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Original Article

Molecular expression and localization of follicle-stimulating hormone and its receptor in rat liver

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Abstract: Objective: We detected immunohistochemistry location of follicle-stimulating hormone (FSH) and its receptor (FSHR), and further studied its gene sequence characteristics, providing morphological basis for studying FSH function in regulating liver metabolic function. Methods: FSH and FSHR localization were analyzed by double-label immunofluorescence method. The levels of FSH and FSH mRNA in rat liver cells were detected by RT-PCR, and the obtained cDNA was used for gene cloning and sequence analysis of the core sequence. Results: FSH and FSHR were expressed in cytoplasm of rat liver cells with different density and degree of immunostaining in liver cells in different parts. Green fluorescence appeared in cytoplasm of FSH positive cells, and red fluorescence appeared in cytoplasm of FSHR positive cells. Some liver cells expressed both FSH and FSHR, showing red and yellow fluorescence. FSH and FSHR gene in liver cells expressed similarly, as revealed by mRNA analysis. Sequence analysis was performed on the specific bands of FSH and FSHR gene amplified from rat liver tissue, and the results suggested that in the amplified products, FSH gene sequence was consistent with genes in rat adenohypophysis; FSHR gene sequence was consistent with rat testis, which was previously reported. Conclusion: FSH and FSHR were expressed in a portion of rat liver cells. FSHR expression level was different in liver cells in different parts. FSH and FSHR only coexisted in a portion of liver cells. FSH may regulate liver functions through autocrine or paracrine.

Keywords: Follicle-stimulating hormone receptor, immunofluorescence histochemistry, liver, RT-PCR, rat

Introduction

It has been confirmed that follicle-stimulating hormone (FSH) is a dimeric glycoprotein consisting of α and β subunits, which is secreted by the anterior pituitary basophilic cells. It functions in gonad through blood circulation and plays important roles in sex hormones production and reproductive processes [1]. Follicle-stimulating hormone receptor (FSHR) is a G protein coupled receptor with seven transmembrane structures and mainly expressed in testicular sertoli cells and ovarian granular cells. FSH contains a large extracellular domain in its N-terminal region for FSH combination. However, more studies have reported that FSH is expressed in several other tissues apart from pituitary gland. FSH is expressed in prostate [2], galactophore [3], spermary [4] and placenta [5] in reproductive system, and also expressed in human gastric parietal cells in non-reproductive systems [6]. FSH and FSHR expression outside endocrine tissue was reported in recent years and remains controversial. Transcriptional and genetic evidences are necessary for supporting this view. However, it is still under investigation. Therefore, FSH and FSHR in rat liver were analyzed by double-label immunofluorescence method and gene sequence analysis based on our premier experimental works [7-9] to demonstrate FSH and FSHR expression and distribution outside endocrine tissue, particularly in liver tissue. The study also provided morphological basis for detecting FSH function in regulating liver function.

Materials and methods

Animals and reagents

Twenty normal male and female SD rats weighing (200±20) g were purchased from Beijing Vital River Laboratory Animal Technology Co.,

Ltd, China. Microscope (BA400) was purchased from Motic Company. Micro-ultraviolet spectrophotometer (Q-5000) was purchased from US Quawell Company and Gel imaging analyzer (WD-9413) was purchased from Beijing Liuyi Instrument Factory. Large horizontal electrophoresis tank (DG-3D) and bistable digital electrophoresis apparatus (DG-III) were both from Beijing Donglinchangsheng Biotechnology Co., Ltd; PCR instrument (9600) was purchased from ABI Company.

Tissue slice preparation

Rats were injected intraperitoneally with pentobarbital (80 mg/kg) for anesthesia and given open-heart surgery rapidly. Intubation was performed via ventriculus sinister to ascending aorta. 100 ml normal saline was used to rinse the blood and then the tissue was perfused for fixation with 0.1 mol/L phosphate buffer saline (PBS pH 7.4) containing 40 g/L paraformaldehyde at 4°C for 2 h. Hepatic tissue was obtained by laparotomy, immersed in 300 g/L sucrose solution at 4°C and allowed to sink. Hepatic tissue was placed in 4% paraformaldehyde and fixed for 6~8 h at room temperature. Fixed tissue was dehydrated in gradient alcohol, placed in xylene and then embedded in paraffin wax. The tissue was cut into 4 μm-thick sections and mounted on APES coated slides. RNase activity was ultimately inhibited in the process of slice preparation.

Double-label immunofluorescence localization

Double-label immunofluorescence histochemical staining: Slices were prepared as follows: Dewaxing and hydration. Slices were immersed in xylene for 6 min and 5 min, respectively; then slices were dehydrated in gradient alcohol, immersed in deionized water for 5 min and rinsed in PBS for 5 min; Antigen retrieval was performed based on microwave heating of slices in citrate antigen retrieval buffers for 12 min. Then slices were rinsed in PBS for 5 min × 3; Serum blocking: slices were dried and added with goat serum for 30 min at room temperature; Primary antibody incubation. Slices were removed of serum and added with FSH/FSHR antibodies prepared with antibody diluent (1:150 and 1:200, respectively). Slices were incubated overnight at 4°C and rinsed in PBS for 5 min × 3; 5) Secondary antibody incubation. Slices were dried and added with TRITC (1:50) labeled goat anti-rabbit working solution for 30 min at room temperature, and then rinsed in PBS for 5 min × 3; Slices were dried and added with FITC (1:50) labeled goat anti-mouse working solution for 30 min at room temperature. Slices were washed in PBS for 5 min × 3; 6) slices were coverslipped with glycerin-TBS. In negative control groups, PBS was used instead of primary antibodies. Positive samples provided by reagents company were used as positive controls.

Laser confocal scanning microscopy (cfssm) examination

Slices were examined with CFSSM (OFSympus FV300) equipped with an objective lens of × 10 (numerical aperture 0.4) and × 20 (numerical aperture 0.7). FITC488 and Cy3548 images were acquired at the wavelength of 488 nm and 548 nm, respectively. Meanwhile, there were few signals in other wavelength. Adjusting signals of FITC488 and Cy3548 appropriately maintained the best experiment condition. FITC488 and Cy3548 images were accurately ranked on the same focal plane.

RT-PCR analysis

Design and synthesis of primers: Full-length sequence of target gene mRNA was obtained from GenBank. Primers were synthesized by Beijing Dingguo Changsheng Biotechnology Co. LTD. Primer information: primer sequence: GAPDH, 5' ACAGCAACAGGGTGGTGGAC 3', 5' TTTGAGGGTGCAGCGAACTT 3', product length 252 bp; FSH 5AGACCAAACACCCAGAA 3', 5' CACCGAAGGAGCAGTAG 3', product length 193 bp, FSHR, 5' TCTGATAGATGATGAACCC 3', 5' CAAAGACAGTGAAAAAGCC 3', product length 413 bp.

RNA extraction: 50-100 mg Gastric tissue samples conserved at -80°C were placed in a mortar and ground in liquid nitrogen for 3 times. 1 mL Trizol was added to every 50-100 mg of tissue and cracked for 5 min at room temperature. The precipitate was removed by centrifugation at 12000 rpm for 5 min; 200 μL chloroform was added to every 1 mL Trizol and mixed at room temperature for 15 min; the upper aqueous phase was removed to another EP tube by centrifugation at 4°C 12000 rpm for 15 min; 0.5 ml isopropanol was then added to 1 mL Trizol and mixed well, conserved at

-20°C for 30 min; the supernatant was removed by centrifugation at 4°C 12000 rpm for 10 min and RNA was at the bottom. 1 ml 75% ethanol was added to 1 mL Trizol and mixed gently to suspend the precipitate. The supernatant was removed by centrifugation at 4°C 8000 rpm for 5 min; and precipitate was air-dried at room temperature or vacuum dried for 5-10 min; RNA sample was dissolved in 30 μl TE buffer at 55-60°C for 5-10 min.

DEPC water was added to total RNA to 300 μL; 300 μl chloroform was added, mixed for 5 min and centrifuged at 4°C 12000 rpm for 10 min. The supernatant was removed to another EP tube treated with DEPC water. 300 μl chloroform was added and the following procedure was repeated. Then isopropanol in the same volume was added and placed at -20°C for 20-30 min. The supernatant was removed by centrifugation at 4°C 12000 rpm for 10 min. 1 ml 75% ethanol was added after precipitation. The supernatant was removed by centrifugation at 4°C 8000 rpm for 5 min; and precipitate was air-dried at room temperature or vacuum dried for 5-10 min; RNA sample was dissolved in DEPC water for RNA purification.

RNA concentration and purity analysis

RNA concentration: 2 μl RNA sample was measured for the absorbance at 260 nm with a micro-ultraviolet spectrophotometer (Q-5000). RNA purity: RNA purity was measured for the absorbance at 260 nm and 280 nm with a micro-ultraviolet spectrophotometer (Q-5000). OD_{260}/OD_{280} was used to assess the RNA purity; $1.7<OD_{260}/OD_{280}<2.0$ was accepted. Samples with the ratio <1.7 indicated protein contaminants and chloroform purification was repeated.

RNA integrity analysis

RNA integrity was evaluated using agarose gel electrophoresis stained with ethidium bromide, which produced a certain banding pattern. RNA was considered of high quality when the ratio of 28S:18S ribosomal RNA (rRNA) bands was about 2.0.

Reverse transcription

The extracted total RNA was treated with DNase and used as templates. cDNA was synthesized by using TOYOBO reverse transcription kit.

RT-PCR analysis PCR products were analyzed by agarose gel electrophoresis in 0.5 × TBE at 5 V/cm for 30 min. The electrophoresis bands were photographed and analyzed.

Cloning and sequence analysis of PCR products

Target fragment recovery: the target band was cut from the agarose gel and placed in sterilized EP tube. Then it was weighed, dissolved, eluted and placed at room temperature for 2 min. Then DNA solution was collected after centrifugation at 13000 rpm for 1 min. A 10 ml ligation system contained T4 DNA ligase 3 mL, recovery products of target gene 2 ml, T-vector 1 ml. The reaction was performed at 16°C overnight. Transformation was performed as follows: 100 μl competent DH5a E. Coli cells in a tube were thawed on ice; ligation system was added to thawed competent cells and placed on ice for 30 min, and then heat shock was performed at 42°C for 1 min and placed on ice for 2 min; the tube was added with 900 μl LB and shaken at 37°C, 100 rpm for 1 h. 900 μl supernatant was removed and cells were collected by centrifugation. Precipitate was suspended in the remaining 100 μl solution for spreading onto LB plates with 100 mmol/L ampicillin, which was incubated at 37°C for 12-16 h. Blue-white screening was then performed; white clones were selected for streak cultivation on LB plates with 100 mmol/L ampicillin for 10-12 h; clones were analyzed with PCR, during which the positive clones were selected for plasmid extraction and electrophoresis. Clones of positive sequences were sequenced by Beijing Dingguo Changsheng Biotechnology Co., LTD.

Results

Immunofluorescent and histochemical localization

FSH was expressed in cytoplasm of rat liver cells. The positive cells showed green fluorescence in cytoplasm while nuclei were negative (**Figure 1A**, **1D**). FSHR was expressed in cytoplasm of rat liver cells. The positive cells showed red fluorescence in cytoplasm while nuclei were negative (**Figure 1B**, **1E**). Some liver cells expressed both FSH and FSHR, show-

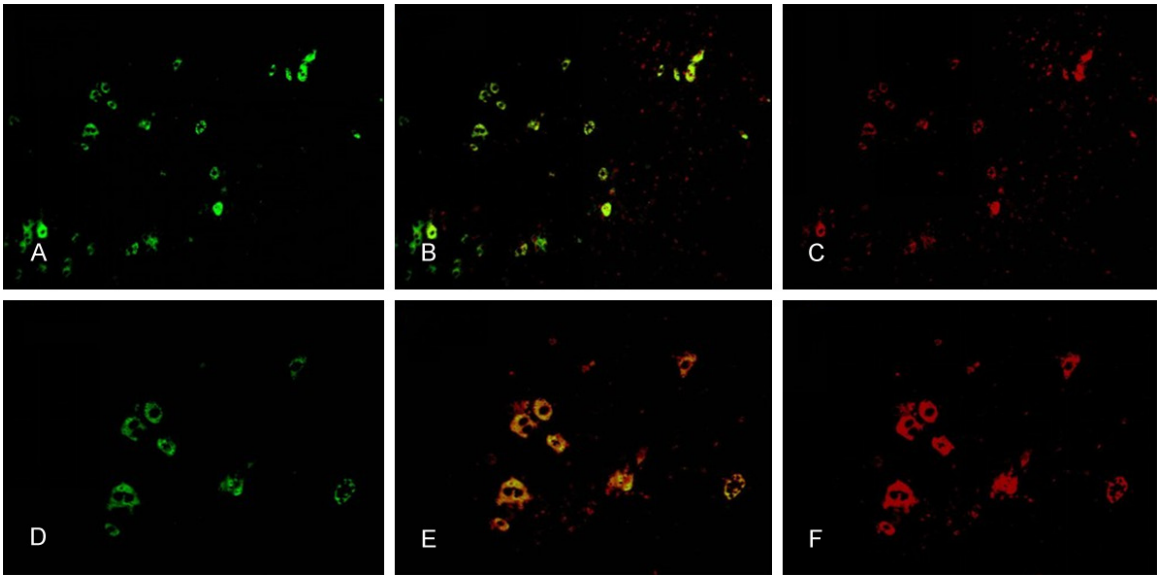

Figure 1. A, D: FSH in rat liver fluorescent labeling, FSH positive products distributed in some livers cells showing green fluorescence in cytoplasm (FITC) × 200, × 400; B, E: Both FSH and FSHR positive products were co-located in some liver cells showing yellow or green and red fluorescence in cytoplasm (FITC, TRITC) × 200, × 400; C, F: FSHR in rat liver fluorescent labeling, FSHR positive products distributed in some liver cells showing red fluorescence in cytoplasm (TRITC) × 200, × 400.

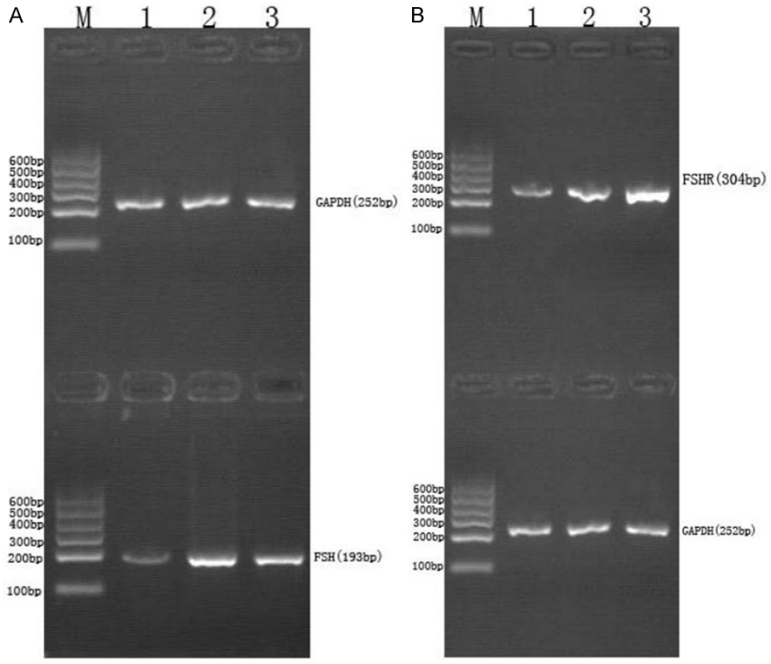

Figure 2. A: Core fragment of rat liver FSH gene amplification product by agarose gel electrophoresis. GAPDH is a reference gene; B: Core fragment of rat liver FSHR gene amplification product by agarose gel electrophoresis. GAPDH is a reference gene.

ing red and yellow fluorescence (**Figure 1C**, **1F**). All results showed no fluorescence or light color in the background, which was easy for recognition. The negative control showed no fluorescence.

RT-PCR results

RT-PCR products were analyzed by 1% agarose gel electrophoresis stained with ethidium bromide. Results suggested that FSH and FSHR had specific fluorescent bands of 193 bp and 304 bp, respectively, consistent with the designed base number (**Figure 2**). Meanwhile, the negative group showed no bands.

Sequencing and homology comparison of positive clones

Two clones were selected from FSH and FSHR positive clones, respectively. The sequencing results were analyzed by homologous search in GenBank. It was found that FSH sequence was fully consistent with rat pituitary FSH gene sequence; FSHR sequence was fully consistent with rat testicle FSHR gene sequence.

Discussion

It has been reported that FSH and FSHR are expressed and distributed in rat pancreas [8, 10] and stomach [7, 11], but there is no report about their distribution in rat liver. In the study, it was discovered that FSH and FSHR were distributed in cytoplasm of a portion of rat liver cells, while some liver cells only had weakly positive immunofluorescence reaction. It was also found that FSH and FSHR immunofluorescent reactants were distributed in a portion of liver cells, probably caused by different FSH and FSHR distribution, or different sensitivity of the two antibodies, as remains to be further investigated. Physiological study revealed that the blood in hepatic lobule flowed from peripheral sites to central sites and then rejoined into the central vein. Thus the liver cells in peripheral and central hepatic lobules were different in sequence and amount of oxygen and nutrient from circular blood, which might lead to different FSH and FSHR expression.

Patients with hepatopathy have decreased inactivation functions of hormones and increased levels of estrogen, aldosterone, antidiuretic hormone, which may cause gynaecomastia, spider nevus, and so on, indicating that several kinds of hormones were metabolized in liver tissue. It was reported recently that FSH not only regulated reproductive function, but also regulated other physiological activities. Our results suggested the existence of FSH and FSHR immunofluorescent positive substances in rat liver cells, which indicated FSH and FSHR expression in rat liver cells. RT-PCR analysis and the cloning and sequence analysis of its products were used to further verify FSH and FSHR expression in liver tissues. The results demonstrated the expression of FSH and FSHR in liver cells from molecular level and similar expression level of the two genes. Sequence analysis was carried out on the specific bands of FSH and FSHR gene amplified from rat liver tissue, and the results suggested that in the amplified products, FSH gene sequence was consistent with genes in rat pancreas, glandula submandibularis [8, 9] and adenohypophysis; FSHR gene sequence was consistent with rat pancreas, glandula submandibularis [8, 9] and testis, which was previously reported. It was demonstrated that FSH and FSHR were produced in liver, which also targeted at the liver. Morphology experimental results showed that a portion of rat liver cells were positive in FSH and FSHR immunofluorescence reaction. It was also discovered that FSH and FSHR can be expressed in one liver cell, indicating the coexistence of FSH and FHSR. It was also demonstrated that FSHR inducted physiological function of FSH. The results indicated that FSH in rat liver may regulate liver functions through autocrine or paracrine approach. But further studies are still required to reveal and verify the specific functions.

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Disclosure of conflict of interest

None.

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