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RESEARCH ARTICLE

Association between expression levels and growth trait-related SNPs located in promoters of the *MC4R* and *MSTN* genes in *Spinibarbus hollandi*

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Abstract

Melanocortin 4 receptor: (*MC4R*) and *Myostatin* (*MSTN*) are two important growth trait-related genes in animals. In this study, we showed that two SNPs, *MC4R*-719A>G and *MSTN*-519C>T, found in the promoters of the *MC4R* and *MSTN* genes, respectively, are both associated with growth traits in *Spinibarbus hollandi*. Furthermore, we observed that there were significant associations between the expression levels of the *MC4R* and *MSTN* genes and these two growth trait-related SNPs. The expression level of *MC4R* gene in brain was lower in GG genotype fish with extremely high growth performance than that in AA genotype fish with extremely low growth performance. Expression level of the *MSTN* gene in muscle was lower in TT genotype fish with extremely high growth performance than that in CC and CT genotype fish with lower growth performance. The results indicated that these SNPs located in the promoters of *MC4R* and *MSTN* are associated with growth-related traits through modification of gene expression levels. The *MSTN* and *MC4R* SNPs may have useful application in effective marker-assisted selection aimed to increase output in *S. hollandi*.

Keywords *Melanocortin-4 receptor* (*MC4R*) · *Myostatin* (*MSTN*) · Expression level · *Spinibarbus hollandi* · Single-nucleotide polymorphism (SNP)

Introduction

Melanocortin-4 receptor (MC4R) is a G-protein-coupled receptor expressed in the appetite-regulating area of brain, which is associated with feed intake regulation and energy balance (Fan et al. 1997; Meidtner et al. 2010). Huszar et al. (1997) observed that knockout of *MC4R* induced symptoms of polyphagia, obesity, and higher levels of insulin secretion in mice. Moreover, mutations of the *MC4R* gene have been found to be the most common cause of hereditary obesity in humans (Carroll et al. 2005). Since *MC4R* links to the control of body weight via regulation of energy homeostasis, it has been considered as a potentially valuable gene for improving growth-related traits in animals.

Myostatin (MSTN) is a negative regulator of muscle development through regulating both the number and growth of muscle fibers (Lee and Mcpherron 1999). Mutations of *MSTN* leading to non-functional proteins have been reported to cause the "double-muscling" phenotype in cattle (Mcpherron and Lee 1997; Kambadur et al. 1997). Similarly, *MSTN* gene knockout was shown to cause a significant increase in muscle mass in mice, as a consequence of muscle cell hypertrophy and hyperplasia (McPherron et al. 1997). Additionally, knockdown of the *MSTN* gene has been shown to give rise to muscular hypertrophy in zebra fish (Lee et al. 2009). This gene has therefore been considered a potential candidate gene for identification of genetic markers and improving growth and meat quality traits in livestock and fish.

Single-nucleotide polymorphism (SNP) is a common form of variation in genes, promoters, and regulatory regions, it is widely distributed throughout genomes. Therefore, some SNPs may affect biological phenotypes through modifying the expression of genes. SNPs have become the focuses of intense research in the fields of fish genetics and breeding (Houston et al. 2012; Poćwierz-Kotus et al. 2014;

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Yang et al. 2016). SNPs of *MSTN* associated with production traits have been widely reported, both in livestock, such as sheep (Hickford et al. 2010), pig (Jiang et al. 2002), and cattle (Sellick et al. 2007), and in fish, such Atlantic salmon (*Salmo salar*; Peñaloza et al. 2013), common carp (*Cyprinus carpio*; Sun et al. 2012), spotted halibut (*Verasper variegatus*; Li et al. 2012), and bighead carp (*Aristichthys nobilis*; Liu et al. 2012). Although polymorphisms located in the *MC4R* gene have been widely demonstrated to be associated with growth traits (Zeng et al. 2014; Kim et al. 2000; Cai et al. 2015; Lee et al. 2013; Fontanesi et al. 2013), to date there have been few reports regarding associations between *MC4R* gene polymorphisms and growth quality traits in fish.

The cyprinid fish *S. hollandi* (Cyprinidae: Cypriniformes) is widely distributed in the south of China, including the provinces of Guangxi, Guangdong, Fujian, and Anhui. As *S. hollandi* has high nutritional and medicinal value, it produces high economic benefit (Cai et al. 2007). In recent years, increasing demand for *S. hollandi* has stimulated considerable research on this species. Most previous studies on *S. hollandi* have focused on genetic resources (Shu et al. 2015), rearing conditions (Lv et al. 2008), ethology (Li et al. 2011), and morphology (Wang 2013), whereas there is considerably less attention paid to analysis of marker-assisted selection.

Therefore, the aims of this study were to: (1) identify SNPs of the *MSTN* and *MC4R* genes and investigate the relationships between *MSTN* and *MC4R* polymorphisms and growth traits in *S. hollandi*, (2) analyze the relationships between expression levels of the *MC4R* and *MSTN* genes, their SNPs and growth traits in *S. hollandi*.

Materials and methods

Materials and phenotypic data collection

The experimental fish were obtained from Shaoguan Fisheries Research Institute in Guangdong, China. The parent fish of the experimental population used for association analysis were selected from the first generation of wild stock collected from the Beijiang River. All fish were hatched at the same time and cultured under the same rearing and management conditions. At the age of 1 year, 235 *S. hollandi* with an average weight of 134.75 ± 38.66 g were randomly selected without differentiating sexes. Five growth traits, including body weight (BWT), body length (BL), total length (TL), body depth (BD), and body width (BWH), were measured in each fish for association analysis. The part of caudal fin of each fish was collected and preserved in 95% ethanol. After completing measurements, each fish was marked and maintained with feeding under pre-measurement conditions in another pool.

PCR amplification and SNP identification

To detect *MSTN* and *MC4R* polymorphisms, two primer pairs, MSTNPF, 5′- ACAGATACGTGAATATTATC-3′, MSTNPR, 5′-TGCGCCGTTATATCTCCATG-3′, and MC4RPF, 5′-TCTTTATGAGTGAATTACTG-3′, MC4RPR, 5′-CAAAGCAGGTGCTGTGTGAG-3′, were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA) based on the DNA sequences of *S. hollandi MSTN* (GenBank: KY853657) and *MC4R* (GenBank: KY022411) genes. PCR amplification was performed in a reaction volume of 50 µL, containing 25 µL 2 × Taq Master Mix (Dye Plus, Vazyme Biotech, Nanjing, China), 20 µL double-distilled water, 3 µL DNA solution, and 1 µL of each primer (10 µM). The PCR amplification reactions were performed using the following thermo cycle program: 94 °C for 10 min, followed by 35 cycles of 94 °C for 45 s, 55 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. Amplification results were verified by 2% agarose gel electrophoresis and PCR fragments of the predicted size were purified using an agarose gel DNA Extraction kit (Generay Biotech Co., Ltd, Shanghai, China).

SNP identification, genotyping, and association analysis

Sequencing of the amplified DNA fragments was performed by Generay Biotech Co., Ltd using an ABI 3730XL sequencer (Applied Biosystems, USA). Differences of gene sequences between individuals were detected using SeqMan version 7.1.0 (DNASTAR Inc., Madison, WI, USA). SNPs were detected and genotyped through observing and comparing chromatogram files using Chromas version 2.33 (Technelysium Pty Ltd., South Brisbane, Australia). Association analyses between genotypes of the *MSTN* gene and the *MC4R* gene and the five selected growth traits were performed using post hoc multiple comparisons (the Duncan method) with SPSS 19.0 software (IBM, Armonk, NY, USA).

Association between gene expression levels and growth trait-related SNPs

On the basis of the results of association analyses, for each genotype of the growth trait-related SNPs, 12 fish were randomly collected from marked fish to analyze the association between gene expression levels and the growth trait-related SNPs. Sampled fish were anesthetized by immersion in 0.1% eugenol for 1–2 min, and these fish were then dissected using stainless steel scissors. Brain or/and muscle samples were frozen immediately in liquid nitrogen and stored at

− 80 °C for RNA isolation. The brains were used to analyze the association between expression levels and the growth trait-related SNP in *MC4R*, whereas both muscle and brains were used for the same analysis in *MSTN*.

RNA isolation and the real-time PCR

Total RNA was isolated from frozen samples using RNA Isolater Total RNA Extraction Reagent (Vazyme Biotech, Nanjing, China) according to the manufacturer's instructions. RNA concentrations were determined at 260 nm. The 260/280 ratio was used to verify the quality of the RNA in each sample. RNA samples were dissolved in diethylpyrocarbonate-treated water and stored at − 80 °C.

Real-time PCR was performed using an ABI 7000 thermal cycler in 20-μL reaction volumes containing the following components: 100 ng of cDNA, 10 μL Power SYBR Green PCR Master Mix (Vazyme Biotech Co., Ltd, Nanjing, China), 0.3 μL of each primer (10 μmolL^{-1}), and 7.4 μL double-distilled water. The primer pairs, QMSTNF, 5′-ATG ACCATGGCCACAGAGCCTG-3′, QMSTNR, 5′-CCGGTC TCAGATGAACCCAGAGC-3′, and QMC4RF, 5′-AGCCGT AGCAGACTTGTTGGTC-3′, QMC4RR, 5′-TGTTCTTGA TGATGCTCTCGCG-3′, were used for RT-PCR amplification of *MSTN* and *MC4R*, respectively. All samples were analyzed in triplicate and the mean value was used to calculate mRNA expression levels. *β-actin* was amplified as the internal control gene with the primers F (5′-CAGCCATCC TTCTTGGGTATG-3′) and R (5′-TCTGCATACGGTCAG CAATGC-3′). The relative mRNA expression levels of each genotype were analyzed using the $2^{-\Delta\Delta C_t}$ method.

Statistical analysis

Genetic analyses, including Hardy–Weinberg equilibrium (HWE), expected heterozygosity (*He*), and observed heterozygosity (*Ho*), were calculated using Haploview software (Broad Institute, America). Polymorphism information content (PIC) was calculated using PIC CALC version 0.6 (Yellow Sea Fisheries Research Institute, Qingdao, China). The effects of different SNP genotypes on the five selected growth traits were analyzed by one-way ANOVA using SPSS 19.0 software. The genotypes of SNPs significantly associated with the growth traits of *S. hollandi* were analyzed through post hoc multiple comparisons (the Duncan method). Analysis of the association between gene expression levels and growth trait-related SNPs was performed using Student's *t*-test. The following statistical model was applied:

$$Y = u + G + e,$$

where Y is the phenotypic value of each trait, u is the population mean value of each growth trait, G is the fixed genotypic effect of each SNP, and e is the random error effect.

Table 1 The genetic polymorphic information of the *MC4R* and *MSTN* SNPs of *Spinibarbus hollandi*

SNPs	*Ho*	*He*	PIC	*p* value (χ^2, HWE)
MC4R-719A>G	0.434	0.498	0.3742	0.06
MSTN-519C>T	0.243	0.466	0.3576	< 0.01

p-value > 0.05 means the loci were in HWE

SNPs single nucleotide polymorphisms, *He* expected heterozygosities, *Ho* observed heterozygosity, *HWE* Hardy–Weinberg equilibrium, *PIC* polymorphism information content

Table 2 The allele and genotype frequencies of SNPs

SNPs	Genotype frequencies (%)			Allele frequencies (%)	
MC4R-719A>G	31.1/AA	43.4/GA	25.5/GG	52.77/A	47.23/G
MSTN-519C>T	50.6/CC	24.7/CT	24.7/TT	62.98/C	37.02/T

SNPs single nucleotide polymorphisms

Results

SNP identification and genotyping

Two SNPs (*MC4R*-719A>G and *MSTN*-519C>T) were detected in the promoters of *MC4R* and *MSTN*, respectively. Chi square tests revealed that *MC4R* -719A>G was in HWE ($p > 0.05$), whereas *MSTN*-519C > T deviated from the HWE ($p < 0.05$), The *He, Ho*, PIC, and Hardy–Weinberg p-values of these two SNPs are shown in Table 1. The two SNPs were classified as being moderately polymorphic loci based on the following criteria: loci with PIC > 0.5 are highly polymorphic; loci with 0.5 > PIC > 0.25 are moderately polymorphic; and loci with PIC < 0.25 have low polymorphism (Vaiman et al. 1994). The allele and genotype frequencies of the SNPs are shown in Table 2.

Analysis of associations between SNPs and growth traits

The results of association analyses between different SNP genotypes and the five selected growth traits are shown in Table 3. For the *MC4R*-719A>G SNP, the five growth traits of the GG genotype fish were significantly higher than those of the AA and AG genotype fish. Measurements of the five growth traits of the AG genotype fish were also higher than those of the AA genotype fish, although the differences were not significant. For the *MSTN*-519C>T SNP, the BWT of the TT genotype fish was significant higher than that of the CC and CT genotype fish, and the BD and BWH of the TT

Table 3 One-way ANOVA analysis of the association between single-nucleotide polymorphisms (SNPs) of the *MC4R* and *MSTN* genes and growth traits in *Spinibarbus hollandi* (mean value ± standard deviation)

SNPs	Genotypes	N	BWT (g)	BL (cm)	TL (cm)	BD (cm)	BWH (cm)
MC4R-719A>G	AA	73	122.47 ± 35.53[a]	18.48 ± 1.64[a]	21.86 ± 1.89[a]	4.37 ± 0.49[a]	2.97 ± 0.39[a]
	AG	102	130.80 ± 33.37[a]	19.21 ± 1.65[a]	22.87 ± 7.80[a]	4.47 ± 0.51[a]	3.01 ± 0.43[a]
	GG	60	156.44 ± 42.28[b]	19.63 ± 1.60[b]	23.18 ± 1.86[b]	4.85 ± 0.55[b]	3.35 ± 0.60[b]
	Total	235	134.75 ± 38.66	19.10 ± 1.69	22.64 ± 1.92	4.53 ± 0.54	3.08 ± 0.49
MSTN-519C>T	CC	119	127.80 ± 33.40[a]	19.05 ± 1.53	22.60 ± 1.88	4.42 ± 0.48[a]	3.01 ± 0.40[a]
	CT	58	134.87 ± 39.99[a]	18.88 ± 1.65	22.35 ± 1.81	4.57 ± 0.56[ab]	3.13 ± 0.61[ab]
	TT	58	148.90 ± 43.83[b]	19.42 ± 1.98	23.01 ± 2.07	4.73 ± 0.95[b]	3.18 ± 0.51[b]
	Total	235	134.75 ± 38.66	19.10 ± 1.69	22.64 ± 1.92	4.53 ± 0.54	3.08 ± 0.49

Significant differences ($p < 0.05$) are shown using different superscripts (a and b)

SNPs single nucleotide polymorphisms, *BWT* body weight, *BL* body length, *TL* total length, *BD* body depth, *BWH* body width

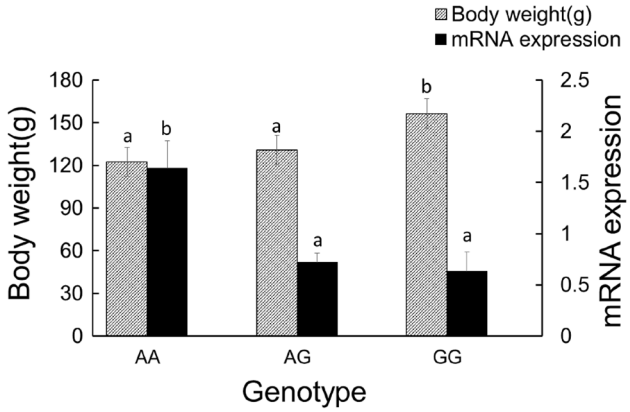

Fig. 1 Body weight and *MC4R* mRNA expression in the brain. Different letters indicate a significant difference ($p < 0.05$). Black columns represent *MC4R* mRNA expression levels, and black dashed columns represent the body weight of each genotype. Results are expressed as means ± standard error

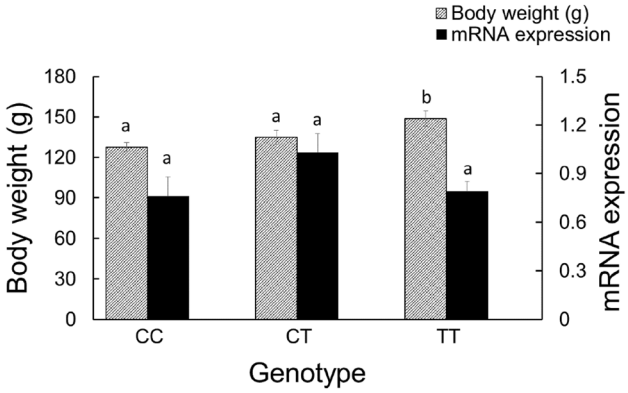

Fig. 2 Body weight and *MSTN* mRNA expression in the brain. Different letters indicate a significant difference ($p < 0.05$). Black columns represent *MSTN* mRNA expression levels, and black dashed columns represent the body weight of the different genotypes. Results are expressed as means ± standard error

genotype fish were significantly higher than those of CC genotype fish. Measurements of the five growth traits were not significantly different between CC and CT genotype fish.

Association between gene expression levels and growth trait-related SNPs

Expression levels of the *MC4R* gene in brain were significantly higher in the AA genotype fish than in the AG and GG genotype fish ($p < 0.05$) and slightly higher in the AG genotype fish than in the GG genotype fish (Fig. 1). The GG genotype fish, which had the highest BWT, had lowest expression of the *MC4R* gene in the brain. The AA genotype fish, which had the lowest BWT, had the highest expression of *MC4R* in the brain.

Body weight and *MSTN* mRNA expressions in the brain were shown in Fig. 2. No significant difference was detected in the *MSTN* expression levels in the brain among the different genotype groups of *MSTN*-519C>T ($p > 0.05$).

The expression levels of the *MSTN* gene in muscle were significantly lower in TT genotype fish than in the CC and CT genotype fish ($p < 0.05$). The BWT of TT genotype fish was significant higher than that of CC and CT genotype fish ($p < 0.05$), whereas there were no significant differences in BTW and expression levels of the *MSTN* gene between CC and CT genotype fish (Fig. 3).

Discussion

In this experiment, we detected two mutations (*MC4R*-719A>G and *MSTN*-519C>T) in the promoters of the *MC4R* and *MSTN* genes of *S. hollandi*, respectively. Both SNPs were moderately polymorphic loci. *MC4R*-719A>G was in HWE, whereas *MSTN*-519C>T deviated from the HWE, it is

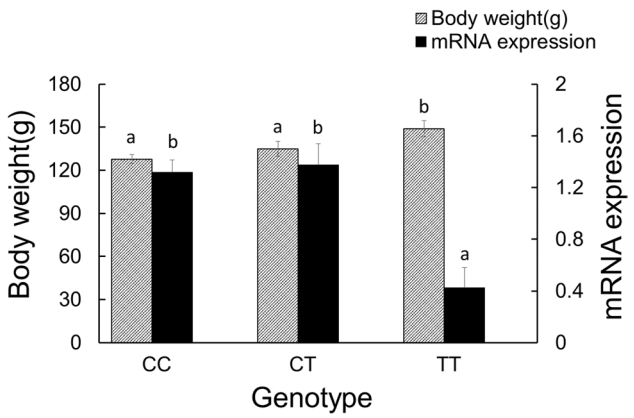

Fig. 3 Body weight and *MSTN* mRNA expression in muscle. Different letters indicate a significant difference ($p < 0.05$). Black columns represent *MSTN* mRNA expression levels, and black dashed columns represent the body weight of the different genotypes. Results are expressed as means ± standard error

possible that the *MSTN*-519C>T, or other variation closely linked to the SNP, was subjected to natural selection pressure. This phenomenon was also found in *Argopecten irradians* (Meng et al. 2017). The two SNPs are both related with growth traits in this fish. The expression levels of the *MC4R* gene in the brain are associated with the growth trait-related SNP, *MC4R*-719A>G, whereas the SNP, *MSTN*-519C>T, is associated with expression levels of the *MSTN* gene in muscle, but not in the brain.

Our results showed that the *MC4R*-719A>G is associated with all five growth traits of *S. hollandi* examined in the present study, with the GG genotype being predominant among the three genotypes. As an important candidate gene affecting growth ratio, numerous association studies have focused on the detection of *MC4R* gene polymorphisms associated with economic traits in domestic animals. Zhang et al. (2006) reported a missense mutation in *MC4R* that was significantly associated with birth weight and average daily gain in cattle. Kim et al. (2000) observed that an SNP, Asp298Asn, located in *MC4R* was significantly associated with growth traits in pigs, and Song et al. (2012) detected four SNPs in the 3′-UTR of *MC4R* that were significantly associated with birth weight in sheep. However, there have been few studies that have focused on the association between *MC4R* SNPs and growth quality traits in fish. Although several SNPs have previously been identified in the *MC4R* gene of *Oreochromis niloticus* (tilapia) and *Takifugu rubripes*, no growth trait-related SNPs were identified in the two species (Liu et al. 2009; Zhang et al. 2012). The growth trait-related SNP identified in the *MC4R* gene in the present study will therefore make a potentially important contribution to the analysis of molecular markers in fish.

In the present study, we observed that the *MC4R*-719A>G is associated with expression levels of the *MC4R* gene in the brain of *S. hollandi*. The GG allele has the lowest expression level and the GG genotype fish have the most predominant growth traits. These observations are consistent with previous observations indicating that inactivation or decreased activity of *MC4R* can result in an increase in body weight (Huszar et al. 1997).We hypothesized that an SNP located in the 5′-flanking region might have an impact on *MC4R* expression and growth performance, by affecting *MC4R* promoter activity. However, we cannot exclude the possibility that the *MC4R* SNP identified in the present study is in linkage disequilibrium with an unidentified causal mutation for *MC4R* expression and growth performance.

In association studies, the *MSTN*-519C>T was also found to be associated with growth traits of *S. hollandi*, with the TT genotype being predominant. Significant associations between *MSTN* polymorphisms and production traits have been widely reported in aquacultural species, including bighead carp (*Aristichthys nobilis*; Liu et al. 2012), yellow catfish (*Pelteobagrus fulvidraco*; Zhu et al. 2012), spotted halibut (*Verasper variegatus*; Li et al. 2012), common carp (*Cyprinus carpio*; Sun et al. 2012), Atlantic bay scallop (*Argopecten irradians*; Guo et al. 2011), and gilthead seabream (*Sparus aurata*; Sánchez-Ramos et al. 2012). Moreover, the *MSTN*-519C>T departed from Hardy–Weinberg equilibrium, and thus it is possible that this SNP is linked to unidentified genes that are affected by natural selection.

The *MSTN*-519C>T is associated with expression levels of the *MSTN* gene in the muscles of *S. hollandi*. This result indicated that *MSTN* is a negative regulator of growth in muscle. Knocking down *MSTN* using antisense morpholinos resulted in the increase of length and width of somites in juvenile zebra fish (Amali et al. 2004). Muscle hyperplasia and hypertrophy were also found in *Myostatin* dsRNA-microinjected zebrafish (Acosta et al. 2005). No significant association between expression level of the *MSTN* gene in brain and its SNP was found, suggesting that *MSTN* expressed in brain may plays another role at the stage of development in *S. hollandi*. Further investigation of this possibility, including RNA-Seq after *MSTN* knockdown and other functional studies, will provide important insights into *MSTN* function. The possibility same as above, the SNP might have a direct impact on *MSTN* expression, as well as is in linkage disequilibrium with unidentified loci that impact on *MSTN* expression.

In conclusion, the findings of this study indicate that SNPs located in the *MSTN* and *MC4R* promoters have effects on growth-related traits via modifications of gene expression. Information obtained in this study on the *MC4R* and *MSTN* SNPs may have potential applications in effective marker-assisted selection to increase body weight and lean meat percentage in *S. hollandi*.

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Compliance with ethical standards

Conflict of interest Yang Yang declares that he does not have conflict of interest. Zhaojun LAN declares that he does not have conflict of interest. Hu Shu declares that he does not have conflict of interest. Huiqiang Zhou declares that he does not have conflict of interest. Xiaolu Jiang declares that she does not have conflict of interest. Liping Hou declares that she does not have conflict of interest. Pinghua Gu declares that he does not have conflict of interest.

Research involving human and animal rights All animal experiments throughout the study were conducted in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals for the Science and Technology Bureau of China.

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