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Molecular insights into the association of obesity with breast cancer risk: relevance to xenobiotic metabolism and CpG island methylation of tumor suppressor genes

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Abstract Obesity, genetic polymorphisms of xenobiotic metabolic pathway, hypermethylation of tumor suppressor genes, and hypomethylation of proapoptotic genes are known to be independent risk factors for breast cancer. The objective of this study is to evaluate the combined effect of these environmental, genetic, and epigenetic risk factors on the susceptibility to breast cancer. PCR–RFLP and multiplex PCR were used for the genetic analysis of six variants of xenobiotic metabolic pathway. Methylation-specific PCR was used for the epigenetic analysis of four genetic loci. Multifactor dimensionality reduction analysis revealed a significant interaction between the body mass index (BMI) and catechol-*O*-methyl transferase H108L variant alone or in combination with cytochrome P450 (CYP) 1A1m1 variant. Women with "Luminal A" breast cancer phenotype had higher BMI compared to other phenotypes and healthy controls. There was no association between the BMI and tumor grade. The post-menopausal obese women exhibited lower glutathione levels. BMI showed a positive association with the methylation of extracellular superoxide dismutase ($r = 0.21$, $p < 0.05$), Ras-association (RalGDS/AF-6) domain family member 1 (RASSF1A) ($r = 0.31$, $p < 0.001$), and breast cancer type 1 susceptibility protein ($r = 0.19$, $p < 0.05$); and inverse association with methylation of BNIP3 ($r = -0.48$, $p < 0.0001$). To conclude based on these results, obesity increases the breast cancer susceptibility by two possible mechanisms: (i) by interacting with xenobiotic genetic polymorphisms in inducing increased oxidative DNA damage and (ii) by altering the methylome of several tumor suppressor genes.

Keywords Obesity · Cytochrome P450 1A1 · Catechol-*O*-methyl transferase · Ras-association (RalGDS/AF-6) domain family member 1 · Breast cancer type 1 susceptibility protein · BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 · Extracellular superoxide dismutase · CpG island methylation

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Introduction

The etiology of breast cancer is complex and involves genetic, epigenetic, and environmental components [1, 2]. Obesity is a well-documented risk factor for breast cancer [3, 4], which is partly attributed to increased peripheral synthesis of estrogens in adipose tissues [5]. The "Phase I" xenobiotic metabolic pathway converts these estrogens into catechol estrogens by the action of cytochrome P450

(CYP) enzymes. Catechol estrogens are converted to semiquinones and quinones, which have the ability to produce mutagenic lesions in DNA through the adduct formation. The "Phase II" xenobiotic metabolic pathway detoxifies catechol estrogens by (i) *O*-methylation to form methoxy estrogens in the presence of catechol-*O*-methyl transferase (COMT); (ii) conjugation with glutathione in the presence of glutathione-*S*-transferases (GSTs). In a recent study, we showed the association of six genetic variants of this pathway i.e., CYP1A1 m1 (rs4646903), CYP1A1 m2 (rs1048943), CYP1A1 m4 (rs1799814), COMT H108L (rs4680), GSTT1 null, GSTM1 null with breast cancer risk [6]. Obesity was shown to induce expression of CYP2E1 thus contributing to increased ROS generation, autophagy, endoplasmic reticulum stress, and inhibited migration in breast cancer cells [7].

A recent study showed a positive association of body mass index (BMI) with LINE-1 methylation [8]. A regulatory subunit of methionine adenosyltransferase (MAT) i.e., MAT2beta was shown to be down regulated in subcutaneous adipose tissue and skeletal muscle of obese pigs compared to lean pigs, suggesting a link between the obesity and impaired synthesis of *S*-adenosylmethionine (SAM) [9]. Based on these evidences, we hypothesized that such impairment in the synthesis of SAM might affect the methylome of certain tumor suppressors thus inflating the breast cancer risk. In our recent studies, we have demonstrated the association of methylation and expression of extracellular superoxide dismutase (Ec-SOD), Ras-association (RalGDS/AF-6) domain family member 1 (RASSF1), breast cancer type 1 susceptibility protein (BRCA1) and BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) with breast cancer susceptibility and disease progression [6, 10, 11]. The Ec-SOD is an important antioxidant defense enzyme involved in the scavenging of superoxide anion. The RASSF1 is a key regulator of apoptosis, growth, and microtubule dynamics during mitotic progression, and its hypermethylation was reported to be associated with different types of cancers [12, 13]. The BRCA1 plays a pivotal role in DNA repair process by forming the BRCA1-associated genome surveillance complex (BASC) by assembling with the other tumor suppressors, DNA damage sensors, and signal transducers [14]. The BNIP3 is a pro-apoptotic protein induced by hypoxia inducible factor 1 (HIF-1) that plays an important role in hypoxia-induced cell death [15].

In the current study, we examined the interaction of obesity with six genetic variants of xenobiotic metabolic pathway in inducing oxidative DNA damage and breast cancer risk. Further, we studied whether obesity affects the methylation of Ec-SOD, RASSF1, BRCA1, and BNIP3 thus inflating breast cancer risk.

Materials and methods

Enrollment of subjects

Breast cancer cases ($n = 341$) were recruited for the study during the period of June 2009 to June 2012 from the Medical and Surgical Oncology Unit, Nizam's Institute of Medical Sciences, Hyderabad, India. The breast cancer diagnosis was based on mammography and histopathological examination. As control subjects, healthy women volunteers ($n = 252$) were enrolled. The study protocol was approved by the Institutional Ethical Committee of Nizam's Institute of Medical Sciences, Hyderabad (EC/NIMS/767/2007, dated 05.09.2008). Informed consent was obtained from all the subjects prior to enrollment. Demographic characteristics of patients were recorded during the enrollment. A standardized and validated food frequency questionnaire-based dietary assessment of folate, vitamin B_2, vitamin B_6, and vitamin B_{12} was done as described earlier [4].

Immunohistochemistry and fluorescent in situ hybridization (FISH)

Immunohistochemistry for ER, PR, HER2, and Ki67 was performed on the serial sections with the standard streptavidin–biotin complex method with 3,3′-diaminobenzidine as the chromogen. The immunohistochemical studies were performed as per the procedure described earlier [16]. For HER2, FISH assay was performed using probes to locus-specific identifier (LSI), HER2/Neu, and centromere 17 using the PathVysion HER2 DNA probe kit (Abbott Molecular, Abbott Park, IL, USA) as per the manufacturer's instructions. Based on the immunohistochemistry and FISH, the breast tumors were classified into six molecular subtypes i.e., luminal A (ER: ++, PR: ++, HER2/Neu: −, Ki67<14 %), luminal B (ER: +, PR: +, HER2/Neu: −, Ki67>14 %), luminal A-HH (ER: ++, PR: ++, HER2/Neu: +, Ki67<14 %), luminal B-HH (ER: +, PR: +, HER2/Neu: +, Ki67>14 %), HER-enriched (ER: −, PR: −, HER2/Neu: +), and Basal-like (ER: −, PR: −, HER: −).

The tumor grading was done according to the Scarff–Bloom–Richardson (SBR) scale and the lymph node involvement was assessed by histopathological examination. Briefly, the grading was done by combining tubule formation (>75 %: 1, 10–75 %: 2, and <10 %: 3), nuclear pleomorphism (small regular uniform cells: 1, moderate nuclear size and variation: 2, and marked nuclear variation: 3), and mitoses/10 hpf (0–9: 1, 10–19: 2, and _20: 3). Tumors grading was done based on the cumulative histological grade [low grade (I): 3–5, intermediate grade (II): 6–7, and high grade (III): 8–9].

Biochemical analysis

Estradiol was estimated by the commercially available competitive ELISA-based procedure described by the manufacturer (DRG Estradiol ELISA kit, EIA-2693). Plasma 8-oxo-2′-deoxyguanosine (8-oxo2dG), a marker of DNA damage, was determined by competitive ELISA kit following the manufacturer's instructions (Northwest Life Sciences, USA). Total plasma homocysteine and glutathione levels were determined by reverse phase HPLC [17].

Genetic analysis

Whole blood samples were collected from all the subjects in EDTA vacutainers. Genomic DNA was isolated using standard phenol–chloroform extraction method.

Analysis of CYP1A1 polymorphisms

The CYP1A1 polymorphisms including CYP1A1 m1 (T3801C), CYP1A1 m2 (A2455G), and CYP1A1m4 (C2453A) were determined using PCR–RFLP approach. The primers used for the analysis of CYP1A1 m1 were forward 5′-CAGTGAAGAGGTGTAGCCGCT-3′ and reverse 5′-TAGGAGTCTTGTCTCATGCCT-3′. For the analysis of CYP1A1m2 and CYP1A1m4 polymorphisms, same set of primers was used: forward 5′-CTGTCT CCCTCTGGTTACAGGAAG-3′ and reverse 5′-TTCCA CCCGTTGCAGCAGGATAGCC-3′. The PCR reaction volume contained 100 ng genomic DNA, 1× PCR buffer with 1.5 mM $MgCl_2$ (Qiagen, Santa Clarita, CA. USA), 0.2 mM each of dNTP, 10 pM of each primer, and 1.0 U of Bangalore GeneiTaq DNA polymerase. The PCR reaction was carried out by an initial denaturation at 95 °C for 10 min, followed by 35 cycles each consisting of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s. The final extension of PCR was done at 72 °C for 7 min. The CYP1A1m1 polymorphism was detected by digesting the 340 bp PCR product with the restriction enzyme, MspI. The presence of polymorphism results into 200 bp and 140 bp products following the digestion, while the uncut 340 bp product indicates the absence of polymorphism. The CYP1A1m2 polymorphism was detected by digesting the 204 bp PCR product with BsrDI restriction enzyme. The lack of polymorphism results into 149 and 55 bp products after digestion, while the undigested 204 bp product suggests the absence of polymorphism. The restriction enzyme BsaI was used to distinguish the CYP1A1m4 polymorphic allele. The PCR product (204 bp) with the wild-type allele generates 139 and 65 bp products after digestion, whereas the presence of mutant allele renders the 204 bp undigested.

Analysis of COMT H108L polymorphism

PCR amplification of the exon 4 (237 bp) of the COMT gene was performed using the following primers: forward 5′-TACTGTGGCTACTCAGCTGTGC-3′ and reverse 5′-GTGAACGTGGTGTGAACACC-3′. The PCR reaction volume contained 100 ng of genomic DNA, primers (each 10 pM), 1× reaction buffer, dNTPs (each 200 μM) and 1 unit of Taq polymerase (Promega). PCR amplification was carried for 30–40 cycles with denaturation at 93 °C for 45 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. An initial 3 min denaturation step at 94 °C and a final 4 min extension at 72 °C were used. The PCR products were digested with the restriction enzyme Nla III (New England Biolabs) for 3 h at 37 °C. The amplicons were resolved by agarose (3 %) gel electrophoresis. The presence of H-allele generates a unique 114 bp fragment, while the occurrence of L-allele resulted in the digestion of the 114 bp fragment into products of 96 and 18 bp, respectively.

Glutathione-*S*-transferase polymorphisms

Deletion status of GSTM1 and GSTT1 was determined by a multiplex PCR method using the following specific primers: forward 5′-GAA CTC CCT GAA AAG CTA AAG C-3′ and reverse 5′-GTT GGG CTC AAA TAT ACG GTG G-3′ for GSTM1 and forward 5′-TTC CTT ACT GGT CCT CAC ATC TC-3′ and reverse 5′-TCA CCG GAT CAT GGC CAG CA-3′ for GSTT1. As an internal control, exon 7 of the CYP1A1 gene was co-amplified using the primers forward 5′-GAA CTG CCA GGC CAG CA-3′ and reverse 5′-CAG CTG CAT TTG GAA GTG CTC-3′. Agarose gel electrophoresis (1 %) resolved the amplified DNA fragments of 480, 312, and 215 bp for GSTT1, CYP1A1, and GSTM1, respectively.

Quality control for genotyping

The cases and matched controls were analyzed in the same set of PCR. For all the genetic analyses, each PCR set was accompanied by a negative control without genomic DNA in order to check the contamination of the components. For the RFLP analysis, a positive control was included in each set, which ensures complete digestion. Genotypes in few specimens were randomly rechecked to rule out the genotyping errors, and 100 % concordance was observed.

Bisulfite treatment and epigenetic studies

Breast tumor specimens ($n = 120$) obtained following surgery were used for genomic DNA isolation and bisulfite conversion. For epigenetic studies, genomic DNA was denatured for 10 min in 2 M NaOH at 37 °C before the addition of 30 μl of 10 mM hydroquinone (SIGMA) and 520 μl of 3 M

Table 1 Demographic characteristics based on BMI

Variable	Underweight ($n = 23$)	Optimal weight ($n = 137$)	Overweight ($n = 107$)	Obese ($n = 74$)	p_{trend}
Age of onset (year)	47.2 ± 16.4	51.3 ± 12.6	50.3 ± 12.5	54.3 ± 14.0	0.09
Age of menarche (year)	14.0 ± 1.6	13.2 ± 1.2	13.0 ± 1.2	13.3 ± 1.3	0.007*
Parity	2.6 ± 2.2	2.6 ± 1.5	2.4 ± 1.1	2.8 ± 1.7	0.51
Pre/post menopause	17/6	77/60	61/46	36/38	0.09
F/H of breast cancer	2/21	10/127	13/94	7/67	0.50

Underweight: BMI <18.5 kg/m^2; Optimal weight: BMI = 18.5–25.0 kg/m^2; Overweight: BMI = 25.1–30.0 kg/m^2; Obese: BMI >30.0 kg/m^2

F/H family history

* Statistically significant

sodium bisulfite (pH 5.0). The mixture was incubated for 16 h at 50 °C. The resultant modified DNA was purified using a Wizard DNA Purification System (Promega, Madison, WI, USA), after which it was again treated with NaOH and precipitated. Finally, the DNA precipitate was resuspended in 20 µl of Millipore water and stored at −20 °C until used. Methylation-specific PCR (MSP) approach was used for CpG island methylation analysis of Ec-SOD, RASSF1, and BRCA1 [6, 10]. Combined bisulfite restriction analysis (COBRA) was used to study BNIP3 methylation [11].

Statistical analysis

The BMI data of all the subjects were categorized into four groups: underweight (<18.5 kg/m^2), optimal BMI (18.5–25.0 kg/m^2), over weight (25.1–30.0 kg/m^2), and obese (>30 kg/m^2) based on the International Classification of adult BMI as per World Health Organization (WHO) guidelines [18]. Analysis of variance (ANOVA) was used for ascertaining the variance of categorical variables across different BMI groups. Spearman rank correlation coefficient was used to study correlation between the BMI and promoter DNA methylation of each locus. All the statistical analysis was performed using computational web page www.statpages.org. Multifactor dimensionality reduction (MDR) analysis (Version 3.0.2) was performed using BMI group and six genetic variables as predictors and the presence or absence of breast cancer as Class. Using the median value of 8-oxodG as cut-off point, we segregated the case–control data in two classes: Class "0": 8-oxodG <4.37 ng/ml and Class "1": 8-oxodG ≥4.37 ng/ml. MDR analysis was carried out using dietary folate, BMI and six genetic polymorphisms of xenobiotic metabolic pathway as "predictor variables" and 8-oxodG groups as "class."

Results

The age of onset of breast cancer, parity, menopausal status, and family history of breast cancer showed no statistically significant differences across the different classes of BMI (Table 1). The age of menarche of underweight women was significantly higher than normal weight, overweight, and obese women (Table 1).

The obese women exhibited higher estradiol compared to women with normal weight in both pre- and post-menopausal groups (Table 2). The pre-menopausal women had higher estradiol levels than post-menopausal women with identical BMI. No significant correlation was observed between the plasma homocysteine levels and BMI. Earlier, we have reported the elevated plasma homocysteine levels in breast cancer cases compared to controls [19]. In the current study, an inverse association of total plasma homocysteine with dietary folate ($r = -0.12$, $p = 0.007$) and vitamin B_2 ($r = -0.11$, $p = 0.01$) was observed. On the other hand, vitamin B_6 ($r = -0.06$, $p = 0.18$) and vitamin B_{12} ($r = 0.02$, $p = 0.66$) showed no association with total plasma homocysteine levels. In pre-menopausal group, BMI had no significant effect on total glutathione, while in post-menopausal women, the total glutathione levels were lower in under weight and obese women.

A significant association was found between the high BMI and luminal A breast cancer phenotype compared to other breast cancer phenotypes and healthy controls (Fig. 1), whereas BMI showed no direct association with tumor grade (Fig. 2).

Genomic DNA samples isolated from whole blood were used for genetic analysis. The bivariate interaction plot showed a significant interaction between the BMI and COMT H108L variant ($p < 0.0001$). The COMT HH (OR 3.00, 95 % CI 1.28–7.15, $p = 0.008$) and COMT HL (OR 2.66, 95 % CI 1.15–6.22, $p = 0.02$) genotypes showed increased breast cancer risk in women with BMI >30 kg/m^2. In contrast, the COMT LL-genotype showed a significant breast cancer risk in women with BMI >25 kg/m^2 (OR 3.05, 95 % CI 1.37–6.85, $p = 0.004$). The CYP1A1 m1 variant showed synergistic effect with BMI and COMT H108L in increasing the breast cancer risk (Fig. 3).

As shown in Fig. 4, the MDR analysis showed COMT H108L, dietary folate deficiency, and BMI as the most

Table 2 Biochemical variables based on BMI

Variable	Underweight ($n = 23$)	Optimal weight ($n = 137$)	Overweight ($n = 107$)	Obese ($n = 74$)	p_{trend}
Estradiol (pg/ml)					
Pre-menopausal	137.4 ± 44.9	132.4 ± 19.2	136.0 ± 20.1	148.5 ± 51.4	0.08
Post-menopausal	117.4 ± 12.4	115.2 ± 24.6	112.0 ± 21.4	120.6 ± 30.5	0.12
Total plasma homocysteine (μmol/L)					
Pre-menopausal	16.7 ± 8.7	16.4 ± 8.2	15.7 ± 7.4	15.8 ± 6.1	0.94
Post-menopausal	14.6 ± 6.5	16.5 ± 8.1	15.6 ± 7.5	16.9 ± 7.7	0.53
Plasma glutathione (μmol/L)					
Pre-menopausal	488 ± 235	481 ± 113	483 ± 98	492 ± 124	1.00
Post-menopausal	254 ± 148	503 ± 216	457 ± 139	415 ± 157	0.04*

Underweight: BMI <18.5 kg/m^2; Optimal weight: BMI = 18.5–25.0 kg/m^2; Overweight: BMI = 25.1–30.0 kg/m^2; Obese: BMI >30.0 kg/m^2

F/H family history

* Statistically significant

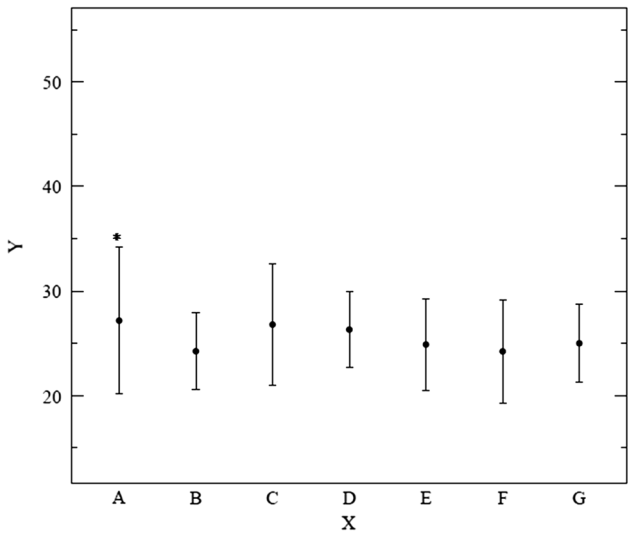


Fig. 1 Analysis of variance to establish the association of BMI with immunohistochemical phenotypes of breast cancer. In *X*-axis, *A* luminal A ($n = 107$); *B* luminal B ($n = 54$); *C* luminal A-HH ($n = 41$); *D* luminal B-HH ($n = 30$); *E* HER-enriched ($n = 40$); *F* basal-like breast cancer ($n = 60$); *G* healthy controls ($n = 252$). *Y*-axis represents mean ± standard deviation of BMI (kg/m^2) of each group. The results of an ANOVA statistical test showed an "*F*" value of 2.45, $p_{trend} = 0.03$. The BMI values of different groups were represented in mean ± SD format. Luminal A: 27.170 ± 7.02 kg/m^2; Luminal B: 24.233 ± 3.65 kg/m^2; Luminal A-HH: 26.786 ± 5.77 kg/m^2; Luminal B-HH: 26.314 ± 3.58 kg/m^2; HER-enriched: 24.879 ± 4.39 kg/m^2; Basal-like: 24.224 ± 4.92 kg/m^2; healthy controls: 24.996 ± 3.76 kg/m^2

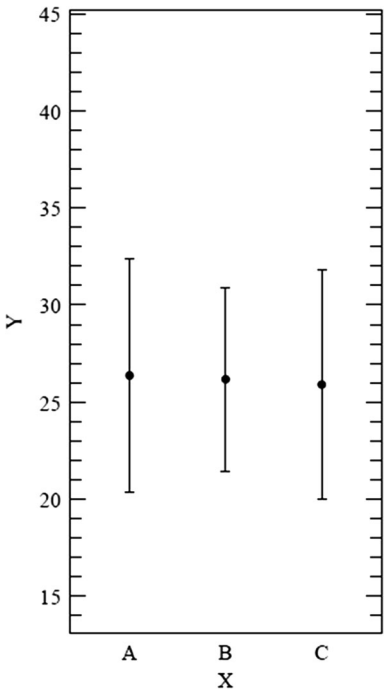


Fig. 2 Analysis of variance to ascertain the association of BMI with the tumor grade. In *X*-axis, *A–C* represent low, intermediate, and high grade tumors. *Y*-axis represents mean ± SD of BMI (kg/m^2) of each group. The results of ANOVA statistical test showed an "*F*" value of 0.03, p_{trend}: 0.97. Low grade ($n = 67$): 26.368 ± 6.01 kg/m^2; Intermediate grade ($n = 134$): 26.173 ± 4.73 kg/m^2; high grade ($n = 140$): 25.897 ± 5.89 kg/m^2

important determinants of 8-oxodG levels. Further, 8-oxodG levels were found to reflect the multiple gene-environmental interactions including the COMT H 108L and BMI, COMT H108L and dietary folate deficiency, as well as BMI and dietary folate deficiency.

Genomic DNA samples that are isolated from breast tumor tissues were used for methylation of Ec-SOD, RASSF1, and BRCA1 genes following bisulfite conversion. The BMI showed positive association with methylation of Ec-SOD ($r = 0.21$, $p < 0.05$), RASSF1 ($r = 0.31$, $p < 0.001$), and BRCA1 ($r = 0.19$, $p < 0.05$). On the other hand, it showed an inverse association with the methylation of BNIP3 ($r = -0.48$, $p < 0.0001$).

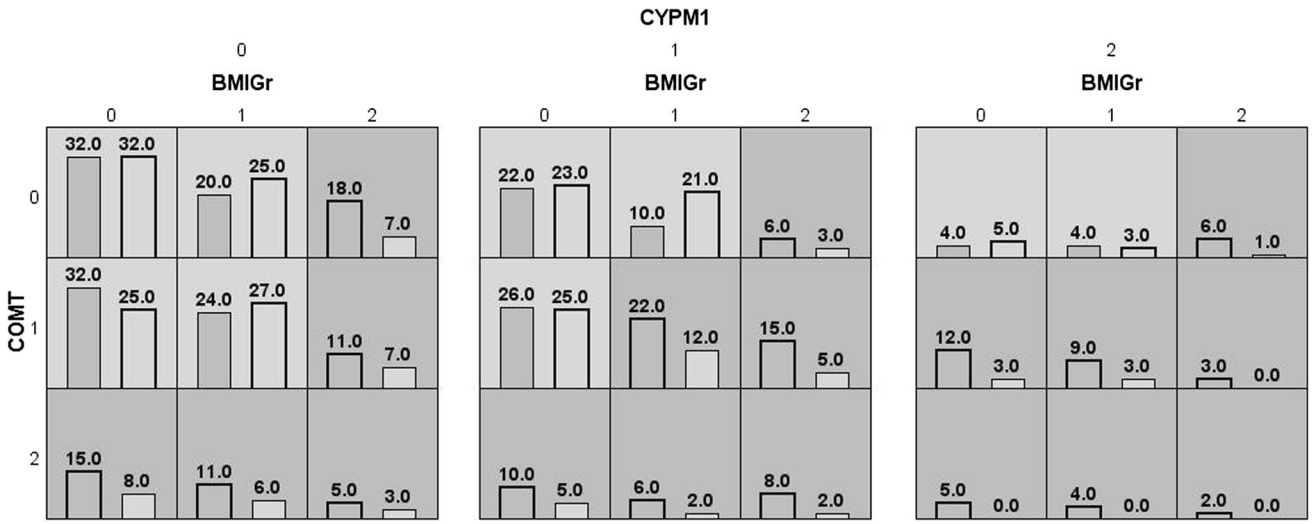


Fig. 3 MDR analysis showing interaction of COMT H108L with obesity. Using CYP1A1 m1, CYP1A1 m2, CYP1A1 m4, COMT H108L, GSTT1 null, GSTT1 null, and BMI group (BM class) as predictor variables and the presence or absence of breast cancer as "Class," MDR analysis was carried out. Potential interactions were observed between BMI groups and COMT genotypes, which were found to modulate breast cancer risk ($p_{\text{interaction}} < 0.0001$). Three way interactions were also observed between CYP1A1 m1, COMT H108L, and BMI In BM class, 0: BMI <25.0 kg/m^2; 1: BMI = 25.1–30.0 kg/m^2; 2: BMI >30 kg/m^2. In COMT, 0, 1 and 2 represent HH, HL and LL-genotypes. IN CYP1A1 m1, 0, 1, and 2 represent wild, heterozygous, and homozygous variant genotypes. *Light* and *dark* backgrounds represent low-risk and high-risk combinations

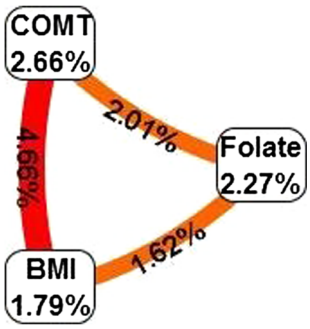


Fig. 4 Fruchterman–Rheingold plot showing interactions between BMI, dietary folate and COMT in modulating oxidative DNA damage Using the median value of 8-oxodG as cut-off point, we segregated the case–control data in two classes: Class "0": 8-oxodG <4.37 ng/ml and Class "1": 8-oxodG ≥4.37 ng/ml. MDR analysis was carried out using dietary folate, BMI and six genetic polymorphisms of xenobiotic metabolic pathway as predictor variables and 8-oxodG groups as class. Three variables showed strong association with DNA damage in the following order of entropy: COMT H108L >dietary folate deficiency >BMI. The interactions between these variables are in the following order: [COMT H 108L × BMI] > [COMT H108L × dietary folate deficiency] > [BMI × dietary folate deficiency]

Discussion

The results of the current study demonstrated that the obesity is associated with the breast cancer risk by interacting with xenobiotic genetic polymorphisms in inducing increased oxidative DNA damage and also by altering the methylome of certain tumor suppressor genes. The specific changes observed in obese women were (i) higher estradiol levels with prolonged exposure time (early menarche and late age of onset), (ii) increased oxidative stress as evidenced by lower glutathione levels and higher 8-oxodG, (iii) interaction of BMI with COMT H108L and CYP1A1 m1 in increasing breast cancer risk by inducing oxidative DNA damage, and (iv) hypermethylation of tumor suppressor genes and hypomethylation of pro-apoptotic gene.

The increased peripheral synthesis of estrogens in obese women coupled with hyper inducibility of CYP1A1m1 might lead to increased catechol estrogen formation [20]. The impaired *O*-methylation of catechol estrogens to methoxy estrogens due to COMT LL-genotype [5, 21] might increase the mutagenicity, conferring the higher risk for breast cancer. Consistent with our findings, COMT inhibition was shown to block 2-methoxy estradiol formation in estradiol-treated MCF-7 cells with subsequent increase in oxidative DNA damage [22]. The association of BMI with oxidative DNA damage as observed in the current study was consistent with a recent study [23]. The association of BMI with luminal A breast cancer phenotype, as observed in the current study, was also demonstrated in two recent studies [24, 25]. We observed no association of BMI with tumor grade. However, in a study, Turkoz et al. [26] showed a borderline positive association of obesity with the tumor grade and lymph node invasion in premenopausal women.

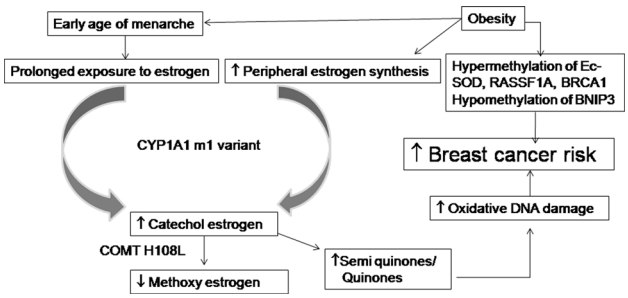
In this study, we observed a positive association of BMI with methylation of Ec-SOD, which is consistent with a

study that showed an inverse association of BMI with the plasma Ec-SOD activity [27]. The positive association of BMI with Ras-association (RalGDS/AF-6) domain family member 1 (RASSF1A) methylation corroborated with the study of Peters et al. [28]. Ghosh et al. [29] observed an inverse correlation between the adipogenesis-induced aromatase expression and BRCA1 expression. Our study was also demonstrated the positive association of BMI with BRCA1 methylation. To the best of our knowledge, for the first time, we reported the association of BMI with BNIP3. The hypomethylation of BNIP3, although not specific to breast cancer alone, coupled with hypermethylation of RASSF1A and BRCA1 might have utility as predictive biomarker for breast cancer. The BNIP3 upregulation was reported to be a useful prognostic marker in invasive duct cell carcinoma and observed to be a poor prognostic marker in duct cell in situ [30]. Severely obese breast cancer patients treated with anthracyclines and taxanes were reported to have a worse prognosis in terms of recurrence, breast cancer mortality, and overall mortality than patients with BMI <25 kg/m^2 [31]. Tao et al. [32] observed a positive association between the waist to hip ratio and DNA promoter methylation of E-cadherin, p16, and RAR-β (2) genes.

The outcome of the pharmacological manipulation of specific CYP450 isoforms in the pathogenesis of breast cancer was evaluated by some studies. Water-soluble extract of *Vernonia amygdalina*, a natural anti-cancer agent, was found to induce expression of microsomal epoxide hydrolase and CYP3A4, but not of CYP1A1 and CYP1A2 in MCF-7 cells [33]. Anti-sense-based inhibition of CYP3A2 was shown to be effective in reducing tamoxifen-DNA adduct formation in rat model [34]. These two studies have provided evidence of possible beneficial effects of pharmacological modulation of CYP enzymes in breast cancers that respond to anti-estrogen therapy.

In the current study, we observed no statistically significant association between the BMI and plasma homocysteine levels. However, an inverse association of plasma homocysteine levels with the dietary folate and vitamin B_2 as observed in the current study was consistent with a recent population-based study from our group [35].

To conclude, the results of the current study demonstrated the association of obesity with luminal A breast cancer phenotype and lack of association of BMI with tumor grade. The obese women exhibited higher estadiol levels than women with optimal weight in pre-menopausal groups. Early age of menarche and late age of onset in obese women support the association of prolonged exposure to estrogen with breast cancer risk. This risk was found to be augmented particularly in the presence of CYP1A1 m1 and COMT H108L polymorphisms via increased oxidative DNA damage. To the best of our knowledge, this is the first report highlighting the impact of obesity on CpG island methylation of Ec-SOD, RASSF1, BRCA1, and BNIP3 (Scheme 1). The current study underscores the importance of healthy life style to lower the breast cancer risk especially in women carrying high-risk alleles of CYP1A1 m1 and COMT H108L. Since the obesity showed the association with hypermethylation of tumor suppressors, it is imperative to investigate whether the obese patients need DNMT inhibitors as adjunct therapy along with standard therapeutic modality.



Scheme 1 The possible mechanism of obesity-mediated breast cancer risk The scheme proposes two possible mechanisms of obesity-mediated breast cancer risk: (i) prolonged exposure to estrogen resulting in elevated catechol estrogen levels due to CYP1A1 m1 and impaired methoxy estrogen levels due to COMT H108L inducing increased oxidative DNA damage (ii) by altering the methylome of Ec-SOD, RASSF1A, BRCA1, and BNIP3

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