Gene copy number alterations in the azoospermia-associated AZFc region and their effect on spermatogenic impairment

Chuncheng Lu1,2,†, Jie Jiang1,2,3,†, Ruyang Zhang2,3,†, Ying Wang1,2, Miaofei Xu1,2, Yufeng Qin1,2, Yuan Lin1,2,3, Xuejiang Guo1, Bixian Ni1,2,3, Yang Zhao3, Nancy Diao4, Feng Chen3, Hongbing Shen1,2,3, Jiahao Sha1, Yankai Xia1,2,e, Zhibin Hu1,2,3, and Xinru Wang1,2

1State Key Laboratory of Reproductive Medicine, Institute of Toxicology, Nanjing Medical University, Nanjing 210029, China 2Key Laboratory of Modern Toxicology of Ministry of Education, School of Public Health, Nanjing Medical University, Nanjing 210029, China 3Department of Epidemiology and Biostatistics, School of Public Health, Nanjing Medical University, Nanjing, China 4Department of Environmental Health, Harvard School of Public Health, Harvard University, Boston, MA, USA

*Correspondence address. Tel: +86-25-86862845; Fax: +86-25-86862847; E-mail: yankaixia@njmu.edu.cn

Submitted on November 22, 2013; resubmitted on May 21, 2014; accepted on June 9, 2014

ABSTRACT: The azoospermia factor c (AZFc) region in the long arm of human Y chromosome is characterized by massive palindromes. It harbors eight multi-copy gene families that are expressed exclusively or predominantly in testis. To assess systematically the role of the AZFc region and these eight gene families in spermatogenesis, we conducted a comprehensive molecular analysis (including Y chromosome haplogrouping, AZFc deletion typing and gene copy quantification) in 654 idiopathic infertile men and 781 healthy controls in a Han Chinese population. The b2/b3 partial deletion (including both deletion-only and deletion-duplication) was consistently associated with spermatogenic impairment. In the subjects without partial AZFc deletions, a notable finding was that the frequency of DAZ and/or BPY2 copy number alterations in the infertile group was significantly higher than in the controls. Combined patterns of DAZ and/or BPY2 copy number abnormality were associated with spermatogenic impairment when compared with the pattern of all AZFc genes with common level copies. In addition, in Y chromosome haplogroup O1 (Y-hg O1), the frequency of copy number alterations of all eight gene families was significantly higher in the case group than that in the control group. Our findings indicate that the DAZ, BPY2 genes may be prominent players in spermatogenesis, and genomic rearrangements may be enriched in individuals belonging to Y-hg O1. Our findings emphasize the necessity of routine molecular analysis of AZFc structural variation during the workup of azoospermia and/or oligozoospermia, which may diminish the genetic risk of assisted reproduction.

Key words: spermatogenic impairment / AZFc / copy number alteration / Y chromosome haplogroup

Introduction

Infertility affects about one in six couples in preparation for pregnancy, with the man being responsible for approximately half of the cases (Guzick et al., 2001). Since the identification of azoospermia factors (AZFs) in 1970s, the AZFc (MIM 415000) region in the distal long arm of human Y chromosome represents a key genetic determinant for spermatogenesis due to its involvement in germ cell development (Tiepolo and Zuffardi, 1976). The AZFc region consists almost entirely of very long repeat units, also known as amplicons (Kuroda-Kawaguchi et al., 2001). The complex structure of AZFc predisposes to a series of genomic rearrangements mediated by non-allelic homologous recombination (NAHR) between amplicons, including deletions, duplications and their combinations (Yen, 2001; Carvalho et al., 2003; Repping et al., 2003, 2004; Vogt, 2005; Lin et al., 2007), which can lead to the copy number alterations of the AZFc genes.

The AZFc region contains eight multi-copy gene families, including deleted in azoospermia (DAZ), basic protein Y2 (BPY2), chima domain on Y (CDY1), Golgi autoantigen, golgin subfamily a2 like Y (GOLGA2LY), chondroitin sulfate proteoglycan 4 like Y (CSPG4LY), testis-specific transcript,
AZFc gene copy number and spermatogenic impairment

Materials and Methods

Studied populations

Study subjects were volunteers from the affiliated hospitals of Nanjing Medical University between July 2007 and July 2010 (NMU Infertile Study). The protocol and consent form were approved by the Institutional Review Boards of Nanjing Medical University. All activities involving human subjects were done under full compliance with government policies and the Helsinki Declaration.

The cases were male patients with infertility, who sought treatment in the Center of Clinical Reproductive Medicine, and were included in this study retrospectively. Some cohorts within the population have been included in previously published data (Wu et al., 2007; Lu et al., 2011). Individuals with obstructive azoospermia, abnormal karyotype, cryptorchidism, secondary sterility cases, medical history of risk factors for infertility and those receiving treatment for infertility were excluded. All the subjects with Y chromosome microdeletions of azoospermia factor regions (AZFa, AZFb and AZFc) were also excluded (Sun et al., 2000; Kuroda-Kawaguchi et al., 2001; Repping et al., 2002).

The semen analysis for sperm concentration, motility and morphology was performed following the World Health Organization criteria (Cooper et al., 2010). Eventually, 654 idopathic infertility patients included both non-obstructive azoospermia (no sperms in the ejaculate even after centrifugation) and oligozoospermia (sperm counts from 0.1 to 15 × 10⁶/ml) were recruited to this study.

The controls were healthy and fertile young men who had fathered one or more healthy children without using assisted reproductive techniques from the same hospital during the same period. Eventually, 781 healthy controls, aged from 26 to 40 years old, were recruited to this study. The fertile controls and infertile cases were well matched for age; mean ± SD, 29.8 ± 3.68 and 29.9 ± 3.76 years, respectively (P > 0.05).

Deletion typing

Details of the deletion typing procedure in AZFc were described in our previous study (Wu et al., 2007). Briefly, nine AZFc-specific sequence-tagged sites (STSs) (sY1191, sY1291, sY1206, sY1201, sY142, sY1258, sY1197, sY1154 and sY1161) were used to identify the types of partial AZFc deletions in all the subjects without classical AZF deletions (Fig. 1A; Repping et al., 2003, 2004). Deletion patterns were identified by the absence of one or more boundary markers, i.e. markers that overlap unique boundaries between two neighboring amplicons that are present within the AZF region.

Y chromosome haplogrouping

Y chromosome haplogroups (Y-hgs) were defined using 14 highly informative polymorphic loci for East Asians: M130, YAP, M89, M9, M231, M120, M119, M268, M95, M176, M175, M122, M134 and M117 (Jin and Su, 2000; Jobling and Tyler-Smith, 2003). Totally, 14 Y-hgs were defined following the nomenclature recommended by the Y Chromosome Consortium (YCC) and its update (Sengupta et al., 2006).

CVN genotyping using the AccuCopy method

The AccuCopy technique, a CNV genotyping method based on multiplex fluorescence competitive amplification, was recently developed by Genesky Biotechnologies (Shanghai, China), and is well described by Du et al. (2012). We used this technology for quantitative analysis of copy numbers of all these eight gene families. To ensure the reliability of our results, we chose three target genomic segments within the CNV region for DAZ (exons 1, 27 and 28) and for TTTY4 (exons 1, 3 and 4) genes. We chose two for BYP2 (intron 3 and exon 7), TTTY17 (intron 1 and exon 2), CDY1 (exons 1 and 2), CSPG4L (exon 1 and intron 1), GOLGA2LY (exon 3 and intron 7) and for TTTY3 (exon 1 and intron 2). According to the reaction conditions and stability, these target segments were divided into two panels. The reference genome sequences were obtained from the UCSC Genome Browser (http://genome.ucsc.edu; genome assembly hg19). Additionally, three reference segments used for normalization were screened and chosen at three loci of POP1, RPP14 and TBX15 (Du et al., 2012). These three loci are housekeeping genes, and their copy numbers are stable across the population. The forward (F) and reverse (R) primers of these segments and the size of PCR products amplified from human genomic DNA are provided in Supplementary data, Table SI.

To minimize the cost for synthesis of fluorescence-labeled primers, the AccuCopy assay was modified as one multiplex competitive PCR amplification followed by one labeling extension, in which one of the PCR primers for each fragment was synthesized with addition of a universal sequence, i.e. 5′-ACACGACCGTAACGCTTAGA-3′ at 3′ end so that the PCR products could be FAM-labeled (6-carboxyfluorescein, 6-FAM, the most commonly used fluorescent dye for labeling oligonucleotides) in a subsequent extension reaction using a 5′-FAM-modified primer, i.e. FAMEF: 5′-FAM-ACCACGCCTTAGA3′. All primers mentioned above were synthesized at Sangon Biotech (Shanghai, China). The competitive DNAs for the three reference and 18 target segments were designed and synthesized in double strand by Genesky Biotechnologies and provided in a 200× mixture. The sequences of synthesized competitive DNAs were almost the same as their reference homologies in the human reference genome except 2 bp deletion introduced.

Briefly, the synthesized competitive DNAs for target and reference segments are first mixed with a defined amount of genomic DNA from the sample under test, and then subjected to a multiplex fluorescence competitive PCR amplification, which can simultaneously amplify all reference and target segments from both the sample DNA and the competitive DNA.
using multiple fluorescence-labeled primer pairs. As the PCR product of competitive DNAs is 2 bp shorter than that of human genomic DNAs amplified by the same pair of primers, they can be distinguished from each other after fluorescence capillary electrophoresis. The experimental procedures, mainly involving multiplex PCR amplification reaction, capillary electrophoresis and data analysis, were described previously (Du et al., 2012) with minor modifications.

**Statistical analysis**

The distributions of Y-hg among cases and controls were assessed by using the Arlequin software (Raymond and Rousset, 1995). Differences in Y-hg frequencies and gene CNVs between cases and controls were calculated and tested with χ² test or Fisher’s exact test using the Intercooled Stata 7.0 (StataCorp, LP). Correlations among these eight gene families in AZFc were evaluated by Pearson correlation and Spearman rank correlation analysis.

We used QVALUE software (http://genomine.org/qvalue/) to calculate the false discovery rate-adjusted P-value (Storey and Tibshirani, 2003). Probability (P) values of ≤0.05 were regarded as statistically significant.

**Results**

**No difference in Y-hg distribution between the case and control groups**

To test for the potential influence of genetic backgrounds, 14 Y chromosome binary markers were used to define 14 Y-hgs in patients and normal subjects (controls) with different spermatogenic phenotypes. No significant difference in the Y-hg distribution was found between the infertile cases and the fertile controls (Supplementary data, Table SII). This suggests that the genetic background and in particular Y-hgs, may not affect the results of the present association study.

**Distributions of partial AZFc deletions between the case and control groups**

After eliminating the cases with classical AZF deletions, we evaluated the distributions of partial AZFc deletions in 654 infertile patients and 781
healthy controls (Supplementary data, Table SIII). Overall, we found 70 gr/gr deletions and 62 b2/b3 deletions in the patients, compared with 67 gr/gr deletions and 49 b2/b3 deletions in the fertile men (Supplementary data, Table SIII). The analysis revealed a significant difference in the frequency of b2/b3 subdeletions [odds ratio (OR), 1.56; 95% confidence interval (CI) 1.06–2.31; \( P = 0.024 \)] between the controls and infertile patients (Supplementary data, Table SIII). We did not observe significant differences in the distribution of the gr/gr deletion between patients and controls.

### Distributions of gene copy numbers in AZFc

Considering the potential influence of partial AZFc deletions on spermatogenic impairment, we have investigated the possible association between AZFc gene copy numbers and spermatogenic impairment in individuals without partial AZFc deletions.

Overall, 522 patients and 665 controls, all without partial AZFc deletion, were further examined for AZFc gene copy numbers using the AccuCopy method. The distributions of DAZ copy number alteration in the case and control groups in the non-deletion population were shown in Table I and Supplementary data, Table SIV. In the non-deletion population, 60 out of 522 patients and 43 out of 665 controls were found to be duplication only (dup-only). We found that DAZ copy number alteration was significantly more frequent in the case group (OR, 1.88; 95% CI 1.25–2.83; \( P = 0.0023 \)) when compared with control group, which was previously reported and associated with Y-hg (Fernandes et al., 2006).

Another gene cluster, in which three genes (BPY2, TTTY4 and TTTY17) lie adjacent to each other, commonly has three copies. According to the copy numbers of these genes, individuals were classified into two copy number patterns: the common level and the over-dosage level. The detailed frequency distributions were shown in Table I and Supplementary data, Table SIV. The frequency of the BPY2 copy number abnormality in the infertile cases (OR, 1.67; 95% CI 1.16–2.39; \( P = 0.0054 \)) was significantly higher than that in the fertile controls. The frequencies of the TTTY4, TTTY17 copy number alterations were also higher in the infertile cases than in the fertile controls, though these differences did not reach a statistical significance (Table I).

The other gene cluster, including the CDY1, CSPG4LY, GOLGA2LY and TTTY3 gene families, commonly has two copies. The frequencies of the CDY1, CSPG4LY, GOLGA2LY and TTTY17 copy number abnormality were higher in the infertility group than those in the control group, although there is no statistical significance (as shown in Table I and Supplementary data, Table SIV).

Further, the correlations (including linear correlation and Spearman’s rank correlation) among these eight gene families, as shown in Fig. 1, were significant with \( P \)-values of < 0.001. This observation prompted us to evaluate the effects of combined CNVs in these eight gene families on spermatogenic impairment. Three subgroups were defined: (i) all eight gene families with common level copy number, (ii) DAZ gene with abnormal level copy number and all other AZFc gene families with the common level and (iii) BPY2 gene with abnormal level copy number and the all other AZFc gene families with the common level. The detailed distribution frequencies are shown in Table II. Compared with the group of all eight gene families with common level copies, the frequencies of DAZ gene with abnormal level copies alone pattern (OR, 1.67; 95% CI 1.68–34.53; \( P = 0.0058 \)) and BPY2 gene with abnormal level copies alone pattern (OR, 2.04; 95% CI 1.09–3.82; \( P = 0.0266 \)) were significantly higher in the infertile group.

### Distributions of CNVs of AZFc gene families in Y-hg

To explore the predisposition of Y-hg or certain gene family to copy number alteration, we analyzed the distribution of copy number abnormality of these eight gene families in 13 Y-hgs (excluding hg-Q1 that is fixed with gr/gr deletion). As shown in Supplementary data, Table SV, the prevalence of AZFc gene copy number alterations varied significantly among Y-hgs. Even in the same Y-hg, we observed different prevalences of AZFc gene copy number abnormality in these eight gene families.

Notably, in hg-O1, the frequency of copy number alterations of all eight gene families was significantly higher in the case group than that in the control group: the \( P \)-value were 0.004 for DAZ gene, 0.0304 for BPY2 gene and 0.021 for TTTY17, TTTY4, TTTY3, CDY1, GOLGA2LY and CSPG4LY gene families, respectively (Table III). The frequency of copy number abnormalities in Y-hgs DE and O3e-I was higher in the infertility

### Table I  Distributions of DAZ, BPY2, TTTY4, TTTY17, CDY1, CSPG4LY, GOLGA2LY and TTTY3 gene copy numbers in subjects without AZFc deletions.

<table>
<thead>
<tr>
<th>Gene cluster</th>
<th>Gene*</th>
<th>Fertile controls (665)</th>
<th>Infertile cases (522)</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Common copy number n (%)</td>
<td>Abnormal copy number n (%)</td>
<td>Common copy number n (%)</td>
<td>Abnormal copy number n (%)</td>
</tr>
<tr>
<td>Commonly four copies</td>
<td>DAZ</td>
<td>622 (93.53)</td>
<td>43 (6.47)</td>
<td>462 (88.51)</td>
<td>60 (11.49)</td>
</tr>
<tr>
<td>Commonly three copies</td>
<td>BPY2</td>
<td>605 (90.98)</td>
<td>60 (9.02)</td>
<td>448 (85.82)</td>
<td>74 (14.18)</td>
</tr>
<tr>
<td></td>
<td>TTTY4</td>
<td>625 (93.98)</td>
<td>40 (6.02)</td>
<td>476 (91.19)</td>
<td>46 (8.81)</td>
</tr>
<tr>
<td></td>
<td>TTTY17</td>
<td>628 (94.44)</td>
<td>37 (5.56)</td>
<td>480 (91.95)</td>
<td>42 (8.05)</td>
</tr>
<tr>
<td>Commonly two copies</td>
<td>CDY1</td>
<td>627 (94.29)</td>
<td>38 (5.71)</td>
<td>480 (91.95)</td>
<td>42 (8.05)</td>
</tr>
<tr>
<td></td>
<td>CSPG4LY</td>
<td>630 (94.74)</td>
<td>35 (5.26)</td>
<td>483 (92.53)</td>
<td>39 (7.47)</td>
</tr>
<tr>
<td></td>
<td>GOLGA2LY</td>
<td>627 (94.29)</td>
<td>38 (5.71)</td>
<td>483 (92.53)</td>
<td>39 (7.47)</td>
</tr>
<tr>
<td></td>
<td>TTTY3</td>
<td>629 (94.59)</td>
<td>36 (6.71)</td>
<td>481 (92.15)</td>
<td>41 (7.85)</td>
</tr>
</tbody>
</table>

*DAZ commonly have four copies; BPY2, TTTY4 and TTTY17 commonly have three copies; CDY1, CSPG4LY, GOLGA2LY and TTTY3 commonly have two copies.

*\( \chi^2 \) test \( (P < 0.05) \) when compared with the control group.
Kuroda-Kawaguchi et al. have reported to cause spermatogenic impairment (Vogt et al., 1996). Complete AZFc deletion (referred to as the b2/b4 deletion), which removes all eight testis-specific expressed gene families in AZFc, has been reported to cause spermatogenic impairment (Lin et al., 2007). Interestingly, it has been recently reported that secondary duplications to partial AZFc deletions can restore the total motile sperm count to normal value (Noordam et al., 2011). However, our previous study in Han Chinese populations showed once again the inconsistent association of AZFc rearrangements with spermatogenic impairment across populations and showed that additional AZFc duplications did not compensate but convey the susceptibility of b2/b3 deletion (one type of partial AZFc deletion) to spermatogenic impairment (Lin et al., 2007). In 2007, Lin et al. reported that partial AZFc duplication resulting in increased AZFc gene copies is a risk factor for spermatogenic impairment (Lin et al., 2007). Interestingly, it has been recently reported that secondary duplications to partial AZFc deletions can restore the total motile sperm count to normal value (Noordam et al., 2011). However, our previous study in Han Chinese populations showed once again the inconsistent association of AZFc rearrangements with spermatogenic impairment across populations and showed that additional AZFc duplications did not compensate but convey the susceptibility of b2/b3 deletion (one type of partial AZFc deletion) to spermatogenic impairment (Lin et al., 2007). To determine the extent to which the multi-copy AZFc genes affect spermatogenic impairment, we evaluated the gene dosage variations of all these eight gene families in this study.

In this study, consistent with our previous study, the b2/b3 partial deletion (including deletion-only and deletion-duplication) was consistently associated with spermatogenic impairment (Wu et al., 2007; Lu et al., 2009).

Discussion

AZFc is composed of several distinct families of long repeats (amplicons) (Fig. 1A) and it is susceptible to recurrence of various rearrangements. Complete AZFc deletion (referred to as the b2/b4 deletion), which removes all eight testis-specific expressed gene families in AZFc, has been reported to cause spermatogenic impairment (Vogt et al., 1996; Kuroda-Kawaguchi et al., 2001; Tyler-Smith and McVean, 2003). The roles of AZFc rearrangements (including partial deletions, duplications and their combinations) in spermatogenesis are controversial and variable among different human populations (Navarro-Costa et al., 2010).

Since the finding of partial AZFc deletions in 2003, copy number reduction of AZFc genes has been found to be associated with spermatogenic impairment in some human populations (mainly European), suggesting that dosage insufficiency of the AZFc genes might be the mechanism for AZFc-associated spermatogenic impairment (Repping et al., 2003; de Llanos et al., 2005; Ferlin et al., 2005). However, this model of dosage insufficiency has been challenged by the evidence that partial AZFc deletions with reduced AZFc gene copies are frequent in healthy males in many other populations, including our findings in East Asians (Machev et al., 2004; Hucklebroich et al., 2005; Zhang et al., 2007; Lu et al., 2009).

In this study, consistent with our previous study, the b2/b3 partial deletion (including deletion-only and deletion-duplication) was consistently associated with spermatogenic impairment (Wu et al., 2007; Lu et al., 2009).
To avoid the potential confounding influence of partial AZFc deletions (including deletion-only and deletion-duplication) on spermatogenic impairment, we focused on the possible association between AZFc gene copy numbers and spermatogenic impairment in individuals without partial AZFc deletions. We found that copy number alterations of DAZ and BPY2 were associated with spermatogenic impairment, while the CNVs in the other genes (TTY4, TTTY17, CDY1, CSPG4LY, GOLGA2LY and TTTY17) showed no association with impaired spermatogenesis, suggesting a potential different role of spermatogenesis between AZFc genes. In previous study, the DAZ1/DAZ2 doublet was considered to be crucial for normal spermatogenesis in Europeans (Ferlin et al., 2002, 2005; Fernandes et al., 2002), while the DAZ3/DAZ4 cluster has been...
demonstrated to be associated with spermatogenic impairment in the Han Chinese population (Wu et al., 2007; Lu et al., 2009). Although AZFc regions are known to be associated with spermatogenic impairment, no definitive conclusion has been reached for the contribution of different copies of the AZFc genes to spermatogenic impairment. Furthermore, because frequent gene conversions exist between AZFc gene copies, it was challenging to exactly define and distinguish between different copies of a multi-copy AZFc gene, even though methods to achieve this have been described (Fernandes et al., 2002; Lu et al., 2009).

When combined CNVs were analyzed, compared with the pattern of all eight gene families having common level copies, the frequency of two abnormal combined patterns (DAZ or BPY2 gene with abnormal level copies only) were significantly higher in the case group than that in the control group. The frequency of another combined pattern (all eight gene families with abnormal level copies) was higher in the case group than that in the control group, but with no statistical significance (OR, 1.60; 95% CI 0.98–2.61; P = 0.0594) (data not shown), which suggested that the potential influence of copy number alterations of other genes to spermatogenic impairment was not excluded.

Although the mechanism underlying the susceptibility of excessive copies of DAZ and/or BPY2 to impaired spermatogenesis remains to be uncovered, a similar observation has also been reported in another Han Chinese Taiwan population (Lin et al., 2007). The findings that men with primary AZFc duplications, i.e. duplications not preceded by any deletion, resulting in Y chromosomes with eight DAZ genes on their Y chromosomes have a severely diminished total motile sperm count, also suggested that over-dosage of DAZ gene might be a disadvantage for spermatogenesis (Noordam et al., 2011).

Our results differed in some aspects compared with previous studies, in which the CDY1A was proved to be a modifier in spermatogenesis (Machet al., 2004; Lu et al., 2009). However, in this study, we did not screen for the potential impact of the CDY1 gene on spermatogenesis. One possible explanation for the observed difference could be due to the difference in the genomic structure of the gene copies that remain after deletion. In the deletion and/or duplication process in AZFc, the NAHR between long repeat sequences (amplicons) on the human Y chromosome can occur between different amplicon pairs (Fig. 2). Subsequently, the deleted or duplicated gene copies and their copy numbers can vary among different rearrangement subtypes. In this study, we screened the CNVs in subjects without AZFc deletions, thus the potentially functional cluster CDY1A still existed. Eventually, the influence of CDY1 copy number increase on spermatogenesis seems very limited.

In addition, we investigated the predisposition of certain Y-hg to gene copy number alteration. We found that the copy number increase of all eight gene families was associated with spermatogenic impairment in Y-hg O1. More individuals with increased gene copy number were identified in cases in Y-hgs DE and O3e1, although the frequency distributions were not significantly different.

In conclusion, further examination of the expression levels of the AZFc genes in human testis may help elucidate the mechanisms of abnormal spermatogenesis. To our knowledge, this study is the first comprehensive investigation regarding the distribution of CNVs of all eight AZFc gene families in patients with spermatogenic impairment and normozoospermic controls without AZFc deletions. Our findings emphasize the necessity of more extensive studies on over-dosage sensitive AZFc genes and susceptible Y-hg, for better understanding of spermatogenesis and its pathology.

**Supplementary data**

Supplementary data are available at http://molehr.oxfordjournals.org/.

**Acknowledgements**

We thank all the study participants, research staff and students who took part in this work.

**Authors’ roles**

X.W., Y.X. and Z.H. directed the study, obtained financial support and were responsible for study design. C.L. performed overall project management with J.J. and R.Z., performed statistical analysis with Y.Q., X.G. and Y.Z., and drafted the initial manuscript. Y.W., M.X., Y.L. and B.N. were responsible for subject recruitment and sample preparation. N.D., F.C., H.S. and J.S. conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

**Funding**

Funding was provided by grants from National 973 Program (2011CB944304, 2013CB911400), National Natural Science Foundation of China (30930079, 81100461), Jiangsu Natural Science Foundation (BK20111774), Research Fund for the Doctoral Program of Higher Education of China (RFDP) (20113234120001), University Natural Science Research Project in Jiangsu Province (11KBJ330001) and the Priority Academic Program for the Development of Jiangsu Higher Education Institutions (Public Health and Preventive Medicine).

**Conflict of interest**

None to declare.

**References**


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