Expression patterns of the creatine metabolism-related molecules AGAT, GAMT and CT1 in adult zebrafish Danio rerio

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AGAT, GAMT and CT1, three creatine synthesis and transport-related molecules, have been widely studied in mammals. To explore their homologous genes in adult zebrafish Danio rerio, the gene expression patterns of these three genes in D. rerio were investigated. The results reveal that AGAT, GAMT and CT1 are expressed widely in diverse tissues of D. rerio where the homologous genes in mammals are also expressed.

Key words: AGAT; CT1; Danio rerio; expression patterns; GAMT; zebrafish.

The creatine–phosphocreatine–creatine kinase system is essential for the buffering and transport of high energy phosphates. Creatine is taken up in the diet or synthesized endogenously by a two-step mechanism involving L-arginine, glycine amidino-transferase (AGAT) and N-guanidinoacetate methyltransferase (GAMT). Creatine is taken up by cells with high energy demand through a specific creatine transporter, creatine transporter 1 (CT1). Any abnormality of AGAT, GAMT or CT1 can cause creatine deficiency syndrome (CDS) (Schulze, 2003; Stromberger et al., 2003). The common clinical features of three kinds of creatine deficiency syndromes are neurological symptoms and severe neurological development delay during childhood (Arias-Dimas et al., 2006; Stockler et al., 2007). GAMT and AGAT deficiency are treatable by oral creatine supplementation, while patients with CT1 deficiency do not respond to this type of treatment (Arias-Dimas et al., 2006). Although patients with AGAT and GAMT deficiencies can be treated partly by taking creatine, brain development and intellectual barriers remain.

Most research about AGAT, GAMT and CT1 has been performed in mammals such as rat (Braissant et al., 2001; da Silva et al., 2009) and mouse (Lee et al., 1994; Torremans et al., 2005; Kan et al., 2007). Zebrafish Danio rerio (Hamilton) creatine

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kinase, which catalyses the formation of phosphocreatine from creatine, has been previously studied (Dickmeis et al., 2001; Harder & McGowan, 2001). The origin of the creatine, however, remained unclear until three homologous genes of AGAT, GAMT and CT1 in *D. rerio* were identified in a previous work (Wang et al., 2007). The high similarities of amino acid sequence and developmental expression patterns of AGAT, GAMT and CT1 between *D. rerio* and mammals established the presence of endogenous creatine synthesis and transport in fish and the evolutionary conservation of creatine metabolism in vertebrates. To further explore the creatine metabolism in *D. rerio*, the expression patterns of AGAT, GAMT and CT1 in adult *D. rerio* were detected using real-time polymerase chain reaction (PCR), *in situ* hybridization and immunohistochemistry on various tissues. These results improve understanding of the nature of creatine metabolism in fish and the evolutionary relationship from fish to mammals, and may provide a new animal model for the study of human CDS.

Total RNA was isolated from dissected tissues using the RNeasy Mini kit (Qiagen; www.qiagen.com). RNA from normal adult *D. rerio* brain, eye, heart, intestine plus pancreas, liver and ovary was isolated following the manufacturer’s protocol. RNA samples were quantified using an Ultrospec 1100 Pro UV-Visible Spectrophotometer (Amersham Biosciences; www.gelifesciences.com) and diluted to the same concentration. Reverse transcription was done on 500 ng of total RNA using the Reverse Transcription System (Promega; www.promega.com) with random primers and oligo dT in the ratio of 9:1. The following primers were used for SYBR Green (QuantiTect SYBR Green PCR Kits, Qiagen) real-time quantitative PCR:

- (F) 5′-CCAGAAAGATGTTCCAGAGCC-3′ and (R) 5′-GTCCTGGGCTACCAACCTTGA-3′ for agat (NM_199531.1),
- (F) 5′-CACACACCTGGAGATCATTG-3′ and (R) 5′-CACGACCCCTTCAACCTTTAG-3′ for gamt (NM_001105595.1),
- (F) 5′-TCTGTCCTCGGATGG-3′ and (R) 5′-CAACCAGCTTCCCTTAAAGAG-3′ for ct1 (XM_690840.2),
- (F) 5′-CTGTATGCTGCTTACGGGC-3′ and (R) 5′-ATGGTGGAAGGAGCAAGAGA-3′ for β-actin1 (NM_131031.1).

Real-time PCR was carried out on an CFX96 Real-Time PCR Detection System (Bio-Rad Labs; www.biospace.com) under the conditions of 95°C for 900 s, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. All RT and PCR were performed in triplicate with and without RT as controls. Cycle threshold [C(t)] values were converted to relative gene expression levels using the 2−ΔC(t) method.

Sense and antisense RNA probes were the same probes as those in a previous study (Wang et al., 2007). Formalin-fixed, paraffin-embedded tissue sections (8 μm thickness) and *in situ* hybridizations were performed according to a previously published protocol (Wang et al., 2008). *Danio rerio* tissue sections were used for immunohistochemistry with specific multiclonal antibody to mouse GAMT (Sanying Biotechnology, Inc., Wuhan Proteintech Group, Hubei, China). Primary antibody had been previously tested in mouse tissues (not shown).

Real-time PCR was carried out to determine the mRNA expression levels of agat, gamt and ct1 in adult *D. rerio*. Fig. 1 shows that agat was abundantly expressed in brain and intestine plus pancreas, while absent in eyes, heart, liver and ovary [Fig. 1(a)]. In contrast, gamt and ct1 were ubiquitously expressed, with the highest level of gamt in heart, followed by intestine plus pancreas, liver and eye, and almost absent in brain and ovary [Fig. 1(b)], and the most abundant ct1 transcript was
Fig. 1. mRNA expression levels of (a) agat, (b) gamt and (c) crf in adult zebrafish Danio rerio detected by SYBR Green real-time PCR.
observed in brain and eye, followed by intestine plus pancreas and heart, and not in liver and ovary [Fig. 1(c)].

To further determine the gene expression patterns of \textit{agat}, \textit{gamt} and \textit{ct1} in different organs of adult \textit{D. rerio}, \textit{in situ} hybridization was performed on the sections using sense and antisense RNA probes. The results showed that \textit{agat}, \textit{gamt} and \textit{ct1} were all detected in the neurons and glia of brain [Fig. 2(a1–a3)], although \textit{gamt} was very weak in this tissue. In the retina, \textit{agat} could not be detected [Fig. 2(b1)], while the positive signals of \textit{gamt} and \textit{ct1} were obvious. The staining of \textit{gamt} was only found in the cone cells and outer limiting membrane [Fig. 2(b2)], while \textit{ct1} was expressed more ubiquitously in the cone cells, outer limiting membrane, outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer and layer of ganglion cells [Fig. 2(b3)]. In addition, \textit{gamt} was located both in the inner and outer segments of the cones, but \textit{ct1} was only observable in the inner segments. No signal of \textit{agat} [Fig. 2(c1)] and \textit{gamt} [Fig. 2(c2)] was found in the lens, while \textit{ct1} was detected richly in the lens epithelium [Fig. 2(c3)]. The transcript of \textit{agat} could not be detected in the heart or bulbus arteriosus [Fig. 2(d1)]. In contrast, \textit{gamt} [Fig. 2(d2)] showed similar expression patterns but higher levels than \textit{ct1} [Fig. 2(d3)] in the myocardium and vascular smooth muscle of the bulbus arteriosus. The positive signals of \textit{agat}, \textit{gamt} and \textit{ct1} were all detected in the small intestine and pancreas. In the small intestine, \textit{agat} was found in the lamina propria, submucosa and serosa [Fig. 2(e1)], and \textit{gamt} was mainly expressed throughout the mucosal epithelial cells, especially in the enteroendocrine cells such as goblet cells [Fig. 2(e2)]. The transcript of \textit{ct1} was detected in the lamina propria, submucosa, serosa and the goblet cells of mucosal epithelium (but not in the columnar epithelial cells) [Fig. 2(e3)]. The dominant \textit{gamt} and \textit{ct1} were expressed in almost the entire pancreas, while \textit{agat} was detected only in the fringes of the organ [Fig. 2(f1–f3)]. In the liver, \textit{agat} and \textit{ct1} were not detected but \textit{gamt} was strongly expressed, particularly in the portal area [Fig. 2(g1–g3)].

Three genes of \textit{agat}, \textit{gamt} and \textit{ct1} were all expressed in the ovaries during the maturation of oocytes. In phase I oocytes, the expression of \textit{agat} and \textit{gamt} was located widely in the cytoplasm separated by organelles and yolk [Fig. 2(h1 & h2)]. In contrast, \textit{ct1} mRNA was found mainly in the region near the nucleus [Fig. 2(h3)]. In phase II oocytes, the expression of \textit{agat} and \textit{gamt} were down-regulated, while \textit{ct1} was up-regulated with dominant signals in the cytoplasm of oocytes [Fig. 2(i1–i3)]. By the phases III [Fig. 2(j1–j3)] and IV [Fig. 2(k1–k3)], the expressions of these three genes were located mainly in the cortex layer of the mature oocytes.

The use of immunochemistry indicated that GAMT protein was detected in the similar locations where \textit{gamt} mRNA was discovered, including the neurons and glia of brain [Fig. 3(a)], cone cells of retina [Fig. 3(b)], myocardium, especially the epicardium of heart [Fig. 3(c)], the smooth muscle of the bulbus arteriosus [Fig. 3(d)], the mucosal epithelium of intestines [Fig. 3(e)], pancreatic cells [Fig. 3(f)], liver [Fig. 3(g)] including portal area [Fig. 3(h)] and the developing oocytes [Fig. 3(i)–(l)].

The expression patterns of AGAT, GAMT and CT1 mRNA in adult \textit{D. rerio} are similar to their homologues in mammals. First, \textit{D. rerio} AGAT, GAMT and CT1 are all detected in adult CNS like the mammalian homologues. The CNS is the major organ affected in patients suffering from CDS, and AGAT, GAMT and CT1 have been found in the brain and CNS of rat (AGAT, GAMT and CT1), mouse (GAMT and CT1) and human (GAMT) (Braissant \textit{et al.}, 2001, 2007; Braissant & Henry, 2008).
FIG. 2. Expression patterns of *agat*, *gamt* and *ctl* in adult *Danio rerio* detected by *in situ* hybridization. The arrow indicates the positive signals (blue staining) in the cones in b2, bulbus arteriosus in d2, goblet cell in e2 and e3, and portal area in g2. The arrowhead indicates the outer segment of cones in b3 and columnar epithelial cell in e3, which are both negatively stained. The arrowhead in f1 indicates the expression of *agat* in the fringes of the pancreas. Br, brain; Re, retina; Le, lens; He, heart; In, small intestine; Pa, pancreas; Li, liver; oI, oII, oIII and oIV, oocytes at stages I, II, III and IV; CO, cones; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, layer of ganglion cells; LEP, lens epithelium; BA, bulbus arteriosus; LP, lamina propria; SM, submucosa; SE, serosa; ME, mucosal epithelium; GOB, goblet cells; CEC, columnar epithelial cells; PO, portal area; Nu, nucleus of oocytes; YK, yolk. Scale bar as indicated.
Fig. 3. Expression patterns of GAMT protein in adult *Danio rerio* detected by immunohistochemical staining. The arrow indicates the positive signals (brown staining) in the cones (CO) in (b), bulbus arteriosus (BA) in (d) and portal area (PO) in (g). The arrowhead in (c) indicates the strong expression of GAMT in the epicardium of heart. Br, brain; Re, retina; He, heart; In, small intestine; Pa, pancreas; Li, liver; oI, oII, oIII and oIV, oocytes at stages I, II, III and IV; ME, mucosal epithelium; Nu, nucleus of oocytes; YK, yolk.

Similarly, *D. rerio* AGAT, GAMT and CT1 mRNA are also expressed in the adult brain tissues. Second, liver is an important organ for creatine metabolism, in which GAMT is strongly expressed, whereas AGAT is not found in liver of either *D. rerio* or mammals, such as rat, mouse, dog, cat and rabbit. Livers of all mammalian species tested so far contain high amounts of GAMT but display only low levels of creatine (Wyss & Schulze, 2002). Rat hepatocytes readily convert guanidinoacetate to creatine (the second step of creatine synthesis catalysed by GAMT), but are unable to produce guanidinoacetate from arginine and glycine (the first step of creatine synthesis is catalysed by AGAT from these two amino acids to guanidinoacetate) (da Silva *et al.*, 2009). The same thing also occurs in *D. rerio* liver. Third, one of the main organs of endogenous synthesis of Cr is pancreas throughout vertebrates. In adult *D. rerio* and most mammals, pancreas contains high levels of AGAT and GAMT (Wyss & Schulze, 2002). Fourth, the maternal source of creatine may be essential until the creatine synthesis, and transport system matures in both fish and mammals. In the spiny mouse, total creatine, AGAT, GAMT and CT1 mRNA significantly increased in the placenta in the second half of pregnancy (Ireland *et al.*, 2009). In *D. rerio* ovaries, these three genes are all continuously expressed during oocyte maturation. Even in the embryos, *agat* and *gamt* also have extremely strong expression in the yolk syncytial layer until the long-pec stage (48 h post-fertilization) (Wang *et al.*, 2010).
No signal of these three genes was found in ovaries before sexual maturation of D. rerio (Fig. 1).

Together with the report of D. rerio creatine kinase and a previous research about agat, gamt and ct1 in D. rerio embryos, this study in D. rerio adults depicts a more complete picture of creatine synthesis, transport and phosphorylation in fish. Because of the similarity in the expression patterns of AGAT, GAMT and CT1 between D. rerio and mammals, these results provide a new animal model for CDS studies, and further study on these genes in D. rerio will provide much new evidence for the pathogenic mechanism, diagnosis and treatment of human CDS.

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References


