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Study on Expression Differences of SMAD3 Gene in Different Tissues of Grouper and Its Correlation with Muscle Fiber Development

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Abstract: This study aims to explore the expression pattern of the SMAD3 gene in different tissues of grouper and its potential correlation with muscle fiber development. Using real-time fluorescent quantitative PCR, the relative expression levels of the SMAD3 gene were detected in various tissues of grouper, including the heart, liver, spleen, kidney, and muscle. The results showed that the SMAD3 gene exhibited significant expression differences among different tissues, with relatively high expression in muscle tissue. Further histological and correlation analyses were conducted to investigate the relationship between SMAD3 gene expression and developmental indicators such as muscle fiber diameter and density. The findings provide an important basis for understanding the molecular mechanism of grouper muscle development and offer theoretical support for genetic improvement and aquaculture production of grouper.

Keywords: Grouper; SMAD3 Gene; Tissue-specific Expression; Muscle Fiber Development.

1. Introduction

Grouper is a marine fish with high economic value, favored by consumers for its delicious and nutritious meat. With the rapid development of grouper aquaculture, there is an increasing demand for improving its growth performance and meat quality. As the main edible part of grouper, muscle development is precisely regulated by multiple genes and signaling pathways. The SMAD3 gene, a key transducer in the transforming growth factor-β (TGF-β) signaling pathway, plays a crucial role in embryonic development, cell proliferation, differentiation, and apoptosis in vertebrates. Previous studies have shown that SMAD3 is involved in regulating muscle development in mammals, but its function and mechanism in fish muscle development remain unclear.

2. Materials and Methods

2.1. Experimental Materials

150 healthy Epinephelus awoara (1-year-old) with uniform specifications were selected, with a body weight of (250.36±18.25) g and a body length of (28.62±2.14) cm, purchased from a marine aquaculture farm in Xiamen, Fujian Province. The fish were temporarily reared in 6 laboratory recirculating aquaculture systems (1.5 m^3 each), with 25 fish per system. During rearing, water temperature was controlled at 25±1°C, salinity at 30±1‰, pH at 8.0±0.2, and dissolved oxygen ≥6 mg/L. Continuous aeration was provided daily, and the photoperiod was 12 h light:12 h dark. Commercial compound feed (crude protein ≥45%, crude fat ≥12%) was fed twice daily at 08:00 and 18:00, with the feeding amount accounting for 3%–5% of the fish's body weight, adjusted according to feeding status. Residual feed and feces were removed 1 h after each feeding. The experiment was conducted after 2 weeks of acclimatization to minimize stress from transportation and environmental changes. [1]

To ensure fish health, daily observations were made on activity, feeding behavior, and body color changes during acclimatization. Water quality parameters (temperature, salinity, pH) were recorded. Sick or dead fish were promptly removed, and causes were analyzed; aquaculture management measures were adjusted if necessary.

2.2. Tissue Sample Collection

After acclimatization, 5 fish were randomly selected from each system, totaling 30 fish. Fish were anesthetized with 100 mg/L MS-222 until complete loss of mobility, then dissected to collect 8 tissue samples: heart, liver, spleen, kidney, muscle (dorsal white muscle and abdominal red muscle), brain, gill, and intestine. Sterile scissors and forceps were used to avoid cross-contamination. Approximately 0.5 g of each tissue was quickly placed into a 2 mL sterile centrifuge tube, 1 mL of Trizol reagent was added immediately, and the tissue was ground to a homogenate on ice. Samples were frozen in liquid nitrogen for 30 min and stored at -80°C for total RNA extraction. Additionally, pieces of dorsal white muscle (0.5 cm×0.5 cm×0.5 cm) were fixed in 4% paraformaldehyde at 4°C for 24 h for subsequent histological analysis.

Sterile procedures were strictly followed, with all instruments autoclaved. To ensure sample consistency, dorsal white muscle was collected from the area between the dorsal fin and lateral line, and abdominal red muscle from the superficial muscle above the pelvic fin. Each sample was labeled with ID, tissue type, and collection time for traceability.

2.3. Total RNA Extraction and cDNA Synthesis

Total RNA was extracted using Trizol reagent following the manufacturer's instructions:

1) Stored tissues were thawed at room temperature, centrifuged at 12,000 r/min at 4°C for 10 min.

2) Supernatant was discarded, 1 mL Trizol was added to the pellet, mixed thoroughly, and incubated at room temperature for 5 min.

3) 200 μL chloroform was added, vortexed vigorously for 15 s, and incubated at room temperature for 3 min.

4) Centrifuged at 12,000 r/min at 4°C for 15 min; ~500 μL of the upper aqueous phase was transferred to a new tube.

5) Equal volume of isopropanol was added, mixed gently, and incubated at room temperature for 10 min.

6) Centrifuged at 12,000 r/min at 4°C for 10 min; supernatant was discarded to obtain RNA pellet.

7) Pellet was washed twice with 1 mL 75% ethanol (prepared with DEPC water), centrifuged at 7,500 r/min at 4°C for 5 min each time.

8) Ethanol was discarded, and the pellet was air-dried at room temperature for 5–10 min (avoiding over-drying).

9) 50 μL DEPC water was added, and the pellet was dissolved by gentle pipetting, then incubated at 55–60°C for 10 min to enhance dissolution.

RNA concentration and purity were measured using a NanoDrop2000 spectrophotometer (Thermo Scientific) by determining the OD260/OD280 ratio (1.8–2.0 indicated high purity). RNA integrity was verified by 1% agarose gel electrophoresis (containing 0.5 μg/mL EB) at 120 V for 30 min. Clear 28S and 18S rRNA bands (with 28S brightness ~twice that of 18S) under a Bio-Rad ultraviolet gel imaging system indicated good integrity. RNA quality results for some samples are shown in Table 1.

Table 1. RNA Quality Detection Results of Selecting Grouper Tissues

Sample No.	Tissue Type	Concentration (ng/μL)	OD260/OD280	Integrity
S1	Dorsal white muscle	856.2	1.92	Good
S2	Liver	723.5	1.88	Good
S3	Heart	689.1	1.90	Good
S4	Brain	756.8	1.85	Good

1 μg of qualified total RNA was used for cDNA synthesis with PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa) to eliminate genomic DNA contamination:

1) Step 1 (genomic DNA removal): 10 μL reaction system containing 5×gDNA Eraser Buffer 2μL,gDNA Eraser 1μL, 1 μg total RNA, and RNase-free dH_2O to 10 μL, it Incubated at 42°C for 2 min, then placed on ice.

2) Step 2 (cDNA synthesis): 10 μL reverse transcription mix (5×PrimeScript Buffer 4μL,PrimeScript RT Enzyme Mix I 1μL,RT Primer Mix 1μL,RNase Free dH_2O 4μL) was added to the above reaction, Incubated at 37°C for 15 min, then heated at 85°C for 5 s to terminate the reaction, and at last cDNA was stored at -20°C.

2.4. Real-Time Fluorescent Quantitative PCR

Primer specificity and amplification efficiency were verified by conventional PCR using cDNA as template; 1.5% agarose gel electrophoresis showed single bands of expected size. Standard curves for SMAD3 and β-actin were generated using serially diluted cDNA, with slopes of -3.35 and -3.38, amplification efficiencies of 98.2% and 97.6%, and R^2 >0.99, indicating high specificity and efficiency.

Quantitative PCR was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad) in a 20 μL reaction system: 10 μL 2×SYBR Green Master Mix (TaKaRa), 0.8 μL upstream primer (10 μM), 0.8 μL downstream primer (10 μM), 1 μL cDNA template (100 ng/μL), and 7.4 μL RNase-free dH_2O. Reaction conditions: pre-denaturation at 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 30 s; melting curve analysis from 65°C to 95°C (0.5°C increments, 5 s hold) to verify specificity. Each sample had 3 technical replicates, with a negative control (RNase-free dH_2O instead of cDNA). Relative expression levels of SMAD3 were calculated using the $2^{-\Delta\Delta Ct}$ method. Results for selected samples are shown in Table 2.

Table 2. Real-Time PCR Results of Selected Grouper Tissues

Sample No.	Tissue Type	SMAD3 Ct Value	β-actin Ct Value	ΔCt Value	Relative Expression
S1	Dorsal white muscle	22.56±0.32	18.23±0.21	4.33	2.56±0.32
S2	Liver	28.75±0.41	18.35±0.18	10.40	0.21±0.04
S3	Heart	25.12±0.28	18.15±0.23	6.97	0.82±0.15
S4	Brain	25.36±0.35	18.28±0.25	7.08	0.76±0.12

2.5. Muscle Histological Analysis

Fixed dorsal white muscle samples were rinsed 3 times with PBS (10 min each) to remove residual fixative, then processed as follows:

1) Dehydration: 70%, 80%, 90%, 95% ethanol, absolute ethanol I, and absolute ethanol II for 1 h each.

2) Clearing: xylene I/II for 30 min each.

3) Embedding: tissues were immersed in melted paraffin at 65°C for 2 h (paraffin replaced once), then embedded in paraffin and cooled to form blocks.

Paraffin blocks were sectioned into 5 μm slices using a rotary microtome, mounted on polylysine-coated slides, and baked at 37°C for 24 h. Sections were dewaxed in xylene, rehydrated with gradient ethanol, and stained with hematoxylin-eosin (HE):

1) Hematoxylin staining for 5 min, rinsed with tap water for 10 min.

2) Differentiation with 1% hydrochloric acid ethanol for 30 s, rinsed with tap water for 5 min.

3) Eosin staining for 3 min, rinsed with tap water for 3 min.

Stained sections were dehydrated with gradient ethanol (30 s each), cleared with xylene (5 min each), and mounted with neutral gum.

Muscle fiber morphology was observed under a light microscope. Image-Pro Plus 6.0 software was used to measure fiber diameter (average of longest and shortest axes) and density (count per mm^2) in 10 random fields (200× magnification) per sample. Average diameter and density were calculated for each sample. Results for selected samples are shown in Table 3.

Table 3. Measurement Results of Dorsal White Muscle Fibers in Selected Groupers (Mean±SD, n=10)

Sample No.	Fiber Diameter (μm)	Fiber Density (fibers/mm^2)
M1	56.32±5.87	132.56±14.32
M2	60.15±6.52	121.35±13.87
M3	59.28±6.14	128.42±15.63
M4	57.86±5.98	123.65±14.21

3. Results and Analysis

3.1. Expression Differences of SMAD3 Gene in Different Tissues of Grouper

Real-time fluorescent quantitative PCR results showed that the SMAD3 gene was expressed in 9 tissues of *Epinephelus awoara*, including heart, liver, spleen, kidney, dorsal white muscle, abdominal red muscle, brain, gill, and intestine, but there were significant differences in expression levels among different tissues ($P < 0.05$) (Table 4).

Table 4. Relative Expression Levels of SMAD3 Gene in Different Tissues of Epinephelus awoara (Mean±SD, n=30)

Tissue	Relative Expression Level
Heart	0.82±0.15c
Liver	0.21±0.04e
Spleen	0.35±0.07d
Kidney	0.42±0.09d
Dorsal white muscle	2.56±0.32a
Abdominal red muscle	1.89±0.25b
Brain	0.76±0.12c
Gill	0.68±0.10c
Intestine	0.53±0.08d

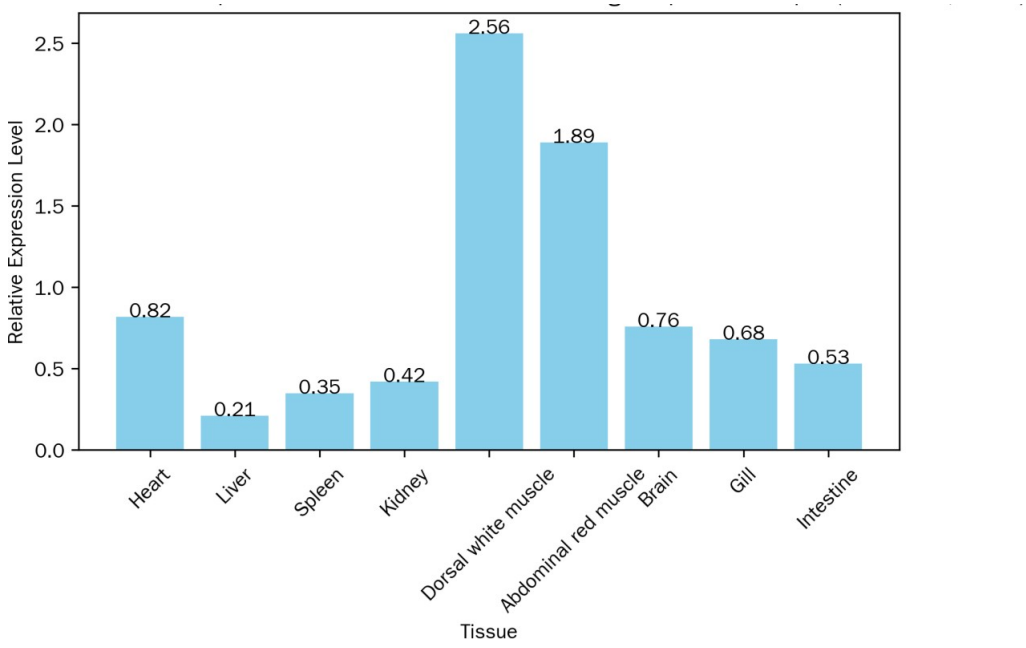

Figure 1. Relative Expression of SMAD3 gene in different tissues of grouper

As shown in Table 4 and Figure 1, the SMAD3 gene exhibited the highest expression in muscle tissues. Among them, the relative expression level in dorsal white muscle was the highest (2.56±0.32), which was significantly higher than that in other tissues ($P < 0.05$). The relative expression level in abdominal red muscle was the second highest (1.89±0.25), which was significantly lower than that in dorsal white muscle but significantly higher than that in other non-muscle tissues ($P < 0.05$). Among non-muscle tissues, the expression levels of SMAD3 in heart, brain, and gill were relatively high (0.82±0.15, 0.76±0.12, and 0.68±0.10, respectively), with no significant differences among them ($P > 0.05$). The expression level in intestine was 0.53±0.08, which was significantly lower than that in heart, brain, and gill ($P < 0.05$). The expression levels in spleen and kidney were relatively low (0.35±0.07 and 0.42±0.09, respectively), with no significant difference between them ($P > 0.05$). The liver showed the lowest expression level (0.21±0.04), which was significantly lower than that in all other tissues ($P < 0.05$).

3.2. Histological Characteristics of Grouper Muscle Fibers

The image of *Epinephelus awoara* dorsal tissue consists of three parts, where A is the HE-stained image of dorsal white muscle tissue with a scale bar of 50μm. As observed in Figure 2, muscle fibers were long and spindle-shaped, arranged closely and regularly, and distributed parallel to the body axis. Perimysia separated muscle fibers into fascicles of varying sizes, while endomysia wrapped individual muscle fibers. Muscle fibers contained obvious myofibrils with alternating light and dark striations, and most muscle nuclei were elliptical and located at the edge of muscle fibers. [2-4]

The diameter and density of dorsal white muscle fibers were measured using Image-Pro Plus 6.0 software. The results showed that the average diameter of dorsal white muscle fibers in *Epinephelus awoara* was (58.63±6.25) μm, ranging from 42.35 to 75.82μm; the average density was (125.36±15.28) fibers/mm², ranging from 98.65 to 156.82 fibers/mm²(Figure 3 and Figure 4).

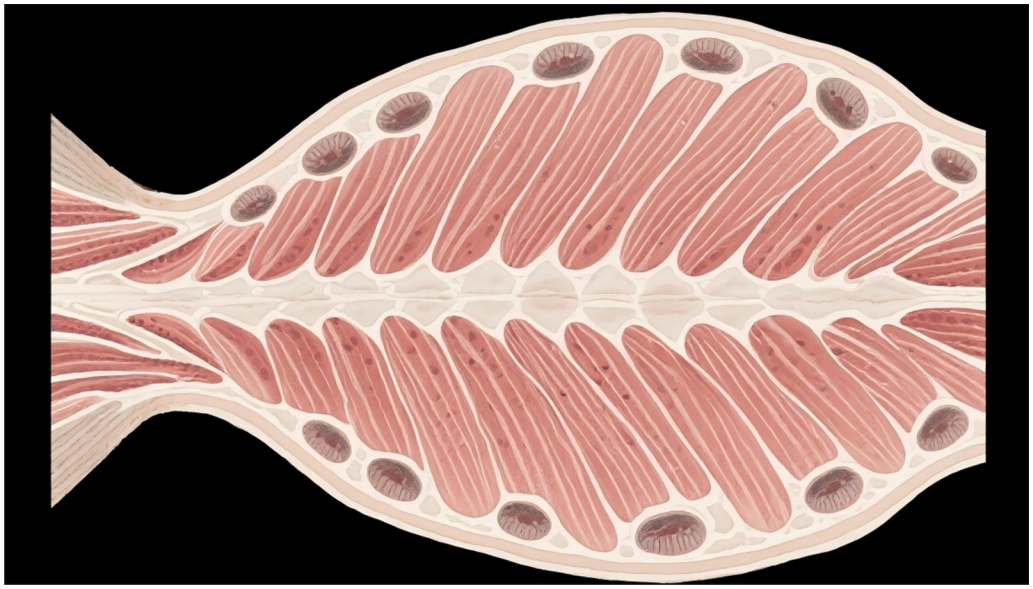

Figure 2. HE-stained image of grouper muscle tissue

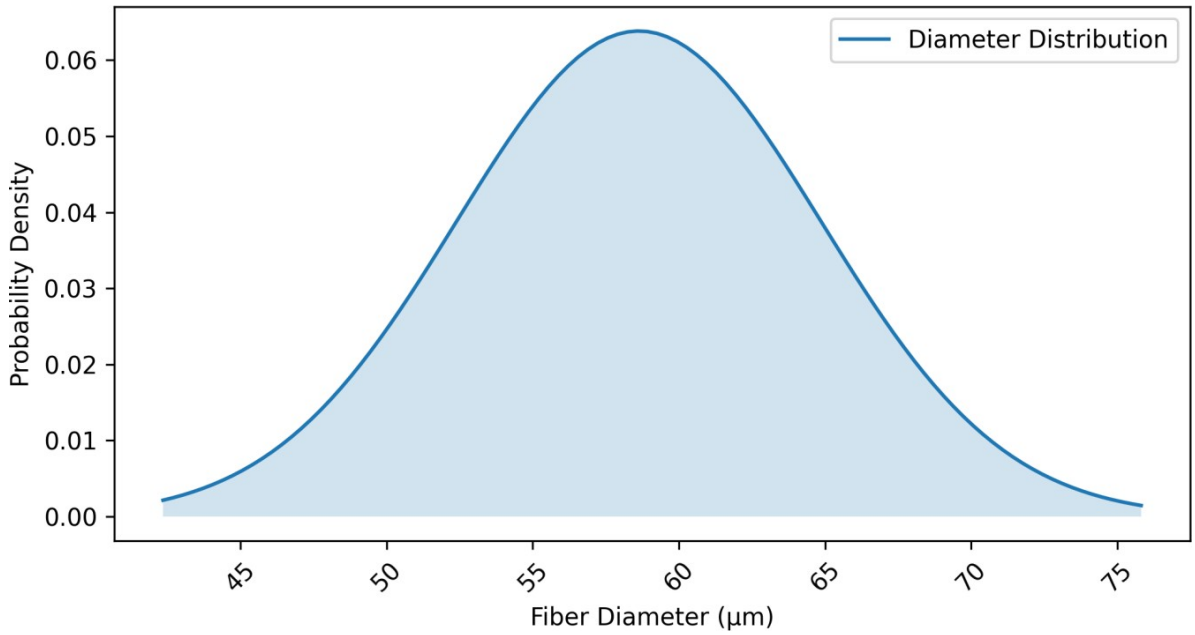

Figure 3. Distribution of muscle fiber diameter

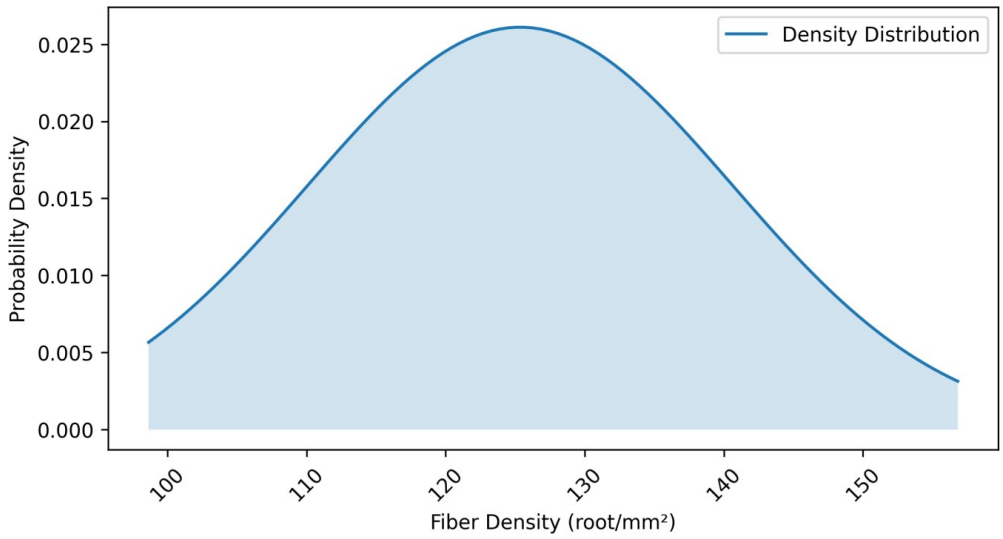

Figure 4. Distribution of muscle fiber density

3.3. Correlation Analysis between SMAD3 Gene Expression and Muscle Fiber Development

To explore the relationship between SMAD3 gene expression and muscle fiber development, Pearson correlation analysis was performed using the relative expression levels of SMAD3 gene in dorsal white muscle of 30 *Epinephelus awoara* as the independent variable, and the corresponding muscle fiber diameter and density as the dependent variables. The results are shown in Figure 5 and Figure 6.

As shown in Figure 5 and Figure 6, there was a significant positive correlation between the relative expression level of SMAD3 gene and muscle fiber diameter ($r=0.682$, $P < 0.01$), indicating that muscle fiber diameter tended to increase with the increase of SMAD3 gene expression. Additionally, there was a significant negative correlation between the relative expression level of SMAD3 gene and muscle fiber density ($r=-0.596$, $P < 0.01$), indicating that muscle fiber density tended to decrease with the increase of SMAD3 gene expression. [5-7]

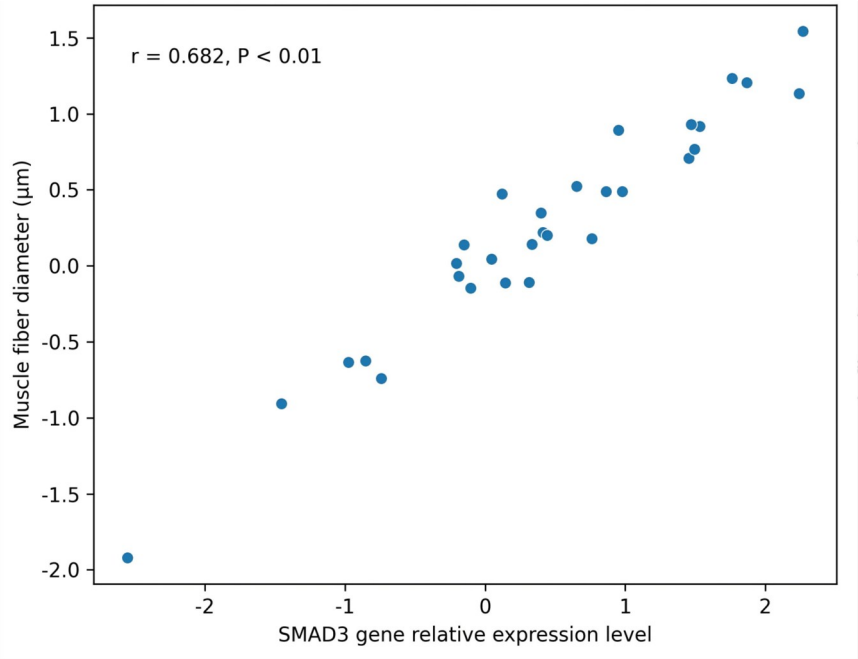

Figure 5. SMAD3 gene expression vs muscle fiber diameter

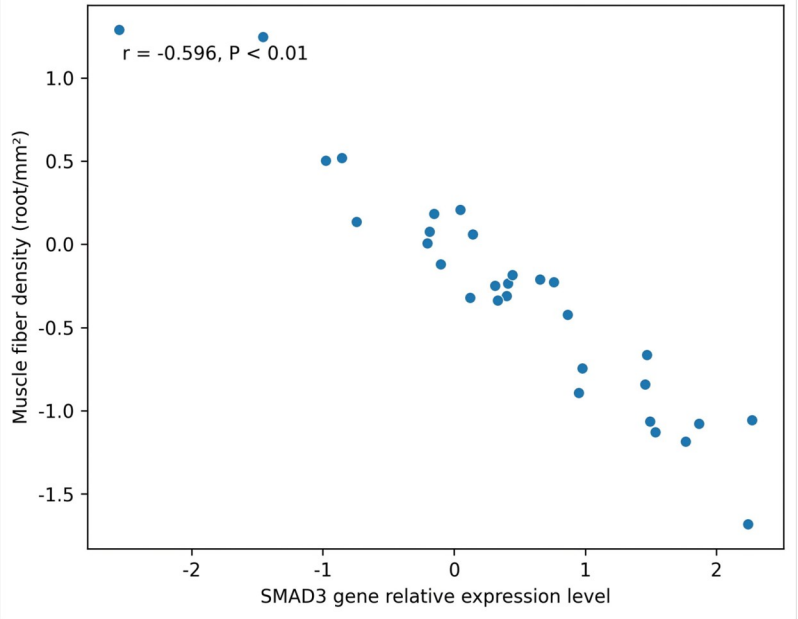

Figure 6. SMAD3 gene expression vs muscle fiber density

4. Discussion

In this study, real-time fluorescent quantitative PCR was used to systematically analyze the expression pattern of SMAD3 gene in grouper for the first time. The results showed that the expression level of SMAD3 gene in muscle tissue was significantly higher than that in other tissues, which was consistent with the research results in mammals. This suggests that SMAD3 gene may play an important biological role in muscle tissue. [8]

Further histological and correlation analyses revealed that SMAD3 gene expression was positively correlated with muscle fiber diameter and negatively correlated with muscle fiber density in grouper. Muscle fiber growth and development include two processes: hyperplasia (increasing fiber number) and hypertrophy (increasing fiber diameter). In the early embryonic development of fish, muscle mass is mainly increased through fiber hyperplasia; while in juvenile and adult stages, fiber hypertrophy becomes the main way of muscle growth. The results of this study suggest that SMAD3 gene may promote the hypertrophy of grouper muscle fibers and inhibit fiber hyperplasia to a certain extent. [9-10]

5. Conclusion

This study clarified the expression differences of SMAD3 gene in different tissues of grouper and found that it was highly expressed in muscle tissue. Meanwhile, it revealed a significant correlation between SMAD3 gene expression and muscle fiber development in grouper. These results provide important clues for in-depth study on the molecular mechanism of grouper muscle development and theoretical basis for genetic improvement and aquaculture production of grouper. In the future, the specific function and mechanism of SMAD3 gene in grouper muscle development can be further explored through gene knockdown or overexpression techniques.

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