

编号：SCIE-H-20260908-29

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标题: Identification and functional analysis of six melanocortin-4-receptor-like (MC4R-like) mutations in goldfish (Carassius auratus)

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期刊: GENERAL AND COMPARATIVE ENDOCRINOLOGY 卷: 360 文献号: 114639 文献类型: Article

DOI: 10.1016/j.ygcen.2024.114639

出版年: JAN 1 2025

在线发表时间:NOV 2024

已索引:2024-11-24

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出版商:ACADEMIC PRESS INC ELSEVIER SCIENCE

Web of Science 类别: Endocrinology & Metabolism ;Zoology

语种: English

入藏号: WOS:001357296000001

ISSN: 0016-6480

eISSN: 1095-6840

Web of Science 核心合集中的 "被引频次":1

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2026 年公布的影响因子: 2.1, JCR 分区情况:

JCR® 类别	类别中的排序	JCR 分区
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ENDOCRINOLOGY & METABOLISM	148/198	Q3

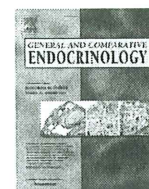
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Research paper

Identification and functional analysis of six melanocortin-4-receptor-like (MC4R-like) mutations in goldfish (*Carassius auratus*)

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ARTICLE INFO

Keywords:

Melanocortin-4-receptor-like (MC4R-like)
Goldfish (*Carassius auratus*)
Natural mutation
Pharmacology

ABSTRACT

Melanocortin receptor-4 (MC4R) belongs to the G protein-coupled receptor family, characterized by a classical structure of seven transmembrane domains (7TMD). They play an important role in food intake and weight regulation. In the present study, we identified *melanocortin-4-receptor-like* (*caMC4RL*) mutants of goldfish from the Qian River in the Qin Ling region and characterized their functional properties, including the constitutive activities of the mutants, ligand-induced cAMP and ERK1/2 accumulation, and AMPK activation. The results show that six *caMC4RL* mutants were identified in goldfish from the Qian River in the Qin Ling region, and are located in the conserved position of the Cyprinidae MC4Rs. The mutations (E57K, P296S, and R302T/K) result in the loss of Gs signaling function. The mutations (P296 and R302T/K) exhibited biased signaling in response to ACTH stimulation in the MAPK/ERK pathway. In addition, the E57K mutant may play a role in weight regulation and could serve as molecular markers for molecular breeding. These data will provide fundamental information for functional studies of teleost GPCR mutants and MC4R isoforms.

1. Introduction

Growth has always been used as an important indicator in aquaculture. It is closely related to food intake and energy homeostasis. Melanocortin receptor 4 (MC4R) plays a crucial role in regulating feeding and energy homeostasis (Li et al., 2019; Matsumura et al., 2022; Park et al., 2016; Wang et al., 2021), making it a promising target gene for enhancing growth-related traits in animals (Coogan et al., 2023, 2022; El-Sabrou and Soliman, 2018; Kim et al., 2000). MC4R is a member of the rhodopsin family belonging to G protein-coupled receptors (GPCRs) with the classical structure of seven transmembrane domains (7TMD) (Gantz et al., 1993; Tao, 2010) and conserved structural domains of DRY, PMY, and N/DPxxY. In *Carassius auratus*, there are multiple isoforms of the MC4R, possibly due to the additional whole genome duplication (WGD) (Chen et al., 2020). These isoforms share the same structural features and conserved structural domains, and thus may have similar physiological functions (unpublished findings from our lab).

MC4R is highly expressed in the central nervous system (CNS) and is an important receptor for regulating appetite (Kishi et al., 2003; Wei et al., 2023). MC4R ligands such as MTII, NDP-MSH, SHU9119, and

HS024 can regulate food intake (Brown et al., 1998; Jonsson et al., 2002; Kask et al., 1998; Murphy et al., 1998). MC4R knockout mice exhibit hyperphagia (Srisai et al., 2011). MC4R also plays an important role in energy homeostasis and body weight regulation. Prolonged stimulation of HS014/HS024 (melanocortin-4 receptor antagonist) leads to obesity in mice (Jonsson et al., 2002; Kask et al., 1999). MC4R polymorphisms are associated with childhood obesity and body energy expenditure (Arrizabalaga et al., 2014; Cole et al., 2010; Kring et al., 2010; Muller et al., 2014), and individuals with loss-of-function (LOF) mutant MC4R are hyperphagic and obese (Wade et al., 2021). MC4R knockout mice become obese at maturity, develop hyperinsulinemia, and hyperglycemia compared to wild-type (WT) mice (Tallam et al., 2005). In farm animals, MC4R polymorphisms were associated with food intake, body weight, body length, carcass metrics, and starvation resistance (El-Sabrou and Soliman, 2018; Kim et al., 2000; Yang et al., 2018, 2017).

MC4R is activated by its natural agonists, the pro-opiomelanocortin-derived melanocortin peptides, α - and β -melanocyte-stimulating hormone (Millington, 2007). Upon activation, it couples to Gs proteins, leading to increased intracellular levels of cyclic adenosine monophosphate (cAMP) and the activation of downstream protein kinase A

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(PKA). In addition, MC4R phosphorylates extracellular signal-regulated kinase 1 and 2 (ERK1/2) and AMP-activated protein kinase (AMPK) to exert various physiological functions (Chen et al., 2018; Daniels et al., 2003; Vongs et al., 2004). Some mutant MC4Rs lead to the loss of Gs signaling function (Tao and Segaloff, 2005), which in turn reduces intracellular cAMP signaling. The V103I and H158R mutants affect the affinity for ligands and biasing of signals (Botha et al., 2023; Paisdzior et al., 2020).

Currently, *MC4R* is used as a target gene for breeding, and most studies have focused on the association of polymorphisms with economic traits (Lampert et al., 2010; Lyadskiy et al., 2011; Song et al., 2012; Yang et al., 2018, 2017; Zeng et al., 2014; Zuo et al., 2014). To the best of our knowledge, there are few narratives on *MC4R* polymorphisms in fish, and no studies on fish *MC4R*-like have emerged. *Carassius auratus* is one of the most important culture fish. According to records, the domestication history of goldfish was first recorded during the Eastern Han Dynasty (AD 25 to 220) (Li et al., 2018). It is a model species in the field of endocrinology and comparative endocrinology, and is widely used in studies on growth, feeding, and neural signaling (Blanco et al., 2018). *Carassius auratus* is a species that holds both scientific research and economic value. Therefore, we identified *melanocortin-4-receptor-like* (*caMC4RL*) mutants of goldfish from the Qian River in the Qin Ling region and characterized its functional properties, including the constitutive activities of the mutant, ligand-induced cAMP and ERK1/2 accumulation, and AMPK activation. This work serves as a reference for understanding the function of GPCR mutants in teleost and will also lay the foundation for molecular breeding of *Carassius auratus*.

2. Materials and methods

2.1. Animals and chemical compounds

The twelve sampling sites were set up based on the elevation changes and hydrological conditions of the Qianhe River. In July 2022, a total of 73 fish were collected using ground cages at five of these sampling sites. These sites include the Caobi River site (E107.009°, N34.776°; 27 goldfish), the Fengjiashan Reservoir kuwei site (E107.156°, N34.619°; 24 goldfish), the Fengjiashan Reservoir baxia site (E107.127°, N34.637°; 7 goldfish), the Wangjiaya Reservoir baxia site (E107.262°, N34.416°; 9 goldfish), and the Ruweikou site (E107.301°, N34.372°; 6 goldfish). A portion of the caudal fin was clipped from fish anaesthetized with MS-222. The samples were preserved in 95 % alcohol for genomic DNA extraction. The ligands [Nle4, D-Phe7]- α -MSH (NDP-MSH), and ACTH (1–24)) which were used for pharmacological assays, were purchased from GenScript Biotechnology (Nanjing, China). Single luciferase reporter vectors (pGL4.33 and pGL4.29) were purchased from Promega (Wisconsin, USA). In this experiment, all primers were designed by primer premier 5 application (PREMIER Biosoft International, Palo Alto, USA).

2.2. DNA extraction and identification of *caMC4RL* mutations

The genomic DNA of goldfish was extracted using the tissue genomic DNA extraction kit (Tiangen, China). Through the application of polymerase chain reaction (PCR) and the Sanger method of DNA sequencing, we identified the presence of two gene paralogues for the melanocortin 4 receptor (*MC4R*) in goldfish. This phenomenon has emerged as a result of an additional whole genome duplication event in some species within the Cyprinidae family, such as goldfish and common carp (Chen et al., 2020). Furthermore, through the application of quantitative reverse transcription polymerase chain reaction (qRT-PCR), we identified two transcripts of *MC4R* paralogues in goldfish (based on unpublished data from our lab). Therefore, it is posited that goldfish possess two distinct types of *MC4R* paralogues.

The *caMC4RL* is a paralogue of the *caMC4R*. This study focuses exclusively on identifying mutations in *caMC4RL*. Therefore, the specific

primers for the coding sequence (CDS) region were designed using the Primer Premier 5 application based on the *caMC4RL* sequence that was cloned and obtained in our lab. We performed a 50 μ L PCR system containing 100 ng of genomic DNA, 0.4 μ M of each primer (sense primer: 5'-ATGAACACCTCAGTTCATCATGGA-3'; antisense primer: 5'-TCACACAAGC TATGCAGTGTAA-3'), and 25 μ L of 2 X Rapid Taq Master Mix (Vazyme, Nanjing, China). PCR products were separated using a 1.2 % agarose gel (voltage for electrophoresis: 120 V; duration of electrophoresis: 30 min) and recovered using the Gel Extraction Kit (Omega, Guangzhou, China) for target-sized PCR products. Single-nucleotide polymorphisms (SNPs) in the coding region of *caMC4RL* were identified by sequencing (Sangon Biotechnology Co. Ltd., Shanghai, China).

2.3. Linkage disequilibrium analysis and construction of mutant *caMC4RL* plasmids

The linkage disequilibrium analysis of SNP mutations in the *caMC4RL* coding region was conducted using the SHEsis website (<http://analysis.bio-x.cn/myAnalysis.php>).

Wild-type (WT) *caMC4RL* was amplified from WT goldfish cDNA (retained in the laboratory) using the primers described above. The PCR product was detected by 1.2 % agarose gel electrophoresis. The PCR-fragment of desired size was purified by gel midi purification kit (TIANGEN, China) and cloned into pEASY-T1 Cloning vector (TranGen Biotech, Beijing, China). The vector was transformed into *E. coli* (*Escherichia coli*) DH5 α (Sangon Biotech, Shanghai, China) and positive clones were sequenced. After confirming that the sequence was accurate, the CDS of *caMC4RL* was sub-cloned into pcDNA3.1 (+) (Invitrogen, CA, USA) by Bio-Transduction Lab Co.Ltd (Wuhan, China), and the cloned vector was sequenced to ensure its correctness. To further clarify the function of the mutations, all mutants were commercially synthesized by Tsingke Biotech Co., Ltd (Beijing, China). The Plasmid DNA was prepared with HiPure Plasmid Mini Kit (Biosharp, Nanjing, China) and sequenced again.

2.4. Cell culture

Human embryonic kidney 293 T (HEK293T) cells are widely used models in GPCR studies and have been applied to the study of gene function in various animals. Therefore, we used HEK293T cells as the model for pharmacological functional studies of the mutant *caMC4RL*.

HEK293T cells ((China Centre for Type Culture Collection (CCTCC), Wuhan, China) were seeded into 4 mL Dulbecco modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (FBS, Beyotime Biotechnology, Shanghai, China): USA), 100U/ml penicillin (Biosharp), and 0.1 mg/ml streptomycin (Biosharp) in 25-cm² cell culture flasks. The cells were cultured at 37 °C, 5 % CO₂ and passaged every two days.

2.5. Mutant constitutive activity assay

HEK293T cells were seeded into a 6-well culture plate at a density of 5×10^5 cells/well and cultured for 24 h. The cells were cultured in serum-free DMEM containing pGL4.29 (1000 ng/well), WT *caMC4RL* (500 ng/well) or one of six mutant *caMC4RL*s (500 ng/well), pEGFP-N1 vector (300 ng/well, serving as the calibrating vector or control for estimating transfection efficiency), and 8 μ L/well PEI (Fushen Biotechnology, Shanghai, China) for transfection. After 6 h, the DMEM with 10 % FBS was added to a 6-well plate. After 24 h of incubation, the intensity of green fluorescence was measured using a fluorescence microscope. Once the success of the transfection was confirmed, the cells were passaged into a 48-well plate and cultured for an additional 24 h. HEK293T cells were lysed with 1X Passive Lysis Buffer (Beyotime Biotechnology). The luciferase activities were quantified with Luciferase Reporter Gene Assay Kit (Yeasen, Shanghai, China) and Multi-Mode Microplate Reader Synergy H1 (Agilent BioTek, Beijing, China).

Relative luciferase activity was calculated as the ratio of the luciferase signal of the groups to the WT group, and the luciferase values were expressed as multiples relative to that of WT group.

2.6. Functional characterization of mutant caMC4RL in HEK293T cells

To investigate the functional differences of mutant caMC4RLs, the luciferase reporter vectors pGL4.33 or pGL4.29 were used to detect the activation of cAMP and MAPK/ERK signaling pathways, respectively. Cells were transfected with plasmid mixture including pGL4.29 (1000 ng/well), the expression vector of WT caMC4RL (500 ng/well) or one of six mutant caMC4RL (500 ng/well), pEGFP-N1 vector (300 ng/well). The NDP-MSH or ACTH (1–24)) (GenScript Biotechnology, Nanjing, China) were diluted with a serum-free DMEM medium to the specified concentration (ACTH(1–24), 1×10^{-10} mol/L to 1×10^{-5} mol/L; NDP-MSH, 1×10^{-13} mol/L to 1×10^{-8} mol/L) and used for treatment. The cells expressed WT caMC4RL were served as the control. After 6 h of drug treatment, HEK293T cells were lysed, and the luciferase activities of the cells were quantified according to the method in section 3.6. Relative luciferase activity was calculated as the ratio of the luciferase signal of the groups to the lowest concentration of WT group, and the luciferase values were expressed as multiples relative to that of WT group.

2.7. AMPK phosphorylation assay

The WT caMC4RL or one of six mutant caMC4RLs was transfected into HEK293T cells. After transfection for 6 h, the DMEM with 10 % FBS was added to a 6-well plate. After 24 h of incubation, the transfected cells were treated with agonists for 6 h and then collected for Western blot analysis. The procedure was as follows: The total protein samples were separated by 12.5 % SDS-PAGE gel and blotted onto PVDF membranes. After PVDF membranes were blocked with 10 % skimmed milk, they were incubated overnight at 4 °C with rabbit Anti-beta Actin antibody (1:1000, Servicebio, Wuhan, China) or Anti-Phospho-AMPK

alpha 1 (T183) + AMPK alpha 2 (T172) Rabbit antibody (1:1000, Servicebio, Wuhan, China). The PVDF membranes were incubated with the horseradish peroxidase-labeled goat anti-rabbit IgG (H + L) (Beyotime Biotechnology) for 1 h at room temperature, protected from light. After TBST washing, the specific bands were exposed using ECL reagents (Zeta, USA). The grayscale of the bands was identified using the ImageJ application, and the level of AMPK phosphorylation was quantified by calculating the ratio of p-AMPK to β -actin.

2.8. Statistical analysis

All experiments were performed in triplicate for statistical significance and the reliability of the results. GraphPad Prism 8.0 and Excel were used to calculate signaling parameters, including the maximal response (R_{max}) and EC_{50} values for cAMP and MAPK/ERK signaling. All data were presented as mean $\pm$ SEM, analyzed using one-way analysis of variance (ANOVA) in SPSS 21.0 application, and Tukey's method was used to determine significant differences ($P < 0.05$, $P < 0.01$).

3. Result

3.1. Identification and functional analysis of five polymorphisms in goldfish

The MC4R-like mutants were amplified from genomic DNA obtained from individuals of five different populations. Sequencing of caMC4RL CDS was used to identify SNPs, and analysis of the sequencing identified five SNP sites (c.C74 > T, c.G169 > A, c.G247 > T, c.G886 > T, c.G905 > A/C) resulting in 6 mutants (Fig. 1A). All 6 mutants were determined to be missense mutations according to the caMC4RL mRNA sequence deposited at Genbank (XM_026207258.1) and obtained through cloning in our lab (Fig. 1B). These mutations are located at highly conserved positions among the Carpiidae MC4Rs/MC4RLs. The corresponding residues of hMC4R were also missense mutated (Fig. 1C), such as hMC4R-S28R/I (P25L in caMC4RL), hMC4R-E61K (E57K in caMC4RL),

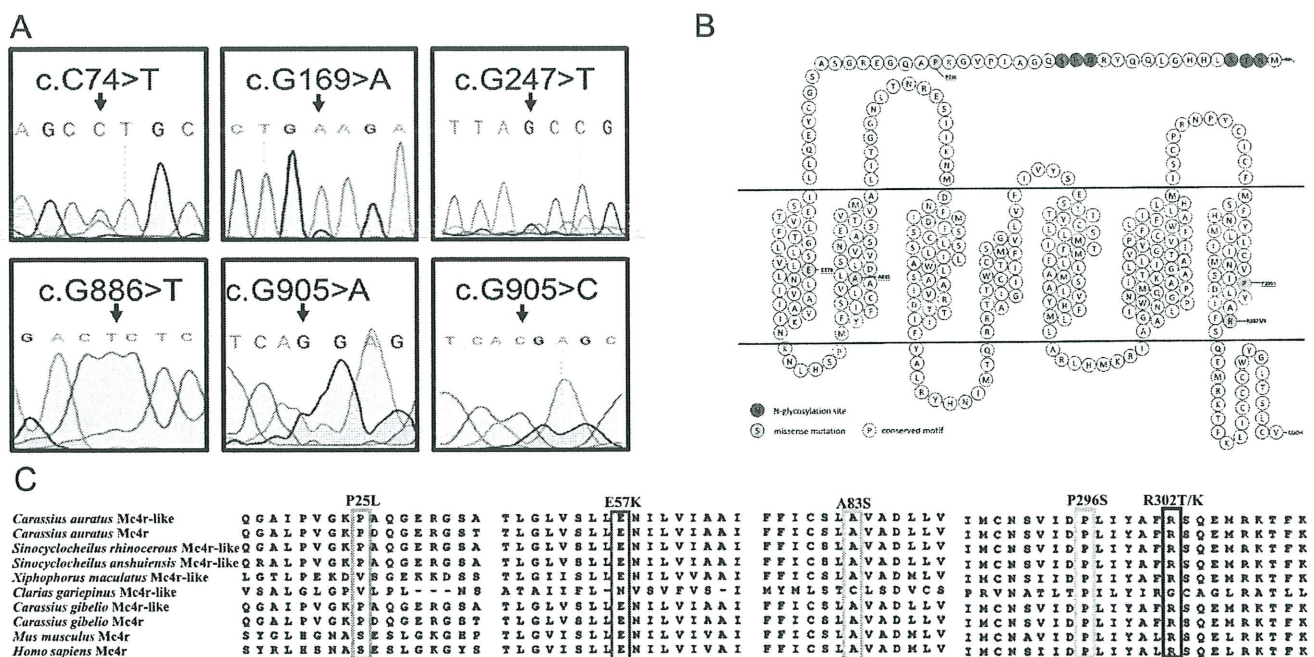


Fig. 1. Identified SNPs and deduced amino acid substitutions in the caMC4RL. (A) Sequence electropherograms for the five SNPs. (B) Alignment of the caMC4RL protein regions around the mutated nucleotides with the same protein regions of other animals MC4R/MC4RL (*Sinocyclocheilus rhinoceros* Mc4r-like, [XP_016394904.1]; *Sinocyclocheilus anshuiensis* Mc4r-like, [XP_016314419.1]; *Xiphophorus maculatus* Mc4r-like, [NP_001303384.1]; *Clarias gariepinus* Mc4r-like, [XP_053366708.1]; *Carassius gibelio* Mc4r-like, [XP_052405144.1]; *Carassius gibelio* Mc4r, [XP_05246620.1]; *Mus musculus* MC4R, [NP_058673.2]; *Homo sapiens* MC4R, [NP_005903.2]). (C) The position of the caMC4RL mutation in the receptor.

hMC4R-A87T (A83S in caMC4RL), P299H/L (P296S in caMC4RL), and R305W (R302T/K in caMC4RL). Residues P25, E57, and A83 are located at the N-terminus, the first transmembrane domain (TM1), and the second transmembrane domain (TM2), respectively. Residues P296 and R302 are located in the seventh transmembrane domain (TM7). More importantly, they are also located at the MC4R conserved motif (N/DPxxY) and Helix 8, respectively (Fig. 1B).

3.2. Genetic parameter analysis and LD analysis of SNPs

The genotype frequencies and allele frequencies of 5 SNPs are displayed in Table 1. c.G169A and c.G247T were in Hardy–Weinberg equilibrium, whereas the other SNPs deviate from Hardy–Weinberg equilibrium. The c.169A and c.247 T alleles are not fixed in the Qian Rivers goldfish breed (no purebred animals).

LD analyses among the 5 SNPs were conducted using the SHEsis website. Due to the low number of samples, the linkage between SNPs was determined by the R^2 value. The estimated R^2 value (Fig. 2) demonstrates weak linkage between all SNPs, and no linkage block was formed.

3.3. Constitutive activity of mutant caMC4RL

Constitutive activity is the ability of a GPCR to adopt an active conformation in the absence of the agonist. In this experiment, the constitutive activity of six mutants was examined (Fig. 3). Mutant P25L significantly increased constitutive activity compared to WT caMC4RL (1.36-fold of WT caMC4RL). The other 5 mutants significantly reduced constitutive activity, with mutants E57K, R302T, and R302K exhibiting the lowest constitutive activity (18 %, 23 %, and 21 % of WT MC4RL).

3.4. Characterizations of cAMP signaling by mutant caMC4RL

To investigate functional differences in the mutant caMC4RL, we examined the levels of cAMP signaling in cells that overexpressed the mutant after agonist activation. NDP-MSH is a synthetic and efficient agonist of melanocortin receptors (MCRs). Therefore, it was used to stimulate the cells in the experiment. As expected, NDP-MSH activated WT caMC4RL and increased intracellular cAMP levels in a dose-dependent manner (Fig. 4A). NDP-MSH could effectively activate both P25L and A83S mutants. Both mutants increased the R_{max} of cAMP compared to WT caMC4RL, but the EC_{50} values of both mutations were higher than those of WT caMC4RL (Table. 2). However, the remaining four mutant MC4RLs (E57K, P296S, R302T, and R302K) failed to respond to NDP-MSH stimulation (Fig. 4A).

ACTH, cleaved from POMC, is an endogenous agonist and is considered a “primitive” ligand. Some studies have reported that ACTH (1–24) exhibits high affinity for fish MCRs (Klovins et al., 2004). This is consistent with the pharmacological functional studies of caMC4RL (unpublished findings from our lab). Similar to the activation effect of NDP-MSH, ACTH (1–24) activated WT caMC4RL and increased intracellular cAMP levels in a dose-dependent manner (Fig. 4B). The maximal

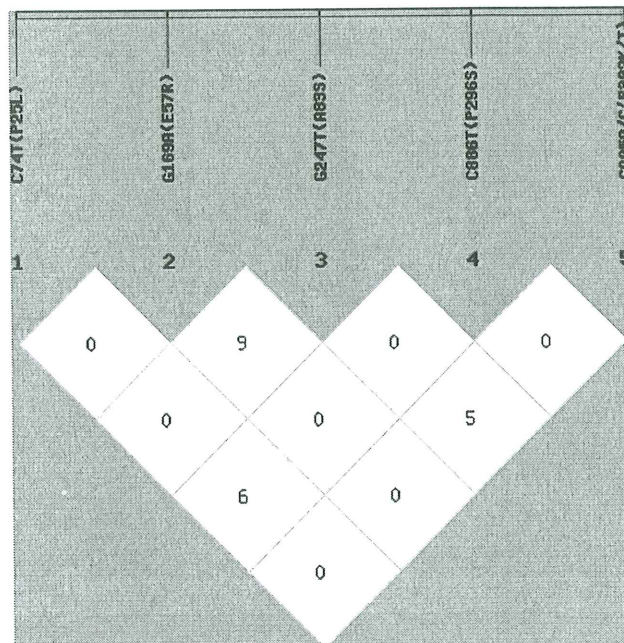


Fig. 2. Linkage disequilibrium (LD) patterns of caMC4RL in goldfish from the Qian River in the Qin Ling region. LD was estimated by R^2 value.

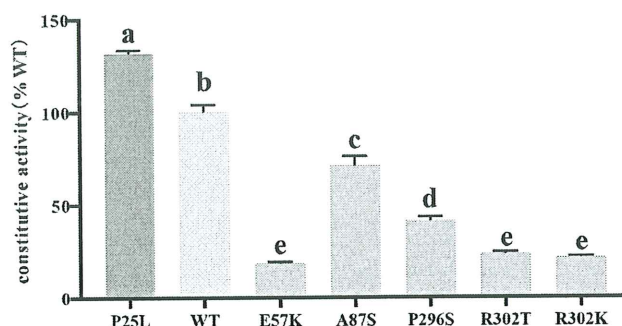


Fig. 3. Constitutive activity of WT caMC4RL and mutant caMC4RL. The results are expressed as percentage of WT constitutive activity. Significant differences are indicated by different letters ($P < 0.05$). Results are expressed as the mean $\pm$ SEM of six experiments.

cAMP response value of P25L is significantly lower than that of WT caMC4RL, but the EC_{50} of the two mutants was significantly higher than that of WT caMC4RL (Table. 2). The other mutants (E57K, P296S, R302T, and R302K) had no measurable signaling (Fig. 4B).

Table 1

Hardy–Weinberg test P values (HW P value) of caMC4RL SNPs and their allele and genotype frequencies.

Loci	Size	Genotypic frequencies				Allele frequencies				Hardy–Weinberg equilibrium (HWE)	
P25L(c.C74T)	71	CC (65)	CT (4)	TT (2)		C	T	P Values			
		0.92	0.06	0.03		0.94	0.06	0.000391			
E57K(c.G169A)	71	GG (64)	GA (7)	AA (0)		G	A	P Values			
		0.90	0.10	0.00		0.95	0.05	0.90896800			
A83S(c.G247T)	73	GG (72)	GT (1)	TT (0)		G	T	P Values			
		0.99	0.01	0.00		0.99	0.01	0.998265477			
P296S(c.C886T)	73	CC (68)	CT (3)	TT (2)		C	T	P Values			
		0.93	0.04	0.03		0.95	0.05	1.61263×10^{-5}			
R302T/K (c.G905C/A)	73	GG (63)	GC (1)	GA (2)	CA	CC (6)	AA (1)	G	C	A	P Values
		0.86	0.01	0.03	0.01	0.08	0.00	0.88	0.09	0.03	3.62911×10^{-15}

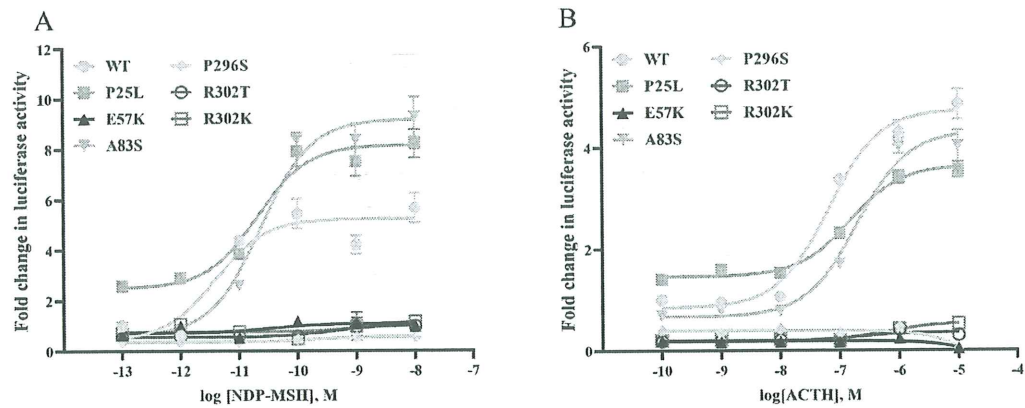


Fig. 4. The cAMP signaling of WT caMC4RL and mutant caMC4RL in HEK293 cells. After stimulating cells with different concentrations of (A) ACTH (1–24), and (B) NDP-MSH, the luciferase activity of the cAMP signaling pathway was detected. Each data point represents the mean ± SEM of three repeated measurements.

Table 2
Signaling properties of WT and mutant caMC4RLs.

caMC4RL construct	NDP-MSH-stimulated cAMP		ACTH (1–24)-stimulated cAMP	
	EC ₅₀ (nM)	R _{max} (% WT)	EC ₅₀ (nM)	R _{max} (% WT)
WT	0.0039 ± 0.0002 ^b	100 ± 6.79 ^b	67.65 ± 11.32 ^b	100 ± 10.68 ^a
P25L	0.0210 ± 0.0004 ^a	144.74 ± 5.70 ^a	154.26 ± 3.54 ^a	73.44 ± 3.11 ^b
E57K	N/D	N/D	N/D	N/D
A83S	0.0239 ± 0.0016 ^a	163.51 ± 8.11 ^a	201.20 ± 20.49 ^a	83.09 ± 6.46 ^{ab}
P296S	N/D	N/D	N/D	N/D
R302T	N/D	N/D	N/D	N/D
R302K	N/D	N/D	N/D	N/D

N/D: Not detectable.
Significant differences are indicated by different letters ($P < 0.05$). Results are expressed as the mean ± SEM of triplicate determinations. All experiments were performed at least three times.

3.5. Mutant caMC4RL on MAPK/ERK signaling activity

In this section, the luciferase reporter vector pGL4.33 was utilized to assess the impact of mutant caMC4RL on the activation of the MAPK/ERK signaling pathway. The results (Fig. 5A) showed that NDP-MSH activated WT caMC4RL and increased intracellular MAPK/ERK levels

in a dose-dependent manner. The maximum response value of MAPK/ERK mediated by the mutant P25L/A83S was higher than that of the WT caMC4RL. However, other mutants (E57K, P296S, R302T, and R302K) do not respond to NDP-MSH stimulation.

Notably, the R302K mutation can respond to low concentrations of 10^{-10} M ACTH stimulation, but the maximal level of MAPK/ERK is not significantly different from that of the WT caMC4R. Mutant E57K was unable to respond to ACTH (1–24) stimulation. Mutants P25L, A83S, P296S, and R302T responded to ACTH (1–24) stimulation and increased MAPK/ERK signaling levels in a dose-dependent manner. Mutant A83S increased the maximal level of MAPK/ERK compared to WT caMC4RL, while mutant R302T resulted in a significantly lower maximal level than that of WT caMC4RL (Fig. 5B).

3.6. AMPK phosphorylation by mutant caMC4RL

AMP-activated protein kinase (AMPK) is an intracellular energy sensor that maintains the energy balance and serves as a key regulator of food intake. To investigate the potential effects of mutant MC4RL on feeding and energy homeostasis in fish, we examined mutant caMC4RL-induced AMPK phosphorylation. The results showed that the levels of AMPK phosphorylation induced by the mutant A83S upon ACTH (1–24)/NDP-MSH stimulation were not significantly different from those of WT caMC4RL (Fig. 6). The levels of AMPK phosphorylation induced by ACTH (1–24) stimulation in the other mutants were lower than those in WT caMC4RL (Fig. 6A, C). However, the levels of AMPK phosphorylation induced by NDP-MSH stimulation were significantly

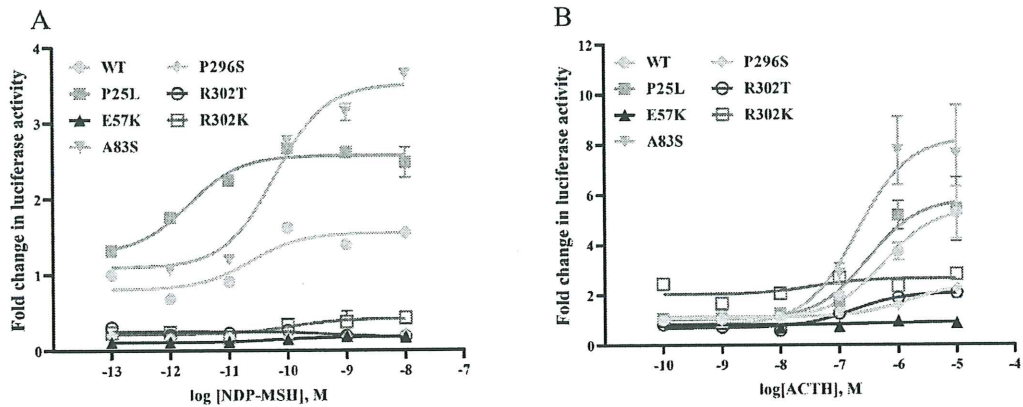


Fig. 5. The MAPK/ERK signaling of WT caMC4RL and mutant caMC4RL in HEK293 cells. After stimulating cells with different concentrations of (A) ACTH (1–24), and (B) NDP-MSH, the luciferase activity of the MAPK/ERK signaling pathway was detected. Each data point represents the mean ± SEM of three repeated measurements.

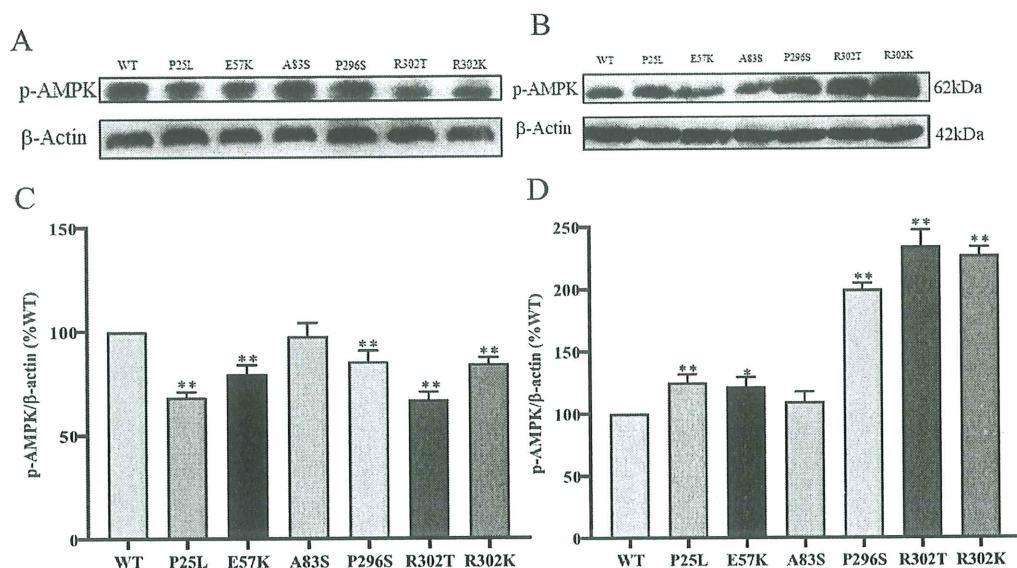


Fig. 6. AMPK phosphorylation level induced by WT caMC4RL and mutant caMC4RL. After stimulating cells with different concentrations of (A) ACTH (1–24), and (B) NDP-MSH, AMPK phosphorylation was detected by western blot. (C) Data of the p-AMPK levels of WT and mutant caMC4RL after ACTH (1–24) stimulation are expressed as percentage of WT p-AMPK level from at least three independent experiments. (D) Data of the p-AMPK levels of WT and mutant caMC4RL after NDP-MSH stimulation are expressed as percentage of WT p-AMPK level from at least three independent experiments. “*” indicates a significant difference from WT group ($P < 0.05$) and “**” indicates an extremely significant difference ($P < 0.01$).

higher than those in WT caMC4RL (Fig. 6B, D).

4. Discussion

Weight is a critical indicator for evaluating human health and the breeding industry. The MC4R expressed in the brain plays a critical role in weight regulation (Kishi et al., 2003; Wei et al., 2023). Loss of the MC4R allele is sufficient to cause severe obesity in rodents and humans (Arrizabalaga et al., 2014; Cole et al., 2010; Kring et al., 2010; Muller et al., 2014). Additionally, LOF MC4R mutations in human can also result in obesity (Wade et al., 2021). MC4R has become an important target for drug development and molecular breeding. Therefore, it is important to study pharmacological functions and signaling transduction of MC4R mutants. MC4R mutations have been extensively studied in humans, but research on MC4R/MC4RL mutations in farmed animals is extremely limited. *Carassius auratus* with a long aquaculture history in East Asia, Europe, and North America is a representative teleost species and a model organism in the fields of endocrinology and comparative endocrinology (Blanco et al., 2018). This work will address the gap in functional research on teleost MC4R/MC4RL mutants. Therefore, in this study, we identified wild goldfish MC4RL mutants from the Qin Ling region of China and analyzed their pharmacological functions and receptor signaling. These data will serve as a reference for the development of pharmacological functional studies and signaling differences of teleost GPCR mutants, as well as providing fundamental information for *Carassius auratus* molecular breeding.

Five SNPs were identified in wild goldfish from the Qian River. c.C74 > T, c.G886T, and c.G905 > A/C deviated from the Hardy-Weinberg equilibrium ($P < 0.05$), suggesting that these three SNPs may be under the influence of natural selection. Pure animals with the c.G169A/c.G247T genotype were not found in goldfish from the Qian River. This absence is likely due to the lethal effects associated with the purity of these two loci.

The five SNPs resulted in six mutants, all of which were located in conserved positions in the Cyprinidae MC4R/MC4RL-like. Among these mutations, E57K, A83S, P296S, and R302T/K were located in the transmembrane domains. P296S is located at the second residue position of the MC4R conserved motif (N/DPxxY). Previous studies have shown

that the hMC3R-P333A mutant located in N/DPxxY cannot respond to NDP-MSH stimulation of cAMP production, and hMC3R-P333A reduces constitutive activity (Yang et al., 2015). The R302T/K mutant is located in Helix 8, which was discovered by Debarshi Mustafi (Mustafi and Palczewski, 2009) and Daniel M. Rosenbaum (Rosenbaum et al., 2009) in GPCR. It plays critical roles in GPCR expression, conformational switching after GPCR activation, G protein coupling, and GPCR internalization (Barak et al., 2002; Barak et al., 1995; Fritze et al., 2003; Prioleau et al., 2002; Santos et al., 2006; Tetsuka et al., 2004; Wess et al., 1993). Previous reports have shown that mutants hMC4R-E61K (equivalent to E57K in caMC4RL) (Akbari et al., 2021; Barton et al., 2021), P299H/L (equivalent to P296S in caMC4RL) (Lubrano-Bertheliet et al., 2003), and R305W (equivalent to R302T/K in caMC4RL) (Morell-Azanza et al., 2019) result in high BMI and adolescent obesity. However, the other mutants have not yet been reported to be associated with the phenotype. Thus, the mutant P296S may not induce cAMP production in response to agonists, and its constitutive activity may be reduced. P296S and R302T/K are involved in regulating body weight regulation.

The constitutive activity is an important pharmacological function of constitutively active GPCRs. The mutations in GPCRs result in the formation of constitutively active mutant (CAM) GPCRs, which have been associated with the development of various diseases (Teidler et al., 2002). MC4R is a constitutive activity GPCR whose constitutive activity is involved in regulating appetite and metabolism in vivo (Kleinau et al., 2020). Mutants E57K, P296S, and R302T/K exhibited significantly lower constitutive activity compared to the wild type and did not induce cAMP production in response to ACTH (1–24) and NDP-MSH stimulation. The results of the R302T/K mutant were similar to those of the mutant hMC4R-R305W. Previous studies have shown that carriers of the R305W mutation develop pubertal obesity (Morell-Azanza et al., 2019), so the R302T/K mutation may be a LOF mutation. R302T/K mutation could potentially promote growth and fat accumulation in goldfish. The two mutants, R302T and R302K, can be utilized in the molecular breeding of goldfish to enhance growth performance and increase feeding rates. Mutant hMC4R-E61K (E57K in caMC4RL) is associated with obesity and high BMI (Akbari et al., 2021; Barton et al., 2021; Gokul et al., 2024; Xiang et al., 2010). Therefore, the E57K mutation may be associated with the body fat percentage and body weight of

goldfish. This mutation could serve as a molecular breeding marker to assist in the regulation of goldfish body weight. These mutants can be utilized in selective breeding through gene editing technology. FGF5, GDF9 and FecBB/TBXT have been modified in goats and sheep, respectively, and individuals carrying the desired mutations have been successfully produced. However, there are still several limitations to the selective breeding of goldfish using gene editing technology. These limitations include the lengthy breeding cycle of goldfish parents and the nascent application of CRISPR/Cas9 for defining point mutations in goldfish.

The results of the P296S mutant confirmed the findings of the bio-informatics analysis and is consistent with findings regarding conserved N/DPxxY motifs in the MCR. Thus, the Pro residue of the conserved N/DPxxY motif in the *Carassius auratus* MCRs family may impact receptor constitutive activity, ligand binding, and signal transduction. LOF MC4R reduces cAMP production, which in turn leads to obesity (Rodríguez Rondón et al., 2024).

In addition to activating the cAMP-PKA pathway through Gs protein, MC4R can also activate the MAPK pathway, particularly extracellular signal-regulated kinase (ERK) 1/2 (Daniels et al., 2003; Vongs et al., 2004). Mo et al. (2012) found that some MC4R mutants (L140G, L140R) are biased receptors, they lack cAMP signaling but can still maintain normal MAPK/ERK signaling. Three mutants (P296S, R302T/K) that are defective in Gs-cAMP signaling retain ERK1/2 signaling in response to ACTH (1–24) stimulation. Therefore, they may be biased receptors. However, three mutants (P296S, R302T/K) are unable to generate ERK1/2 signaling in response to NDP-MSH stimulation. Ligand bias (also known as “biased agonism” or “ligand functional selectivity”) is the ability of ligands to differentially activate a subset of receptor signaling pathways (Gundry et al., 2017) and related with the target receptor conformation (Violin et al., 2014). This bias can cause GPCR functional selectivity (Gundry et al., 2017; Kelly, 2013; Violin et al., 2014). Amino acid residue alterations affect the proteins conformation and ligand bias (Hothersall et al., 2017), so mutation-induced ligand bias may be responsible for the failure to respond to NDP-MSH stimulation.

Mutants P25L increase cAMP or MAPK/ERK signaling levels after NDP-MSH stimulation compared with WT caMC4RL. However, AMPK activity was higher in HEK293T-P25L than in HEK293T-WT caMC4RL after NDP-MSH stimulation. Mutants E57K and R302T resulted in significantly lower levels of cAMP or MAPK/ERK signals following ACTH (1–24) stimulation compared to those produced by WT caMC4RL. However, the AMPK activity in HEK293T-E57K/R302T after stimulation was significantly lower than the AMPK activity in HEK293T-WT caMC4RL. Ellen Dam found that the inhibition of AMPK activity by melanocortin in hypothalamic GT1-7 cells depends on PKA-mediated activation of ERK-1/2 (Damm et al., 2012). We found that some mutants exhibited cAMP or MAPK/ERK signaling change without a corresponding change in their regulation of AMPK activity. Our results provide further support for Yang and Tao's argument (Yang and Tao, 2016) that the inhibitory effect of AMPK activity may not be entirely ERK-dependent. Further experiments are needed in the future to clarify their relationship.

Some limitations of this study need to be noted. Due to lacking the fish cellular expression system, mammalian cell lines were adopted for pharmacological analysis. Although this approach could be a general method, there are high chances that different cell lines may significantly impact the activation potencies of different ligands on their receptor for a signaling pathway.

In summary, our work elucidates the function of six caMC4RL mutants in goldfish in the Qian River. All six mutants are in the conserved position of the Cyprinidae MC4Rs, and mutations E57K, P296S, and R302T/K result in the loss of Gs signaling function. P296 and R302T/K mutants exhibited biased signaling in response to ACTH stimulation in the MAPK/ERK pathway. In addition, the E57K mutants may play a role in weight regulation. These data will provide fundamental information for functional studies of teleost GPCR mutants and MC4R isoforms, as

well as for molecular breeding of *Carassius auratus*.

6. Consent for publication

All authors review and approve the manuscript for publication.

Funding statement

This Research Project was supported by Shaanxi Provincial Agricultural Products Quality and Safety Project (20200824000001), National Natural Science Foundation of China (32202909) and the Natural Science Foundation of Shaanxi Province (2023-JC-YB-155).

CRediT authorship contribution statement

Ying Wang: Writing – original draft. **Tianze Yang:** Writing – original draft. **Haolin Mo:** Methodology. **Mingxing Yao:** Resources. **Qingchuan Song:** Resources. **Huixia Yu:** Methodology. **Yuyou Du:** Data curation. **Yang Li:** Writing – review & editing, Project administration. **Jiajia Yu:** Project administration. **Lixin Wang:** Writing – review & editing, Funding acquisition, Conceptualization.

Ethics approval

The Faculty Animal Policy and Welfare Committee of Northwest A&F University under contract (NWAUFU-314022039) approved this experiment. The experimental process complied with protocols of international guidelines for the ethical use of animals in research.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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获奖项目：金鱼黑素皮质素样受体-4（MC4R-like）自然突变的药理学功能解析

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证书编号：CULSC2025CY0233



全国大学生生命科学竞赛委员会
二〇二五年七月