Child's Nervous System (2019) 35:965–972
https://doi.org/10.1007/s00381-019-04131-1

ORIGINAL ARTICLE

# A functional indel polymorphism rs34396413 in *TFAP2A* intron-5 significantly increases female encephalocele risk in Han Chinese population

Ke Su[1] · Shuxia Chen[1] · Jianhong Ye[1] · Lele Kuang[1] · Ting Zhang[2] · Hongyan Wang[1,3,4] · Xueyan Yang[1]

Received: 21 December 2018 / Accepted: 18 March 2019 / Published online: 24 April 2019


## Abstract

**Purpose** Transcription factor AP-2 alpha (TFAP2A) is an important transcriptional factor involved in various aspects of embryo development including neural tube closure. *Tfap2a* deficiency led to the failure of cranial neural-tube closure in mice and other model organisms. However, it remains largely unknown about the relationship between *TFAP2A* variants and human cranial neural tube defects (NTDs). The aim of this study was to find the association between *TFAP2A* intronic SNP rs3439413 and NTDs and to explore its function.

**Methods** We found an indel polymorphism rs3439413 in *TFAP2A* intron-5 from our previous target sequencing project. In this study, we validate its association with human NTDs in Shanxi group containing 266 NTD cases and 295 matched controls. Then, we investigated its function on transcriptional activity by dual-luciferase assays and EMSA.

**Results** The minor allele of rs34396413 significantly increased the risk of NTD in a Han Chinese population of Shanxi Province ($P = 0.0082$, OR = 1.45, 95%CI = 1.10–1.90), especially the risk of encephalocele for female ($P = 0.0064$, OR = 2.46, 95%CI = 1.22–4.94). Functional analysis revealed the minor allele of rs34396413 decreases transcriptional activity and attenuates transcription factor binding affinity.

**Conclusion** We have demonstrated that the minor allele of rs34396413 was a risk factor of NTD in the Shanxi group, providing new insight into the study of NTD etiology.

**Keywords** Neural tube defects · *TFAP2A* · Intronic variant · rs34396413 · Enhancer

## Introduction

Neural tube defects (NTDs) are malformations due to the failure of the neural tube closure, which are among the most common and severe birth defects. The worldwide prevalence of NTDs is approximately 0.5–2 per 1000 established pregnancies [3]. Their incidence varies in different populations [7]. In particular, Shanxi Province in Northern China has the highest incidence of NTD (13.9/1000 births) in the world [10].The etiology of NTD is multifactorial and complex, determined by both environmental and genetic factors [18]. Previous evidence showed that heritability of NTDs was up to 70% [3]. However, the genetic background of NTD susceptibility remains largely unknown.

Transcription factor AP-2 alpha (*TFAP2A*, also called *AP-2*, *Tcfap2a*) encodes a member of the AP-2 transcription factors, which regulates various aspects of tissue development during embryogenesis [19]. In 1996, two groups reported that *Tfap2a* deficiency led to failure of cranial neural-tube closure

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s00381-019-04131-1) contains supplementary material, which is available to authorized users.

✉ Xueyan Yang
Xueyanyang@fudan.edu.cn

[1] The State Key Laboratory of Genetic Engineering and MOE Key Laboratory of Contemporary Anthropology, School of Life Sciences, Fudan University, Shanghai 200438, China

[2] Beijing Municipal Key Laboratory of Child Development and Nutriomics, Capital Institute of Pediatrics, Beijing 100020, China

[3] Institute of Reproduction and Development, Obstetrics and Gynecology Hospital of Fudan University, Shanghai 200011, China

[4] Key Laboratory of Reproduction Regulation of NPFPC, Obstetrics and Gynecology Hospital of Fudan University, Shanghai 200032, China

in mice [16, 20]. The AP-2 null mice exhibited anencephaly, craniofacial defects, and thoraco-abdominoschisis, which died prenatally. In chimeric mice with both wildtype cells and AP-2-null cells, AP-2 was independently required for the formation of the neural tube, body wall, and craniofacial skeleton [13]. In zebrafish, null mutation in AP-2 also caused severe craniofacial defects, and defects in early neural crest specification [2, 5, 9]. In human, *TFAP2A* gene deletion or mutations usually resulted in branchio-oculo-facial syndrome with cutaneous anomalies, ocular anomalies, and characteristic facial appearance [1, 12, 15]. Although there were few case-control studies on *TFAP2A* variants and human NTD risk, only *TFAP2A* rs3798691 increased NTD risk in Hispanics ($P = 0.015$, OR = 1.78, 95%CI = 1.13–2.87, 143 cases and 122 controls) [8, 11].

In our previous study, we target-sequenced 285 NTD-related genes among 352 NTD patients and 224 healthy controls [14]. Through association analysis, we identified rs34396413, an insertion-deletion polymorphism in *TFAP2A* gene intron 5, significantly increased NTD risk. In this study, we validated the association between rs34396413 and NTDs by multiplex fluorescent PCR and further explored the effect of rs34396413 on transcriptional regulation.

## Materials and methods

### Subjects

The detailed information of 352 NTDs and 224 control samples used for target sequencing was described in our previous report [14]. In this study, we picked out 266 Shanxi NTD patients from previous case groups and extended region-matched healthy controls to 295 to validate by multiplex fluorescent PCR. All miscarriage or stillborn neural tube defect patient ($n = 266$, $23.2 \pm 6.2$ weeks) samples were collected in hospitals in Shanxi Province of China from the 1990s to the 2010s. A total of 295 unrelated healthy controls were also from Shanxi Province, including 85 randomly selected healthy infants born at the same local hospitals and 210 healthy adults who went for routine physical examination at the same hospitals. The detailed information was summarized in Table 1.

All participants provided informed consent and the study protocol was reviewed and approved by the Ethics Committee of the School of Life Sciences, Fudan University. Detailed clinical information was collected by locally trained doctors.

### DNA extraction and genotyping

Approximately 2 mL of peripheral blood or abortive tissue samples was collected from each subject. Genomic DNA was isolated from peripheral blood or tissue samples, using conventional reagents, and quantified using a NanoDrop2000 (Thermo Scientific).

**Table 1** Demographic and phenotypic characteristics of cases and controls

| | Cases | | Controls | |
|---|---|---|---|---|
| | $n = 266$ | | $n = 295$ | |
| Characteristic | *n* or mean | % or SD | *n* or mean | % or SD |
| Age | | | | |
| Fetuses (weeks) (mean, SD) | 23.2 | 2.6 | 20.6 | 3.1 |
| | 264 | 99.2% | 85 | 28.8% |
| Adults (≥ 18 years) | 0 | 0.0% | 210 | 71.2% |
| Unknown | 2 | 0.8% | 0 | 0.0% |
| Gender | | | | |
| Male | 114 | 42.8% | 127 | 43.1% |
| Female | 150 | 56.4% | 168 | 56.9% |
| Unknown | 2 | 0.8% | | |
| NTD classification | | | | |
| Craniorachischisis | 19 | 7.1% | | |
| Encephalocele | 69 | 25.9% | | |
| Anencephaly | 59 | 22.2% | | |
| Exencephaly | 1 | 0.4% | | |
| Spina bifida | 118 | 44.4% | | |

*SD*, standard deviation

Rs34396413 was genotyped by multiplex fluorescent PCR method. The primers used for genotyping were listed in Online Resource Table 1. The PCR products were diluted ten times and then tested by capillary electrophoresis detection with ABI3730xl. Then, the data files were analyzed using GeneMapper 4.1 (Applied Biosystems). The entire process was carried out by Shanghai Genesky Biotechnologies Inc.

### Plasmid constructs, cell culture, and luciferase assays

To construct the luciferase reporter plasmid, we cloned the 1792 bp fragment and the 652 bp fragment from IVS5-1802 to IVS5-11 of *TFAP2A* intron 5 by PCR from genomic DNA (Fig. 1a). The primers used are presented in Online Resource Table 1. Then, the fragment was subcloned into the MluI and BglII restriction sites of the pGL3-Basic vector. The minor allele of rs34396413 was generated by site-directed mutagenesis with the KOD-Plus enzyme (TOYOBO, Japan). All recombinant clones were confirmed by DNA sequencing.

Human embryonic kidney 293T (HEK293T) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and seeded in 24-well culture plates 24 h before the transfections. The cells were transfected with 350 ng/well pGL3-Basic vector, intron5-major, intron5-minor, intron5–652-major, and intron5–652-minor plasmids, respectively. A total of 5 ng/well pRL-TK plasmid (Promega, USA) was co-transfected as an internal

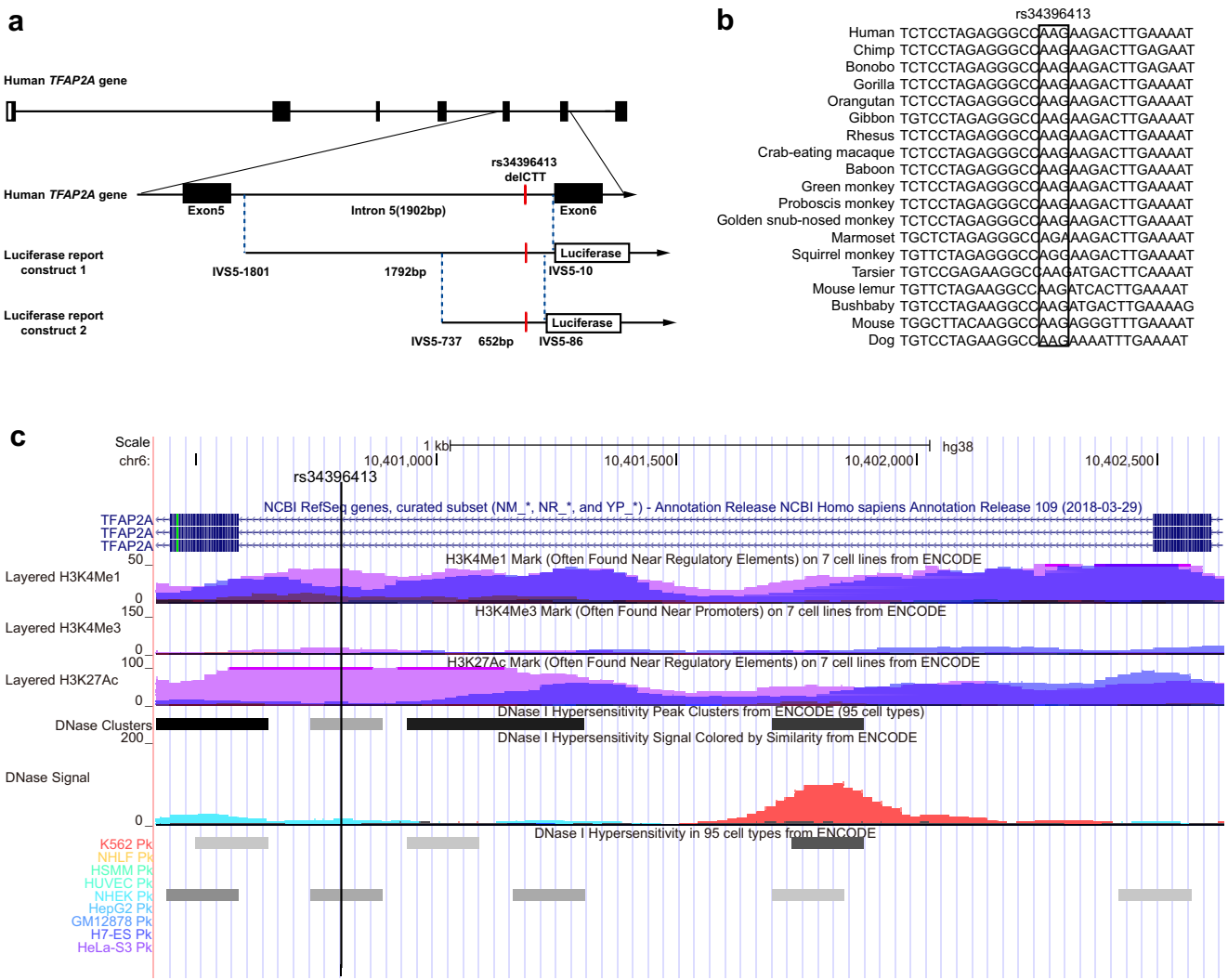


**Fig. 1** Rs34396413 was located in the highly conserved intron-5 region of *TFAP2A*. **a** The constructs of luciferase reporter plasmids containing rs34396413. **b** The rs34396413 residue was highly conserved among mammals. **c** UCSC genome browser image of rs34396413 in *TFAP2A* gene suggested that this region was DNaseI hypersensitive and enriched histone makers

control, and 145 ng/well salmon sperm DNA (ssDNA) to fill the total 500 ng/well plasmid. Cell transfection used Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. At 24 h post-transfection, the cells were lysed in 100 μL lysis buffer (Promega, USA), incubated on ice for 15 min and centrifuged at 3000 rpm/min for 5 min. The supernatant (25 μL) was used for luciferase assay with the Dual-Luciferase Reporter Assay System (Promega, USA). Three independent transfection experiments were performed, and the luciferase assay of each transfection was carried out in triplicate. GraphPad Prism 6.01 and Adobe Photoshop CC 2017 were used to create Fig. 2a.

## EMSA

Electrophoretic mobility shift assay (EMSA) probes with 5′-Biotin labeling were listed in Online Resource Table 1. Nuclear protein was extracted from HEK293T cells using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific, USA). Protein concentration was measured with NanoDrop2000 (Thermo Fisher Scientific, USA). The probes were incubated with nuclear protein and ran on a 6% native-polyacrylamide gel. All the procedures followed the EMSA kit instructions (Thermo Fisher Scientific, USA). Adobe Photoshop CC 2017 was used to create Figs. 1 and 2b.

## Statistical analysis

The Hardy–Weinberg equilibrium (HWE) was assessed by chi-square analysis using data from controls only. Odds ratios (OR) and 95% confidence intervals (CI) for SNP genotypes were calculated using logistic regression analyses. The association study between the SNP and NTD risk was performed using the SNPStats (http://bioinfo.iconcologia.net/snpstats/start.htm). All statistical tests were two-tailed and $P < 0.05$ was set as the significant threshold.

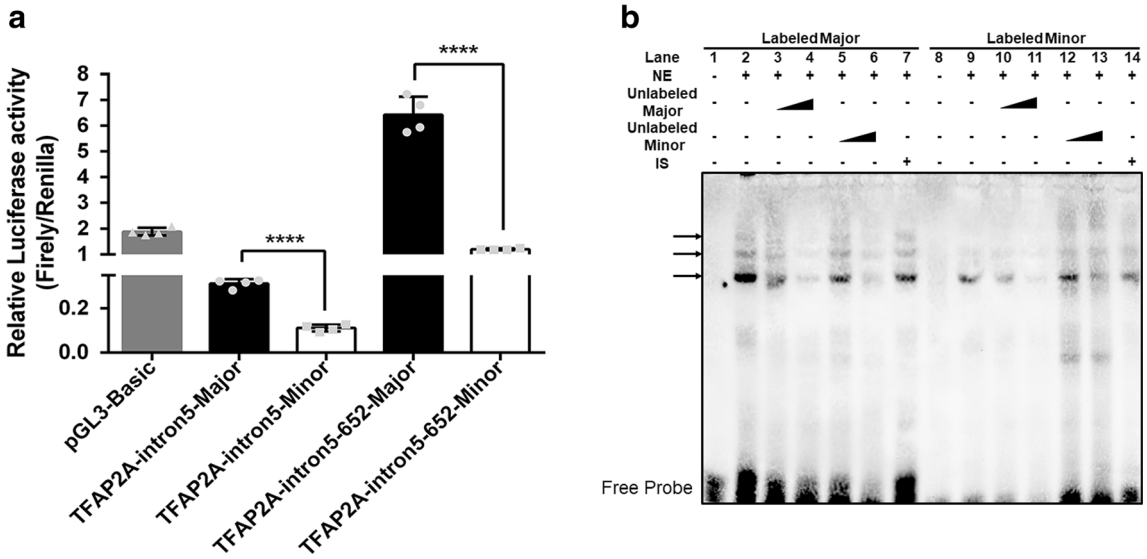


**Fig. 2** Rs34396413 decreased transcriptional activity through attenuating its binding affinity to certain nuclear proteins. **a** Dual-luciferase assays showed that the minor allele of rs34396413 significantly decreased transcriptional activities both in full-length intron-5 or 652 bp intron-5 fragment. **b** Binding affinity of major/minor probes with HEK 293T nuclear proteins. Three arrows highlight three specific nuclear proteins that interact with major/minor probes. All three proteins have lower binding affinity with minor probe.NE, nuclear extract; IS, irrelevant sequence

## Results

### *TFAP2A* intronic variant rs34396413 significantly increased the risk of NTDs in a Han Chinese population

In our previous study, we target-sequenced 285 NTD-related genes among 352 NTD patients and 224 healthy controls [14]. After association analysis, we identified rs34396413, an insertion-deletion polymorphism in *TFAP2A* gene intron 5, significantly increased NTD risk ($P = 0.0014503$). In order to validate if rs34396413 was associated with NTD risk, we genotyped this variant by multiplex fluorescence PCR in a NTD cohort including 266 NTD cases and 295 controls. All subjects were Han Chinese from Shanxi Province. The detailed information was listed in Table 1.

Our results suggested that the minor G allele of rs34396413 significantly increased NTD risk in Han Chinese in Shanxi Province ($P = 0.0082$, OR = 1.45, 95%CI = 1.10–1.90, log-additive) (Table 2). And we found that rs34396413 only increased NTD susceptibility in female ($P = 0.0086$, OR = 1.62, 95%CI = 1.12–2.34, log-additive) (Table 3), but not in male ($P > 0.05$ in all genetic models) (Online Resource Table 2). Furthermore, we performed stratification analysis according to the standard NTD classifications [6]. According to the results, rs34396413 only contributed to the susceptibility of encephalocele ($P = 0.01$, OR = 1.82, 95%CI = 1.12–2.95, log-additive) (Table 4). Thus, the risk of encephalocele for female was most significant in all categories ($P = 0.0064$, OR = 2.46, 95%CI = 1.22–4.94, log-additive) (Table 5).

All genotype frequencies were in accordance with the Hardy–Weinberg expectation among the control subjects ($P > 0.05$).

### Rs34396413 minor allele suppressed transcriptional activity

Rs34396413 located in the fifth intron of *TFAP2A* and was highly conserved among mammals (Fig. 1b). According to the ENCODE Project database, this region enriched the active enhancer-associated H3K27ac and H3K4me1 marks and showed DNaseI hypersensitivity (Fig. 1c). Recent research in mouse model also suggested that there were tissue-specific enhancers in human *TFAP2A* intron 5 [21]. These evidences supported this region may be an enhancer and play roles in *TFAP2A* transcriptional activity.

Thus, we performed luciferase assays in HEK 293T cells. The full length of human *TFAP2A* gene intron 5 is 1902 bp. We amplified both the full length of the intron 5 and the relatively conservative region containing the rs34396413 (labeled as intron5–652). Then, we generated corresponding minor alleles by point mutagenesis. We observed that the plasmids containing the minor G allele significantly reduced luciferase expression compared with the major CTTG allele, with 65% reduction in full-length intron5 construct and 81% reduction in conserved intron5–652 construct (Fig. 2a).

### The rs34396413 minor allele attenuated transcriptional factors affinity

Since the rs34396413 minor allele significantly reduced the transcriptional activity, we wondered if it was due to a different binding affinity to certain transcription factors. We carried out EMSA using biotin-labeled probes with either rs34396413 major allele or minor allele (labeled as major probe/minor probe). We observed three specific shifted bands (Fig. 2b). The shifted

**Table 2** Association study of rs34396413 in the Shanxi group

| Model | Genotype | Case | | Control | | OR (95% CI) | *P* value | *P* (HWE)* |
|---|---|---|---|---|---|---|---|---|
| | | *n* | % | *n* | % | | | |
| Codominant | CTTG/CTTG | 170 | 63.9 | 161 | 54.6 | 1.00 | *0.026* | 0.3 |
| | G/CTTG | 85 | 31.9 | 109 | 37.0 | 1.35 (0.95–1.93) | | |
| | G/G | 11 | 4.1 | 25 | 8.5 | *2.4 (1.14–5.04)* | | |
| Dominant | CTTG/CTTG | 170 | 63.9 | 161 | 54.6 | 1.00 | *0.025* | |
| | G/CTTG-G/G | 96 | 36.1 | 134 | 45.4 | *1.47 (1.05–2.07)* | | |
| Recessive | CTTG/CTTG-G/CTTG | 255 | 95.9 | 270 | 91.5 | 1.00 | *0.034* | |
| | G/G | 11 | 4.1 | 25 | 8.5 | *2.15 (1.03–4.45)* | | |
| Overdominant | CTTG/CTTG-G/G | 181 | 68.0 | 186 | 63.0 | 1.00 | 0.21 | |
| | G/CTTG | 85 | 31.9 | 109 | 37.0 | 1.25 (0.88–1.77) | | |
| Log-additive | – | – | | – | | *1.45 (1.10–1.90)* | *0.0082* | |

The italicized items indicate $P<0.05$

*$P$ value for deviation from the Hardy–Weinberg equilibrium in control group. *OR*, odds ratio; *CI*, confidence interval

bands suggested the minor G allele has a lower binding affinity with certain nuclear proteins. Competition assays using unlabeled probes also demonstrated the minor G allele has lower binding affinity to these proteins. Thus, we presumed that those specific binding proteins were probably transcription activators because the minor G allele shows decreased transcriptional activity compared with the major CTTG allele, while details deserved further investigation.

## Discussion

*TFAP2A* performed vital roles in morphogenetic process critical for mammalian development including the formation of the neural tube, face, eyes, body walls, limb buds, and cardiovascular system [20]. Thus, it was one of the important candidate genes for human birth defects, such as neural tube defects (NTDs) and cleft lip. There were several studies investigating the role of *TFAP2A* gene SNPs in the pathogenesis of NTDs [8, 11, 17]. However, they were unable to show a significant role of the *TFAP2A* gene in the etiology of human neural tube defects except *TFAP2A* rs3798691 increased NTD risk in Hispanics ($P = 0.015$, OR = 1.78, 95%CI = 1.13–2.87, 143 cases and 122 controls) [11]. We speculate it might due to the composition of their NTD cases because the majority of them were spina bifida. Null mutation of the mice *Tfap2a* exhibited exencephaly and thoraco-abdominoschisis, which suggested that it may contribute to cranial NTDs instead of spina bifida. Here, we identified rs34396413, an insertion-deletion polymorphism in *TFAP2A* gene intron 5 from our previous target sequencing and validated that it significantly increase NTD risk in Shanxi case-control group. In

**Table 3** Stratification analysis of rs34396413 in female patients and controls in the Shanxi group

| Model | Genotype | Case | | Control | | OR (95% CI) | *P* value | *P* (HWE)* |
|---|---|---|---|---|---|---|---|---|
| | | *n* | % | *n* | % | | | |
| Codominant | CTTG/CTTG | 97 | 64.7 | 83 | 49.4 | 1.00 | *0.022* | 1 |
| | G/CTTG | 45 | 30 | 71 | 42.3 | *1.84 (1.15–2.96)* | | |
| | G/G | 8 | 5.3 | 14 | 8.3 | 2.05(0.82–5.12) | | |
| Dominant | CTTG/CTTG | 97 | 64.7 | 83 | 49.4 | 1.00 | *0.006* | |
| | G/CTTG-G/G | 53 | 35.3 | 85 | 50.6 | *1.87 (1.19–2.94)* | | |
| Recessive | CTTG/CTTG-G/CTTG | 142 | 94.7 | 154 | 91.7 | 1.00 | 0.29 | |
| | G/G | 8 | 5.3 | 14 | 8.3 | 1.61 (0.66–3.96) | | |
| Overdominant | CTTG/CTTG-G/G | 105 | 70.0 | 97 | 57.7 | 1.00 | *0.023* | |
| | G/CTTG | 45 | 30 | 71 | 42.3 | *1.71 (1.07–2.72)* | | |
| Log-additive | – | – | | – | | *1.62 (1.12–2.34)* | *0.0086* | |

The italicized items indicate $P<0.05$

*$P$ value for deviation from the Hardy–Weinberg equilibrium in control group. *OR*, odds ratio; *CI*, confidence interval

**Table 4** Stratification analysis of rs34396413 in NTD subtypes in the Shanxi group

| Model | Genotype | Control | | Spina Bifida | | | | Anencephaly | | | | Encephalocele | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *n* | % | *n* | % | OR (95% CI) | *P* | *n* | % | OR (95% CI) | *P* | *n* | % | OR (95% CI) | *P* |
| Codominant | CTTG/CTTG | 161 | 54.6 | 72 | 61 | 1.0 | 0.33 | 38 | 64.4 | 1.00 | 0.33 | 46 | 66.7 | 1.00 | 0.0024 |
| | G/CTTG | 109 | 37.0 | 40 | 33.9 | 1.22 (0.77–1.92) | | 18 | 30.5 | 1.43 (0.78–2.63) | | 23 | 33.3 | 1.35 (0.78–2.36) | |
| | G/G | 25 | 8.5 | 6 | 5.1 | 1.86 (0.73–4.74) | | 3 | 5.1 | 1.97 (0.56–6.86) | | 0 | 0 | NA (0.00–NA) | |
| Dominant | CTTG/CTTG | 161 | 54.6 | 72 | 61 | 1.00 | 0.23 | 38 | 64.4 | 1.00 | 0.16 | 46 | 66.7 | 1.00 | 0.065 |
| | G/CTTG-G/G | 134 | 45.4 | 46 | 39 | 1.30 (0.84–2.01) | | 21 | 35.6 | 1.51 (0.84–2.69) | | 23 | 33.3 | 1.66 (0.96–2.89) | |
| Recessive | CTTG/CTTG-G/CTTG | 270 | 91.5 | 112 | 94.9 | 1.00 | 0.22 | 56 | 94.9 | 1.00 | 0.35 | 69 | 100 | 1.00 | 9e–04 |
| | G/G | 25 | 8.5 | 6 | 5.1 | 1.73 (0.69–4.33) | | 3 | 5.1 | 1.73 (0.50–5.92) | | 0 | 0 | NA (0.00–NA) | |
| Overdominant | CTTG/CTTG-G/G | 186 | 63.0 | 78 | 66.1 | 1.00 | 0.56 | 41 | 69.5 | 1.00 | 0.34 | 46 | 66.7 | 1.00 | 0.57 |
| | G/CTTG | 109 | 37.0 | 40 | 33.9 | 1.14 (0.73–1.79) | | 18 | 30.5 | 1.33 (0.73–2.44) | | 23 | 33.3 | 1.17 (0.67–2.04) | |
| Log-additive | – | – | | – | | 1.29 (0.91–1.83) | 0.15 | – | | 1.42 (0.88–2.27) | 0.14 | – | | *1.82 (1.12–2.95)* | *0.01* |

The italicized items indicate $P<0.05$

our Shanxi group, half of NTD cases were cranial NTDs, mainly anencephaly and encephalocele. Further stratification study also suggested rs34396413 specifically increased encephalocele risk instead of spina bifida or anencephaly risk, especially in female ($P = 0.0064$, OR = 2.46, 95%CI = 1.22–4.94). It is highly consistent with mice *Tfap2a* expression exclusively in head ectoderm [21]. Another concern we wanted to mention was that the MAF of rs34396413 varied dramatically in different populations. The minor allele frequency counted to be 24% in EAS compared with less than 2% in EUR from the 1000 genome project. Thus, this SNP might contribute to NTD risk specifically to Eastern Asia population such as Han Chinese. However, it required further investigation in large cohorts.

**Table 5** Subtype analysis of rs34396413 in female patients and controls in the Shanxi group

| Model | Genotype | Control | | Spina Bifida | | | | Anencephaly | | | | Encephalocele | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *n* | % | *n* | % | OR (95% CI) | *P* | *n* | % | OR (95% CI) | *P* | *n* | % | OR (95% CI) | *P* |
| Codominant | CTTG/CTTG | 83 | 49.4 | 42 | 62.1 | 1.00 | 0.18 | 21 | 63.6 | 1.00 | 0.25 | 26 | 70.3 | 1.00 | 0.01 |
| | G/CTTG | 71 | 42.3 | 22 | 33.3 | 1.59 (0.87–2.93) | | 9 | 27.3 | 2.00 (0.86–4.64) | | 11 | 29.7 | 2.02 (0.93–4.38) | |
| | G/G | 14 | 8.3 | 3 | 4.5 | 2.31 (0.63–8.47) | | 3 | 9.1 | 1.18 (0.31–4.49) | | 0 | 0 | NA (0–NA) | |
| Dominant | CTTG/CTTG | 83 | 49.4 | 41 | 62.1 | 1.00 | 0.078 | 21 | 63.6 | 1.00 | 0.13 | 26 | 70.3 | 1.00 | *0.02* |
| | G/CTTG-G/G | 85 | 50.6 | 25 | 37.9 | 1.68 (0.94–3.01) | | 12 | 36.4 | 1.79 (0.83–3.87) | | 11 | 29.7 | *2.42 (1.12–5.21)* | |
| Recessive | CTTG/CTTG-G/CTTG | 154 | 91.7 | 63 | 95.5 | 1.00 | 0.29 | 30 | 90.9 | 1.00 | 0.89 | 37 | 100 | 1.00 | 0.016 |
| | G/G | 14 | 8.3 | 3 | 4.5 | 1.91 (0.53–6.87) | | 3 | 9.1 | 0.91 (0.25–3.36) | | 0 | 0 | NA (0–NA) | |
| Overdominant | CTTG/CTTG-G/G | 97 | 57.7 | 44 | 66.7 | 1.00 | 0.21 | 24 | 72.7 | 1.00 | 0.1 | 26 | 70.3 | 1.00 | 0.15 |
| | G/CTTG | 71 | 42.3 | 22 | 33.3 | 1.46 (0.81–2.66) | | 9 | 27.3 | 1.95 (0.86–4.45) | | 11 | 29.7 | 1.73 (0.80–3.73) | |
| Log-additive | – | – | | – | | 1.56 (0.96–2.53) | 0.065 | – | | 1.41 (0.76–2.62) | 0.26 | – | | *2.46 (1.22–4.94)* | *0.0064* |

The italicized items indicate $P<0.05$

Rs34396413 located in the fifth intron of *TFAP2A* and this region was highly conserved in mammalians and contained H3K27ac and H3K4me1 histone marks. It is interesting that previous study suggested that the cis-regulation elements to direct appropriate tissue-specific expression of *TFAP2A* fall in the 24 kb of genomic DNA extending downstream from exon five instead of up to 20 kb of genomic sequence 5′ to the transcriptional start site [21]. Further investigation suggested there were conserved enhancer element in *TFAP2A* intron-5 required for expression in facial and limb bud mesenchyme [4]. Thus, we reckoned that rs34396413 might influence *TFAP2A* transcriptional activity. Our results suggested that the minor allele of rs34396413 did decrease transcriptional activity by losing its binding to certain transcriptional activators. While relative luciferase activity of the full-length intron-5 was lower than the 652 bp fragment of intron-5 pGL3-Basic vector might due to the full-length intron-5 contains much more regulatory elements, some of those might be transcriptional suppressors.

To summarize, we demonstrated the minor allele of rs34396413 was associated with NTD, especially female encephalocele in the Shanxi group. Functional studies showed that the minor allele decreased transcriptional activity and had a lower binding affinity with several nuclear proteins. Our study provides new insight into the relationship between important transcription factor *TFAP2A* and human NTDs. That may provide a new candidate site for prenatal screening to prevent certain birth defects.

**Acknowledgments** We sincerely thank all the patients and control individuals for their participation in this study.

**Funding information** This work is supported by grants from the National Key Basic Research Program of China (2016YFC1000502 to X. Yang and H. Wang), the National Natural Science Foundation of China (81472050 to X. Yang and 81430005, 31521003, 81741048 to H. Wang), and Open project of National Population and Family Planning Key Laboratory of Contraceptive Drugs and Devices (2016KF04 to X Yang).

**Data availability** The datasets during and analyzed during the current study could be available from the corresponding author on reasonable request.

## Compliance with ethical standards

**Conflict of interest** On behalf of all authors, the corresponding author states that there is no conflict of interest.

**Ethical approval** All procedures performed in studies involving human participants were approved by the Ethics Committee of the School of Life Sciences, Fudan University.

**Research involving human participants and animals** This article did not contain any studies with animals performed by any of the authors.

**Informed consent** Informed consent was obtained from all individual participants included in this study.

## References

1. Aliferis K, Stoetzel C, Pelletier V, Helle S, Angioi-Duprez K, Vigneron J, Leheup B, Marion V, Dollfus H (2011) A novel TFAP2A mutation in familial branchio-oculo-facial syndrome with predominant ocular phenotype. Ophthalmic Genet 32:250–255
2. Barrallo-Gimeno A, Holzschuh J, Driever W, Knapik EW (2004) Neural crest survival and differentiation in zebrafish depends on mont blanc/tfap2a gene function. DEVELOPMENT 131:1463–1477
3. Copp AJ, Greene ND (2010) Genetics and development of neural tube defects. J Pathol 220:217–230
4. Feng W, Huang J, Zhang J, Williams T (2008) Identification and analysis of a conserved Tcfap2a intronic enhancer element required for expression in facial and limb bud mesenchyme. Mol Cell Biol 28:315–325
5. Gunes N, Cengiz FB, Duman D, Dervisoglu S, Tekin M, Tuysuz B (2014) Branchio-oculo-facial syndrome in a newborn caused by a novel TFAP2A mutation. GENET COUNSEL 25:41–47
6. Hook EB (2000) Neural-tube defects. N Engl J Med 342:1136–1137
7. Kibar Z, Capra V, Gros P (2007) Toward understanding the genetic basis of neural tube defects. Clin Genet 71:295–310
8. Klootwijk R, Hol FA, Wu M, Willemen JJ, Groenen P, Hamel B, Straatman H, Steegers-Theunissen RP, Mariman EC, Franke B (2003) Genetic variation analysis of MLP, TFAP2A, and CSK in patients with neural tube defects. J Med Genet 40:e43–e443
9. Knight RD, Nair S, Nelson SS, Afshar A, Javidan Y, Geisler R, Rauch GJ, Schilling TF (2003) Lockjaw encodes a zebrafish tfap2a required for early neural crest development. DEVELOPMENT 130:5755–5768
10. Li Z, Ren A, Zhang L, Ye R, Li S, Zheng J, Hong S, Wang T, Li Z (2006) Extremely high prevalence of neural tube defects in a 4-county area in Shanxi Province, China. Birth Defects Res A Clin Mol Teratol 76:237–240
11. Lu W, Guzman AR, Yang W, Chapa CJ, Shaw GM, Greene RM, Pisano MM, Lammer EJ, Finnell RH, Zhu H (2010) Genes encoding critical transcriptional activators for murine neural tube development and human spina bifida: a case-control study. BMC Med Genet 11:141
12. Milunsky JM, Maher TA, Zhao G, Roberts AE, Stalker HJ, Zori RT, Burch MN, Clemens M, Mulliken JB, Smith R, Lin AE (2008) TFAP2A mutations result in branchio-oculo-facial syndrome. Am J Hum Genet 82:1171–1177
13. Nottoli T, Hagopian-Donaldson S, Zhang J, Perkins A, Williams T (1998) AP-2-null cells disrupt morphogenesis of the eye, face, and limbs in chimeric mice. Proc Natl Acad Sci U S A 95:13714–13719
14. Qiao X, Liu Y, Li P, Chen Z, Li H, Yang X, Finnell RH, Yang Z, Zhang T, Qiao B, Zheng Y, Wang H (2016) Genetic analysis of rare coding mutations in CELSR1-3 in Chinese congenital heart and neural tube defects. Clin Sci (Lond)
15. Sato T, Samura O, Kato N, Taniguchi K, Takahashi K, Ito Y, Aoki H, Kobayashi M, Migita O, Okamoto A, Hata K (2018) Novel TFAP2A mutation in a Japanese family with branchio-oculo-facial syndrome. Hum Genome Var 5:5
16. Schorle H, Meier P, Buchert M, Jaenisch R, Mitchell PJ (1996) Transcription factor AP-2 essential for cranial closure and craniofacial development. NATURE 381:235–238
17. Stegmann K, Boecker J, Richter B, Capra V, Finnell RH, Ngo E, Strehl E, Ermert A, Koch MC (2001) A screen for mutations in human homologues of mice exencephaly genes Tfap2 alpha and Msx2 in patients with neural tube defects. TERATOLOGY 63:167–175
18. Wallingford JB, Niswander LA, Shaw GM, Finnell RH (2013) The continuing challenge of understanding, preventing, and treating neural tube defects. SCIENCE 339:1222002

19. Xu X, Liu Z, Huang H, Zheng K, Hu X, Zhang Z, Qiu M (2017) AP-2alpha and AP-2beta regulate dorsal interneuron specification in the spinal cord. NEUROSCIENCE 340:232–242
20. Zhang J, Hagopian-Donaldson S, Serbedzija G, Elsemore J, Plehn-Dujowich D, McMahon AP, Flavell RA, Williams T (1996) Neural tube, skeletal and body wall defects in mice lacking transcription factor AP-2. NATURE 381:238–241
21. Zhang J, Williams T (2003) Identification and regulation of tissue-specific cis-acting elements associated with the human AP-2alpha gene. Dev Dyn 228:194–207