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Senescence marker protein 30 (SMP30)/regucalcin (RGN) expression decreases with aging, acute liver injuries and tumors in zebrafish

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ABSTRACT

Senescence marker protein 30 (SMP30)/regucalcin (RGN) is known to be related to aging, hepatocyte proliferation and tumorigenesis. However, expression and function of non-mammalian SMP30/RGN is poorly understood. We found that zebrafish SMP30/RGN mRNA expression decreases with aging, partial hepatectomy and thioacetamide-induced acute liver injury. SMP30/RGN expression was also greatly decreased in a zebrafish liver cell line. In addition, we induced liver tumors in adult zebrafish by administering diethylnitrosamine. Decreased expression was observed in foci, hepatocellular carcinomas, cholangiocellular carcinomas and mixed tumors as compared to the surrounding area. We thus showed the importance of SMP30/RGN in liver proliferation and tumorigenesis.

1. Introduction

Senescence marker protein 30 (SMP30) was originally identified by two-dimensional gel electrophoresis as a 34 KDa protein of which expression decreases by up to 40% in the aged rat liver without androgen dependence [1]. But it was subsequently found to be the same as regucalcin (RGN), a calcium-related protein without an EF-hand motif [2]. RGN has been intensively studied by Yamaguchi and Takahashi who reported that RGN regulates intracellular Ca^{2+} homeostasis by activating Ca^{2+} pump activity in the cell membrane [3]. Moreover, SMP30/RGN inhibits Ca^{2+}-dependent protein kinase, protein phosphatase and nitric oxidase synthase, and thereby inhibits cell proliferation [4]. SMP30/RGN is known to exist in a broad range of species from mammals to bacteria, and its expression in mammals is high in hepatic parenchymal cells and the renal cortex [5]. Compared with normal mice, SMP30/RGN knockout mice age more rapidly and have increased fat droplets and lysosomes in their livers. Furthermore, SMP30/RGN was shown to be gluconolactonase, an enzyme indispensable for vitamin C synthesis, and the relationship between vitamin C and aging appears to be important [6,7]. SMP30/RGN mRNA expression is reportedly decreased in chemically-induced tumors as compared to surrounding normal liver tissue, based on in situ hybridization [8]. Microarray analysis showed SMP30/RGN to be one of the down-regulated genes in the GST-P positive area [9]. Although overexpression of SMP30/RGN in hepatoma cells down-regulates oncogenes such as c-myc, Haras and c-src [10], details of how SMP30/RGN takes part in the development and progression of cancer remain unknown. Zebrafish are the simplest vertebrate model because of their low breeding costs and the capacity for high-through-put screening [11,12]. Zebrafish provide a good cancer model because various diethylnitrosamine (DEN)-induced tumors resemble human tumors [13]. There are no reports concerning zebrafish SMP30/RGN except a study on dioxin administration to embryos [14]. Moreover, the expression of SMP30/RGN in mixed tumors and cholangiocellular carcinomas has not previously been reported. In this study, we examined the importance of SMP30/RGN in aging, liver proliferation and liver tumorigenesis using the zebrafish model.

2. Materials and methods

2.1. Animals

Zebrafish were maintained in accordance with the Animal care Guidelines of Yamaguchi University. Fish were kept in tap water in plastic tanks and illuminated with fluorescent lighting set at 16 h light and 8 h dark.

2.2. Cell culture

An adult zebrafish liver cell line (ATCC CRL-2643) was cultured in ZFL medium as described by Ghosh et al. [15].

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2.3. DEN exposure

One-year old fish were exposed to 200 ppm DEN for 2 months. DEN solutions were changed every week to compensate for degradation. Following exposure, the fish were maintained in tap water without DEN for 4 months.

2.4. Quantitative RT-PCR

Total RNA was isolated with TRIzol (Invitrogen), and treated with Turbo DNase (Ambion). RT-PCR was performed by utilizing the step one plus real time PCR system and Fast SYBR Green Master Mix (Applied Biosystems). Specific primers for SMP30/RGN (5′-ACT ATG ACA TCC AAA CTG GAG GA-3′ and 5′-CTT CTG TGT CTA TGC ACA TAC CG-3′) were used. Elongation factor 1-alpha was used as an internal control.

2.5. Tissue collection and histology

Fish were killed and opened from the anal vent to the gills. The entire body was fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The liver was dissected, dehydrated in alcohol and embedded in paraffin according to routine procedures. Serial sections were cut at a thickness of 3–5 μm. Staining was performed using hematoxylin and eosin (HE).

2.6. Immunohistochemistry (IHC)

Antibody for SMP30/RGN was obtained from Shima Laboratories (Tokyo, Japan). SMP30/RGN was immunohistochemically assessed using the avidin–biotin–peroxidase complex method, as described previously [16].

2.7. Western blot analysis

Samples were prepared by the same methods as previously reported [17]. The blots were incubated for 1 h at room temperature with primary antibodies against SMP30/RGN (Shima), and β-actin (Sigma) in blocking buffer. After being washed, the blots were incubated for 1 h at room temperature with secondary antibodies. Reactive bands were identified using an enhanced chemiluminescence kit (Amersham Biosciences) and autoradiography according to the manufacturer's instructions.

2.8. Partial hepatectomy

Liver regeneration was induced by partial hepatectomy. The ventral lobes of zebrafish livers were removed by the methods reported by Sadler et al. [18].

2.9. Thioacetamide (TAA) treatment

Six-month-old female zebrafish were injected with 300 mg/kg body TAA intraperitoneally. Two days after injection, the livers were collected and used for further examinations.

2.10. Statistical analysis

All data are expressed as means ± S.D. One way ANOVA followed by the Dunnett post hoc multiple comparison test was performed to assess the statistical significance of differences in SMP30/RGN expression with aging. The Kruskal–Wallis test followed by the Steel method was performed to assess the statistical significance of SMP30/RGN expression changes caused by partial hepatectomy. Student's *t*-test was performed to assess the results of other examinations. *P* values less than 0.05 were considered to be significant.

3. Results

3.1. Expression of SMP30/RGN in normal zebrafish tissues

SMP30/RGN is reportedly expressed in the livers and kidneys of mammals. We examined SMP30/RGN expression in various zebrafish tissues by Western blotting methods. The 35 KDa form was observed in livers, intestine and kidneys (Fig. 1A). We then examined immunohistochemical expression patterns and found that SMP30/RGN was expressed in hepatic parenchymal cells and the renal cortex of adult zebrafish (Fig. 1B).

3.2. SMP30/RGN expression changes with aging

Mammalian SMP30/RGN expression is known to decrease with aging. We examined SMP30/RGN expression changes in the liver with aging. We used 3-month-old, 6-month-old, 1-year-old and 3-year-old zebrafish. Body lengths were 19.6 ± 1.9, 22.9 ± 1.9, 28.7 ± 1.5, and 30.3 ± 3.2 mm (Fig. 2A), weights.7 ± 8.3, 295.8 ± 84.7, 493.0 ± 108.3, and 721.3 ± 259.3 mg (Fig. 2B), body mass index (BMI) 0.034 ± 0.002, 0.055 ± 0.009, 0.063 ± 0.008, and 0.076 ± 0.010 g/cm^2 (Fig. 2C), respectively. Three-year old zebrafish had spinal curvature which is a feature of advanced age (Fig. 2D). Fold changes in SMP30/RGN mRNA expression by quantitative RT-PCR were 1.00 ± 0.41,.0 0.43 ± 0.25, 0.41 ± 0.27, and 0.17 ± 0.05, respectively (Fig. 2E). SMP30/RGN expression thus decreased significantly with aging.

3.3. SMP30/RGN expression changes during regeneration after partial hepatectomy

We isolated total RNA from partially hepatectomized livers and regenerating livers after partial hepatectomy, and examined SMP30/RGN mRNA expressions by quantitative PCR with EF1a expression as an internal control. Expression of SMP30/RGN mRNA was significantly decreased to 50.0% ± 13.6% and 50.9% ± 10.4% 1

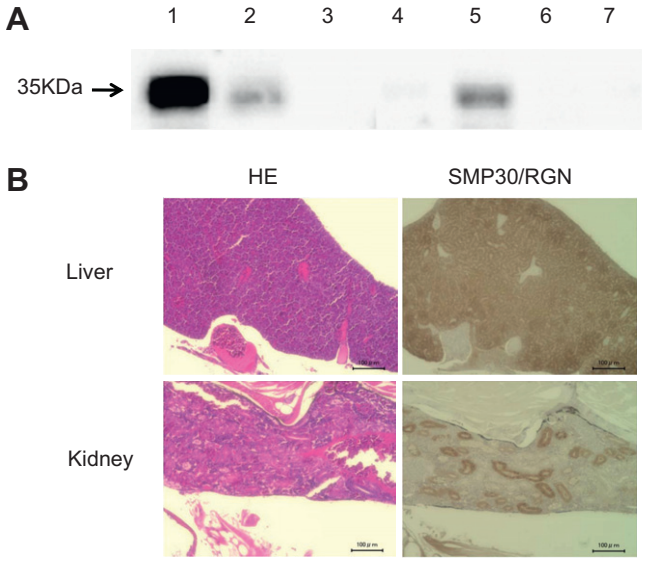

Fig. 1. SMP30/RGN expressions in various tissues in zebrafish. (A) SMP30/RGN expressions in various tissues. 1, liver; 2, intestine; 3, muscle; 4, brain; 5, kidney; 6, heart; 7, testicle. (B) IHC staining of zebrafish liver and kidney with polyclonal antibody for SMP30/RGN. Left: HE staining, right: anti-SMP30/RGN antibody, bar: 100 μm.