



Expression and clinical significance of obesity-associated gene *STEAP4* in obese children

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ABSTRACT. The aim of this study was to investigate the expression and clinical significance of the obesity-associated gene *STEAP4* in obese children. Fifty-three obese children and 33 children with a standard body weight (control) from our hospital were recruited to this study. The expression of *STEAP4* mRNA and protein in the adipose tissue were detected by reverse transcriptase polymerase chain reaction and

enzyme-linked immunosorbent assay, respectively, in order to analyze the relationship between *STEAP4* mRNA and protein levels and blood pressure, blood lipid profile, blood glucose levels, and inflammation in obese children. Obese children showed significantly lower levels of *STEAP4* mRNA and protein in the adipose tissue compared to the control subjects ($P < 0.05$). The obese subjects exhibited significantly higher diastolic blood pressure (DBP), systolic blood pressure (SBP), total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL), fasting plasma glucose (FPG), interleukin (IL)-6, and tumor necrosis factor (TNF)- α levels, and a significantly lower high-density lipoprotein (HDL) level, compared to the control subjects ($P < 0.05$). Correlation analysis revealed that *STEAP4* expression was negatively correlated with the DBP, SBP, TC, TG, LDL, FPG, IL-6, and TNF- α levels, and was positively correlated with the HDL level ($P < 0.05$). In conclusion, the expression of *STEAP4* was significantly downregulated in the adipose tissue of obese children and was closely related to the blood pressure, blood lipid, blood glucose, and inflammation in these patients; therefore, these results could provide a theoretical basis for the treatment of childhood obesity.

Key words: *STEAP4*; Obese children; Lipid; TNF- α ; IL-6; Blood pressure

INTRODUCTION

Obesity is a chronic dystrophy characterized by excess weight. It is caused by excessive energy intake over a long period, leading to the excessive accumulation of adipose tissue in the body (Kruse and Leifeld, 2015; Kobylak et al., 2016). Obesity tends to develop at a younger age. In fact, recent surveys have shown a rapid increase in the number of overweight and obese preschool children worldwide (Small and Aplasca, 2016; Ulijaszek et al., 2016). Obesity is correlated with the incidence and long-term mortality of several diseases. For example, obesity directly damages heart and lung functions and causes serious mental disorders, mental stress, and abnormal behavior in children (Kawarazaki and Fujita, 2016; Mandviwala et al., 2016). In addition, childhood obesity could develop into chronic diseases such as hypertension and diabetes if not addressed at an early stage. Despite being the focus of a number of research studies, the molecular mechanism of obesity remains to be elucidated. Recent studies have characterized obesity as a systemic chronic low-grade inflammation caused by various inflammatory factors (Esser et al., 2014; Lee and Lee, 2014; Kawarazaki and Fujita, 2016). In addition to storing energy and adipose, adipose tissues are believed to function as an endocrine organ, secreting a host of factors such as interleukin (IL)-6, tumor necrosis factor (TNF)- α , leptin, and adiponectin (Calder et al., 2011; Wensveen et al., 2015). Imbalances in these factors may lead to obesity. *STEAP4* is a member of the TNF- α inducible protein family, whose function remains to be elucidated. Previous studies have indicated a possible correlation between *STEAP4* and human obesity (Zhang et al., 2008; Catalán et al., 2013). However, the exact mechanism of the correlation remains unclear. The purpose of this study was to analyze the expression of *STEAP4* in obese children to explore its role in obesity, and to provide a theoretical basis for its clinical treatment.

MATERIAL AND METHODS

General data

Fifty-three obese children (31 male and 22 female; age range: 5-10 years; average age: 6.78 ± 2.43 years) who visited our hospital between June 2013 and December 2015 were recruited to this study. The subjects were included based on the following inclusion criteria: children aged between 5 and 10 years with no family history of genetic disease and with a body-mass index ≥ 23 kg/m²; children without malignant tumors; children with diabetes (based on the medical history and fasting blood glucose levels) and/or abnormal thyroid function (according to the medical history and thyroid-stimulating hormone levels); children without any liver or kidney insufficiency; children without a serious disease affecting the digestive system or chronic diarrhea; and children without anorexia (or who have not started a diet immediately prior to the study). Thirty-three normal children (20 male and 13 female; age range: 5-10 years; average age: 6.78 ± 2.43 years) were simultaneously recruited to the control group. The age, gender, and other indices did not differ significantly between the groups ($P < 0.05$). This study was conducted in accordance with the Declaration of Helsinki and with approval from the Ethics Committee of the Children's Hospital of Kaifeng City. Written informed consent was obtained from all participants or their guardians.

Specimen collection

Adipose tissue was obtained from the obese children via a laparotomy and from normal children via an abdominal operation. The obtained tissues were preserved at -80°C until further use.

Real-time fluorescence quantitative polymerase chain reaction (PCR)

The adipose tissue was homogenized in a 1.5-mL centrifuge tube with 1 mL RNase-free TRIzol (TaKaRa Bio Inc., Otsu, Japan); the homogenized tissue was vortexed with 200 μL chloroform until layers were obtained. The sample was centrifuged at 15,000 rpm for 15 min and the supernatant was added to a new 500- μL centrifuge tube. This was incubated on ice for 30 min with isometric isopropanol, and the mixture was centrifuged again at 15,000 rpm for 15 min. The supernatant was discarded, while the precipitate was washed once with the pre-cooled 70% ethanol and centrifuged at 15,000 rpm for 6 min. The obtained precipitate was dissolved in RNase-free double-distilled water, and the RNA concentration was measured. The RNA was reverse transcribed to cDNA using the appropriate kit (TaKaRa Bio Inc.). The PCR primers were designed based on the *STEAP4* mRNA sequences provided in GenBank as follows: *STEAP4-F*, 5'-CGAAACTTCCCTCTACCCG-3'; *STEAP4-R*, 5'-ACACAAACACCTGCCGACTT-3'; *GAPDH-F*, 5'-CCTTCCGTGTTCTACCC-3'; and *GAPDH-F*, 5'-CAACCTGGTCCCTCAGTGTAG-3'. The primers were diluted, and the 20- μL reaction system was prepared as follows: 10 μL 2X SYGreen RT-PCR Mix (TaKaRa Bio Inc.), 8 μL deionized water, 0.5 μL upstream primer, 0.5 μL downstream primer, 1 μL template, and 1 μL synthesized cDNA. The reaction was performed in the CFX96 fluorescent quantitative thermal cycler (Bio-Rad, Hercules, CA, USA) using the following reaction conditions: initial denaturation at 95°C for 10 min; and 40 cycles of denaturation at 95°C for 10 min, annealing

at 55°C for 20 s, and extension at 72°C for 20 s. One negative control was set for each group and each sample was amplified in triplicate. Data were directly read from the instrument.

Western blot analysis

Adipose tissue (1 g) was obtained from both obese and normal children and homogenized with 500 μ L. The homogenized mixture was mixed (by inverting 10 times) with 1/9 100% TCA, incubated on ice for 30 min, and subsequently centrifuged at 15,000 rpm for 20 min, obtaining a dark brown precipitate. The supernatant was discarded and the EP tube was gently inverted on absorbent paper and subsequently baked in a 37°C oven for 10-20 min to remove all the residual liquid from the orifice. The sample was centrifuged at 15,000 rpm for 10-20 min and the residual liquid was removed with a 20- μ L micropipette. The precipitate was dissolved in sodium dodecyl sulfate (SDS)-loading buffer and incubated in boiling water for 20 min. The partially dissolved precipitate was then separated by SDS-polyacrylamide gel electrophoresis. The separated protein was transferred to a polyvinylidene difluoride membrane; the membrane was blocked with 5% skim milk powder for 1 h, incubated overnight with rabbit anti-STEAP4 antibody (1: 1000; Boster, Wuhan, China), and subsequently washed thrice with TBST for 5 min. The membrane was then incubated with a sheep-anti-rabbit horseradish peroxidase-labeled secondary antibody (1:500; ZSGB-Bio, Beijing, China) for 1 h and finally washed thrice with TBST for 5 min (each). GAPDH was used as the internal reference and the membranes were photographed.

Relationship between STEAP4 level and blood pressure, blood glucose, and blood lipid levels

The blood pressure of all obese and normal subjects was measured in triplicate using the Omron Hem-770A electronic sphygmomanometer (Omron, Hoofddorp, Netherlands) and the average blood pressure was calculated. Hypertension was characterized by a systolic blood pressure (SBP) \geq 140 mmHg or a diastolic blood pressure (DBP) \geq 90 mmHg. Serum was separated from venous blood extracted from fasting children in the early morning. The blood lipid and blood glucose levels were measured using an automatic biochemical analyzer (AU5800; Beckman-Coulter, Miami, FL, USA). The blood lipid measurement included the measurement of total cholesterol (TC), triglyceride levels (TG), high-density lipoprotein (HDL), and low-density lipoprotein (LDL). Hyperlipidemia was characterized by TG \geq 1.7 mM or HDL $<$ 0.91 mM. Blood glucose analysis included the measurement of fasting plasma glucose (FPG) levels (hyperglycemia was characterized by fasting blood glucose \geq 7 mM).

Relationship between STEAP4, IL-6, and TNF- α expression

IL-6 and TNF- α levels were detected using a standard enzyme-linked immunosorbent assay (ELISA) kit (Amyjet Inc., Wuhan, China), according to the manufacturer protocols.

Statistical analysis

All data were analyzed using the SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). Data are reported as means \pm standard deviation of three independent measurements

and compared using the *t*-test. Differences with *P* values < 0.05 were considered statistically significant.

RESULTS

Downregulation of *STEAP4* mRNA in the adipose cells of obese patients

The fluorescence quantitative amplification of *STEAP4* mRNA revealed that the primers had good specificity, as no miscellaneous bands were observed in the agarose gel. Quantitative analysis showed that the *STEAP4* mRNA levels were significantly downregulated in the adipose tissues of obese children compared to those in normal control children (*P* < 0.05) (Figure 1).

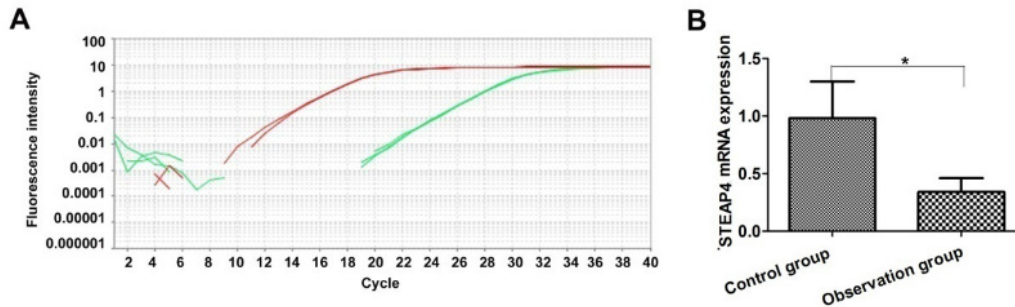


Figure 1. Comparison of *STEAP4* mRNA expression in the adipose cells of obese and normal children. **A.** Fluorescence quantitative PCR amplification curve of *STEAP4* and *GAPDH*; **B.** RT-PCR analysis showed the *STEAP4* mRNA expression in the adipose cells of obese and normal children.

Downregulation of *STEAP4* protein in adipose cells of obese patients

Total protein was extracted from the adipose cells of normal and obese children using TCA and the levels of *STEAP4* in the two groups were determined by western blot. We observed a significant decrease in the *STEAP4* levels in the adipose cells of obese patients compared to those in normal patients (*P* < 0.05) (Figure 2).

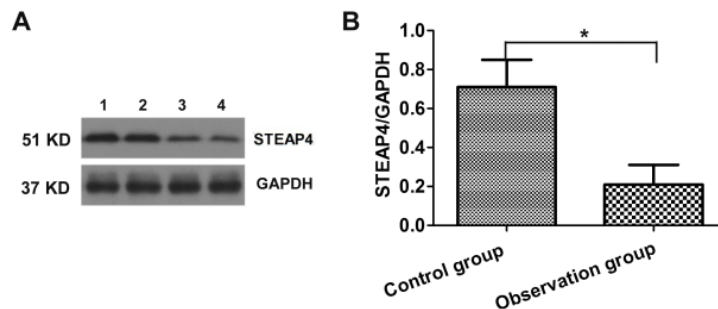


Figure 2. Comparison of *STEAP4* expression in the adipose cells of obese and normal children. Results of the western blot analysis (**A**) and quantitative analysis (**B**) showed the *STEAP4* expression in the adipose cells of obese and normal children.

STEAP4 expression is negatively correlated with blood pressure, blood lipid, and blood glucose

The obese subjects exhibited significantly higher DBP and SBP levels than the control subjects ($P < 0.05$) (Table 1). Moreover, significantly higher TC, TG, LDL, as well as fasting blood glucose levels were recorded in patients belonging to the observation group (compared to the control subjects) ($P < 0.05$). However, the obese subjects exhibited significantly lower HDL levels compared to the control subjects ($P < 0.05$). A statistical analysis of these results showed a negative correlation between the STEAP4 level and the DBP, SBP, TG, TC, LDL, and FPG levels ($r = -0.019, -0.025, -0.127, -0.078, -0.099, \text{ and } -0.211$, respectively; all $P < 0.05$), and a positive correlation between the STEAP4 and HDL levels ($r = 0.183, P < 0.05$).

Table 1. Comparison of blood pressure, blood lipid, and blood glucose between obese and normal subjects.

Groups	N	Blood pressure			Blood lipid			FPG (mM)
		SBP (mmHg)	DBP (mmHg)	TC (mM)	TG (mM)	HDL (mM)	LDL (mM)	
Control group	33	113.5 ± 15.5	79.2 ± 12.1	5.27 ± 0.50	1.93 ± 0.78	1.72 ± 0.32	3.23 ± 0.34	4.51 ± 0.67
Obese group	53	139.2 ± 17.2	90.2 ± 14.3	5.66 ± 0.61	2.34 ± 0.98	1.45 ± 0.25	3.67 ± 0.39	5.12 ± 0.74
P value		0.012*	0.019*	0.032*	0.0268	0.038*	0.041*	0.010*

SBP: systolic blood pressure; DBP: diastolic blood pressure; TC: total cholesterol; TG: triglyceride; HDL: high-density lipoprotein; LDL: low-density lipoprotein; FPG: fasting plasma glucose.

Relationship between STEAP4 expression and IL-6 and TNF- α levels

The ELISA results showed that obese patients had significantly higher IL-6 and TNF- α levels compared to the control subjects ($P < 0.05$). Moreover, the correlation analysis showed a negative correlation between the STEAP4 expression and the IL-6 and TNF- α levels in obese subjects ($r = -0.102 \text{ and } -0.153$, respectively; $P < 0.05$) (Table 2).

Table 2. Comparison of interleukin (IL)-6 and tumor necrosis factor (TNF)- α between obese and control subjects.

Groups	N	IL-6 (ng/L)	TNF- α (ng/L)
Control group	33	13.25 ± 1.96	112.14 ± 17.15
Obese group	53	20.14 ± 2.14	150.24 ± 18.21
P value		<0.05	<0.05

IL-6: interleukin-6; TNF- α : tumor necrosis factor- α .

DISCUSSION

Obesity is a nutritional and metabolic disorder caused by the accumulation or abnormal distribution of adipose in the body due to the imbalance between energy intake and consumption (which in turn can be attributed to genetic and environmental factors) (Zhang et al., 2012; Crane and McGowan, 2016; Floyd et al., 2016). In recent years, the rapid improvement in living conditions has led to a significant increase in the number of obese children worldwide. Obesity is a major factor influencing the development of several diseases that affect the growth and development of children, including diabetes, cardiovascular diseases, cerebrovascular diseases, and disorders in the lipid metabolism (Ng et al., 2014;

Basil et al., 2016). However, the molecular mechanism and primary cause of obesity remains to be elucidated. Recent developments in molecular biological techniques could facilitate research into obesity and its development at the genetic level. At present, obesity has been predominantly attributed to chronic inflammation. Previous studies have reported significantly abnormal levels of inflammatory factors such as IL-6 and TNF- α in the adipose cells of obese patients (Park et al., 2010; Todendi et al., 2015). However, there is no explanation for the upregulated phenotypes of these inflammatory factors.

STEAP4, or TNF-AIP9, is a member of the tumor necrosis factor- α inducible protein family. Previous studies have reported that *TNFAIP9* knockout mice spontaneously developed tendonitis and synovitis, showed significantly higher levels of IL-6 in the peripheral blood and CD11B cells in the spleen, and exhibited significantly enhanced Th1 cell activation and Th17 cell response (Takai et al., 2015). This suggested that STEAP4 played an important role in the inflammatory process. Recent studies have demonstrated a close relationship between STEAP4 expression and obesity. Therefore, the expression and function of STEAP4 in obese children was discussed in this study. Fluorescence quantitative PCR and ELISA analyses revealed a significant downregulation in the STEAP4 mRNA and protein levels in the adipose tissue of obese children. However, the IL-6 and TNF- α levels were significantly increased in the peripheral blood of obese children. This result was consistent with the phenotype observed in the knockout mice, suggesting that STEAP4 might be a negative regulator of inflammation, and played an important role in maintaining the inflammatory balance.

We also analyzed the relationship between STEAP4 levels and the blood pressure, blood lipid, and blood glucose levels in obese children. Research showed that obese children exhibited a 10-fold higher (abnormal) incidence of compared to normal children (Matsumoto et al., 2014). Our analysis showed a negative correlation between STEAP4 expression and DBP and SBP levels in obese children. Obese children also showed lipid metabolism disorder; for example, the blood lipid analysis showed significantly higher levels of TC, TG, and LDL in the obese subjects, compared to the normal subjects. Moreover, the correlation analysis showed a negative correlation between STEAP4 expression and the TC, TG, and LDL levels. Alternately, the HDL expression significantly decreased and correlated with the STEAP4 level in obese patients. HDL is mainly responsible for the transport of cholesterol released from extrahepatic cells into the liver, which prevents the accumulation of cholesterol in blood, as well as atherosclerosis. Increased HDL levels increases the chance of atherosclerosis in obese children. Therefore, obesity is a major risk factor of cardiovascular and cerebrovascular diseases, diabetes, and hypertension.

The results of this study are subject to certain limitations. For example, a relatively small sample size was employed, and the study only specifies the correlation between the analyzed factors. Therefore, further studies with larger sample sizes are mandated; moreover, the role of STEAP4 in obesity and the inflammatory response must be further investigated *in vitro* and/or *in vivo*.

In conclusion, STEAP4 expression was downregulated in the adipose cells of obese children. This inflammatory factor may regulate the inflammatory response of adipose cells, and could be used as a therapeutic target for the treatment of obesity.

Conflicts of interest

The authors declare no conflict of interest.

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