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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[1]. Although several causes have been identified for impaired male fertility[2], 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[3].

Sperms are susceptible to oxidative damage and excessive reactive oxygen species (ROS) generation may lead to subfertility or infertility[4]. 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[5]. 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[6,7]. 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[8]. 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[9–25]. 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)[a]	Case (n = 1476)[b]
Age (years, mean ± SD)	29.89 ± 4.34	29.90 ± 4.89
BMI (kg/m², mean ± SD)[c]	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 (%)]		
<4	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 (10^6/ml)[e]	41.42 (29.22–68.90)	34.98 (6.60–75.59)*
Sperm motility (%)[e]	62.72 (39.86–78.07)	43.67 (16.35–65.60)*

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

studies till date have analyzed these polymorphisms in small sample size, leading to over-estimation of the association. Therefore, we analyzed *GSTM1* and *GSTT1* polymorphisms in a large sample size (n = 2371) to elucidate the correlation between this polymorphic variants and male infertility in Han Chinese population. Additionally, the expression levels of GSTM1 and GSTT1 were examined in serum of idiopathic infertile males and fertile controls with different genotypes. To estimate the effect of polymorphisms and risk of male infertility, as well as to quantify the potential between-study heterogeneity, we also conducted a meta-analysis on 19 eligible and published case-control studies. Together with our data, this meta-analysis has a total of 3981 cases and 2953 controls.

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; $P = 0.004$). However, there were 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 (%)	OR (95% CI)	*P*
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**

Abbreviations: OR, odds ratio; 95% CI, 95% confidence interval.
All *P*-values were adjusted for age, smoking, drinking, BMI and Abs.

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

GSTM1	GSTT1	Control n = 895 (%)	Case n = 1476 (%)	OR (95% CI)	*P*
+	+	373 (41.7)	460 (31.2)	reference	
+	−	196 (21.9)	433 (29.3)	1.79 (1.44–2.23)	<0.0001
−	+	163 (18.2)	345 (23.4)	1.71 (1.36–2.16)	<0.0001
−	−	163 (18.2)	238 (16.1)	1.18 (0.93–1.51)	0.171

Abbreviations: +, positive genotype; −, null genotype; OR, odds ratio; 95% CI, 95% confidence interval.

Table 4 | Genotype frequencies of *GSTM1* and *GSTT1* among infertile patients and fertile controls and their association with male infertility

	Control n = 895 (%)	Case n = 1476 (%)								
		Normozoospermia n = 991 (%)	OR (95% CI)	*P*	Oligozoospermia n = 238 (%)	OR (95% CI)	*P*	Azoospermia n = 247 (%)	OR (95% CI)	*P*
GSTM1										
Present	569 (63.6)	604 (61.0)			126 (52.9)			163 (66.0)		
Null	326 (36.4)	387 (39.0)	1.12 (0.93–1.35)	0.231	112 (47.1)	**1.55 (1.15, 2.08)**	**0.004**	84 (34.0)	0.92 (0.68–1.24)	0.579
GSTT1										
Present	536 (59.9)	543 (54.8)			136 (57.1)			126 (51.0)		
Null	359 (40.1)	448 (45.2)	**1.23 (1.03–1.48)**	**0.025**	102 (42.9)	1.11 (0.82–1.48)	0.504	121 (49.0)	**1.58 (1.18–2.11)**	**0.002**

All *P*-values were adjusted for age, smoking, drinking, BMI and Abs.

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 studies[9–25] 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 studies[9–25] 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 azoospermia (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, 1.10–1.90; $P = 0.009$) (Figure S2). However, the association was not observed in the overall

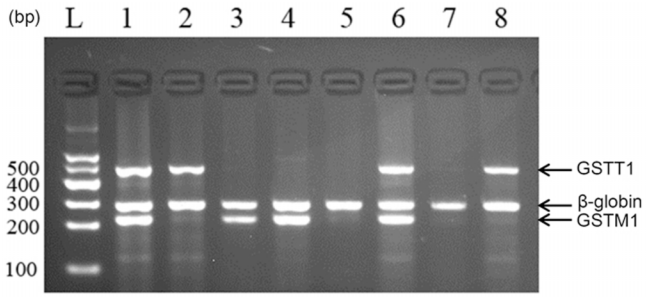

Figure 1 | A representative image of multiplex PCR analysis of *GSTM1* and *GSTT1* gene polymorphisms. Lane: L, 100 bp DNA; lanes 1 and 6, *GSTT1+/GSTM1+*; lanes 2 and 8, *GSTT1+/GSTM1-*; lanes 3 and 4, *GSTT1-/GSTM1+*; lanes 6 and 7, *GSTT1-/GSTM1-*. A fragment of 268 bp indicates the internal control.

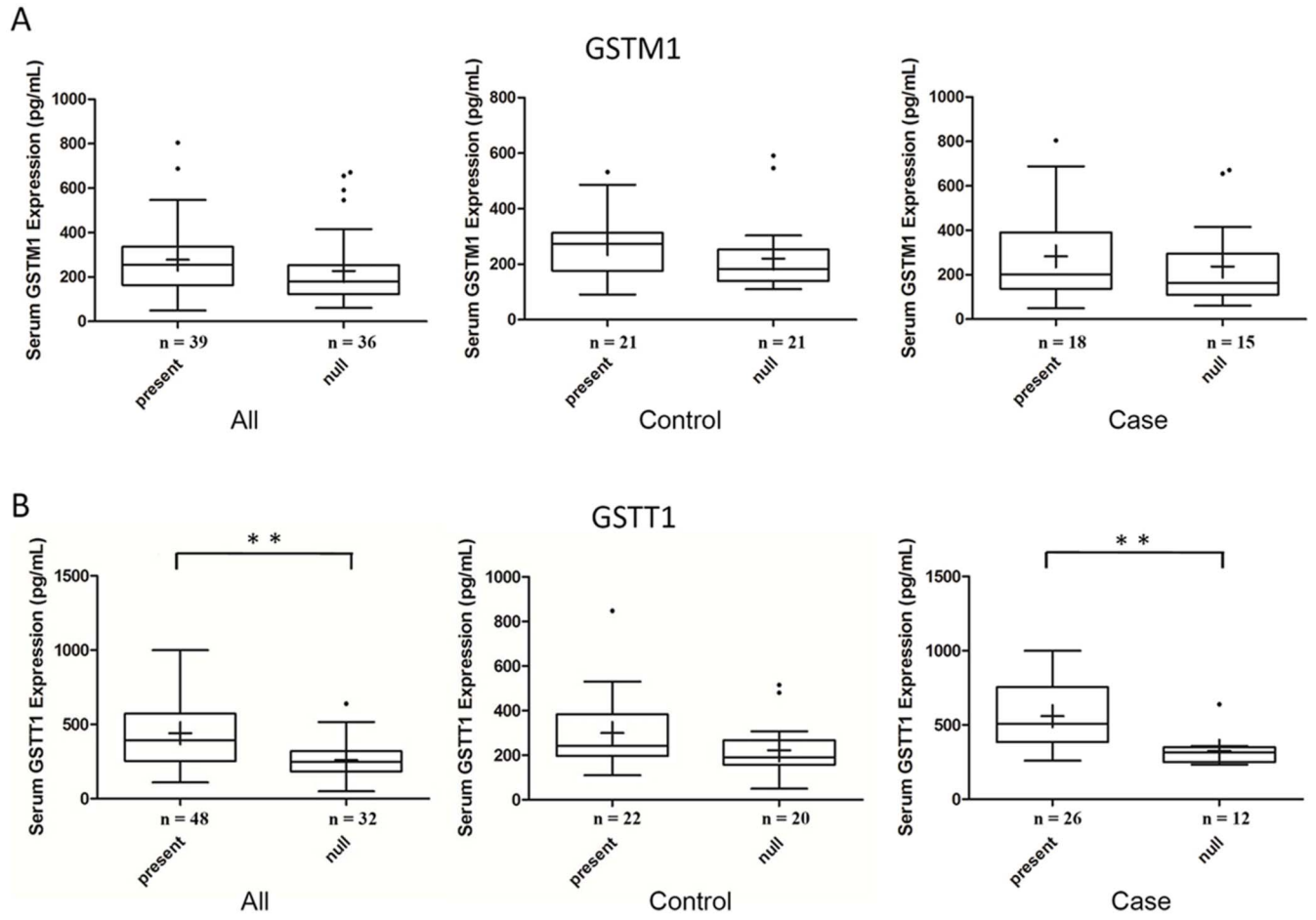

Figure 2 | The expression levels of the serum *GSTM1* (A) and *GSTT1* (B) in different genotypes. Data are given in Tukey Box plots showing median (−) and mean (+) values. Significant difference is marked with $^{**}P < 0.01$.

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 Eggar's test ($P = 0.269$) did not imply any evidence of publication bias. For *GSTT1*, neigher Begg's test ($P = 0.189$) nor Eggar'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 infertile. 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.

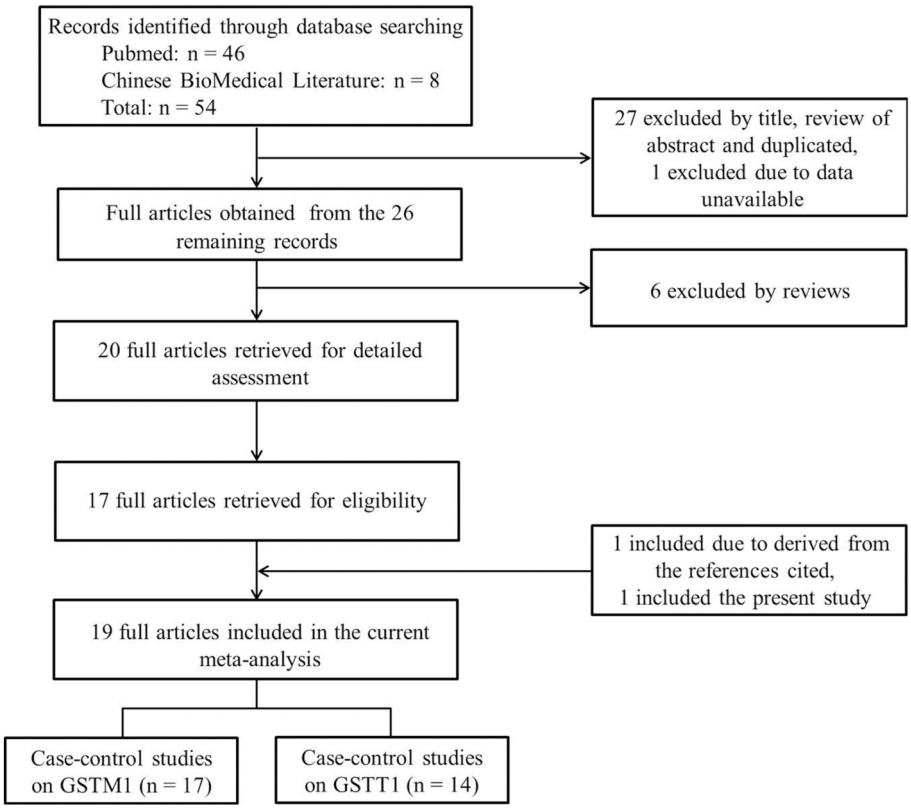

Figure 3 | **Flow diagram of the study selection process.**

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

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

							Case		Control	
First author	Year	Country	Ethnicity	Case	Control	Group	Present	Null	Present	Null
Chen SS	2002	China	Asian	96	46	Total	50 (52.1)	46 (47.9)	31 (67.4)	15 (32.6)
Paracchini V	2006	Italy	Caucasian	57	44	Total	25 (43.9)	32 (56.1)	16 (35.6)	29 (64.4)
Aydemir B	2007	Turkey	Caucasian	52	60	Total	25 (48.1)	27 (51.9)	32 (53.3)	28 (46.7)
Dhillon VS	2007	India	Asian	179	200	Total	120 (67.0)	59 (33.0)	124 (62.0)	76 (38.0)
Aydos SE	2009	Turkey	Caucasian	110	105	Total	51 (46.4)	59 (53.6)	63 (60.0)	42 (40.0)
Ichioka K	2009	Japan	Asian	274	101	Total	115 (42.0)	159 (58.0)	48 (47.5)	53 (52.5)
Finotti AC	2009	Brasil	Caucasian	128	105	Total	40 (31.2)	88 (68.8)	41 (39.0)	64 (61.0)
						OAT[a]	34 (30.6)	77 (69.4)		
						Azoospermia	6 (35.3)	11 (64.7)		
Polonikov AV	2010	Russian	Caucasian	203	227	Total	89 (43.8)	114 (56.2)	107 (47.1)	120 (52.9)
Chen W	2010	China	Asian	75	36	Total	31 (41.3)	44 (58.7)	24 (66.7)	12 (33.3)
Safarinejad MR	2010	Iranian	Asian	166	166	Total	93 (56.0)	73 (44.0)	120 (72.3)	46 (27.7)
Tirumala Vani G	2010	India	Asian	42	43	Total	23 (54.8)	19 (45.2)	34 (79.1)	9 (20. 9)
Volk M	2011	Slovenia	Caucasian	187	194	Total	90 (48.1)	97 (51.9)	102 (52.6)	92 (47.4)
						OAT[a]	44 (43.6)	57 (56.4)		
						Azoospermia	46 (53.5)	40 (46.5)		
Salehi Z	2011	Iran	Asian	150	200	Total	58 (38.7)	92 (61.3)	134 (67.0)	66 (33.0)
Jaiswal D	2012	India	Asian	113	91	Total	84 (73.8)	29 (26.2)	60 (65.9)	31 (34.1)
Tang K	2012	China	Asian	65	30	Total	34 (52.3)	31 (47.7)	17 (56.7)	13 (43.3)
Xu XB	2013	China	Asian	353	201	Total	115 (32.6)	238 (67.4)	85 (42.3)	116 (57.7)
						OAT[a]	64 (31.7)	138 (68.3)		
						Azoospermia	51 (33.8)	100 (66.2)		
This study	2012	China	Asian	1476	895	Total	920 (62.3)	556 (37.7)	523 (58.4)	372 (41.6)
						OAT[a]	145 (60.9)	93 (39.1)		
						Azoospermia	171 (69.2)	76 (30.8)		

OAT: oligoasthenoteratozoospermia.

Table 6 | Main characteristics of all studies of *GSTT1* genotypes included in the meta-analysis

First author	Year	Country	Ethnicity	Case	Control	Group	Case		Control	
							Present	Null	Present	Null
Wu QF	2007	China	Asian	74	53	Total	19 (33.1)	55 (66.9)	26 (50.9)	27 (49.1)
						OAT[a]	11 (27.5)	29 (72.5)		
						Azoospermia	8 (23.5)	26 (76.5)		
Wu QF	2008	China	Asian	181	156	Total	60 (33.1)	121 (66.9)	80 (51.3)	76 (48.7)
						OAT[a]	36 (35.0)	67 (65.0)		
						Azoospermia	24 (30.8)	54 (69.2)		
Aydos SE	2009	Turkey	Caucasian	110	105	Total	90 (81.8)	20 (18.2)	85 (81.0)	20 (19.0)
Ichioka 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)
						OAT[a]	46 (38.0)	75 (62.0)		
						Azoospermia	8 (47.1)	9 (52.9)		
Polonikov AV	2010	Russian	Caucasian	203	227	Total	202 (99.5)	1 (0.05)	198 (87.2)	29 (12.8)
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	166	166	Total	119 (71.7)	47 (28.3)	134 (80.7)	32 (19.3)
Volk M	2011	Slovenia	Caucasian	187	194	Total	152 (81.3)	35 (18.7)	148 (76.3)	46 (23.7)
						OAT[a]	81 (80.2)	20 (19.8)		
						Azoospermia	71 (82.6)	15 (17.4)		
Salehi Z	2011	Iran	Asian	150	200	Total	99 (66.0)	51 (34.0)	166 (83.0)	34 (17.0)
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)
						OAT[a]	85 (42.1)	117 (57.9)		
						Azoospermia	50 (33.1)	101 (66.9)		
This study	2012	China	Asian	1476	895	Total	805 (54.5)	671 (45.5)	536 (59.9)	359 (40.1)
						OAT[a]	136 (57.1)	102 (42.9)		
						Azoospermia	126 (51.0)	121 (49.0)		

[a]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[32]. 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.

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 *GSTT1* 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 *GSTT1* null genotype and male

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)**[a]	**0.001**	<0.001	64.0	14	1.28 (0.97–1.69)[a]	0.082	<0.001	76.7
Ethnic groups										
Asian	11	**1.51 (1.13–2.01)**[a]	**0.005**	<0.001	75.2	10	**1.44 (1.01–1.90)**[a]	**0.009**	0.001	69.6
Caucasian	6	**1.24 (1.00–1.52)**	**0.046**	0.580	0.0	4	0.72 (0.27–1.91)[a]	0.505	<0.001	86.9
Sperm concentration of case group										
OAT	4	**1.53 (1.25–1.86)**	**<0.001**	0.988	0.0	6	1.41 (0.68–1.91)[a]	0.616	<0.001	85.6
Azoospermia	4	1.04 (0.83–1.30)	0.752	0.370	4.5	6	1.28 (0.65–2.50)[a]	0.477	<0.001	88.7

[a]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.

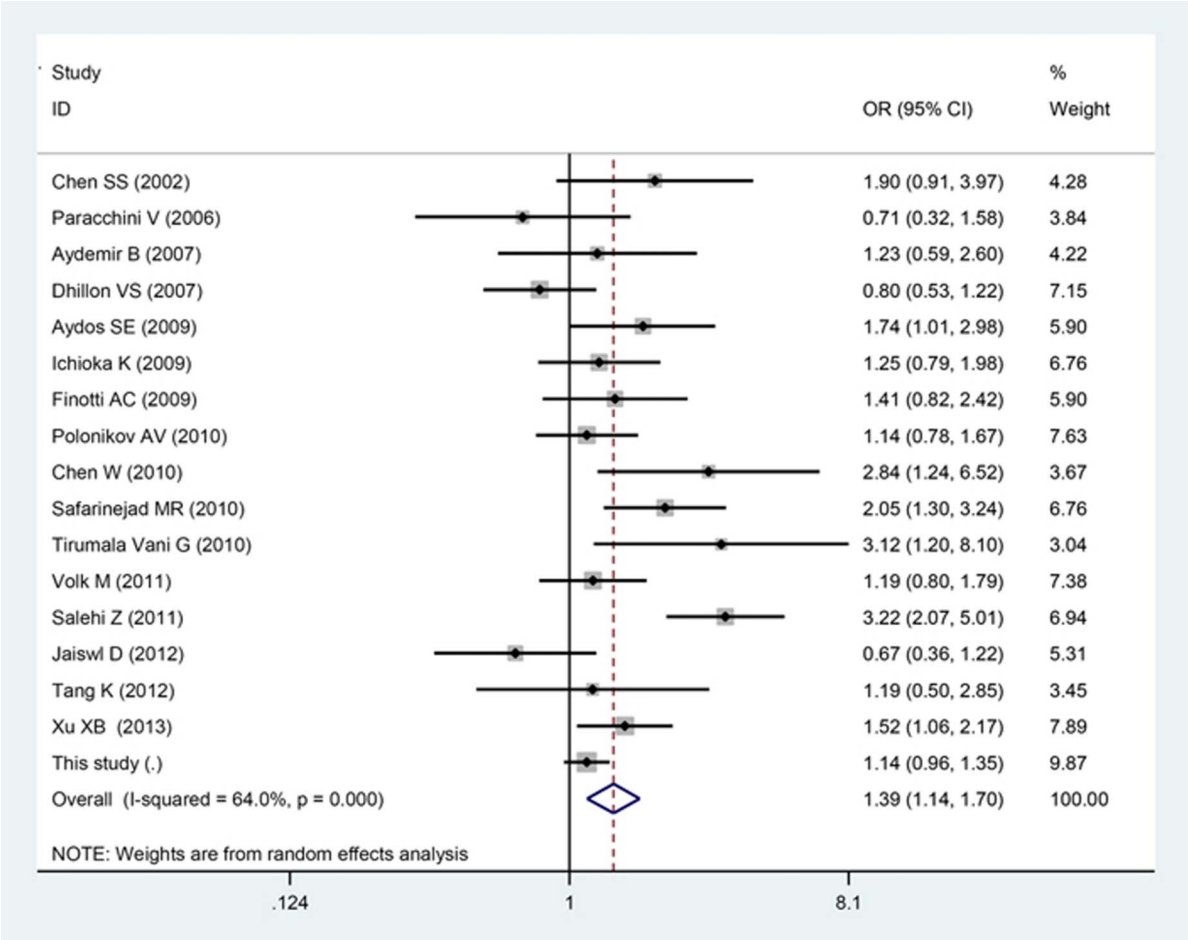

Figure 4 | Forest plot of the *GSTM1* null polymorphism and male infertility risk in overall analysis. Studies are plotted according to the last name of the first author and followed by the publication year in parentheses. Horizontal lines represent 95% CI. Each square represents the OR point estimate 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.

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[14]. 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 $\geq 15 \times 10^6$ spermatozoa/ml), progressive motility (normal $\geq 32\%$) and sperm morphology using strict criteria (normal $\geq 14\%$). All infertile males underwent serum

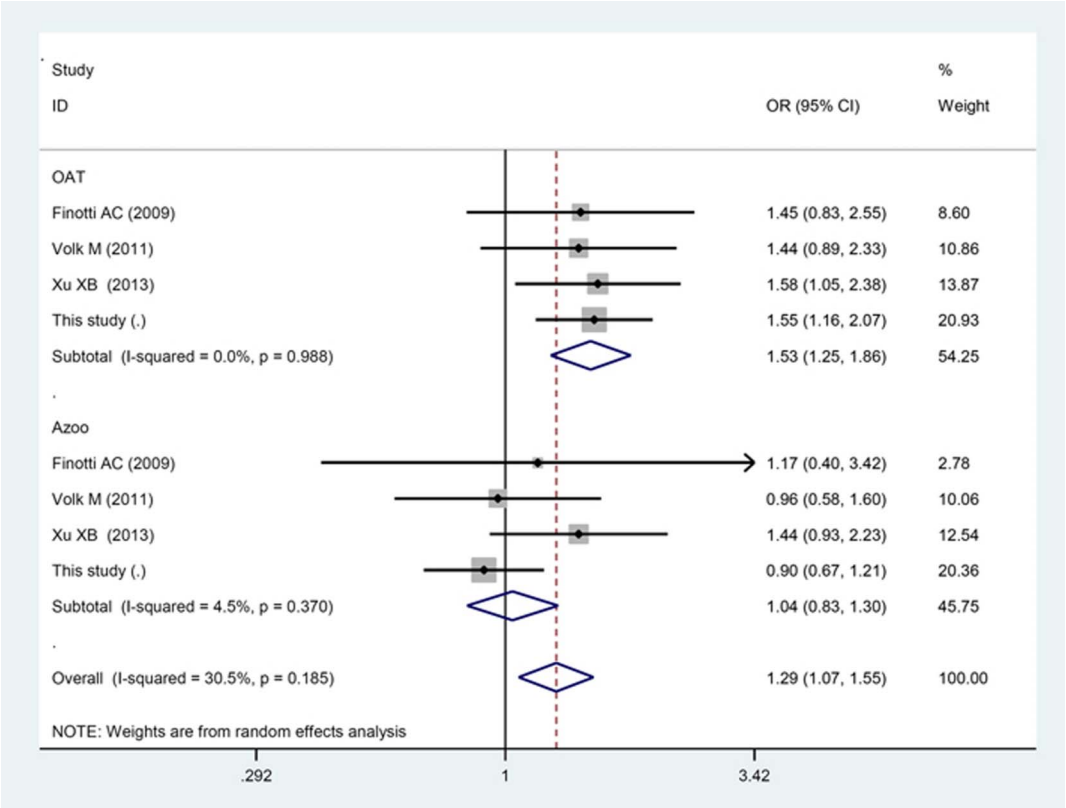

Figure 5 | Forest plot of the *GSTM1* null polymorphism and male infertility risk in subgroup analysis. Studies are plotted according to the last name of the first author and followed by the publication year in parentheses. Horizontal lines represent 95% CI. Each square represents the OR point estimate 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.

determination of estradiol (E_2), 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

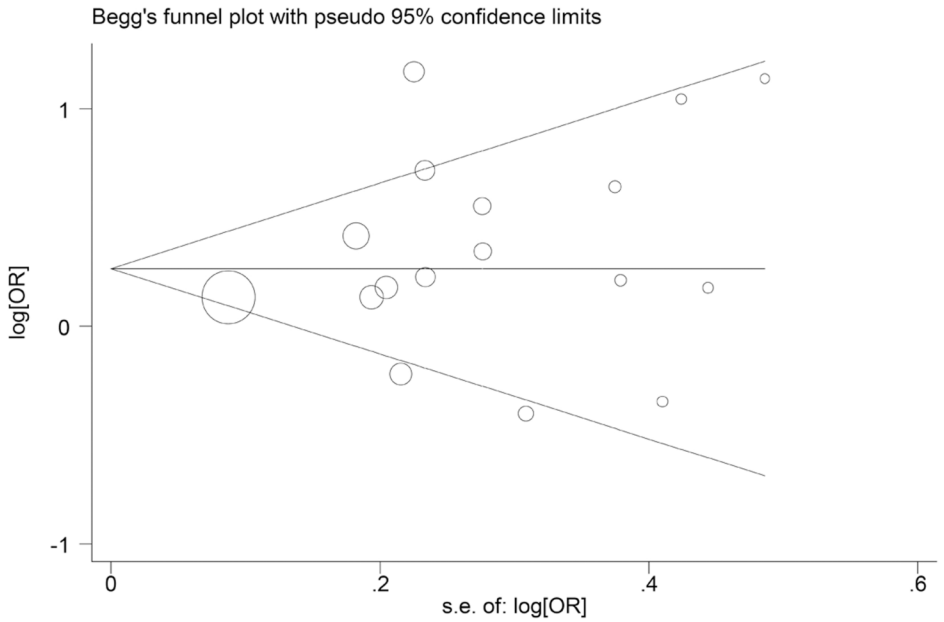

Figure 6 | Funnel plot for *GSTM1* analysis to detect publication bias. Each dot represents a separate study for the indicated association. Location outside the delineated triangle (pseudo 95% confidence intervals) suggests a publication bias.

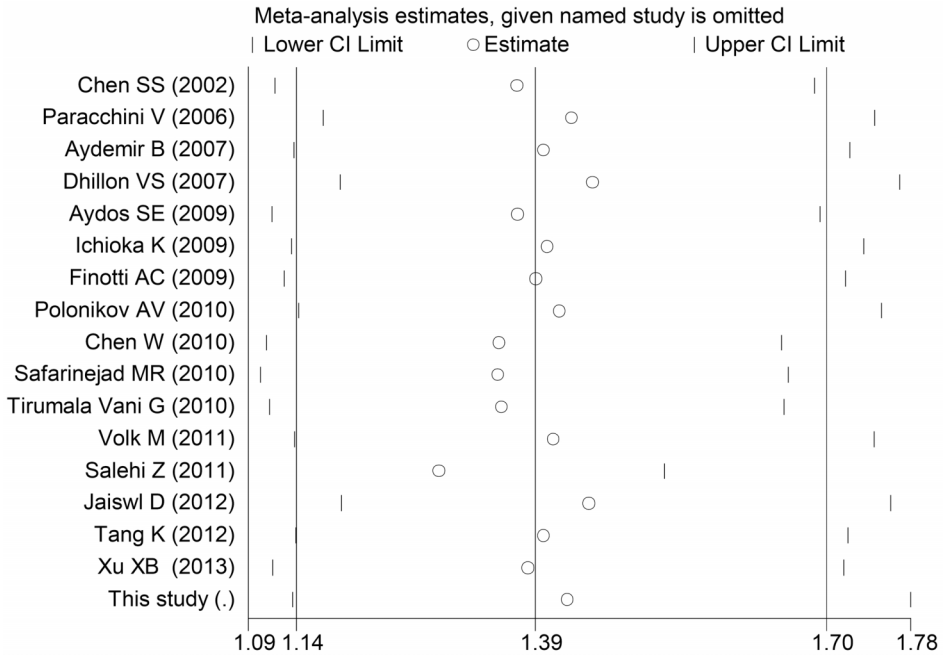

Figure 7 | Sensitivity of *GSTM1* individual studies used in the meta-analysis. The circles represent odds ratio of the overall analyses if the individual study is not included. The lines represent the confidence intervals for the individual study.

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. Primer sequences for *GSTM1* were 5'-GAACTCCCTGAAAAGCTAAAGC-3' (forward primer) and 5'-GTTGGGCTCAAATATACGGTGG-3' (reverse primer), which produced a 219 bp band. The *GSTT1* primers were 5' -TTCCTTACTGGTCCTCACATCTC-3' (forward primer) and 5'-TCACCGGATCATGGCCAGCA-3' (reverse primer), which produced a 480 bp band[38]. The presence of 219 bp and 480 bp fragment 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'-CAACTTCATCCACGTTCACC- 3' (forward primer) and 5' –GAAGAGCCAAGGACAGTTAC-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 (Takara Bio, Tokyo, Japan) and 100 ng template DNA under the following conditions: 10 min of denaturation at 95°C followed by 40 cycles of 1 minute at 95°C, 1 minute at 56°C, 1 minute at 72°C and a final extension for 10 minute at 72°C. The amplification products were separated on 2% agarose gels, stained with ethidium bromide. Representative examples are shown in Figure 1.

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 (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 χ^2 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 χ^2 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 $I^2 < 50\%$, 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 $I^2 \geq 50\%$, 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), Azoospermia). 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)[39]. All statistical analyses were carried out using STATA 12.0 (STATA Corp, LP) and $P < 0.05$ was considered to be significant.

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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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