GSTM1 and GSTT1 null polymorphisms and male infertility risk: an updated meta-analysis encompassing 6934 subjects

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Published data on the association between the GST genes polymorphisms and male infertility risk are inconclusive. We investigated GST genes polymorphisms in a large sample size case-control study, and conducted a literature-based meta-analysis of 6934 individuals. Our case-control study showed the GSTM1 null genotype was significantly associated with idiopathic oligozoospermia, while the null genotype of GSTT1 was significantly associated with normozoospermia and azoospermia. Additionally, significantly elevated GSTT1 expression levels were observed in present genotype compared with null genotype. In the meta-analysis, the null genotype of GSTM1 was associated with a significantly increased risk of male infertility. Furthermore, a stratification analysis showed that the risk of GSTM1 polymorphism was associated with male infertility in both Asian and Caucasian groups. Further studies of GSTM1 and GSTT1 with their biological functions are needed to understand the role of these genes in the development of male infertility.

Infertility is a worldwide reproductive health problem which affects 10%–15% of couples and about half of the cases are due to male factors. Although several causes have been identified for impaired male fertility, the aetiology remains unknown in nearly half of all cases. It is currently accepted that genetics contributes to spermatogenetic failure for about 30% of idiopathic infertility in males.

Sperms are susceptible to oxidative damage and excessive reactive oxygen species (ROS) generation may lead to subfertility or infertility. Glutathione S-transferases (GSTs) represent an important superfamily of phase II metabolic enzymes that catalyze the conjugation of reduced glutathione with electrophilic groups of a wide variety of environmental compounds. GSTs are responsible for detoxification of many xenobiotics and endogenous ROS by catalyzing the conjugation of reduced glutathione to the substrate or sequestering toxic compounds, and play a key role in protecting cells against oxidative stress. Human GSTs are divided into eight distinct classes as alpha, kappa, mu, omega, pi, sigma, theta, and zeta based on the similarity of amino acid sequence and antibody cross-reactivity. The GSTM1 and GSTT1 gene have been located on chromosome 1p13.3 and 22q11, respectively. Homozygotes for the null alleles (deletion) of GSTM1 and GSTT1 lack activity of the respective enzymes. As a result, GSTs decrease the reactivity of electrophilic substrates, which can affect spermatogenesis and spermatozoa function with cellular macromolecules, such as nucleonic acid, lipid and protein. The enzymatic deficiency in isoforms of GSTs is correlated with increased risk to develop certain diseases associated with oxidative damage. In this case an association between the genotypes of GSTM1, GSTT1 and risk of idiopathic infertility is possible.

Recently, a number of molecular epidemiological studies have been conducted to examine the association between GSTM1 and GSTT1 null polymorphisms and male infertility in diverse populations. However, the results of these studies are inconsistent or even contradictory. Each individual study with small sample sizes may be underpowered to detect the effect of GSTM1 and GSTT1 genotype on the susceptibility of male infertility. Most
Table 1 | Characteristics of infertile patients and fertile controls

| Characteristic          | Control (n = 895) | Case (n = 1476) |
|-------------------------|-------------------|-----------------|
| Age (years, mean ± SD)  | 29.89 ± 4.34      | 29.90 ± 4.89    |
| BMI (kg/m², mean ± SD)  | 23.81 ± 2.89      | 23.62 ± 3.37    |
| Smoking status [n (%)]  |                   |                 |
| Yes                     | 399 (44.6)        | 678 (46.0)      |
| No                      | 496 (55.4)        | 798 (54.0)      |
| Alcohol intake [n (%)]  |                   |                 |
| Yes                     | 166 (18.5)        | 320 (21.7)      |
| No                      | 729 (81.5)        | 1156 (78.3)     |
| Abs [days, mean ± SD]   | 4.99 ± 0.30       | 5.15 ± 3.17     |
| Abs [n (%)]             |                   |                 |
| <6                      | 7 (0.8)           | 323 (21.9)      |
| 4–7                     | 888 (99.2)        | 1033 (70.0)*    |
| >7                      | 0 (0.0)           | 120 (8.1)       |
| Ejaculate volume (ml, mean ± SD) | 3.06 ± 1.30        | 3.05 ± 1.40     |
| Sperm concentration (10⁶/ml)* | 41.42 (29.22–68.90) | 34.98 (6.60–75.59)* |
| Sperm motility (%)*     | 62.72 (39.86–78.07)| 43.67 (16.35–65.60)* |

<Control: fertile men who have at least one child and lacked any history of requiring assisted reproduction technology.
*Case: non-obstructive infertile men.
*BMI: body mass index.
Ab: Abstinence time.
Values are given as median and interquartile range (IQR).
P < 0.05 when compared between case and control groups.

Results
Case-control study. The relevant characteristics of the subjects. Demographic categories by fertility and sperm concentration are described in Table 1. No significant differences of age, BMI, drinking, smoking and ejaculate volume were observed between cases and controls.

GSTM1 and GSTT1 polymorphisms and male infertility. The distributions of GSTM1 and GSTT1 genotypes in cases and controls are shown in Table 2. The GSTM1 null phenotype frequency was 39.6% and 36.4% for infertile patients and fertile controls. The GSTT1 null allele frequency was 45.5% and 40.1% respectively for cases and controls. The frequency of null genotype of GSTT1 was significantly higher in infertile patients compared with fertile controls (P < 0.05) (Table 2). Logistic regression analyses revealed that the null genotype of GSTT1 significantly increased the risk of idiopathic male infertility (OR = 1.26; 95%CI, 1.07–1.50; P = 0.007), while no significant association was detected between the null genotype of GSTM1 and idiopathic male infertility risk (OR = 1.15; 95%CI, 0.97–1.36; P = 0.116) (Table 2).

For GSTM1 and GSTT1 genotypes, four combinations were obtained ([+/-]; [+/-]; [-/-] and [-/-]). The combination genotype (+/-) of GSTM1 and GSTT1 showed significant association with idiopathic male infertility (OR = 1.79; 95%CI, 1.44–2.23; P < 0.001). Similarly, the combination genotype (+/-) was also found to be prevalent in infertile males, which was significantly associated with increased risk of infertility (OR = 1.71; 95%CI, 1.36–2.16; P < 0.001). However, no association was found between the combination genotype (+/-) of GSTT1 and GSTM1 and male infertility (Table 3).

Next, the case group was subdivided into three subgroups: normozoospermia, oligozoospermia and azoospermia. The frequency of GSTM1 and GSTT1 genotypes in the control and case groups was shown in Table 4. For the null genotype of GSTM1 polymorphism, significantly elevated risk was observed in oligozoospermia (OR = 1.55; 95%CI, 1.15–2.08; P = 0.007), while no significant associations between null genotype of GSTT1 and normozoospermia (OR = 1.23; 95%CI, 1.03–1.48; P = 0.025) and azoospermia (OR = 1.58; 95%CI, 1.18–2.11; P = 0.002), but not in group of oligozoospermia (OR = 1.11; 95%CI, 0.82–1.48; P = 0.504).

Table 2 | Genotype distribution of GSTM1 and GSTT1 in infertile patients and fertile controls

|          | Control (n = 895) | Case (n = 1476) |
|----------|------------------|-----------------|
| GSTM1    |                  |                 |
| Present  | 569 (63.6)       | 893 (60.5)      |
| Null     | 326 (36.4)       | 583 (39.5)      |
|           | 1.15 (0.97–1.36) | 0.116           |
| GSTT1    |                  |                 |
| Present  | 536 (59.9)       | 805 (54.5)      |
| Null     | 359 (40.1)       | 671 (45.5)      |
|           | 1.26 (1.07–1.50) | 0.007           |

Table 3 | Genotype distribution of combined GSTM1 and GSTT1 in infertile patients and fertile controls

|          | Control (n = 895) | Case (n = 1476) |
|----------|------------------|-----------------|
| GSTM1    |                  |                 |
| +        | 373 (41.7)       | 460 (31.2)      |
| –        | 196 (21.9)       | 433 (29.3)      |
|           | 1.79 (1.44–2.23) | <0.0001         |
| GSTT1    |                  |                 |
| +        | 163 (18.2)       | 345 (23.4)      |
| –        | 163 (18.2)       | 238 (16.1)      |
|           | 1.71 (1.36–2.16) | <0.0001         |

Abbreviations: OR, odds ratio; 95% CI, 95% confidence interval.
Associations between GSTM1, GSTT1 expression levels in serum and GSTM1, GSTT1 genotypes. The serum GSTM1 expression level was conducted in 39 subjects with GSTM1 present genotype and 36 null genotype. No significant difference in the expression level of GSTM1 was observed between different genotypes (Figure 2A). In addition, 48 subjects with present genotype of GSTT1 and 32 subjects with null genotype were examined for the expression level of GSTT1. In accordance with the result of population study, the expression level of GSTT1 was significantly decreased in subjects with GSTT1 null genotype compared with present genotype (Figure 2B).

Meta-analysis. Study characteristics. Through the literature search and selection based on inclusion criteria, nineteen articles were identified by reviewing potentially relevant articles (Figure 3). The characteristics of the selected studies are shown in Table 5 and Table 6. Publication dates range from 2002–2013.

GSTM1 polymorphism. A total of 17 studies9–25 were included in the meta-analysis with 3726 cases and 2744 controls. The number of cases included in the studies varied from 42 to 1476, with a mean of 219, and the number of controls varied from 30 to 895, with a mean of 161.

GSTT1 polymorphism. In total, fourteen studies9–25 met the inclusion criteria and were selected for the meta-analysis with 3555 cases and 2560 controls. The number of cases included in the studies varied from 65 to 1476, with a mean of 253, and the number of controls varied from 30 to 895, with a mean of 183.

Meta-analysis of GSTM1 polymorphism and male infertility. The evaluation of the association between GSTM1 polymorphism and idiopathic male infertility risk is summarized in Table 7. In the overall analysis, significant association was found between GSTM1 null genotype and elevated risk of male infertility (OR = 1.39; 95%CI, 1.14–1.70; P = 0.001) (Figure 4). Moreover, subgroup analyses showed that there were significant association among Asians (OR = 1.51; 95%CI, 1.13–2.10; P = 0.005) and Caucasians (OR = 1.24; 95%CI, 1.00–1.52; P = 0.046) (Figure S1). When stratified by sperm concentration of case, the stratified analysis showed that GSTM1 null genotype was associated with significant increasing in the risk of OAT (OR = 1.53; 95%CI, 1.25–1.89; P < 0.001), but not azoosperma (Figure 5).

Meta-analysis of GSTT1 polymorphism and male infertility. The evaluation of the association between GSTT1 polymorphism and male infertility risk is shown in Table 7. The null genotype of GSTT1 was associated with a significantly increased risk of male infertility in Asian (OR = 1.44; 95%CI, 1.10–1.80; P = 0.009) (Figure S2). However, the association was not observed in the overall
analysis and subgroup analyses according to sperm concentration of case (Figure S3, Figure S4).

Publication Bias. Begg’s funnel plot and Egger’s test were performed to assess the publication bias of literatures. For GSTM1, the shape of the funnel plot did not reveal any evidence of obvious asymmetry ($P = 0.434$) (Figure 6). Moreover, the Egger’s test ($P = 0.269$) did not imply any evidence of publication bias. For GSTT1, neither Begg’s test ($P = 0.189$) nor Egger’s test ($P = 0.475$) suggest any evidence of publication bias (Figure S5).

Sensitive analysis. Sensitivity analyses were conducted to determine whether modification of the inclusion criteria of the meta-analysis affected the final results (Figure 7). Although sample size for cases and controls in 19 studies ranged from 30 to 1476, the corresponding pooled OR were not qualitatively altered with or without the study of small sample. In addition, no other single study influenced the pooled OR qualitatively, as indicated by sensitivity analyses.

Discussion

Spermatogenesis is an elaborate and closely regulated cell-differentiation process. Mutations or polymorphisms in the genes regulating spermatogenesis process may lead to male infertility$^{26,27}$. Recently, the importance of oxidative stress in spermatogenesis has received increasing attention. Oxidative stress is a result of the imbalance between ROS and antioxidants in the body. It is a powerful mechanism that may lead to sperm damage, deformity and eventually, male infertility. The GST enzymes are known to protect sperm against oxidative stress$^{28}$. The GST superfamily represents a major group of detoxification and antioxidant enzymes. The possible role in male infertility has been already suggested for GSTM1 and GSTT1 gene variants, whereas published data are inconsistent. With applying standardize unbiased genotyping methods on large sample size, we found that the null genotype of GSTM1 and GSTT1 were significantly associated with different spermatogenic status of male infertility in Chinese population. This was also supported by meta-analysis on previously published studies including our results.

GSTs have been considered to constitute the major defensive antioxidant system against oxidative stress by reducing ROS, one of the major factors leading to an infertile status, to less reactive metabolites$^{29}$. Recently, several epidemiological studies have reported that the GSTM1 and GSTT1 null genotypes that result in a lack of functional protein are correlated with an increased susceptibility to diseases associated with oxidative stress$^{30,31}$. Sperms are susceptible to oxidative damage and excessive ROS generation may lead to subfertility or infertility$^{30,31}$. The patients simultaneously carrying the GSTM1 and GSTT1 null genotype could be subjected to increased oxidative stress, which may account for the increased risk of male infertility. In our present study, we found that GSTT1 null genotype is significantly associated with lower level of GSTT1 expression level, which may cause increased vulnerability to oxidative DNA damage and excessive ROS generation. Our observations suggest that GSTT1 may play important role in male infertility. Our results added further epidemiologic evidence to the hypothesis that the genetic variant could alter GSTT1 levels in vivo. Future studies of determining the functional significance of differing GSTT1 levels in the etiology of spermatogenic failure will help clarify the hypothetically causal relationship between GSTT1 genotypes, GSTT1 expression levels, and male infertility.
These results suggest that GST gene polymorphisms has a relationship with male infertility, but the exact molecular mechanisms of GSTM1 and GSTT1 null polymorphisms on male infertility are unclear. In order to clarify, the variant region was further analyzed for putative transcriptional factor-binding sites through AliBaba version 2.1 software (http://www.gene-regulation.com/pub/programs/alibaba2/index.html). As shown in Figure S6 and Figure S7, the null genotype deletes a binding site for several transcription factors, such as:

Table 5 | Main characteristics of all studies of GSTM1 genotypes included in the meta-analysis

| First author | Year | Country | Ethnicity | Case | Control | Group | Present | Null | Present | Control |
|--------------|------|---------|-----------|------|---------|-------|--------|------|--------|--------|
| Chen SS      | 2002 | China   | Asian     | 96   | 46      | Total | 50     | 46    | 31     | 46     |
| Paracchini V | 2006 | Italy   | Caucasian | 57   | 44      | Total | 25     | 32    | 16     | 35     |
| Aydemir B    | 2007 | Turkey  | Caucasian | 52   | 60      | Total | 25     | 27    | 32     | 53     |
| Dhillon VS   | 2007 | India   | Asian     | 179  | 200     | Total | 120    | 59    | 124    | 62     |
| Aydos SE     | 2009 | Turkey  | Caucasian | 110  | 105     | Total | 51     | 59    | 63     | 60     |
| Ichikawa K   | 2009 | Japan   | Asian     | 274  | 101     | Total | 115    | 159   | 48     | 53     |
| Finotti AC   | 2009 | Brasil  | Caucasian | 128  | 105     | Total | 40     | 88    | 41     | 64     |
| OAT+         |      |         |           |      |         |       | 34     | 77    | 64     | 61     |
| Polonikov AV | 2010 | Russian | Caucasian | 203  | 227     | Total | 89     | 114   | 107    | 120    |
| Chen W       | 2010 | China   | Asian     | 75   | 36      | Total | 31     | 44    | 24     | 12     |
| Safarinejad MR| 2010| Iranian | Asian     | 166  | 166     | Total | 93     | 73    | 120    | 46     |
| Tirumala Vani G| 2010| India   | Asian     | 42   | 43      | Total | 23     | 19    | 34     | 9      |
| Volk M       | 2011 | Slovenia| Caucasian | 187  | 194     | Total | 90     | 97    | 102    | 92     |
| OAT+         |      |         |           |      |         |       | 44     | 57    | 56     | 44     |
| Salehi Z     | 2011 | Iran    | Asian     | 150  | 200     | Total | 58     | 92    | 134    | 66     |
| Jaiswal D    | 2012 | India   | Asian     | 113  | 91      | Total | 84     | 29    | 60     | 31     |
| Tang K       | 2012 | China   | Asian     | 65   | 30      | Total | 34     | 31    | 17     | 13     |
| Xu XB        | 2013 | China   | Asian     | 353  | 201     | Total | 115    | 238   | 85     | 116    |
| OAT+         |      |         |           |      |         |       | 64     | 138   | 88     | 116    |
| This study   | 2012 | China   | Asian     | 1476 | 895     | Total | 920    | 556   | 523    | 372    |
| OAT+         |      |         |           |      |         |       | 145    | 93    | 93     | 93     |
| Azooospermia |      |         |           |      |         |       | 171    | 76    | 171    | 76     |

OAT: oligoasthenoteratozoospermia.
In our stratified analyses of significant association between the genotype and male infertility, subgroup analysis stratified by ethnicity, they did not found any SCIENTIFIC REPORTS as NF-1, SP1 and SRF. For NF-1, in vitro experiments have shown that it acts both as transcriptional repressor and activator, depending on the context of target gene expression\(^{33}\). SP1, one of the most important transcription factor in the absence encoding protein, is known to influence on cell growth, apoptosis, and chromatin remodeling\(^{33}\). In this case, we infer that the GSTM1 null polymorphism, we only found significant influence on male infertility. Meta-analysis showed that GSTM1 null polymorphism could regulate relevant gene expression by deletion of transcription factors and in turn affect the sperm maturation process and male fertility. Whether this indeed the case requires further investigation.

A recent meta-analysis on GSTM1 polymorphism included fourteen studies and concluded the association between this polymorphism and male infertility\(^{34}\), the overall analysis is similar to ours. In the subgroup analysis stratified by ethnicity, they did not found any significant association between the genotype and male infertility. In our stratified analyses of GSTM1 null polymorphism, we found a significant influence on male infertility risk in both Asian and Caucasian groups. Additionally, in the stratified analyses of GSTTI null polymorphism, we only found significant influence on male infertility risk in Asian. Inclusion in our meta-analysis of few recent studies and data from our case-control study could be responsible for differences of results.

To the best of our knowledge, this is the largest and most comprehensive meta-analysis undertaken so far for quantitative analyses between the role of the GSTM1 and GSTT1 polymorphisms and male infertility risk. The present meta-analysis, including 3981 cases and 2953 controls from 19 case-control studies, explored the association between GSTM1 and GSTT1 null/present polymorphisms and male infertility. Meta-analysis showed that GSTM1 null genotype was significantly associated with male infertility risk, which was consistent with four previous meta-analysis\(^{34–37}\). Nevertheless, a significant association was only found between GSTTI null genotype and male

| Table 6 | Main characteristics of all studies of GSTT1 genotypes included in the meta-analysis |
|---------|----------------------------------|--------|--------|--------|--------|--------|
| First author | Year | Country | Ethnicity | Case | Control | Group | Present | Null | Present | Null |
| Wu QF | 2007 | China | Asian | 74 | 53 | Total | 19 (33.1) | 55 (66.9) | 26 (50.9) | 27 (49.1) |
| Wu QF | 2008 | China | Asian | 181 | 156 | Total | 60 (33.1) | 121 (66.9) | 80 (51.3) | 76 (48.7) |
| Aydos SE | 2009 | Turkey | Caucasian | 110 | 105 | Total | 90 (81.8) | 20 (18.2) | 85 (81.0) | 20 (19.0) |
| Ichikawa K | 2009 | Japan | Asian | 274 | 101 | Total | 148 (54.0) | 126 (46) | 50 (49.5) | 51 (50.5) |
| Finotti AC | 2009 | Brasil | Caucasian | 128 | 105 | Total | 54 (42.2) | 74 (57.8) | 65 (61.9) | 40 (38.1) |
| Polonikov AV | 2010 | Russian | Caucasian | 203 | 227 | Total | 202 (95.9) | 1 (0.05) | 198 (87.2) | 29 (12.8) |
| Jaiswal D | 2010 | India | Asian | 113 | 91 | Total | 119 (71.7) | 47 (28.3) | 134 (65.9) | 34 (34.1) |
| Salehi Z | 2011 | Iran | Asian | 150 | 200 | Total | 152 (81.3) | 35 (18.7) | 148 (76.3) | 46 (23.7) |
| Volk M | 2011 | Slovenia | Caucasian | 187 | 194 | Total | 202 (99.5) | 1 (0.05) | 198 (87.2) | 29 (12.8) |
| Wu QF | 2007 | China | Asian | 74 | 53 | Total | 19 (33.1) | 55 (66.9) | 26 (50.9) | 27 (49.1) |
| Chen W | 2010 | China | Asian | 75 | 36 | Total | 27 (36.0) | 48 (64.0) | 15 (41.7) | 21 (58.3) |
| Safarinejad MR | 2010 | Iranian | Asian | 116 | 166 | Total | 119 (71.7) | 47 (28.3) | 134 (80.7) | 34 (19.3) |
| Volk M | 2011 | Slovenia | Caucasian | 187 | 194 | Total | 152 (81.3) | 35 (18.7) | 148 (76.3) | 46 (23.7) |
| Salehi Z | 2011 | Iran | Asian | 150 | 200 | Total | 152 (81.3) | 35 (18.7) | 148 (76.3) | 46 (23.7) |
| Jaiswal D | 2012 | India | Asian | 113 | 91 | Total | 107 (94.3) | 6 (5.7) | 79 (65.9) | 12 (34.1) |
| Tang K | 2012 | China | Asian | 65 | 30 | Total | 36 (55.4) | 29 (44.6) | 15 (50.0) | 15 (50.0) |
| Xu XB | 2013 | China | Asian | 353 | 201 | Total | 135 (38.2) | 218 (61.8) | 107 (53.2) | 94 (46.8) |
| This study | 2012 | China | Asian | 1476 | 895 | Total | 805 (54.5) | 671 (45.5) | 536 (59.9) | 359 (40.1) |

*OAT: oligoasthenoteratozoospermia.

as NF-1, SP1 and SRF. For NF-1, in vitro experiments have shown that it acts both as transcriptional repressor and activator, depending on the context of target gene expression\(^{33}\). SP1, one of the most important transcription factor in the absence encoding protein, is involved in many cellular processes, including cell differentiation, cell growth, apoptosis, and chromatin remodeling\(^{33}\). In this case, we infer that the GSTM1 and GSTT1 null polymorphism could regulate relevant gene expression by deletion of transcription factors and in turn affect the sperm maturation process and male fertility. Whether this indeed the case requires further investigation.

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| Table 7 | Main results for the GSTT1 and GSTM1 polymorphisms in the meta-analysis |
|---------|-----------------|--------|--------|--------|--------|--------|--------|--------|--------|
| GSTM1 | GSTT1 | Studies | OR [95% CI] | P | P for heterogeneity | I^2 (%) | Studies | OR [95% CI] | P | P for heterogeneity | I^2 (%) |
| Total | | 17 | 1.39 (1.14–1.70)* | <0.001 | 64.0 | 14 | 1.28 (0.97–1.69)* | 0.082 | <0.001 | 76.7 |
| Ethnic groups | | | | | | | | | | |
| Asian | | 11 | 1.51 (1.13–2.01)* | <0.001 | 75.2 | 10 | 1.44 (1.01–1.90)* | 0.009 | 0.001 | 69.6 |
| Caucasian | | 6 | 1.24 (1.00–1.52) | 0.580 | 0.0 | 4 | 0.72 (0.27–1.91)* | 0.505 | <0.001 | 86.9 |
| Sperm concentration | | | | | | | | | |
| OAT | | 4 | 1.53 (1.25–1.86)* | 0.001 | 0.988 | 0.0 | 6 | 1.41 (0.68–2.91)* | 0.616 | <0.001 | 85.6 |
| Azoozoospermia | | 4 | 1.04 (0.83–1.30) | 0.752 | 0.370 | 4.5 | 1.28 (0.65–2.50)* | 0.477 | <0.001 | 88.7 |

*Random-effects model was used when the P-value for heterogeneity test was ≤ 0.1 or I^2 ≥ 50%, otherwise the fixed-effect model was used.
infertility among Asians. Here, we speculate the following three hypotheses. Firstly, because of the function changes of GSTT1, those compounds which could have been decomposed by GSTT1 detoxification may have more impact on spermatogenesis. Secondly, GSTs are crucial for protecting testis against oxidative stress. Perhaps, the deletion of GSTT1 gene may be more likely to decline the protective role and increase the level of oxidative stress. Thirdly, due to the induction or repression changes of GSTT1 in sperm surface, it slightly results in the decreased level of sperm motility. Future research is needed to elucidate the mechanism of the impact of null polymorphism of GSTT1 gene on male infertility.

Several factors must be considered in the design of a reliable case-control study in the future. Large sample size with adequate power is one of the most important factors. The choice of the control population is also considered to be a crucial factor, because the possible different exposure to environmental toxicants should be considered. The use of population-based controls is more appropriate in the association study. In addition, some limitations of this meta-analysis should be acknowledged. First, in the subgroup analysis by sperm concentration of case, the number of OAT and azoospermia subgroups were relatively small, may not having enough statistical power to explore the real association. Second, our results were based on unadjusted estimates, while a more precise analysis should be conducted if all individual data was available, which would allow for the adjustment by other co-variants including age, BMI, smoking status, drinking status, environmental exposures and other lifestyle factors. Third, there were not enough studies in the Caucasian population in this meta-analysis. Finally, gene-gene and gene-environmental factors interactions were not addressed in this meta-analysis for the lack of sufficient data. Future studies may further assess the gene-gene and gene-environmental interactions. Additionally, concerning male infertility with multifactorial etiology, more studies or complete case-control studies, especially stratified by different ethnic background, environmental exposure or other risk factors, should be performed to clarify possible roles of GSTM1 and GSTT1 null polymorphisms in the pathogenesis of male infertility in the future.

Methods
Case-control study. Subject recruitment and sample collection. The study was approved by the Institutional Ethics Committee of Nanjing Medical University. All activities involving human subjects were done under full compliance with government policies and the Helsinki Declaration. Written informed consent was obtained from all study subjects. We sampled 1476 infertile men with normozoospermia, oligozoospermia and non-obstructive azoospermia, as well as 895 fertile controls in this study. These donors all came from the Affiliated Hospitals of Nanjing Medical University between March 2006 and July 2011 (NJMU Infertility Study). Infertile men had an infertility history of at least two years with confirmed normal gynecological assessment. Semen samples were obtained in private by masturbation into a sterile wide mouth and metal-free glass container after a recommended at least 3-day sexual abstinence (Abs). Routine semen analysis was carried out by light microscopy according to World Health Organization (WHO, 2010) guidelines with regard to sperm concentration (normal ≥ 15 x 10⁶ spermatozoa/ml), progressive motility (normal ≥ 32%) and sperm morphology using strict criteria (normal ≥ 14%). All infertile males underwent serum...
determination of estradiol (E2), testosterone (T), prolactin (PRL), luteinizing hormone (LH), and follicle-stimulating hormone (FSH). Men with a history of testicular carcinoma, microdeletions in Y-chromosomal AZF region, obstruction, varicocele, infection or other diseases that could affect fertility were excluded. In total, 2051 infertile patients were assessed, however, the inclusion criteria allowed us to select 1476 patients. The controls were healthy men with normal sperm parameters who had fathered at least one healthy child within a year without assisted reproductive measures during the same period as those of cases recruited in the same hospital. A scheduled interview was arranged for each subject to collect information, including personal background, lifestyle factors, occupational and environmental exposures, sexual and reproduction status, genetic risk factors, medical history and physical activity (e.g., exercise status). After interview, each subject donated a 5-mL

Figure 5 | Forest plot of the GSTM1 null polymorphism and male infertility risk in subgroup analysis. Studies are plotted according to the last name of the first author and followed by the publication year in parentheses. Horizontal lines represent 95% CI. Each square represents the OR point estimate and its size is proportional to the weight of the study. The diamond (and broken line) represents the overall summary estimate, with confidence interval given by its width. The unbroken vertical line is at the null value (OR = 1.0). CI, confidence interval; OR, odds ratio.

Figure 6 | Funnel plot for GSTM1 analysis to detect publication bias. Each dot represents a separate study for the indicated association. Location outside the delineated triangle (pseudo 95% confidence intervals) suggests a publication bias.
Peripheral blood sample for genetic testing. All of the infertile patients and healthy donors were all ethnically Han Chinese from East China.

**Genotyping.** Genomic DNA was isolated from leukocyte pellets of venous blood by proteinase K digestion and followed by phenol-chloroform extraction. Primers sequences for GSTM1 were 5'-GAACTCCCTGAAAAGCTAAAGC-3' (forward primer) and 5'-TGTTGGGCCTCAATAATACGGTGG-3' (reverse primer), which produced a 219 bp band. The GSTT1 primers were 5'-TTCCTTACTGGCCTCACATCATC-3' (forward primer) and 5'-TCACCGGTACATGCGGCAAGCA-3' (reverse primer), which produced a 480 bp band\(^2\). The presence of 219 bp and 480 bp fragments represent GSTM1 present genotype (+) and GSTT1 present phenotype (+), respectively. GSTM1 and GSTT1 null genotypes (−) are indicated by the absence of a 219 bp band and 480 bp band, respectively. As an internal positive control for successful PCR, β-globin (268 bp) was amplified with the primers 5'-GAACTCCCTGAAAAGCTAAAGC-3' (forward primer) and 5'-GAAGACCAGGACCGGACGATAC-3' (reverse primer). The PCR reactions were performed in a 25 μl reaction containing 0.5 μM each of forward and reverse primer, 200 μM dNTPs, 1× PCR buffer, 1.25 U of Taq Hot Start DNA Polymerase (BIOTECH CO., Ltd). The optical density was determined by measuring the absorbance at 562 nm. The absorbance was correlated against a standard curve.

**Enzyme-linked immunosorbent assay (ELISA).** Serum samples were stored at −20°C until serum GSTM1 and GSTT1 levels were measured by ELISA kit (CUSABIO BIOTECH CO., Ltd). The optical density was determined by measuring the absorbance at 450 nm. The absorbance was correlated against a standard curve.

**Meta-analysis. Identification of studies.** Studies addressing the association between polymorphisms of GSTM1 and GSTT1 and risk of idiopathic male infertility were identified by searching for articles in the PubMed and Chinese Biomedical Literature Database (CBM) Database (the main Chinese medical literature retrieval system), until 1 Jun 2013. A systematic search was done on published literature using the keywords ‘(GSTM1 or GSTT1) and (polymorphism or polymorphisms or variant or mutation) and (male infertility or azoospermia or oligozoospermia)’. Additional studies were identified by a hand search from reference of original studies or review articles on this topic.

**Eligibility criteria.** Studies included in our meta-analysis had to meet all of the following criteria: (i) studied on human beings; (ii) each trial is an independent case-control study; (iii) had detailed genotype frequency of cases and controls or could be calculated from the article text; (iv) inclusion of the patients was done according to the standard diagnosis parameter. We identified 54 potentially relevant research papers using our search strategies, but 37 did not meet the inclusion criteria. Along with the present study from China, data for meta-analysis were available from 19 studies, including 3981 cases and 2953 controls.

**Data extraction.** Information was carefully extracted from all eligible publications by investigators according to the inclusion criteria listed above. The following information were extracted from each study: name of the first author, year of publication, country, journal, racial descent of study population, demographics, number of cases and controls, genotyping methods, genotype of origin, ethnicity, number of cases and controls, infertility type and allele distributions.

**Statistical analysis.** Differences in selected demographic variables, smoking and alcohol status between cases and controls were evaluated by the χ² test. Student’s t-test was used to evaluate continuous variables, including age and body mass index (BMI). Hardy-Weinberg equilibrium was tested by a goodness-of-fit χ² test. Statistical significance of differences was estimated as odds ratios (OR) and 95% confidence intervals (95% CI) using unconditional multivariate logistic regression adjusted for age, BMI, smoking status, alcohol drinking and Abs. Differences in GSTM1 and GSTT1 expression levels of serum were tested using Mann-Whitney test. In meta-analysis, a fixed-effect model using the Mantel-Haenszel method and a random-effects model using the DerSimonian and Laird method were used to combine values from studies. If the P value for heterogeneity was > 0.10 and I² < 50%, indicating an absence of heterogeneity between studies, we used the fixed-effect model to evaluate the summary OR. In contrast, if the P value for heterogeneity was ≤ 0.10 or I² ≥ 50%, indicating a high extent of heterogeneity between studies, we used the random-effect model to evaluate the summary OR. Subgroup analyses were further performed by ethnicity (Asian, Caucasian) and case types (oligoasthenoteratozoospermia (OAT), Azoosperma). Meta-regression was applied to illustrate potential reasons for the between-study heterogeneity. Begg’s and Egger’s test and inverted funnel plots were utilized to provide a diagnosis of publication bias (linear regression asymmetry test). All statistical analyses were carried out using STATA 12.0 (STATA Corp, LP) and P < 0.05 was considered to be significant.

Figure 7 | Sensitivity of GSTM1 individual studies used in the meta-analysis. The circles represent odds ratio of the overall analyses if the individual study is not included. The lines represent the confidence intervals for the individual study.

| Study | Lower CI Limit | Estimate | Upper CI Limit |
|-------|---------------|----------|---------------|
| Chen SS (2002) | 1.09 | 1.14 | |
| Paracchini V (2006) | 1.39 | | 1.70 |
| Aydemir B (2007) | | | |
| Dhillon VS (2007) | | | |
| Aydos SE (2009) | | | |
| Ichikawa K (2009) | | | |
| Finotti AC (2009) | | | |
| Polonikov AV (2010) | | | |
| Chen W (2010) | | | |
| Safarinejad MR (2010) | | | |
| Tirumala Vani G (2010) | | | |
| Volk M (2011) | | | |
| Salehi Z (2011) | | | |
| Jaiswal D (2012) | | | |
| Tang K (2012) | | | |
| Xu XB (2013) | | | |
| This study ( ) | | | |

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Author contributions

Conceived and designed the experiments: WW JL. Searched for and selected the publications: BY JL QT. Analyzed the data: SZ JL CL DW SH. Contributed reagents/materials/analysis tools: WW DC YX DC JS XW. Wrote and revised the paper: WW.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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CORRIGENDUM: GSTM1 and GSTT1 null polymorphisms and male infertility risk: an updated meta-analysis encompassing 6934 subjects

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In the original version of the Article the affiliations for the authors Simin Zhang, Beilei Yuan, Jing Li, Di Wu, Chuncheng Lu, Yankai Xia and Xinru Wang were incorrectly listed to include 1State Key Laboratory of Reproductive Medicine, Wuxi Maternity and Child Health Care Hospital Affiliated to Nanjing Medical University, Wuxi 214002, China. In addition the affiliation for Daozhen Chen was incorrectly listed as 3Nanjing Maternal and Child Health Care Hospital Affiliated to Nanjing Medical University, Nanjing 210004, China. The correct affiliations appear above, and have been corrected in the HTML and PDF versions of this Article.