Genetic polymorphisms (rs10636 and rs28366003) in metallothionein 2A increase breast cancer risk in Chinese Han population

Di Liu1*, Meng Wang1*, Tian Tian1*, Xi-Jing Wang1, Hua-Feng Kang1, Tian-Bo Jin2, Shu-Qun Zhang1, Hai-Tao Guan1, Peng-Tao Yang1, Kang Liu1, Xing-Han Liu1, Peng Xu1, Yi Zheng1, Zhi-Jun Dai1

1Department of Oncology, Second Affiliated Hospital of Xi’an Jiaotong University, Xi’an 710004, China
2National Engineering Research Center for Miniaturized Detection Systems, School of Life Sciences, Northwest University, Xi’an 710069, China
*Equal contribution

Correspondence to: Zhi-Jun Dai; email: dzj0911@126.com

Keywords: metallothionein 2A; polymorphism; breast cancer

Received: November 14, 2016       Accepted: February 9, 2017       Published: February 22, 2017

ABSTRACT

Genetic polymorphisms of MT2A are frequently observed in many different cancers. We performed this case-control study, including 459 breast cancer (BC) patients and 549 healthy controls from Northwest China, to evaluate the associations between two common MT2A polymorphisms (rs10636 and rs28366003) and BC risk. The MT2A polymorphisms were genotyped via Sequenom MassARRAY. The individuals with the rs28366003 A/G, A/G-G/G genotypes underwent a higher risk of BC (P < 0.0001). And, the minor allele G of rs28366003 was related to an increased BC risk (P < 0.0001). We also found a significantly increased BC risk with rs10636 polymorphism among homozygote and recessive models (P < 0.05). Further subgroup analysis by clinical characteristics of BC patients showed that Scarff, Bloom and Richardson tumor grade (SBR) 1-2 have a higher expression of the minor allele of these two MT2A loci than SBR 3. Our results indicated that the rs10636 and rs28366003 polymorphisms in MT2A increased BC risk in Northwest Chinese Han population.

INTRODUCTION

Breast cancer (BC) is the most frequent cancer among women, expecting to be 252,710 new BC cases and 40,610 death cases among American women in 2017 [1]. And, the incidence of BC increased dramatically in Asian population in recent years [2]. It is widely known that genetic factors contribute to BC susceptibility [3-7]. Recent studies showed the expression of the Metallothionein 2A (MT2A) gene increased in some human neoplasms [8-10]. And, it has been reported that the high expression of MT2A associated with the stage and prognosis of tumors [9, 11, 12]. MT2 belongs to metallothioneins (MT), which have four groups (MT1, MT2, MT3 and MT4 proteins) and are encoded by a family of genes located on chromosome 16q13 [13]. The expression of MT1 and MT2 are the most active isoforms in human cells [14]. And, the researchers found that MT1/MT2 may play an important role in tumors via several crucial mechanisms including modulating p53 zinc-dependent activity, inhibiting NF-κB signaling, and regulating the PIK3/AKT and Rb/E2F pathways [15-17]. The functional isoforms of MT2A mRNA transcript have been reported to express at the highest levels in breast tissues and is positively related to the cell proliferation and histological grade of BC [18].

Rs28366003 and rs10636 polymorphisms are the most common loci of MT2A, having been found to be associated with many different cancers. Rs28366003 locates in the core promoter region, while rs10636 polymorphism is in the 3’UTR region of the MT2A gene [17]. A recent study found that the single nucleotide polymorphism (SNP) rs28366003 stood apart from rs1610216 and rs10636 is significantly related to the laryngeal cancer risk in a Polish population [12]. Forma studied the association between MT2A (rs28366003,
rs1610216, and rs10636) and the risk of prostate cancer. And only the rs28366003 SNP in MT2A was observed to be correlated with the prostate cancer risk in Polish population [19]. Similarly, Krzeslak’s data suggested that the rs28366003 polymorphism in MT2A was related to the BC risk in a Polish population [13]. Although several poly-morphisms have been identified in MT2A, the function of its polymorphisms in BC not being fully understood [20]. In this study, we investigated and comprehensive-ly assessed the association between two SNPs of MT2A (rs10636 and rs28366003) and BC risk in Chinese Han population.

RESULTS

Characteristics of the study population

No significant difference was observed in the distribution of age, menopausal status, body mass index, and procreative times between the cases and controls ($P > 0.05$), which suggested that the cases and controls of this study were matched adequately on general characteristics (Table 1). The genotypic frequencies of the MT2A rs10636 and rs28366003 polymorphisms among the controls were in accord with HWE ($P = 0.343$ and $P = 0.363$, respectively).

Table 1. The characteristics of breast cancer cases and cancer-free controls.

| Characteristics                        | Cases   | Control | $P$ value |
|----------------------------------------|---------|---------|-----------|
| Number                                 | 459     | 549     |           |
| Age (mean ± SD)                        | 49.09±11.02 | 48.80±8.28 | 0.61 |
| Age of menarche (mean ± SD)            | 14.37±1.57     |         |           |
| Menopausal status                      |         |         |           |
| Premenopausal                          | 237     | 267     | 0.376     |
| Postmenopausal                         | 222     | 282     |           |
| Body mass index (kg/m2) (mean ± SD)    | 23.06±2.92 | 22.45±2.53 | 0.274 |
| Procreative times                      |         |         |           |
| <2                                     | 242     | 298     | 0.657     |
| ≥2                                     | 217     | 251     |           |
| Tumor size                             |         |         |           |
| <2 cm                                  | 152     |         |           |
| ≥2 cm                                  | 307     |         |           |
| Lymph node involvement                 |         |         |           |
| Negative                               | 184     |         |           |
| Positive                               | 275     |         |           |
| Histological grade                     |         |         |           |
| SBR 1-2                                | 244     |         |           |
| SBR 3                                  | 215     |         |           |
| Venous invasion                        |         |         |           |
| None–little                            | 292     |         |           |
| Moderate–severe                         | 167     |         |           |
| Immunohistochemistry results           |         |         |           |
| ER                                     |         |         |           |
| –                                      | 202     |         |           |
| +                                      | 257     |         |           |
| PR                                     |         |         |           |
| –                                      | 208     |         |           |
| +                                      | 251     |         |           |
| Her-2                                  |         |         |           |
| –                                      | 330     |         |           |
| +                                      | 129     |         |           |
| Ki67                                   |         |         |           |
| ≥14%                                   | 294     |         |           |
| <14%                                   | 165     |         |           |

ER: Estrogen receptor; PR: Progesterone receptor; Her-2: human epidermal growth factor receptor 2; SBR: Scarff, Bloom and Richardson tumor grade
MT2A polymorphisms and the breast cancer risk

In terms of genotype and allele distributions of MT2A polymorphisms, two polymorphisms in MT2A (rs10636 and rs28366003) showed positive associations with BC risk (Table 2). Rs10636 was identified to increase the BC risk in codominant and recessive models (C/C vs. G/G: OR = 1.86, 95% CI: 1.17-2.94, P = 0.008; C/C vs. G/G+G/C: OR = 1.96, 95% CI: 1.26-3.05, P = 0.003). And, the minor allele G of rs28366993 was also related to increasing BC risk (G vs. A, OR = 2.87, 95% CI = 1.97-4.2, P < 0.0001). The same result was found in the analysis of the rs28366003 polymorphism and the histological grade of BC (G/G vs. A/A, OR = 0.52, 95% CI = 0.33-0.82, P = 0.005, shown in Table 4).

MT 2A polymorphisms and the clinical features of breast cancer

We also analyzed the relationships between MT2A SNPs and clinical features of BC, including tumor size, lymph node metastasis, ER/PR/HER-2 status, histological grade, and venous invasion. As shown in Table 3, when the G/G genotype was used as a reference, there was no significant relation between the rs10636 polymorphism and clinical parameters except the histological grade (C/C vs. G/G: OR = 1.70-3.96, P = 0.005, shown in Table 4).
Similarly, no significant correlation was detected between the rs28366003 polymorphism and other clinical features.

**DISCUSSION**

It was reported that MT acts as a regulator in cell proliferation, apoptosis, and differentiation, which imply that MT may involve in carcinogenesis of BC [9, 11, 21-24]. The biological effects of MT are connected with its physiochemical properties [25, 26]. Under the stress situation, MT regulates cell apoptosis, inhibits cell death and improves cell survival. Recent studies suggested the regulation of MT were completed by preventing oxidative progress and binding with apoptotic signal [27, 28], in which MT could suppress free-radical-induced oxidative damage of tissues and cells.

The dysfunction of MT2A might be linked with the zinc release blockage and the reduction of intracellular zinc concentrations, which resulted in an increased risk of oxidative damage, as well as abnormal breast cells genesis [25]. As few studies concern to the relationship between the polymorphisms of MT2A and BC, we performed this case-control study in a Chinese population. Krzeslak, et al. have reported an association between rs28366003 polymorphism and the risk of ductal BC, prostate cancer, and laryngeal cancer in a Polish population [12, 13, 19]. We observed significant differences in genotypes distribution and allele...
frequencies of two MT2A gene polymorphisms locus (rs10636 and rs28366003) between BC patients and control groups \((P < 0.05)\). The MT2A SNP rs28366003 is an A/G substitution that is situated in the core promoter region of the MT2A gene sequence between the TATA box and transcription initiation site [13]. The transition may reduce the binding to the core promoter region, which is a nuclear molecule regulates MT2A gene transcription [29]. In this way, MT2A SNPs might be associated with functional changes, which imply that they may be involved in the interactions with the behavior of BC cell. Finally, the biological features of BC cells are gradually influenced. In addition, MT2A rs10636 polymorphism is located in the 3’ untranslated region, which implies that it may be involved in interactions with other nucleotide polymorphisms [29]. Our results suggest that both of the two MT2A SNPs rs10636 and rs28366003 significantly influence the susceptibility of BC.

As indicated by Ki-67 immunohistochemistry, MT2A is related to the proliferation of BC[25]. And, the down-regulation of MT2A arrests growth in MCF-7 cell lines also suggested the involvement of MT2A in the proliferation of BC [29, 30]. Moreover, it has been demonstrated that MT2A regulates endothelial cell migration through transcriptional regulation of the expression of vascular endothelial growth factor-c (VEGF-c) [31]. And it is believed that MT2A affects the histological differentiation grade in BC. The expression of MT2A mRNA in histological grade 3 tumors were higher than grade 1 and 2 tumors [20]. But in our study, BC patients with SBR 1-2 have a higher expression of the minor allele of MT2A SNPs loci than patients with SBR 3. So, we assume MT2A poly-morphisms may mitigate the aggressive behavior of BC cell. However, according to our results, there were no associations between ER/PR/HER-2 status, lymph node metastasis and MT2A polymorphisms (rs10636 and rs28366003).

### Table 4. The associations between the MT-2A rs28366003 polymorphism and clinical characteristics of BC patients.

| Variable            | GG(%) | GC(%) | p    | OR(95% CI) | CC(%) | p    | OR(95% CI) | GC+CC(%) | p    | OR(95% CI) |
|---------------------|-------|-------|------|------------|-------|------|------------|---------|------|------------|
| Tumor size          |       |       |      |            |       |      |            |         |      |            |
| <2CM                | 57    | 54    | 0.63 | 1.12(0.7-1.78) | 41    | 0.11 | 1.49(0.92-2.42) | 95       | 0.23 | 1.14(0.74-1.75) |
| ≥2CM                | 98    | 104   | 0.33 | 0.94(0.56-1.56) | 105   | 0.84 | 1.05(0.66-1.68) | 209      | 0.98 | 1.01(0.65-1.55) |
| LN metastasis       |       |       |      |            |       |      |            |         |      |            |
| Negative            | 45    | 56    | 0.8  | 1.37(0.87-2.18) | 83    | 0.07 | 1.51(0.96-2.39) | 139      | 0.07 | 1.44(0.97-2.16) |
| Positive            | 67    | 78    | 0.2  | 0.86(0.54-1.39) | 100   | 0.26 | 0.77(0.49-1.22) | 188      |       |            |
| ER                  |       |       |      |            |       |      |            |         |      |            |
| Negative            | 70    | 65    | 0.18 | 1.37(0.87-2.18) | 67    | 0.07 | 1.51(0.96-2.39) | 132      |       |            |
| Positive            | 69    | 88    | 0.54 | 0.86(0.54-1.39) | 93    | 0.26 | 0.77(0.49-1.22) | 174      | 0.32 | 0.81(0.54-1.22) |
| PR                  |       |       |      |            |       |      |            |         |      |            |
| Negative            | 55    | 67    | 0.49 | 0.82(0.46-1.45) | 86    | 0.26 | 0.77(0.49-1.22) | 153      | 0.32 | 0.81(0.54-1.22) |
| Positive            | 77    | 81    | 0.54 | 0.86(0.54-1.39) | 93    | 0.26 | 0.77(0.49-1.22) | 174      | 0.32 | 0.81(0.54-1.22) |
| Her-2               |       |       |      |            |       |      |            |         |      |            |
| Negative            | 71    | 82    | 0.2  | 1.37(0.87-2.18) | 177   | 0.16 | 0.7(0.43-1.15) | 259      | 0.2  | 0.76(0.46-1.18) |
| Positive            | 35    | 33    | 0.49 | 0.82(0.46-1.45) | 61    | 0.16 | 0.7(0.43-1.15) | 94       | 0.2  | 0.76(0.46-1.18) |
| Ki-67               |       |       |      |            |       |      |            |         |      |            |
| <14%                | 44    | 105   | 0.98 | 0.99(0.56-1.76) | 145   | 0.52 | 0.83(0.48-1.45) | 138      | 0.69 | 0.9(0.53-1.52) |
| ≥14%                | 27    | 64    | 0.33 | 0.99(0.56-1.76) | 74    | 0.52 | 0.83(0.48-1.45) | 138      | 0.69 | 0.9(0.53-1.52) |
In terms of the current study limitations, the sample size was inadequate for a stratified analysis and analysis of mix-type BC. Besides, we did not investigate other predisposing factors, including high-dose radiation exposure, alcohol consumption, and postmenopausal obesity. The further study should assess these factors as well for a more precise evaluation.

In conclusion, this study showed that MT2A polymorphisms rs10636 and rs28366003 are associated with BC risk in Chinese Han population. And, the relationship of MT2A polymorphisms and the histological grade may guide us to judge prognosis of BC. Further functional studies and large population-based prospective studies are needed to provide accurate evidence about the influence of MT2A variants on BC.

**MATERIALS AND METHODS**

**Ethics statement**

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Xi’an Jiaotong university (Xi’an, China). The research protocol was completed according to the approved guidelines.

**Table 5. Primers used for this study.**

| SNP_ID   | 1st-PCRP                  | 2nd-PCRP                  | UEP_SEQ                      |
|----------|---------------------------|---------------------------|------------------------------|
| rs10636  | ACGTTGGATGAGAAACG         | ACGTTGGATGACATAGAAAAAGG   | GACGGAATATAGCAAAA CCGTCA     |
|          | CGACTTCCACAAAC           | AATATAGC                 |                              |
| rs28366003 | GCCGCCCTACATCGCG         | GAAAGCAACGCGCTTTGGAGGAG  | AACTCAGGTCACCTGG ATGCA       |
|          | GTCAGGGAACTGT            | GCGTGTT                  |                              |

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**Subjects**

The consent of all the participants was obtained after they were informed that their blood samples would be used for research purpose. The blood samples of 459 Chinese women with sporadic BC (mean age: 49.09 ± 11.02 years) were selected from the Second Affiliated Hospital of Xi’an Jiaotong University, Shaanxi Province, China. In addition, 549 age- and sex- matched healthy individuals (mean age: 48.80 ± 8.28 years) without any history of autoimmune or malignant diseases formed the control group. All of the BC cases and controls were Han population and from Northwest China. All the patients were diagnosed by histology or pathology, as described in our previous studies [32]. The data of clinicopathological characteristics of patients, including tumor size, clinical stages, lymph node metastasis, menopausal status, procreative times, estrogen receptor (ER) status, progesterone receptor (PR) status, and human epidermal growth factor receptor type2 (HER-2) status, were obtained from the patients’ medical records (Table 1).

**DNA extraction and genotyping**

The samples were contained in tubes coating with ethylene-diaminetetraacetic acid and were stored at
−80°C after centrifugated until analysis. Genomic DNA was extracted from whole blood using the Universal Genomic DNA Extraction Kit Ver. 3.0 (TaKaRa Bio Inc., Shiga, Japan). DNA concentration was measured by spectrophotometry (DU530 UV/VIS spectro-photometer, Beckman Instruments, Fullerton, CA, USA). Two tag-SNPs (rs10636 and rs28366003) were selected in this study. A multiplexed SNP MassEXTEND assay was designed by Sequenom MassARRAY Assay Design 3.0 Software (Agena Bioscience, Inc., San Diego, CA, USA). SNP genotyping was performed by the Sequenom MassARRAY RS1000, and the primers were listed in Table 5. The data analyses were accomplished by Sequenom Type 4.0 [33].

**Statistical analysis**

All the statistical analyses were completed using the SPSS software package (version 20.0; SPSS Inc., Chicago, IL, USA). Hardy-Weinberg equilibrium (HWE) was examined by comparing expected and observed frequencies using Arlquin 3.1 program (L. Excoffier, CMPG, University of Bern, Switzerland). The genotype frequencies of observed values were compared with expected values obtained from HWE theory (p^2+2pq+q^2=1; p is the frequency of the wild-type allele and q is the frequency of the variant allele). The calculation was performed by $\chi^2$ test and the degree of freedom was 1 in the cases and controls. The significant difference in allele and genotype frequencies between cases and controls was determined by Pearson’s $\chi^2$ test [34, 35]. And the cancer risk linked with alleles and genotypes was calculated with an odds ratio (OR) and 95% confidence interval (CI). We evaluated the risk in the codominant model (Aa vs. AA and aa vs. AA), dominant model (AA+ Aa vs. aa), recessive model (aa vs. Aa+AA), overdominant model (AA+ aa vs. AA) and the allele model (a vs. A) respectively (A: the major allele, a: the minor allele). A two-sided P-value < 0.05 was considered statistically significant in all the tests.

**Abbreviations**

MT2A: metallothioneins 2A; BC: breast cancer; SNP: single nucleotide polymorphism; OR: odds ratio CI: confidence interval; HWE: Hardy-Weinberg equilibrium; ER, Estrogen receptor; PR: Progesterone receptor; Her-2: human epidermal growth factor receptor 2.

**AUTHOR CONTRIBUTIONS**

Zhi-Jun Dai and Di Liu conceived and designed the experiments. Di Liu, Meng Wang, Tian Tian, Peng-Tao Yang, Kang Liu, Xing-Han Liu, Peng Xu, Yi Zheng collected and assembled the data. Zhi-Jun Dai, Di Liu and Tian-Bo Jin analyzed and interpreted the data. Di Liu, Meng Wang, Hua-Feng Kang, Shu-Qun Zhang, Hai-Tao Guan, contributed materials/analysis tools. Zhi-Jun Dai supervised the entire study.

**CONFLICTS OF INTEREST**

The authors have no conflict of interests to declare.

**FUNDING**

This work was supported by National Natural Science Foundation of China (No. 81471670/81274136); China Postdoctoral Science Foundation funded Projects (No. 2014M560791/2015T81037); the Fundamental Research Funds for the Central Universities, China (No.2014gjhz11); and the Funds of the Second Affiliated Hospital of Xi’an Jiaotong University for Young Scientists, China [YJ(QN)201305].

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