Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 29(3): 1839 – 1851 (2025)
www.ejabf.journals.ekb.eg

The Association Between Certain Liver Enzymes, Hematological Parameters, and Growth in the Common Carp (*Cyprinus carpio*) and Genetic Mutations of MSTN

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ARTICLE INFO

Article History:
Received: April 10, 2025
Accepted: May 20, 2025
Online: June 2, 2025

Keywords:
Common carp,
Myostatin gene,
AST,
Mutation

ABSTRACT

Myostatin (MSTN), a negative regulator of skeletal muscle development, controls the size and number of muscle fibers through autocrine and paracrine signaling. This study aimed to investigate the effects of MSTN gene mutations on liver enzyme levels in the common carp (*Cyprinus carpio*) with an average weight of 250 ± 2.1g. The MSTN gene segment responsible for molecular detection and polymorphism analysis, as well as phenotypic variation arising from MSTN mutations, was amplified using the PCR technique. Sequencing revealed two genotypes: AG and GG, with distribution ratios of 17.65 and 82.35%, respectively ($P< 0.01$). Allelic frequencies were A = 0.09 and G = 0.91. The mean (±SE) levels of liver enzymes at the AG site were ALT: 25.08 ± 1.27a and AST: 117.05 ± 9.04a, whereas for genotype GG they were ALT: 22.13 ± 0.97a and AST: 85.71 ± 7.36b. While AST levels differed significantly between genotypes ($P< 0.05$), ALT levels did not show a significant difference. In terms of growth performance, the GG genotype was recorded with significantly higher values in weight gain (WG), daily growth rate (DGR), relative growth rate (RGR), and specific growth rate (SGR) compared to the AG genotype. Similar trends were observed for hematological parameters, including red blood cells (RBCs), white blood cells (WBCs), packed cell volume (PCV), and hemoglobin (Hb). This study contributes to a deeper understanding of the role of MSTN in muscle development and its broader implications for aquaculture and agricultural genetics. The findings may support future research on the genetic regulation of skeletal muscle formation and selective breeding strategies in fish.

INTRODUCTION

Aquaculture is a rapidly expanding industry and a vital contributor to the global food supply. A substantial portion of global finfish aquaculture production—approximately 50%—originates from the Cyprinidae family, which includes a diverse array of fish species **(FAO, 2022)**. One of the most significant species in this family is

the common carp (*Cyprinus carpio*), which is extensively farmed and holds considerable market value as a food fish, both within its native range and in regions where it has been introduced. In 2020, the common carp alone accounted for 8.6% of global fish production **(Docherty *et al.*, 2017; FAO, 2022; Sayed-Lafi *et al.*, 2022)**.

Skeletal muscle growth and development are negatively regulated by myostatin (MSTN), also known as growth differentiation factor 8 (GDF8), a member of the TGF-β superfamily **(Segev-Hadar *et al.*, 2020)**. According to **Sharma *et al.* (2015)**, MSTN is synthesized as a precursor protein that is later cleaved into a mature MSTN peptide and a latency-associated peptide. Unlike mammals, which possess a single MSTN gene that is mainly expressed in skeletal muscle but also found in the brain, eyes, gills, stomach, and other tissues, most fish species possess two or more MSTN gene copies in their genomes **(Maccatrozzo *et al.*, 2001; Rodgers *et al.*, 2001)**.

Elliott *et al.* (2012) suggested that MSTN may act via endocrine, autocrine, or paracrine signaling pathways. The critical function of MSTN in regulating muscle development is further supported by its high degree of conservation among vertebrate species **(Xu *et al.*, 2013; Kang *et al.*, 2017; Segev-Hadar *et al.*, 2020)**. Moreover, this gene regulates fat and glucose metabolism **(Gao *et al.*, 2016)**.

In several fish species, the deletion of the MSTN gene has resulted in significant increases in both myofiber size and myofiber cell number when compared to wild-type (WT) phenotypes **(Yeh *et al.*, 2017; Kim *et al.*, 2019)**. These findings align with observations in various mammalian species, where MSTN functions as a potent inhibitor of muscle growth. Naturally occurring MSTN mutations in species such as Piedmontese and Belgian blue cattle, sheep, and dogs are associated with a double-muscled phenotype, due to gene inactivation and altered protein activity **(Clop *et al.*, 2006; Mosher *et al.*, 2007)**.

Further interest in MSTN was sparked by the discovery of a child with a MSTN gene mutation leading to extreme muscle hypertrophy **(Schuelke *et al.*, 2004)**. In addition to its developmental role, MSTN also maintains muscle homeostasis postnatally. Elevated MSTN protein levels have been observed in patients with muscle-wasting conditions such as cachexia, muscular dystrophy, and other degenerative muscle diseases **(Jespersen *et al.*, 2006; Joulia-Ekaza & Cabello, 2007)**. Modifications of MSTN function via dominant-negative receptors or neutralizing antibodies have also been shown to affect muscle physiology **(Takata *et al.*, 2007)**.

Thus, MSTN has emerged as a promising target in both human medicine and agricultural biotechnology. Technologies aimed at modulating MSTN expression or activity may offer novel approaches to treating muscle disorders or enhancing livestock growth performance **(Grade *et al.*, 2019)**.

Based on this background, the present study aimed to evaluate the levels of liver enzymes ALT and AST in relation to MSTN gene mutations, as well as to assess growth performance and hematological parameters in the common carp (*Cyprinus carpio*).

MATERIALS AND METHODS

Preparing fish and PCR

For five weeks, juvenile common carp (*Cyprinus carpio*) with an average weight of 250 ± 2.1g were reared in 250-liter tanks, with seven to eight fish allocated per treatment group. Water temperature was consistently maintained at 24–26 °C, and nitrite and ammonia levels were regularly monitored to ensure optimal water quality. Fish were fed a commercial diet *ad libitum* twice daily (Table 1). Following the rearing period, genetic analyses were conducted to determine the genotype distribution and allele frequencies of the MSTN gene. Additionally, the relationship between MSTN genotypes and selected physiological parameters was investigated.

Table 1. Ingredients (%) of the experimental basal diet

Component	**Percentage %**
Protein	26.8**%**
Lipid	1.4**%**
Energy	3165.5 kcal
N.F.E (mg/100g) *	66.7**%**
Humidity	5.5**%**
Ash	5.1 %
Aflatoxin test	2.5 (ppb)

* The difference-based nitrogen-free extract is equal to 100-(protein + lipid + ash).

The polymerase chain reaction (PCR) for MSTN was performed using the following thermal cycling protocol: an initial hot-start activation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72 °C for 30 seconds. This was followed by a final extension step at 72°C for 7 minutes and a single incubation cycle at 4°C.

The PCR utilized the following primers:

- **Forward**: 5′-AGCCTACCATAAAAGGTGTGTG-3′
- **Reverse**: 5′-TCAATAGTGTCCATTCCCAAGT-3′

These primers target intron 2 of the MSTN gene (GenBank accession number: GQ214770.1) and were designed to detect phenotypic variation arising from mutations in the gene **(Sun *et al.*, 2012; Yanhong *et al.*, 2012)**. The gene segments used for analysis were obtained from the National Center for Biotechnology Information (NCBI) and verified for accuracy using electronic genome browsers. The primers were provided by Macrogen, Korea.

Following DNA amplification, 5µl of each PCR product was subjected to agarose gel electrophoresis. A 1.5% agarose gel was prepared, and electrophoresis was performed for 80 minutes at 60 volts and 40 amps. The target DNA fragment ranged between 100 and 1500 base pairs (bp) in size.

Subsequent genetic analysis, including single nucleotide polymorphism (SNP) identification, was carried out by Promega Corporation (2800 Woods Hollow Road, Madison, WI, USA), and sequencing was completed via Macrogen, Korea.

Growth and survival assessment

During the 42-day experimental period, the body weight of each fingerling was measured weekly, along with survival counts. After gently blotting the fish with absorbent paper to remove excess moisture, wet weight and total length were recorded to the nearest 0.01 mg. The following mathematical formulas were used to compute the growth performance indicators—weight gain, daily growth rate, relative growth rate, specific growth rate, and survival rate—that fingerlings fed experimental diets displayed:

$$Weight\ gain = \frac{Final\ weight - Initial\ weight}{Initial\ weight} \times 100$$

$$Daily\ growth\ rate = \frac{Maen\ final\ weight - Mean\ initial\ weight}{Rearing\ peroid}$$

$$Relative\ growth\ rate = \frac{Weight\ gain}{Initial\ \times Rearing\ peroid} \times 100$$

$$Specific\ growth\ rate = \frac{Ln\ (final\ weight) - Ln\ (Initial\ weight)}{Rearing\ period\ (Days)} \times 100$$

$$Survival\ rate = \frac{Final\ count}{Initial\ count} \times 100$$

Analysis of blood serum

Blood samples were collected directly from the heart using sterile medical syringes. A volume of 2ml of blood was drawn from each fish, categorized based on their identified MSTN genotypes. Two biological replicates were collected for each genotype.

The blood was transferred into 5ml tubes containing the anticoagulant EDTA to prevent clotting.

Each sample was divided into two portions:

1. **Hematological analysis**

One portion was used to assess hematological parameters using a Mindray automated hematology analyzer (manufactured in China). A volume of 20µl of fresh blood was inserted into the analyzer's sampling needle. The instrument provided readings for red blood cells (RBCs), white blood cells (WBCs), packed cell volume (PCV), and hemoglobin (Hb), which were recorded directly from the device's display.

2. **Biochemical analysis**

The second portion was transferred into sterile tubes for the analysis of liver enzymes: aspartate aminotransferase (AST) and alanine transaminase (ALT). These biochemical parameters were measured using the Reflotron Plus analyzer (manufactured in Germany), following standard clinical biochemistry protocols.

Statistical analysis

To investigate the impact of MSTN polymorphism following the mathematical model below, data were statistically analyzed using the Statistical Analysis System **(SAS, 2012)**. The SAS's least squares means approach was then used to compare the variations in the averages by using **Steele and Torrie's (1990)** completely randomized design (CRD) as follows:

$$Yij = \mu + Gi + eij$$

Where, eij is the naturally distributed random error with an average of zero and a variation of $\sigma 2e$; μ is the overall mean of the characteristic; Gi is the influence of polymorphism of the mstn gene A2232G (AG, GG); and Yij is the value of the genotype I. A meaningful comparison of the means was made using **Duncan**'s **(1995)** multiple-range tests. The Hardy-Weinberg law was used to determine the initial redundancy in the mutation using the chi-square ($\chi 2$) test.

RESULTS

Polymorphisms through the sequencing of the nitrogen bases

Nitrogen base sequencing of a 1000 bp segment of the MSTN gene was performed, and genetic mutations were identified. Genotypes were distinguished based on chromatogram peak patterns: genotype GG was represented by a single yellow peak, while genotype AG was indicated by overlapping red and yellow double peaks (Fig. 1).

Comparison of the sequencing results with reference sequences from the NCBI database revealed a novel single-nucleotide polymorphism (SNP) located in intron 2,

specifically at position 2232 bp. This mutation involved a substitution of adenine (A) with guanine (G) (Fig. 2).

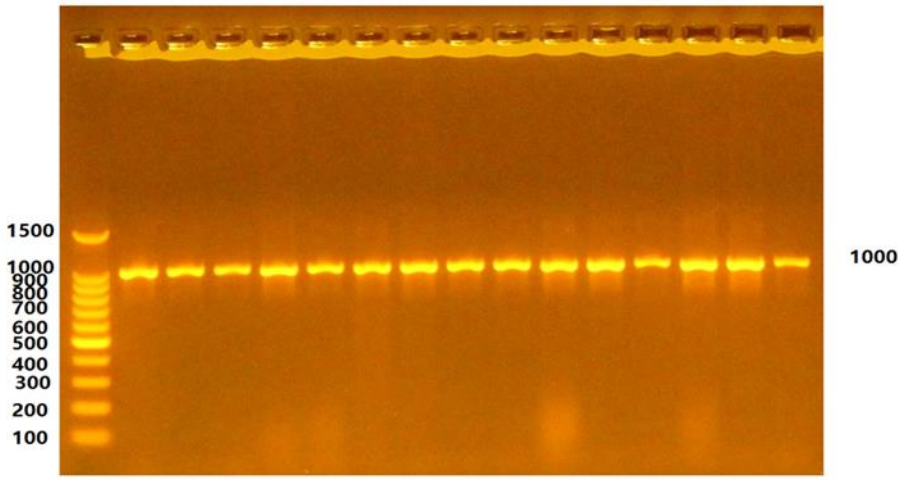

Fig 1. Size of MSTN segments extracted by PCR technology

TACTGTTCGGGAGAATGCGACTACATGCACCTGCAAAAATATCCCCACACCCATCT
GGTGAACAAGGCCAATCCGCGAGGCACCGCCGGGCCCTGCTGCACCCCCACCAA
GATGTCTCCCATCAACATGCTTTACTTCAACGGCAAAGAGCAGATCATCTACGGCA
AGATCCCCTCAATGGTAGTAGACCGCTGTGGCTGCTCGTGAACCAGTGCCCAGAC
AGGACTCGATCCGTCTCACAGACCCGGACATCTGATCACACCATCCACCATCCATT
ATCAGTGCTTTCCGCAAGACACTGTGCAATAGAAGGACGCTCACTCACTCTCTGGG
CACCGCTTCATTTGACTATGTTTTTTGTCATTTTCCTCTAAATCAGTATCTCTGCCAC
AGGAGTCCAATCTTACATGG**G**ATGTACTAAAAGGAATGTCATATACTGGCTGGACT
GGAGAGGACCCCCATTTATAAGAAAAAAAGTGGGAATTTGTGGGG

Fig 2. The nucleotide of the MSTN gene changed from A to G at a distance of 2230 bp in the second intron

Genotypes of the MSTN location of mutation

The results confirm the compatibility and functional presence of the MSTN gene in common carp (*Cyprinus carpio*). Sequencing revealed multiple genetic variants, with the majority of mutations located in the second intron. A key finding was the identification of a single-nucleotide polymorphism (SNP) at position 2232 bp, where adenine (A) was substituted by guanine (G).

Genotype analysis was performed using the Geneious software (version 10.1.3) (Geneious, 2017). The sequence obtained, identified as LOC109091639, was aligned

with the wild-type genotype of the common carp from the NCBI GenBank. Alignment results confirmed the presence of two genotypes at the target site within the MSTN gene: AG (heterozygous) and GG (homozygous mutant). Notably, the AA wild-type genotype was absent in the sample population, while all homozygous individuals exhibited the GG genotype structure.

Genotype distribution and frequencies are presented in Table (2). The AG genotype was detected in 17.65% of the individuals, while the GG genotype appeared in 82.35%. Using the Hardy–Weinberg equilibrium model, allele frequencies were calculated, showing 0.09 for the A allele and 0.91 for the G allele, indicating a strong dominance of the mutant allele in the population.

Table 2. Allelic repeats and genotype count for the MSTN (mutation A2232G)

Genotype	**Number**	**Percentage (%)**
AG	12	17.65
GG	56	82.35
Total	**68**	**100**
Chi-square value ($\chi 2$)	---	90.007**
Allele		Frequency
A		0.09
G		0.91

The correlation between MSTN genotypes and liver enzyme levels (ALT and AST) is summarized in Table (3). A statistically significant difference ($P< 0.05$) was observed in AST levels between the two genotypes at the mutation site. Fish with the AG genotype exhibited a higher AST level (117.05IU/ L), whereas those with the GG genotype showed a significantly lower AST level (85.71IU/ L). However, no significant difference was detected in ALT levels between the genotypes.

Table 3. Relationship between the MSTN mutation and the AST and ALT enzymes: Mean ± Standard error (mg/100ml)

Genotypre	**AST (U/I)**	**ALT (U/I)**
AG	117.05±9.04a	25.08±1.27a
GG	85.71±7.36b	22.13±0.97a
Level significant	*	N. S

a,b values within a row with different superscripts differ significantly at $P<0.05$

The current findings indicate that fish with the GG genotype exhibited significantly enhanced growth performance ($P< 0.01$), demonstrating that the MSTN mutation had a notable impact on growth. This is clearly reflected in the data presented in Table (4).

The influence of the MSTN genotype on hematological parameters is shown in Table (5). The genetic mutation led to a significant increase ($P< 0.01$) in red blood cells (RBCs), white blood cells (WBCs), and packed cell volume (PCV). While hemoglobin (Hb) levels also increased in the GG genotype, the difference was less pronounced and not statistically significant ($P> 0.05$).

Table 4. Effect of polymorphisms of the MSTN on the growth traits of common carp during the duration of the experiment (mean ± standard error)

Traits	Genotype		Significance level
	AG	GG	
IW	117.83± 3.24b	128.82± 2.14a	**
FW	161.33± 4.67b	205.57± 4.87a	**
WG	43.50± 5.07b	76.75± 4.74a	**
DGR	0.547± 0.06b	0. 981± 0.28a	**
RGR	39.91± 1.02b	59.57± 2.34a	**
RGS	0.162± 0.03b	0.214± 0.03a	**
SR	100	100	N.S

IW = Initial body weight (gm), FW = Final body weight (gm), WG= Weight gain (gm), SGR= Specific growth rate (%/day), RGR = Relative Growth Rate (%). Note: indicates that there is a significant difference between the letters in a row ($P<0.01$) **.

Table 5. Relationship of the genotype of the MSTN to haematological parameters

Genotype	RBC ($\times^{106}$/mm3)	WBC ($\times^{103}$/mm3)	PCV (%)	Hb(mg/100ml)
AG	0.96± 0.04b	17.16± 0.75b	17.03± 0.89b	6.20± 0.52b
GG	1.21± 0.01a	23.84± 0.53a	25.14± 0.32a	9.18± 0.16a
Significance level	**	**	**	*

Note: indicates that there is a significant difference between the letters in a row ($P<0.05$) *, ($P<0.01$) **.

DISCUSSION

Myostatin (MSTN), a secreted protein and member of the TGF-β superfamily, is known to negatively regulate skeletal muscle hypertrophy in numerous animal species, including humans and fish, by controlling both the size and number of muscle fibers. In the current study, a novel SNP in intron 2 of the MSTN gene at position 2232 bp (A → G) was identified in the common carp (*Cyprinus carpio*), revealing two genotypes: AG and GG. Notably, the wild-type AA genotype was absent.

Previous studies support this polymorphic variation in MSTN. For example, in the Nile tilapia (*Oreochromis niloticus*), genotype frequencies were reported as 80.00% for AB and 20.00% for BB, with allele frequencies of 0.4 (A) and 0.6 (B) **(Elkatatny, 2016)**. In common carp, the MSTN genotype distribution was previously observed as 20.37% (AA), 59.88% (AG), and 19.75% (GG), with allelic frequencies of 0.51 (A) and 0.49 (G) **(Sun *et al.*, 2012)**. In the Atlantic salmon (*Salmo salar*), the TT, TC, and CC genotypes of MSTN-1b were distributed at 39.11%, 27.49%, and 33.40%, respectively, with T and C alleles occurring at 0.29 and 0.71% **(Yanhong *et al.*, 2012)**.

In the present study, fish carrying the GG mutant genotype exhibited significantly lower AST levels compared to the AG genotype. This decrease may be attributed to increased fat metabolism and higher uptake of fatty acids and amino acids, as suggested by **Vijayan *et al.* (2001)**. These authors also reported typical AST levels in fish blood plasma to range from 51 to 433IU/ L. Supporting this, **Mohapatra *et al.* (2012)** found that decreased AST activity correlates with reduced synthesis of total and albumin proteins in river fish, likely due to AST's abundance in tissues such as the kidneys, heart, muscle, and RBCs.

In contrast, no significant differences in ALT levels were observed between the genotypes ($P > 0.05$), aligning with its tissue distribution pattern—primarily in the liver, but also in the heart, kidney, and skeletal muscle **(Das *et al.*, 2004)**. Its typical range in fish plasma is reported to be 17–136 IU/L.

Galt *et al.* (2014) showed in the rainbow trout (*Oncorhynchus mykiss*) that MSTN expression affects white muscle fat distribution, liver indices, and glucose levels—supporting MSTN's role in metabolism. Environmental conditions, stress, and disease may also influence enzyme levels and MSTN expression. Similarly, **He *et al.* (2018)** observed in goats that MSTN mutations improved growth performance and metabolic activity, though no significant differences in serum AST or ALT levels were found.

In line with these findings, the present study revealed that the GG genotype significantly outperformed AG in growth traits—weight gain (WG), daily growth rate (DGR), relative growth rate (RGR), and specific growth rate (SGR). This agrees with studies on the bighead carp (*Hypophthalmichthys nobilis*) where SNPs in MSTN were significantly associated with growth performance **(Liu *et al.*, 2012; Pang *et al.*, 2018)**. MSTN mutations in coding regions are used to develop genomic markers for selective

breeding programs aimed at improving muscle mass in aquaculture species **(Sun *et al.*, 2012)**.

Given that maximizing growth within a short period is a key goal in aquaculture, MSTN represents a crucial target gene. The current findings confirm that the GG mutant genotype enhances muscle growth, likely due to reduced MSTN activity, which allows for greater expression of muscle-building proteins. This supports previous work in the Nile tilapia by **Elkatatny *et al.* (2016)**, who reported significantly increased body weight ($P < 0.01$) in individuals with MSTN mutations.

Additionally, the increase in RBC and WBC counts in GG fish may reflect elevated oxygen demand and immune function to support enhanced muscle development. **Garikipati *et al.* (2006)** found similar trends in the rainbow trout, noting increased hematological parameters in MSTN mutants. Likewise, **Rescan *et al.* (2001)** linked greater cell density to rapid muscle growth and higher protein synthesis activity in *O. mykiss*.

Elevated hemoglobin (Hb) levels in the GG genotype could be explained by enhanced MSTN expression in hematopoietic organs such as the spleen, facilitating increased oxygen delivery to support protein synthesis. This mechanism was proposed by **Eames *et al.* (2010)** in the zebrafish (*Danio rerio*), where increased MSTN expression coincided with higher Hb levels and tissue oxygenation.

CONCLUSION

In summary, two single-nucleotide polymorphisms (SNPs) were identified within the MSTN gene of the common carp (*Cyprinus carpio*). Among these, the A2232G mutation was notably associated with an enhanced growth performance. Fish carrying the GG genotype demonstrated superior growth traits compared to heterozygous individuals.

The results of this study provide a strong evidence for a correlation between MSTN polymorphisms and key growth parameters in the common carp. These findings support the potential use of MSTN as a candidate gene in marker-assisted selection (MAS) programs aimed at improving growth traits in aquaculture. The identification of such genetic markers is crucial for advancing selective breeding strategies and enhancing the productivity and sustainability of finfish aquaculture.

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