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Identification of differentially methylated regions (DMRs) of neuronatin in mice

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Abstract

Background: Neuronatin (*NNAT*) is a paternal-inherited imprinted gene, first discovered in the rat neonatal brain, where it plays vital roles for neuronal growth, brain development, and metabolic regulation. The maternal imprint of *NNAT* has been identified in mice; however, the differentially methylated regions (DMRs) involved in the monoallelic expression of *NNAT* have not yet been investigated.

Results: In this study, we confirmed expression of two isoforms of the *NNAT* (α and β) in the mice brain via quantitative RT-PCR. Additionally, the methylation profile of the CpG island located in the *NNAT* gene locus was determined in the mice liver, brain, sperm, and the MII oocyte via bisulfite sequencing PCR.

Conclusion: In summary, we provide the first evidence for tissue- and gamete-specific methylation patterns of CpG3 that are located on exon 1, to be putative DMR of *NNAT* in mice.

Keywords: *NNAT*, DMR, Mice, Isoform

Background

Genomic imprinting leads to unequal expression of paternal and maternal alleles in offspring, which is essential for normal embryogenesis, fetal growth, and functional postnatal behavior (Surani 1998; Li et al. 1999; Das et al. 2013). The differentially methylated regions (DMRs) are established during gametogenesis and regulate the parent-specific expression of imprinted genes. DMRs with allele specific methylation have also been used in cancer diagnosis (Ushijima 2005; Bonin et al. 2016). Although 125 imprinted genes have been identified in mice to date (according to the Gene imprint database; <http://www.geneimprint.com/>), very little has been published about the DMRs of those imprinted genes (Gu et al. 2014).

NNAT is a highly conserved imprinted gene among humans, mice, cattle, and pigs, containing two alternatively spliced transcripts (α and β) (Cheng et al. 2007;

Schulz et al. 2009). Previous studies have revealed the parental-specific expression of *NNAT* to be associated with the methylation status of the CpG island located in the *NNAT* promoter sequence of pigs (Chen et al. 2014; Gu et al. 2014) and rabbits (Duan et al. 2015). A second intronic DNA sequence within the mouse *NNAT* with a length of 250 bp was defined as putative DMR and was revealed to act as a transcriptional activator in *Drosophila* (Sowpati et al. 2008). In addition, the hyper methylation of human *NNAT* frequently occurs in pediatric acute leukemia and Wilms Tumors (Kuerbitz et al. 2002; Hubertus et al. 2013).

All of these previously published findings suggest that there is no definite DMR of *NNAT* in mice. With this background, we sought to identify the DMRs of mice *NNAT* via quantitative real-time PCR (q-PCR) and bisulfite sequencing PCR (BSP) analyses.

Results and discussion

In this study, we proposed a model of two *NNAT* isoforms (α and β) using GenBank and Ensembl databases. Furthermore, putative DMRs of *NNAT* (CpG 1 and

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CpG 2, CpG 3, CpG I2) were identified via comparative sequencing analysis and methyprimer (Fig. 1a). The results of q-PCR and RT-PCR demonstrated high expression of both of the transcripts (α and β) in the brain of mice, but not in their liver, which is consistent with the UniGene database (Fig. 1b, c). Previous studies have found two transcripts of *NNAT* that are widely expressed in most of tissues, including in livers and kidneys of cattle (both fetal and adult) and in 2-month-old pigs (Zaitoun and Khatib 2006; Cheng et al. 2007), suggesting different *NNAT* expression profiles between species.

To identify the DMR of mice *NNAT*, methylation profiles of CpG 1, CpG 2, CpG 3, and CpG I2 were determined in both the brain and the liver using BSP. The results revealed hypermethylation of CpG1 (Fig. 2a vs. e), CpG2 (Fig. 2b vs. f), and CpG I2 (Fig. 2d vs. h) in both brain and liver. However, we found tissue-specific methylation patterns of CpG3 in the brain (29.33%) and liver (66%) (Fig. 2c vs. g). These results point towards the methylation status of CpG3 accounting for the expression difference of *NNAT* in liver vs. brain. In addition, tissue-specific methylation patterns revealed CpG3 (located on exon 1) to be a putative DMR of the *NNAT* in mice (Fig. 2c).

Generally, DNA methylation patterns of imprinted genes were established in both sperm and oocyte, which were reprogrammed subsequent after fertilization (Seisenberger et al. 2013). To identify the gamete-specific methylation pattern of CpG3 in mice *NNAT*, the DNA methylation profile of CpG3 in sperm and oocyte was determined via BSP. The results reveal hypomethylation

in sperm (10%) and hypermethylation in MII oocytes (90%) (Fig. 3). In summary, the gamete-specific methylation patterns suggest that the imprinting marks of *NNAT* are established by a sex-specific mechanism, confirming CpG3 as the DMR of *NNAT*.

Conclusions

Here, we studied the expression patterns of two isoforms of mice *NNAT* (α and β), and identified the CpG3 of mice *NNAT* (located on exon 1) as putative DMR. The data revealed hypermethylation of exon 1 to be associated with the silencing of mice *NNAT*, suggesting the *NNAT* gene transcriptional status to be correlated with the methylation status of exon 1, while being independent of both intron and 5' UTR.

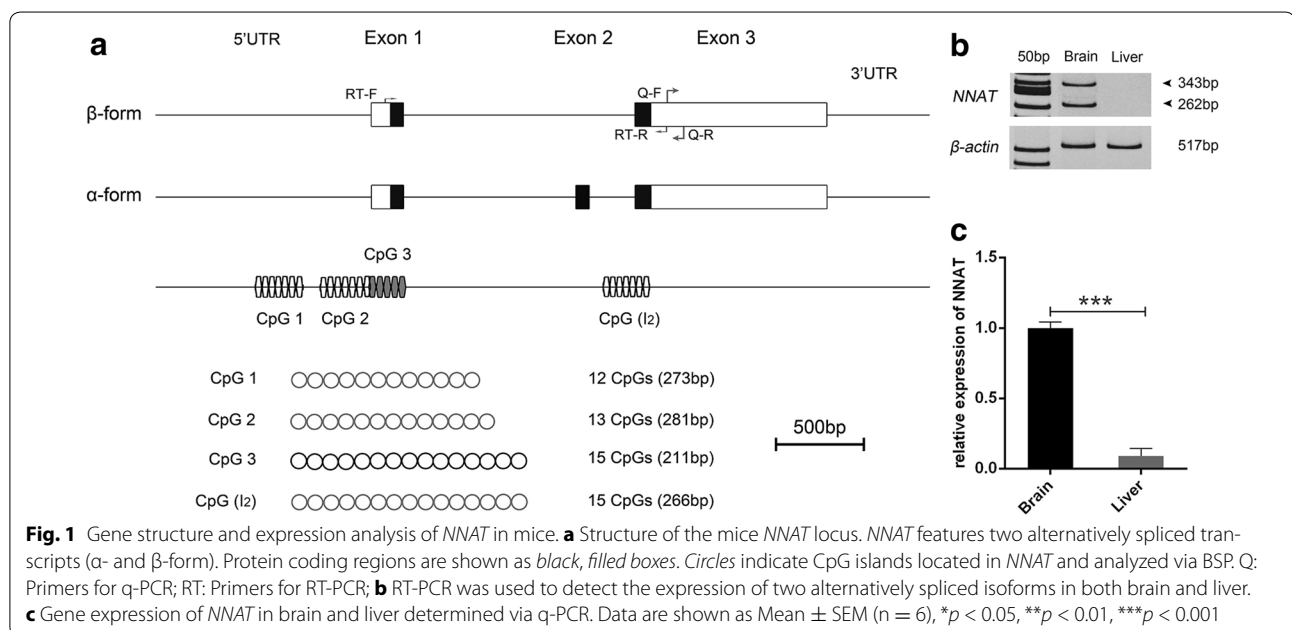
Methods

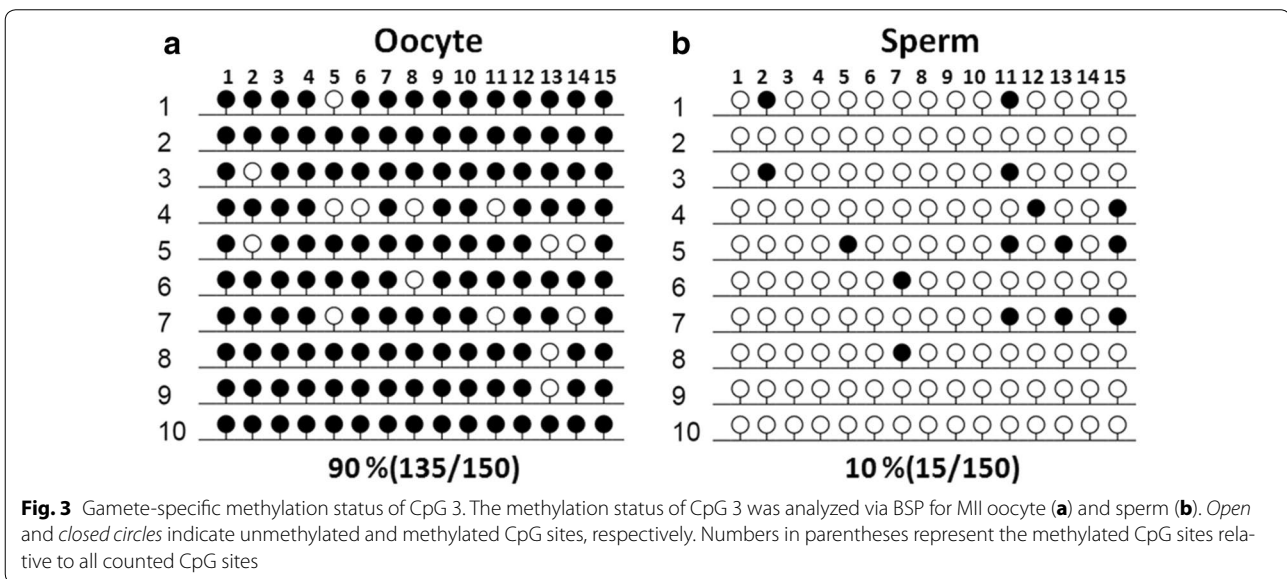
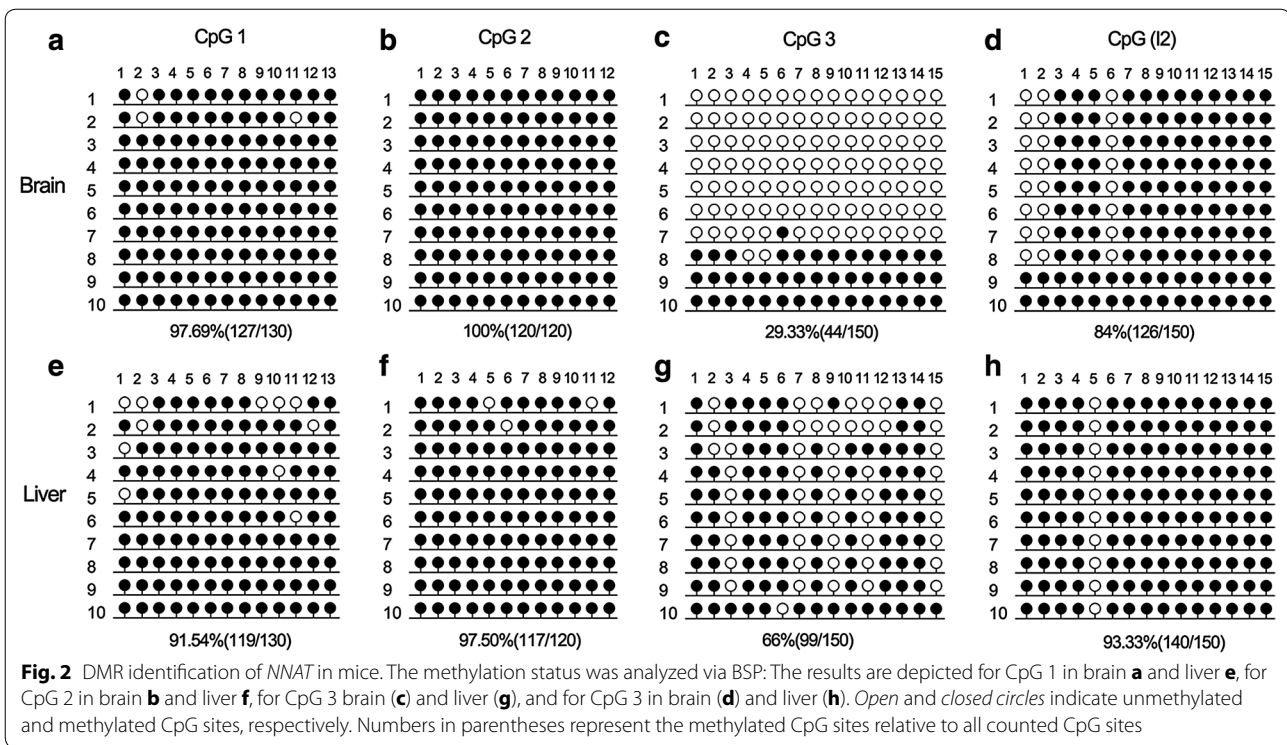
Tissue samples

The brain and liver were collected from two-month old ICR mice and immediately stored in liquid nitrogen until further use. Spermatozoa and MII oocytes were harvested using previously published protocols (Chen et al. 2014).

RT-PCR and quantitative real-time PCR (q-PCR)

Total RNA from liver and brain ($n = 5$) was isolated with TRNzol-A+ reagent (TIANGEN, Beijing, China) according to manufacturer's instructions. The cDNA was synthesized with DNase I (Fermentas, Shanghai, China) treated total RNA via the BioRTcDNA First Stand Synthesis Kit (Bioer Technology, Hangzhou, China).





The BioEasy SYBR Green I Real Time PCR Kit (Bioer Technology, Hangzhou, China) was used to perform q-PCR, using the BIO-RAD Iq5 Multicolor Real-Time PCR Detection System. The reaction conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s for DNA denaturation, 60 °C for 15 s for primer annealing, and 72 °C for 30 s for extension. Relative gene expression normalized to β -actin was determined via

the $2^{-\Delta\Delta CT}$ formula. All experiments on gene expression were performed in triplicate. The gene expression data was presented as mean \pm SEM. Primers used for q-PCR and RT-PCR are listed in Table 1.

Bisulfite sequencing PCR

Bisulfite sequencing PCR (BSP) was performed to determine methylation of *NNAT* CpG islands. The CpGenome

Table 1 Primers for q-PCR and RT-PCR

Gene	Primer sequence (5' → 3')	T _{ann} (°C)	Length (bp)
NNAT-RT	F:GCTCATCATCGGCTGGTACA	60	343
	R:CTTGCCAAGTCTCCTCTGA		262
β-actin-RT	F:ATATCGCTGCGCTGGTCGTC	60	517
	R:AGGATGGCGTGAGGGAGAGC		
NNAT-q	F:GTCCTCTGTGTTCCCTCGTC	60	81
	R:TGTCGGTGCTGCTTTTCTGG		
Actin-q	F:GGCACCACACYTTCTACAATG	60	133
	R:GGGGTGTGAAGGTCTCAAAC		

T_{ann} the annealing temperature, RT the primers for RT-PCR, Q the primers for q-PCR

TM Turbo Bisulfite Modification Kit (Millipore, Jaffrey, NH, USA) was used for bisulfate treatment of genomic DNA of both liver and brain according to the manufacturer's instructions. Bisulfite-treatment of the DNA of sperms (n = 1 × 10³) and matured oocytes (n = 100) was done according to the instructions of the EZ DNA Methylation-Direct™ Kit (Zymo Research, CA). Nested PCR was used to amplify CpG islands, followed by T-vector cloning (positive clones, n = 10) and subsequent sequencing analysis. The BSP primers are listed in Table 2.

Table 2 Primers for BSP

Gene	Primer sequence (5' → 3')	T _{ann} (°C)	Length (bp)
CpG1-BSP	O-F:TTTTGTGTTTAGTTGTATAGCGAA	55	504
	O-R:AATACAAACCTCTTAATTCGACACA		
	I-F:TAGAGTTCGTATTTGTTTCGTAG	55	275
	I-R:TTTTTCTACATTCTACTAATCCGT		
CpG2-BSP	O-F:GAGCGGGAATTAATAGTTAGAAAAG	55	526
	O-R:ACTAATCTCGAAATCCGCTACTAAA		
	I-F:GTATGTAGAATTTGAGGTTTGGG	54	282
	I-R:CTCTTACCACCTAAATACGCATAC		
CpG3-BSP	O-F:GGTAGAGTAGAATTTTTTGGGA	58	590
	O-R:CACCCTAAATCTTTATTTCCC		
	I-F:TTTAGGTGTAAGAGGGTATTTAAGGTA	60	211
	I-R:AATACATACTCACCTACAACA		
I2-BSP	O-F:TTTGGAATGTTGATTTATTGGGTAGGA	55	558
	O-R:CCCCTCACTAACCTTAACAAATACTCCTC		
	I-F:TAGTTGTTTTGATTGGTGGATAAGT	56	266
	I-R:AACTCGCTACTACGCTCCC		

T_{ann} the annealing temperature, O outside primers of the BSP, I inside primers of the BSP

Statistical analysis

Data were analyzed using student's *t*-tests via SPSS 22.0 software (SPSS Inc., Chicago, IL, USA) and a *p* < 0.05 was considered as statistically significant. DNAMAN and online software tools Methprimer (<http://www.urogene.org/methprimer/>) and Bio Analyzer (<http://biq-analyzer.bioinf.mpi-inf.mpg.de/tools/MethylationDiagrams/index.php>) were used for the methylation analysis.

Abbreviations

NNAT: neuronatin; PCR: polymerase chain reaction; qPCR: quantitative real-time PCR; BSP: bisulfite sequencing PCR; DMR: differentially methylated region; RT-PCR: reverse transcription PCR.

Authors' contributions

YX, TW and ZL conceived, designed and performed the experiments and wrote the manuscript. XC and JD collected the samples. MC performed the statistical analysis. ZL provided guidance. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

All experiments about mice were carried out in accordance with the guidelines on animal care and supervised by the Animal Care Center and Use Committee of Jilin University. All experimental protocols were approved by the Ethics Committee of Jilin University.

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