breast cancer (Hollmann *et al.*, 1997). In general, WHR (Waist to Hip Ratio) is one of the markers for insulin resistance and hyperinsulinemia, and an elevated ratio of WHR represents a higher abdominal fat distribution, which in turn is associated with both incidence and mortality of breast cancer (Borugian *et al.*, 2003; Considine *et al.*, 1996). Thus, both obesity and insulin involve in the development of breast cancer (Chen *et al.*, 2006; Kang *et al.*, 2007; Kelesidis *et al.*, 2006; Korner *et al.*, 2007; Mantzoros *et al.*, 2004; Miyoshi *et al.*, 2003; Maeda *et al.*, 1996; Yang *et al.*, 2009).

Leptin is the peptide hormone product of the obesity gene, it is mainly secreted from adipose tissue, and the secondary sources are placenta, stomach, and skeletal muscles (Zhang *et al.*, 1994; Garaulet *et al.*, 2000; Goodwin *et al.*, 2005). It is involved in the regulation of body composition and weight, thus said to be as an important mediator of obesity (Schwartz *et al.*, 1999). A single transmembrane protein, leptin receptor (superfamily of cytokines) exists in many tissues, and mediates the physiologic action of leptin. The interaction between leptin and leptin receptor plays an important role in angiogenesis, body weight homeostasis, hematopoiesis, immune processes and reproduction (Israel and Chua, 2010; Suzukawa, 2011).

Although, leptins have been inconclusive with breast cancer in some studies, both leptin and its receptors are shown to be over expressed in breast cancer, especially in high grade tumors (Han *et al.*, 2008; Mantzoros *et al.*, 1999; Petridou *et al.*, 2000; Stattin *et al.*, 2004; Ishikawa *et al.*, 2004; Surmacz, 2007). The expression of leptin and its receptor is found in 70–80% of breast cancer cases (Bozcuk *et al.*, 2004). In animal and human cell lines, leptin receptors are reported to be associated with increased tumor cell proliferation, and promotion of angiogenesis in benign and malignant epithelial breast cells, (Dieudonne, 2002; Hu, 2002; Li *et al.*, 2004; Lim, 2005; Qian, 2011). The various SNPs (Single Nucleotide Polymorphisms) have been identified in the adiponectin, leptin and leptin receptor genes. The association studies between the SNPs in the adiponectin, leptin, leptin receptor and their possible association with breast cancer have shown little consistency (Cleveland *et al.*, 2010; Kaklamani *et al.*, 2008; Okobia *et al.*, 2008; Snoussi *et al.*, 2006; Teras *et al.*, 2009; Woo *et al.*, 2009).

Therefore, in order to study the association of SNPs in adiponectin and leptin receptors with respect to breast cancer, two SNPs in adiponectin gene (rs2241766 (45T/G) and rs1501299 (276G/T)), one SNP in leptin gene (rs7799039 (2548G/A) and two in leptin receptor SNPs (rs1137100 (109A/G) and rs1137101 (223A/G) were taken in to consideration.

MATERIAL AND METHODS

SAMPLE COLLECTION:

In this study, 5 ml blood samples were collected in EDTA vials from 205 northeast women patient with breast cancer diagnosed at the Northeast Cancer Hospital, Assam, India and 205 healthy Northeast women from Health Screening Center of Northeast Cancer Hospital, Assam, India between the period February 2013 to December 2014. All cases had never received surgery, chemotherapy or radiation therapy, and the study has been approved by Gauhati University ethical committee (GUEC-04/2015) and the written informed consent to participate (i.e., case and controls) in the study was obtained from all subjects.

DNA ISOLATION AND PCR-RFLP

Phenol-chloroform method was used to isolate genomic DNA (Deoxyribonucleic acid) from the collected blood, and the quantity of DNA was determined by spectrophotometer. Amplification of the gene polymorphism regions was carried out by PCR (Polymerase Chain Reaction) and the RFLP (Restriction Fragment Length Polymorphism) was used to identify the Single nucleotide polymorphism regions of adiponectin (45T/G and 276G/T), leptin (2548G/A), and leptin receptor (109A/G and 223A/G). The PCR primer sequences (Invitrogen, India) used for adiponectin SNPs (45T/G and 276G/T); leptin SNP (2548G/A), and leptin receptor SNPs (109A/G

Table 1: Characteristics of Patients and Control		
Percent %	**Case No %**	**Control No %**
Age (years) means±sd	47.59± 18.73	45.07 ± 17.55
BMI (Body Mass Index) (Kg/m2)	27.9 ± 6	26.0 ±3
Age of Diagnosis		
21-40	54 (26.7%)	82 (40%)
41-60	123 (60%)	100 (48.9%)
Above 61	28 (13.3%)	23 (11.1%)
Smoking women	36 (17.8%)	32 (15.6%)
women with previous smoking	9 (4.4%)	18 (8.9%)
Non smoking women	160 (77.8%)	155 (75.5%)
Waist Circumference(WC) (cm)	102±15	102.5±9
Systolic blood pressure (mmHg)	131±20	130±12
Diastolic blood pressure (mmHg)	80.6±6	81±8
Total Cholesterol(mmol/L)	6±1	6.6±1.1
HDL (mmol/L)	1.2±0.5	1.2±0.07
LDL (mmol/L)	3.9±2	4.4±0.6
White women	37 (18%)	42 (20.5%)
Black women	98 (47.8%)	86 (42%)
Other	70 (34.2%)	77 (37.5%)
Drinking women	53 (25.85%)	23 (11.2%)
Women with previous drinking	24 (11.7%)	15 (7.3%)
Non drinking women	128 (62.45%)	167 (81.5%)

and 223A/G) are shown in Table 2. PCR was standardized and carried out for about 34 cycles. The PCR products were separated on 1.5% agarose gel, visualized with ethidium bromide. The different restriction enzymes (genome diagnostics private limited, India) used to study the respective gene polymorphisms by RFLP method was mentioned in Table 2.

The restriction enzyme digestion was carried out for 37^{C} (overnight; 16 hours) for each gene separately, and the products were visualized on 2% agarose gel stained with ethidium bromide.

STATISTICAL ANALYSIS

The allele frequency differences between case and control groups were obtained using used X^2 (Chi- square) test. For each SNP the OR (Odds Ratio) with CI (Confidence Interval) 95% were calculated and the identification of

Table 2: PCR Primer Sequences and the Restriction Enzyme Digestion Used to Study the Different Gene Polymorphisms			
Gene	**Polymorphisms**	**PCR (F = Forward , R= Reverse)**	**Restriction enzymes used for RFLP**
Adiponectin	rs2241766	F: 5'- GAAGTAGACTCTGCTGAGATGG - 3'	SmaI
		R:5'-TATCAGTGTAGGAGGTCTGTGATG-3'	
	rs1501299	F : 5'- ATGCAGCAAAGCCAAAGTCT - 3'	BsmI
		R : 5'- CCTGGTGAGAAGGGTGAGAA - 3'	
Leptin	rs7799039	F : 5'- TGTGTGTTCCCTGGTTCAAG - 3'	HhaI
		R : 5'- TTCCTGCAACATCTCAGCAC - 3'	
Leptin Receptor	rs1137100	F : 5'- CACTGTTGCTTTCGGAGTGA - 3'	BsgI
		R:5'-TCATAGCCATAAGACATCTATTTCA- 3'	
	rs1137101	F : 5'- CAGCCAAACTCAACGACACT -3'	BstEII
		R : 5'- GCCACTCTTAATACCCCCAGT - 3'	

Table 3: Distribution of Genotype Frequencies of adiponectin 45T/G, 276G/T Polymorphisms in Breast Cancer Patients

Genotype	Case %	Control %	95% CI	OR	P-Value
Adiponectin gene : rs2241766 (45T/G)					
TT	132 (64.5 %)	146 (71.2 %)	0.31 – 1.03	0.56	0.05
TG	64 (31.1 %)	50 (24.4 %)	0.96 – 3.17	1.77	0.05
GG	9 (4.4 %)	9 (4.4 %)	0.16 – 9.02	1.26	0.81
Allele					
T	328 (80 %)	342 (83.4 %)		1	
			0.99 -3.14	1.75	0.05
G	82 (20 %)	68 (16.6 %)			
Adiponectin gene : rs1501299 (276G/T)					
GG	110 (53.4 %)	141 (64.5 %)	0.36 – 0.92	0.57	0.02
GT	91 (44.5 %)	59 (33.4 %)	1.00 – 2.62	1.64	0.04
TT	4 (2.1 %)	5 (2.1 %)	0.42 – 34.02	3.75	0.23
Allele					
G	311 (75.85 %)	341 (83.1 %)		1	
T	99 (24.15%)	69 (16.9 %)	0.96 – 2.67	1.67	0.04

P≤0.05 were shown in bold; OR- Odds ratio; CI – confidence interval

genotypes risk was performed using logistic regression analysis. Pearson X^2 statistics with threshold of P<0.05 for each SNP was used to test the Hardy-Weinberg equilibrium. SPSS (Statistical Package for Social Sciences) 16 software was used to perform the statistical analysis and the possibility less than 0.05, was considered significant. Power and Sample Size Calculations was used to estimate the Power (http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize)..

RESULTS

Distribution of genotype frequencies of polymorphisms of adiponectin gene (45T/G, 276G/T), leptin gene (2548G/A), and leptin receptor gene (109A/G, 223A/G) in breast cancer patients were in Hardy-Weinberg equilibrium (Table 3). Among these five polymorphisms tested, significant association with breast cancer was observed in adiponectin gene rs1501299 (276G/T) and leptin gene rs7799039 (2548G/A) polymorphisms among northeast women patients. However, it was noticed that the genotype frequencies of polymorphism of adiponectin gene (276G/T) and leptin gene (2548G/A) were found to be P=0.005 and P=0.019, and showed significantly different in the breast cancer patients and normal controls respectively. The frequency of the risk allele "T" in adiponectin gene (276G/T) was significantly higher in patients with breast cancer (24.1%) in comparison with the normal controls (16.8%) and the allelic odds ratio was found to be 1.57 (95% CI: 1.12-2.22, P=0.009). The frequency of the risk allele "A" in leptin gene polymorphism (2548G/A) was significantly higher in patients with breast cancer (42.0%) in comparison with the normal controls (32.4%) and the allelic odds ratio was found to be 1.51 (95% CI: 1.13-2.00, P=0.005).

The logistic regression analysis was performed to compare the frequencies of genotypes of cases and controls under an additive model, and the findings are presented in Table 3. Similar to the allelic association with breast cancer, the genotypes of the 276G/T (OR = 1.64, 95% CI: 1.00 – 2.62, P = 0.04) and 2548G/A polymorphisms (OR = 1.44, 95% CI: 0.98 – 2.13, P = 0.04) showed a significant susceptibility to breast cancer respectively. However, the association with breast cancer was not observed in the other polymorphisms namely, adiponectin gene (45T/G) and leptin receptor gene (109A/G, 223A/G).

DISCUSSION

According to WHO (World Health organization), obesity is one of the 21st greatest public challenges, and by 2015, it was estimated that more than 700 million adults will be obese. Obesity is found to be one of the major risk factor for breast cancer (Holmes and Willett, 2004); apart from this it is also associated with the increased risk for the number of cancers such as colon, prostate, liver. The cell signaling proteins or cytokines produced by adipose tissue are adipocytokines (i.e., adiponectin and leptin), which plays an important role in obesity

Table 4: Distribution of Genotype Frequencies of leptin 2548G/A and leptin receptor 109A/G, 223A/G Polymorphisms in Breast Cancer Patients

Genotype	Case %	Control %	95% CI	OR	P-Value
Leptin gene : rs7799039 (2548G/A)					
GG	69 (33.3 %)	95 (46.7 %)		1	
GA	100(48.9 %)	87 (42.2 %)	0.98–2.13	1.44	0.04
AA	36 (17.8 %)	23 (11.1 %)	1.45–6.95	3.15	0.001
Allele					
G	238(58.04%)	277(67.5 %)		1	
A	172(41.96 %)	133 (32.5 %)	1.17–2.05	1.56	0.04
Leptin receptor : rs1137100 (109A/G)					
AA	9 (4.4 %)	13 (6.7 %)		1	
AG	59 (28.9 %)	64 (31.1 %)	0.43–2.91	1.05	0.87
GG	137 (66.7 %)	128 (62.2 %)	0.48–2.21	1.02	0.95
Allele					
A	77 (18.78 %)	90 (21.95 %)		1	
G	333(81.22 %)	320(78.05%)	0.39–2.94	1.03	0.87
Leptin receptor : rs1137101 (223A/G)					
AA	5 (2.2 %)	9 (4.4 %)		1	
AG	54 (26.7 %)	37 (17.8 %)	0.17–1.83	0.56	0.31
GG	146 (71.1 %)	159 (77.8 %)	0.72–1.69	1.1	0.66
Allele					
A	64 (15.6 %)	55 (13.42 %)		1	
G	346 (84.4 %)	355(86.58 %)	0.85–1.3	1.05	0.67

P≤0.05 were shown in bold; OR- Odds ratio; CI – confidence interval

related cancers, including breast cancer. These molecules are also known to be one of the causes for the association between obesity and breast cancer risks.

In the present study, we report that the genetic variation in adiponectin (45T/G and 276G/T) and leptin gene (2548G/A) showed significant association with breast cancer among northeast women patients. In this study we demonstrated that the genetic variation in the adiponectin and leptin may be genetic markers for breast cancer, and these genetic markers may be prognostic factors to predict breast cancer recurrence and death in northeast women population. According to the result of this study, polymorphisms in adiponectin (i.e., 45T/G and 276G/T) and leptin (i.e., 2548G/A) have shown increase risk for breast cancer.

In 2011, Al Khaldi *et al.* found that adiponectin gene 45T/G is associated with breast cancer, and also mentioned that the two polymorphisms (45T/G and 276G/T) of adiponectin gene might be the predisposing factors in various cancers in the Kuwait population (Al Khaldi *et al.*, 2011). In the same year, Nyante *et al.* found that 45T/G and 276G/T polymorphisms were associated with breast cancer in whites and African Americans (Nyante *et al.*, 2011). Apart, from breast cancer related studies, it is known that the adiponectin gene polymorphism (45T/G) showed a significant association for polycystic ovary syndrome risk (Ranjzad *et al.*, 2012). Whereas, this polymorphism in type 2 diabetes did not show significant association in Yi and Han people of China (Wang *et al.*, 2011).

In the present study, leptin gene polymorphism (2548G/A) shows significant association with breast cancer in northeast obese women in India. However, previous studies indicated the polymorphism in leptin did not show any association with breast cancer risk and also leptin serum levels showed no relationship with respect to breast cancer in old women (Mantzoros *et al.*, 1999; Petridou *et al.*, 2000).

In contrast, this polymorphism resulted in high leptin secretion and also associated with high risk of prostate cancer (Hoffstedt *et al.*, 2002; Le Stunff *et al.*, 2000; Kote-Jerai *et al.*, 2003; Ribeiro *et al.*, 2004). Although, this study did not measure serum leptin levels it is known that elevated serum leptin levels showed increased risk of developing breast cancer in comparison with normal levels (Han *et al.*, 2005).

The present study did not show any significant association of leptin receptor polymorphism (223A/G and

109A/G), which is in agreement with the unexplanation of the association of this polymorphism in breast cancer (Woo *et al.*, 2006). On the other hand, Quinton *et al.* indicated that leptin receptor SNPs 223A/G and 109A/G were associated with lower circulating leptin level (Quinton *et al.*, 2001). Yiannakouris *et al.* have studied that carriers of the leptin receptor polymorphisms associated significantly with higher leptin level than persons with non carriers (Yiannakouris *et al.*, 2001).

In previously, eight small breast cancer studies conducted in different countries evaluated seven candidate leptin receptor SNPs with inconsistent results. Han and Snoussi found a roughly fold statistically significant risk of breast cancer for 223A/G (Han *et al.*, 2008; Snoussi *et al.*, 2006). In others study, Woo and Gallicchio found no association between leptin receptor SNPs (223A/G, 109A/G) and breast cancer (Woo *et al.*, 2006; Gallicchio *et al.*, 2007). Liu, *et al.*reported a suggestion of an association between 109A/G polymorphism and breast tumor size but among premenopausal women only (Liu *et al.*, 2007). In addition, Petridou *et al.* have found no relationship between leptin serum levels and breast cancer in old women (Petridou *et al.*, 2000). A recent study has demonstrated that subjects with elevated serum leptin levels displayed increased risk of developing breast cancer than those with the normal levels (Han *et al.*, 2005). Thus, association between hormone levels and the respective gene polymorphism would give better insight into the etiology of the breast cancer risk and may help to identify the women who are at risk.

CONCLUSION

In this study we investigated the association of two adiponectin gene SNPs (45T/G and 276G/T), leptin gene SNP (2548G/A), and two leptin receptor SNPs (109A/G, 223A/G) and their possible association with breast cancer in a Northeast women population. Our results indicates polymorphisms in adiponectin gene (276G/T) and leptin gene (2548G/A) have shown increased risk of breast cancer, and might be the genetic markers for breast cancer. The addition of remaining SNPs in adiponectin, leptin, leptin receptor genes along with hormonal level measurement could be useful as genetic markers for breast cancer. To our knowledge, our study provides information on the adiponectin SNPs (45T/G, 276G/T), leptin SNP2548G/A, leptin receptor SNPs (109A/G, 223A/G) and the risk of breast cancer in Northeast women population.

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