Macrosomia is associated with FTO promoter methylation in placenta and cord blood: a hospital-based case-control study.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

YX, WY contributed in the experimental design. HY contributed in the technical development. LZ, ZJ performed the experiments. LZ analyzed the data and wrote the manuscript. SH helped perform the data analysis. All authors approved the final version of the manuscript.

Keywords

Macrosomia, Fat mass and obesity-associated (FTO), methylation, mRNA, Placenta, cord blood

Abstract

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Macrosomia means an infant born with a birth weight ≥ 4000g. Infant birth weight has been proved to be negatively associated with fat mass and obesity-associated (FTO) gene methylation in the placenta. However, no direct connection between FTO associated epigenetic variants and the occurrence of macrosomia has been made. The aim of this study is to explore the association between macrosomia and FTO promoter methylation in placenta and cord blood. A hospital-based case control study design was adopted in the present study. Macrosomia(n=63) and normal birth weight infants (n=72) and their mothers recruited from 1 January to 31 December in 2012 at Wenzhou, China. Placental tissues and cord blood were collected. Methylation of CpG sites in FTO promoter was measured by MALDI-TOF-MS. Placental FTO mRNA transcription was measured by quantitative PCR. The results of the present study the average methylation of CpG sites in FTO promoter of placenta was 3.55±0.67% in macrosomia and 3.73±0.87% in control, P= 0.283. Methylation of CpG16 site was lower in macrosomia than in control (0.81% vs 1.48%, P= 0.034). In cord blood, the average methylation rate of CpG sites was 2.65±0.36% in macrosomia and 2.81±0.52% in control, P= 0.060. Methylation of CpG6.7.8.9 site was lower in macrosomia than in control (6.98% vs 7.94%, P= 0.005). Macrosomia had a higher placental FTO mRNA expression level than control (1.33±0.59 vs 1.05±0.43), P= 0.011). Finally, multivariate logistic regression results showed that the methylation rate of CpG16 in placenta was negatively associated with macrosomia (OR=0.68, 95%CI: 0.47~0.97, P=0.034). Methylation of CpG6.7.8.9 in cord blood was negatively associated with macrosomia (OR=0.78, 95%CI:0.62~0.97, P=0.026). Placenta FTO mRNA expression was positively associated with macrosomia (OR=3.28, 95%CI: 1.20~8.99, P=0.021). In summary, CpG sites in FTO promoter showed a hypo-methylation status in macrosomia and normal birth weight infant's placenta tissues and cord blood. Macrosomia has lower methylation levels of CpG 16 site in placenta and CpG 6.7.8.9 sites in cord blood, and a higher level of placental FTO mRNA expression.

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Ethics approval was granted by the Institutional Review Boards of Wenzhou Medical University.

Macrosomia is associated with *FTO* promoter methylation in placenta and cord blood: a hospital-based case-control study

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Keywords: Macrosomia, fat mass and obesity-associated (*FTO*), methylation, mRNA, placenta, cord blood.

Abstract

Macrosomia means an infant born with a birth weight ≥ 4000g. Infant birth weight has been proved to be negatively associated with fat mass and obesity-associated (*FTO*) gene methylation in the placenta. However, no direct connection between *FTO* associated epigenetic variants and the occurrence of macrosomia has been made. The aim of this study is to explore the association between macrosomia and *FTO* promoter methylation in placenta and cord blood. A hospital-based case control study design was adopted in the present study. Macrosomia(n=63) and normal birth weight infants (n=72) and their mothers recruited from 1 January to 31 December in 2012 at Wenzhou, China. Placental tissues and cord blood were collected. Methylation of CpG sites in *FTO* promoter was measured by MALDI-TOF-MS. Placental *FTO* mRNA transcription was measured by quantitative PCR. The results of the present study the average methylation of CpG sites in *FTO* promoter of placenta was 3.55±0.67% in macrosomia and 3.73±0.87% in control, *P*= 0.283. Methylation of CpG16 site was lower in macrosomia than in control (0.81% *vs* 1.48%, *P*= 0.034). In cord blood, the average methylation rate of CpG sites was 2.65±0.36% in macrosomia and 2.81±0.52% in control, *P*= 0.060. Methylation of CpG6.7.8.9 site was lower in macrosomia than in control (6.98% vs 7.94%, *P*= 0.005). Macrosomia had a higher placental *FTO* mRNA expression level than control (1.33±0.59 *vs* 1.05±0.43), *P*= 0.011). Finally, multivariate logistic regression results showed that the methylation rate of CpG16 in placenta was negatively associated with

macrosomia (OR=0.68, 95%CI: 0.47~0.97, *P*=0.034). Methylation of CpG6.7.8.9 in cord blood was negatively associated with macrosomia (OR=0.78, 95%CI:0.62~0.97, *P*=0.026). Placenta *FTO* mRNA expression was positively associated with macrosomia (OR=3.28, 95%CI: 1.20~8.99, *P*=0.021). In summary, CpG sites in *FTO* promoter showed a hypo-methylation status in macrosomia and normal birth weight infant's placenta tissues and cord blood. Macrosomia has lower methylation levels of CpG 16 site in placenta and CpG 6.7.8.9 sites in cord blood, and a higher level of placental *FTO* mRNA expression.

1 Introduction

Macrosomia refers to an infant born with a birth weight equal to or greater than 4000g (Koyanagi et al., 2013). The prevalence of macrosomia was 5.0%~20.0% in developed countries and 4.1%~13.4% in developing countries (Henriksen, 2008). In China, the morbidity of macrosomia was 7.3% and keep increasing (Li et al., 2014). Overlarge fetus not only increases the risk of maternal and infant obstetrical complications, but also increases the long-term health problems of infant in later life (Hediger et al., 1999; Oral et al., 2001; Boney et al., 2005; Ross, 2006; Giapros et al., 2007; Sprehe et al., 2010). Macrosomia is believed to be the integrated results of genetic and environmental factors with an unclear pathogenesis. There is also still a lack of adequate acknowledge in the occurrence, causes, and prevention of macrosomia. Imageology examinations are effective clinical tools and regularly used to detect macrosomia in early. In molecular and genetic diagnosis, there is still lacking in effective and stable genetic markers or biomarkers to predict the occurrence of macrosomia, which has enormous potential value in the clinical application.

The biochemical markers and gene characteristics in the placenta and cord blood were believed to be related to fetal health status and could be used to predict fetal growth after birth. For instance, alteration in expression and methylation of the gene related to growth and development in placenta and umbilical cord blood, such as IGF2 and H19, has been proved to be associated with macrosomia (Su et al., 2016). Placenta tissue and cord blood are good and feasible research material in genetic study with regard to newborns. *FTO* gene is expressed in the placenta tissues **and** is associated with fetal growth (Fischer et al., 2009; McMurray et al., 2013). A birth cohort study (n=147) indicated that *FTO* was highly expressed in placenta and was associated with increased fetal weight and length in Caucasian (Bassols et al., 2010). A study in French newborns showed that the placental *FTO* mRNA expression was reduced in intrauterine growth restriction fetuses (n=8) compared to normal pregnancies (n=11) and was not associated with risk of macrosomia (n=5) (Mayeur et al., 2013). These results indicate that placental *FTO* expression may be associated with infant birth weight gain. In our previous study, we found that placental *FTO* mRNA expression was increased in infants with birth weight larger than 3500g (Liu et al., 2016). A recent study reported that the *FTO* genotype was not associated with *FTO* expression in placenta but a higher placental *FTO* expression was associated with a larger fetal size(Barton et al., 2016). Based on the previous evidence, we speculate that macrosomia may be positively associated with placental FTO mRNA expression.

FTO has been proved to play an important role in tissue development and energy metabolism, which is also involved in programming epigenetic alteration of other genes during embryonic development (Li et al., 2016; Mauer et al., 2017). Infant birth weight is not only associated with *FTO* mRNA

expression but also related to its epigenetic alteration in DNA. Hypermethylated CpG dinucleotides in the 5' regulatory region of gene promoter was considered as a potential marker for the silence of the gene expression. We found that the decreased methylation of CpG dinucleotides in the promoter of *FTO* in placenta was associated with the increased mRNA expression, which was also related to increased infant birth weight (Liu et al., 2016). Although methylation alteration of *FTO* in the placenta is associated with infant birth weight, it is unknown that whether the methylated status of *FTO* promoter is involved in the occurrence of macrosomia. In addition, there is no study report the *FTO* promoter methylation status in cord blood and its association with macrosomia. Hence, we want to explore the association between macrosomia and *FTO* promoter methylation in placenta and cord blood.

The aim of this study is to adopt epigenetic epidemiology study design to compare macrosomia to normal birth weight infants in *FTO* promoter methylation in the placenta and cord blood, *FTO* mRNA level in the placenta. Firstly, to show the *FTO* methylation status in macrosomia`s placenta and cord blood. Secondly, to show the *FTO* mRNA expression level in macrosomia`s placenta tissue. Thirdly, to explore the association between macrosomia and *FTO* promoter methylation, and the association between macrosomia and placental *FTO* mRNA level. We hope that our finding would provide scientific cues for further studies with regard to biomarker exploring or pathogenesis of macrosomia.

2 Materials and methods

2.1 Subjects

A hospital-based case control study was conducted. Ethics approval was granted by the Institutional Review Boards of Wenzhou Medical University. Participants were consecutively recruited from Yuying Children's Hospital of Wenzhou Medical University, Zhejiang, China (1 January 2012~31 December 2012). Eligibility criteria included: singleton infants born at term (≥37 weeks of gestation), mother with normal oral fasting glucose tolerance test results. Macrosomia had a birth weight ≥4000g. Controls had a birth weight 2500~3999g and selected from those born within three days before or after a case macrosomia was born. Excluded criteria were: Neonates from pregnancy in preeclampsia, with other pediatric diseases, with congenital malformation. In sum, 63 of macrosomia and 72 of normal birth weight infants and their mothers recruited in our study.

2.2 Data collection

Interviewers visited each subject`s mother or father. A face-to-face interview was conducted after obtaining written informed consent from each subject. Parental socio-demographic information was collected by self-developed questionnaire. Maternal anthropometry, medical status, and pregnancy outcome were retrieved from the medical records. Maternal weight before pregnancy was the weight on the day of the last physical examination before the pregnancy diagnosis. Gestational weight gain was calculated as body weight one week before delivery minus the weight before pregnancy.

2.3 Placenta tissue and cord blood sample collection

Immediately after delivery of the placenta, a chorionic villous biopsy (~1g) was excised and put into frozen tubes pretreated with diethyl pyrocarbonate. Placental tissue was preserved in RNAlater (Ambion, Austin, TX, USA) at 4°C overnight and then stored at −80°C until analysis. Cord blood was stored in vacutainer tubes containing EDTA at −80°C.

2.4 DNA methylation analysis of the *FTO* promoter

Methylation status of *FTO* gene was quantitatively measured by the Matrix-Assisted Laser Desorption/ Ionization Time of Flight Mass Spectrometry method. All laboratory processes were performed by the Sequenom MassARRAY platform (CapitalBio, Beijing, China). The specific procedure was given in our previous study (Liu et al., 2016). Two CpG islands were found in the *FTO* sequence (promoter, exon1, and intron1). Length of CpG was 290 bp (–180 to 110 bp) in island1 and 238 bp (115 to 352 bp) in island2. Each island contained 23 CpG sites. We just measure the methylation of CpG site in island 1 which entirely located in the promoter region of *FTO*.

2.5 Reverse transcription PCR (RT-PCR) analysis

Total RNA was isolated from placental tissue by the Trizol reagent (Invitrogen, USA). RNA quality was assessed by agarose gel electrophoresis and by measuring the absorbance at 260 and 280nm. Only samples with an A260/A280 ratio >1.8 and an A260/A230 ratio ≥2.0 were included in the mRNA expression analysis. cDNA was prepared by the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). Primers of genes (*GAPDH* and *FTO*) and reaction condition have been given in the previous study (Liu et al., 2016). Target genes were relatively quantified by the Syber green dye method. Quantitative analysis of target gene expression data was based on the $2^{-\Delta\Delta CT}$ method.

2.6 Statistical analysis

Double data entry and data cleaning were performed using EpiData software (version 3.1, Odense, Denmark). The 2-sample *t*-test or the non-parametric Mann–Whitney test was used to compare continuous variables between macrosomia group and control. The chi-square test was used to compare proportions. Logistic regression analyses and ORs were used to evaluate and estimate relative risks. All tests were two-tailed, and $P<0.05$ was set as the level of statistical significance. Statistical analyses were performed using SPSS version 22.0 software (SPSS Inc., Chicago, Illinois).

3 Results

3.1 Participants characteristics

In this study, 63 of macrosomia, and 72 of normal birth weight infants were included. The average birth weight in macrosomia was 4298.7g (SD: 231.8), which was 3497.4g (SD: 335.8) in the control group. Among the macrosomia, male infant accounted for 74.6%. The maternal age ranged from 22 to 41 years. Among the controls, male infant accounted for 52.8%. The maternal age ranged from 19 to 38. Other information was given in Table1.

Table1 is inserted here.

3.2 *FTO* promoter methylation status in the placenta

Forty-three of placenta tissue from macrosomia and 46 from controls were included in the methylation analyses. There is no difference in age(P=0.202), gender(P=0.460), pre-pregnant

BMI(*P*=0.106), weight gain during pregnancy(*P*=0.402) between included and unincluded subjects. Results of analyses showed that the average DNA methylation level of CpG island1 in the placenta of macrosomia was 3.55% (SD: 0.67) and control was 3.73% (SD: 0.87) (*P*=0.283). Macrosomia had a lower methylated rate of CpG16 site than control (0.81% versus 1.48%, *P*=0.034). There was no statistical significance reported in other sites (*P*=0.071 to 0.659). See Figure1.

Figure1 is inserted here.

3.3 *FTO* promoter methylation status in cord blood

The average DNA methylation level of CpG island1 in cord blood of macrosomia was 2.65% (SD: 0.36) and control was 2.81% (SD: 0.52) (*P*=0.060). Macrosomia had a lower methylation rate of CpG6.7.8.9 than that of control (6.98% vs 7.94%, *t*=2.892, *P*=0.005). See Figure 2.

Figure2 is inserted here.

3.4 *FTO* mRNA expression in placenta

The results showed that *FTO* mRNA expression level was higher among macrosomia than that of control (1.33±0.59 *vs* 1.05±0.43), *t* = –2.591, *P*= 0.011) (Figure 3).

Figure3 is inserted here.

3.5 Association between macrosomia and *FTO* promoter methylation

Univariate logistic regression model showed that methylation rate of CpG16 in placenta (OR=0.72, 95%CI:0.52~0.99, *P*=0.040), CpG6.7.8.9 in cord blood (OR=0.75, 95%CI:0.61~0.92, *P*=0.007), placental *FTO* expression (OR=2.99, 95%CI:1.25~7.19, *P*=0.014) were associated with macrosomia. The result of multivariate logistic regression model 1 showed that placenta *FTO* expression was positively associated with macrosomia (OR=3.74, 95%CI: 1.19~11.74). after controlling the effect of maternal age, educational attainment, BMI, parity, weight gain during pregnancy, gestational age, infant gender and placental *FTO* mRNA. The result of model 2 showed that methylation rate of CpG16 in placenta was negatively associated with risk of having macrosomia (OR=0.65, 95%CI: 0.42~1.00), with a marginal statistical significance. Model 3 showed that CpG6.7.8.9 in cord blood were negatively associated with macrosomia (OR=0.74, 95%CI:0.58~0.94). See Table2

Table2 is inserted here.

4 Discussion

4.1 Main finding

In this study, we found that both macrosomia and normal birth weight infant had very low methylated levels in CpG island 1 of *FTO* promoter. There was no statistical difference in the average methylation rates of *FTO* promoter in placenta and cord blood between macrosomia and control. But methylation rates of CpG16 in placenta and CpG6.7.8.9 in cord blood are lower among macrosomia than control. In addition, placental *FTO* expression was higher among macrosomia. After adjusting for maternal age, parity, pre-pregnant BMI, weight gain during pregnancy, educational attainment, and infant gender, the risk of macrosomia is negatively associated with the methylation rates of CpG16 and CpG6.7.8.9, and positively associated with placental *FTO* expression.

4.2 Macrosomia and *FTO* mRNA expression

Our finding of the present study showed that the placental *FTO* mRNA expression was increased among macrosomia. The result of multivariate logistic regression showed that the OR of *FTO* expression was 3.41. In the previous study, we reported that placental *FTO* mRNA expression was positively associated with infant birth weight (Liu et al., 2016). This indicates that *FTO* mRNA level in the placenta is associated with the occurrence of macrosomia. Studies in *vitro* and *vivo* have indicated that *FTO* expression was closely associated with cellular lipolytic activity and energy expenditure (Dahlman et al., 2007; Fischer et al., 2009; Church et al., 2010; McMurray et al., 2013). A higher *FTO* expression level can prevent adipose tissue from reducing the size and lean mass weight loss. Therefore, increased placental FTO mRNA expression may contribute to having a macrosomia.

4.3 Macrosomia and *FTO* promoter methylation

We found that the average methylation rate of CpG island in the promoter was very low (lower than 5%). CpG16 site in placenta and CpG6.7.8.9 site in cord blood showed a lower methylation among macrosomia than that of normal birth weight infant. Methylation of these two CpG sites was negatively associated with macrosomia, which indicates that lower methylation added more risk for boring a macrosomia. From molecular biologic aspect, methylation of CpG dinucleotides in the 5′promoter regions of genes is generally considered indicative of transcription silencing (Bird, 2002). Lower methylation level of the promoter ensures the stabilized mRNA transcription of *FTO*. The mRNA expression is higher among macrosomia than that of control in the present study. This was consistent with the tendency of methylation change. In addition, CpG6.7.8.9 and CpG16 sites were located near putative transcription factor binding sites, such as transcription factor Sp1and AP-2alpha. However, there is no study reported the association between *FTO* transcription and SP1 or AP-2alpha. We speculate that the hypo-methylated status of CpG sites in the *FTO* promoter serves to combine with transcription factors. Thereby maintaining higher *FTO* transcriptional and protein levels in macrosomia.

Strength and limitation. One strength is that we adopted an epigenetic epidemiology study method to explore the *FTO* gene epigenetic characteristics in macrosomia and showed the association between *FTO* promoter methylation and macrosomia, compared to normal birth weight infant. We also used the multivariate regression model to control various confound regard to demographic characteristics of the population. Thus, we reported reliable associations between molecular or epigenetic change and macrosomia. For limitation, one was that all subjects were recruited from the hospital. The

representativeness of macrosomia and mother may be limited. As far as we know, with the development and improvement of maternal and child health care system, nowadays all pregnant give birth in the hospital except Only few of pregnancy would give birth to other places. All subjects in our study came from the Yuying Children's Hospital of Wenzhou Medical University. The hospital is a large comprehensive hospital and the patients came from all eleven counties in the Wenzhou region. Our samples can be representative of the population from Wenzhou region to a certain extent. Another limitation is that we only measured the *FTO* methylation status in placenta and cord blood at one time point. It cannot be representative of the methylation status during the whole pregnancy. At last, our data have not yet proved the causal relationship between methylation alteration and macrosomia.

5 Conclusion

In summary, CpG sites in *FTO* promoter showed a hypo-methylation status in macrosomia and normal birth weight infant's placenta tissues and cord blood. Methylation of CpG 16 site in placenta and CpG 6.7.8.9 sites in cord blood are lower among macrosomia than that of normal birth weight infant. Placental *FTO* mRNA expression is higher in macrosomia. Macrosomia is negatively associated with methylation of CpG 16 site in placenta and CpG 6.7.8.9 sites in cord blood, but positively associated with placental *FTO* mRNA expression.

6 Figure legends

Figure 1. Median values of methylation rates for CG dinucleotides from CpG island in placenta. * $P < 0.05$.

Figure 2. Median values for CG dinucleotides from CpG island in cord blood. * $P < 0.05$.

Figure 3. Placental expression of *FTO* mRNA was measured by RT-PCR. Quantitative analysis of target gene expression data was calculated by the $2^{-\Delta\Delta CT}$ method. * $P < 0.05$.

7 Conflict of Interest

The authors have no conflicts of interest to declare.

8 Author Contributions

YX, WY contributed in the experimental design. HY contributed in the technical development. LZ, ZJ performed the experiments. LZ analyzed the data and wrote the manuscript. SH helped perform the data analysis. All authors approved the final version of the manuscript.

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11 Data Availability Statement

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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Figure 1.TIF

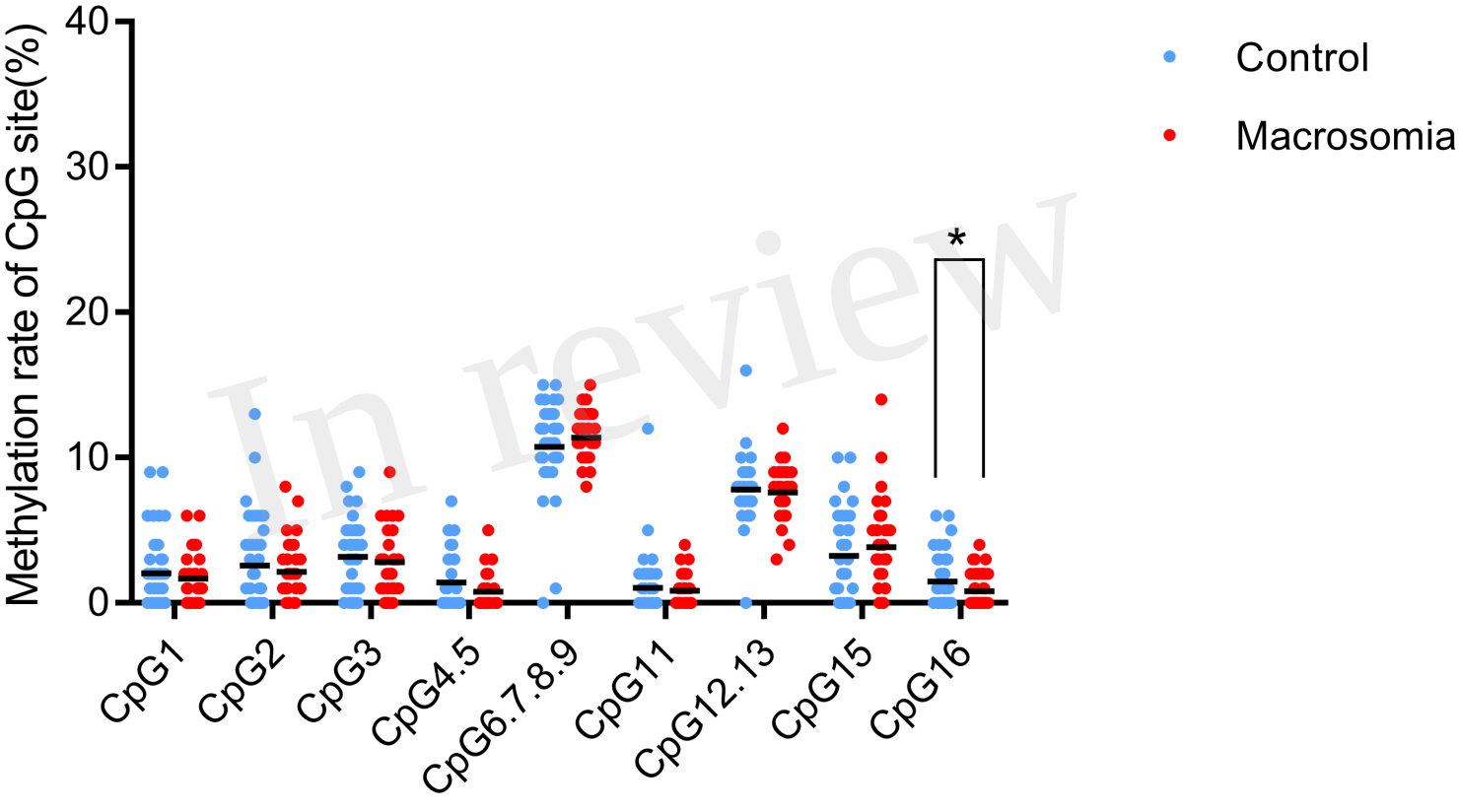

Figure 2.TIF

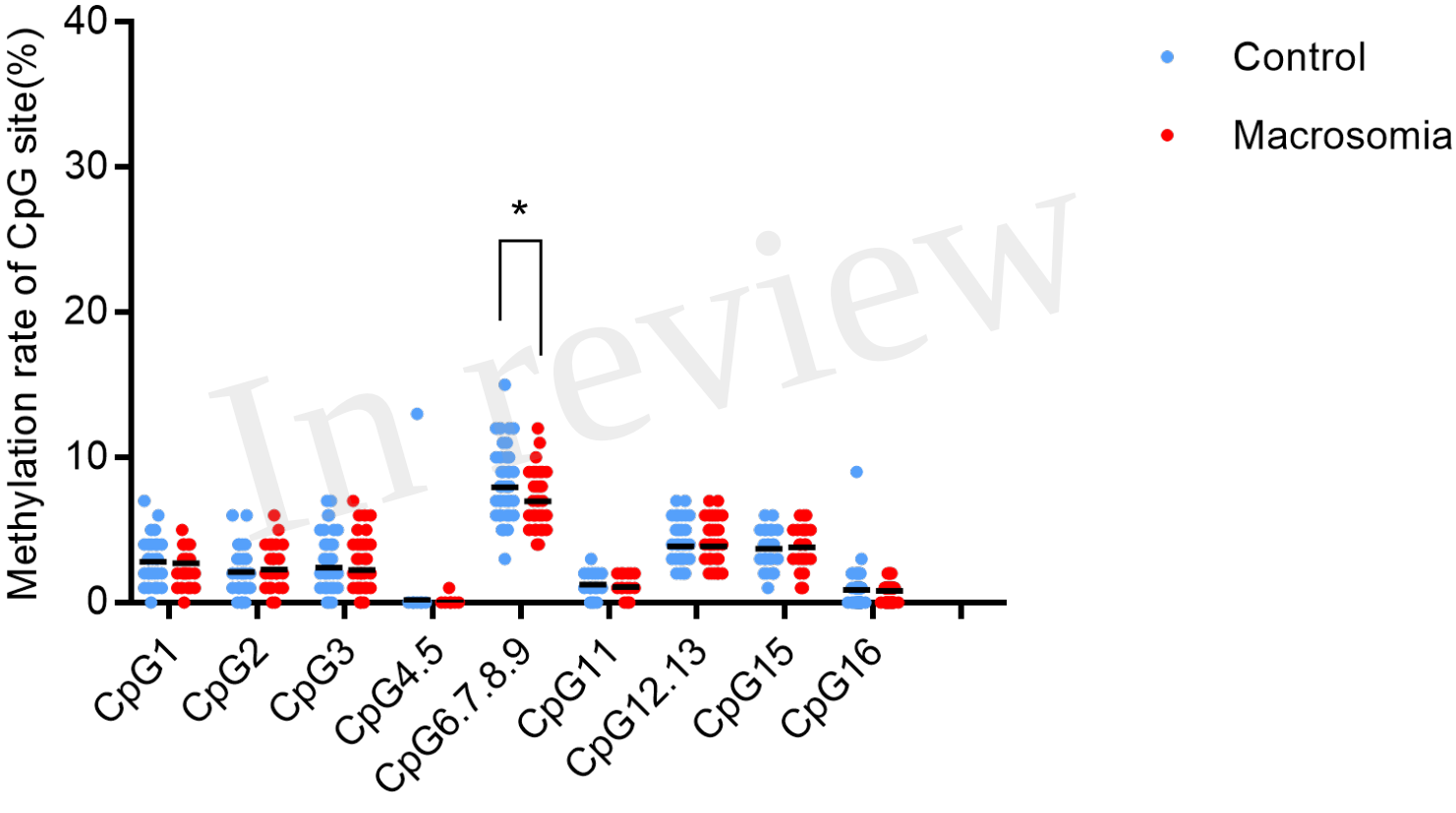

Figure 3.TIF