

Adenosine deaminase-related genes: Molecular identification, tissue expression pattern and truncated alternative splice isoform in adult zebrafish (*Danio rerio*)

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Abstract

Adenosine deaminase (ADA) is responsible for cleaving the neuromodulator adenosine to inosine. Two members of ADA subfamilies, known as ADA1 and ADA2, were described and evidence demonstrated another similar protein group named ADAL (adenosine deaminase “like”). Although the identification of ADA members seems to be consistent, the expression profile of *ADA1*, *ADA2* and *ADAL* genes in zebrafish has not yet been reported. The aim of the present study was to map the expression pattern of ADA-related genes in various tissues of adult zebrafish (*Danio rerio*). An extensive search on zebrafish genome followed by a phylogenetic analysis confirmed the presence of distinct ADA-related genes (*ADA1*, *ADAL* and two orthologous genes of *ADA2*). Specific primers for each ADA member were designed, optimized semi-quantitative RT-PCR experiments were conducted and the relative amount of transcripts was determined. The tissue samples (brain, gills, heart, liver, skeletal muscle and kidney) were collected and the expression of *ADA1*, *ADAL* and *ADA2* genes was characterized. *ADA1* had a similar expression pattern, whereas *ADAL* was less expressed in the heart. The highest relative amount of *ADA2-1* transcripts was observed in the brain, liver and gills and it was less expressed in the heart. RT-PCR assays revealed that the other *ADA2* form (*ADA2-2*) was expressed ubiquitously and at comparable levels in zebrafish tissues. The strategy adopted also allowed the identification of an *ADA2-1* truncated alternative splice isoform (*ADA2-1/T*), which was expressed at different intensities. These findings demonstrated the existence of different ADA-related genes, their distinct expression pattern and a truncated *ADA2-1* isoform, which suggest a high degree of complexity in zebrafish adenosinergic system.

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Introduction

Adenosine is a nucleoside that exerts several actions in many tissues. Its effects are elicited by specific P1 metabotropic

receptors, named A_1 , A_{2A} , A_{2B} and A_3 (Fredholm et al., 2005). The inhibitory actions of adenosine are mediated by A_1 and A_3 , whereas facilitatory mechanisms involve the activation of A_{2A} and A_{2B} receptors (Latini and Pedata, 2001; Rebola et al., 2005). This nucleoside plays several roles in the fast purinergic signaling among tissues, which include neuromodulation, exocrine and endocrine secretion, platelet aggregation, vascular endothelial cell-mediated vasodilatation and nociceptive mechanosensory transduction (Burnstock, 2006). Extracellular

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adenosine concentrations may increase dramatically after seizures, hypoxia and ischemia, playing neuroprotective roles (Cunha, 2001; Dunwiddie and Masino, 2001). Extracellular concentrations of adenosine might be regulated by neural cell uptake through bidirectional nucleoside transporters (Pinto-Duarte et al., 2005) and ecto-nucleotidase activity (Latini and Pedata, 2001). Adenosine uptake followed by its phosphorylation to AMP by adenosine kinase (AK) or deamination to inosine by adenosine deaminase (ADA) are two possible mechanisms able to promote the inactivation of adenosine signaling (Latini and Pedata, 2001; Fredholm et al., 2005).

Adenosine deaminase (ADA, EC 3.5.4.4) is an important enzyme that promotes the irreversible hydrolytic deamination of adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine, respectively. Although inosine had been considered an inactive metabolite, recent evidence suggested that it may exert a function as a natural antioxidant and radioprotector for mice exposed to lethal doses of gamma-radiation (Gudkov et al., 2006) with antiinflammatory (Haskó et al., 2000) and neuroprotective actions against brain injury (Tsuda, 2005).

There are different ADA members in animal cells. These enzymes belong to the adenylation-deaminase family, being grouped consistently with AMP deaminase (AMPD) and adenine deaminase (ADE) as distinct subfamilies (Maier et al., 2005). In humans, it has been shown that almost all ADA activity is attributed to ADA1 (Zavialov and Engstrom, 2005). Deficiency of this protein may lead to severe combined immunodeficiency (SCID) in childhood due to the importance of ADA1 in the immune response mediated by T-lymphocytes (Pacheco et al., 2005; Ozdemir, 2006). This enzyme may be found anchored to the cell membrane in a complex with CD26 (DPPIV — dipeptidyl-peptidase IV) as an ecto-ADA, responsible to breakdown extracellular adenosine (Franco et al., 1997). Studies have demonstrated that this ADA form is also colocalized with A₁ receptors in CNS, suggesting that the modulation of adenosine signaling through A₁R is mediated in part by ecto-ADA in neuronal and non-neuronal cells (Franco et al., 1998; Ruiz et al., 2000).

The other member, ADA2, is most abundant in human plasma and has different kinetic properties when compared to ADA1 (Iwaki-Egawa et al., 2006). This ADA member had already been purified and characterized in chicken liver (Iwaki-Egawa et al., 2004). Evidence has shown that ADA2 belongs to a new family of growth factors named adenosine deaminase-related growth factors (ADGFs) with ADA activity, encoded by cat-eye syndrome critical region candidate 1 (*CECR1*) gene in vertebrates (Zavialov and Engstrom, 2005).

Study performing a phylogenetic analysis of ADA subfamilies revealed that there is another protein with high similarity to the classic members of ADA, however, it is consistently grouped in a different manner. This member was named ADAL (adenosine deaminase “like”) and its genetics, biochemical and physiological properties are still unknown. It has been speculated that it may present ADA activity, because ADAL deduced amino acid sequence has catalytically important sites (Maier et al., 2005).

Zebrafish (*Danio rerio*) is a teleost widely used as a vertebrate model in biochemical, genetic and neurochemical studies

(Grunwald and Eisen, 2002; Lieschke and Currie, 2007) and the genome of this fish shares many similarities with the human genome (Lieschke and Currie, 2007). Purinergic signaling studies have already been performed in zebrafish (Kucenas et al., 2003) and ecto-nucleotidases, enzymes able to form extracellular adenosine through ATP catabolism, were recently characterized in zebrafish brain membranes (Rico et al., 2003; Senger et al., 2004). Moreover, it has been demonstrated that these enzymes may be differently modulated by contaminants, such as pesticides and metals (Senger et al., 2005; Rosenberg et al., 2007) and it has been shown that NTPDase1 and three distinct forms of NTPDase2 are able to contribute for ATP and ADP hydrolysis in zebrafish brain (Rico et al., 2006).

The expression pattern of murine *ADA1* had already been determined in different tissues (Chinsky et al., 1989) and the amount of *ADA2* transcripts had been also investigated during *Drosophila* development stages (Maier et al., 2001) and in the cabbage armyworm, *Mamestra brassicae* (Zhang and Takeda, 2007). However, as *ADA1*, *ADA2* and *ADAL* are grouped in three ADA subfamilies, it is possible that they might have a distinct expression pattern, which could reveal a different contribution for deamination activity among tissues. Thus, the aim of the present study was to map the expression profile of *ADA1*, *ADA2* and *ADAL* genes in different tissues from adult zebrafish to show how the ADA genes could be a fine-tuning regulation for adenosine activity in the zebrafish adenosinergic system.

Materials and methods

Animals

Adult wild-type zebrafish (*D. rerio*) of both sexes (around 3 months old) were obtained from commercial supplier and acclimated for 2 weeks in a 50-L thermostated aquarium. The water was kept between 26±2 °C under a 12-h light–dark controlled photoperiod and the animals were fed with commercial fish pellet twice a day. The use and maintenance of zebrafish were according to the National Institute of Health Guide for Care and Use of Laboratory Animals.

Sequences analysis and primers design

The identification of *ADA1*, *ADA2* and *ADAL* was performed in NCBI Blast searches of GenBank, using the *Homo sapiens*, *Mus musculus*, *Gallus gallus* and *Xenopus laevis* proteins as queries. The obtained sequences (supported by mRNA or EST data) were compared with zebrafish protein database at Zebrafish Information Network (ZFIN) (University of Oregon, Eugene, OR 97403-5274; World Wide Web URL: <http://zfin.org>) and the alignment was performed using ClustalX program (Thompson et al., 1997). A phylogenetic tree was constructed according to Neighbor-Joining method (Saitou and Nei, 1987) using proportional (*p*) distance with MEGA 2.1 program (Ryu et al., 2002).

In order to compare the zebrafish deduced amino acid sequences, an alignment was performed using ClustalX. To

minimize problems due to the sequence divergence observed between ADA1/ADAL and ADA2, manual adjustments were made using the BioEdit 7.0.9 program. The sequences were analyzed using the tools available at CBS Prediction Servers (<http://www.cbs.dtu.dk/services/>).

Zebrafish DNA sequences encoding to each one of ADA members were retrieved from GenBank database and aligned using ClustalX program. Regions with low scores of similarity among the sequences were used for searching specific primers, which were designed using the program Oligos 9.6. In order to confirm the primers specificity, each primer was compared with zebrafish genome and it was able to recognize only its specific target sequence. Thus, the strategy adopted to construct the primers did not allow cross-amplification. The β -actin primers were designed as described previously (Chen et al., 2004) and the optimal PCR conditions were determined (Table 1).

Reverse transcription-polymerase chain reaction (RT-PCR)

Zebrafish brain, gills, heart, liver, skeletal muscle and kidney were dissected under sterile conditions and immediately frozen in liquid nitrogen. RNA was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. In order to achieve a similar weight (around 50 mg) from different tissues, the number of animals used for the experiments was: brain ($n=5$), gills ($n=5$), heart ($n=10$), liver ($n=5$), muscle ($n=5$) and kidney ($n=12$). In brief, the tissue samples were homogenized in 500 μ l of TRIzol. Chloroform (100 μ l) was added and the mixture was centrifuged at 10.600 $\times g$ for 10 min at 5 $^{\circ}C$. The upper aqueous phase was collected and the RNA was precipitated by addition of isopropyl alcohol (250 μ l), followed by a centrifugation at 10.600 $\times g$ for 10 min at 5 $^{\circ}C$. The pellets were further washed with 500 μ l of 75% cold ethanol and centrifuged at 6.800 $\times g$ for 5 min at 5 $^{\circ}C$. The samples were dried out and resuspended in 15 μ l RNase-free water. Before storing at $-70^{\circ}C$, 0.4 μ l of RNaseOUT Ribonuclease Inhibitor (Recombinant) (Invitrogen, USA) was added. RNA purity was quantified spectrophotometrically calculating the ratio between absorbance values at 260 and 280 nm and 2 μ l were tested by electrophoresis in a 1.0% agarose gel containing ethidium bromide. All samples were adjusted to 160 ng/ μ l and cDNA species were synthesized with SuperScriptTM III First-Strand Synthesis SuperMix (Invitrogen,

USA). Each RNA sample was mixed with 1 μ l of 50 μ M Oligo (dt) and 1 μ l Annealing Buffer (final volume of 8 μ l), being incubated in a thermal cycler at 65 $^{\circ}C$ for 5 min. Immediately, the samples were placed on ice for 1 min and 10 μ l 2X First-Strand Reaction Mix and 2 μ l SuperScriptTM III/RNaseOUTTM Enzyme Mix were added. The products were mixed, incubated by 50 min at 50 $^{\circ}C$ and the reaction was finished at 85 $^{\circ}C$ for 5 min.

RT-PCR conditions were optimized in order to determine the number of cycles that would allow product detection within the linear phase of mRNA transcripts amplification. The reaction for β -actin gene was performed using in a total volume of 20 μ l, 0.1 μ M primers (Table 1), 0.2 μ M dNTP, 2 mM MgCl₂ and 0.5 U Taq DNA polymerase (Invitrogen, USA). ADA1 and ADAL PCR assays were performed in a total volume of 25 μ l, 0.08 μ M primer (Table 1), 0.2 μ M dNTP, 3 mM MgCl₂ and 1 U Taq DNA polymerase. ADA2-1 PCR reactions were performed at these same conditions, except that 2 mM MgCl₂ was employed. The amplification of ADA2-2 was carried out at the same condition of ADA1 and ADAL. The ADA2-2 PCR assay was optimized using 0.5 M betain and 1 U Taq Platinum DNA polymerase (Invitrogen, USA). ADA1 and ADA2-2 PCR assays were carried out using 2 μ l cDNA as template, whereas ADA2-1, ADAL and β -actin were conducted using 1 μ l cDNA. The following conditions were used for PCR reactions: 1 min at 94 $^{\circ}C$, 1 min for annealing temperature (see Table 1), 1 min at 72 $^{\circ}C$ for 28 cycles (ADA1) and 35 cycles (ADAL, ADA2-1, ADA2-2 and β -actin). A postextension cycle at 72 $^{\circ}C$ was performed for 10 min. For each set of PCR reactions, a negative control was included. PCR products were resolved by a 1.0% agarose gel containing ethidium bromide and visualized with ultraviolet light. The fragments length of PCR reactions was confirmed with Low DNA Mass Ladder (Invitrogen, USA) and β -actin was carried out as an internal standard. The relative abundance of each mRNA versus β -actin was determined in the organs studied by densitometry using the freeware ImageJ 1.37 for Windows.

Each experiment was repeated four times using RNA isolated from independent extractions. The expression analysis in each tissue was performed in replicate and representative findings were shown. The normalized expression levels of ADA-related genes at different tissues were expressed as mean \pm S.E. and statistically compared by one-way analysis of variance

Table 1
Primer sequences and PCR amplification conditions

ADA member	GenBank accession number	ZFIN ID	Primers (5'–3')	T _m ($^{\circ}C$)	Cycles
ADA1	AAH76532	ZDB-GENE-040718-393	F — CAGGTCCATTCTGTGCTGCATGCGTC R — AAGTGTGTGGTATCCGTGCCCAATGC	58	28
ADA2-1 ^(a)	AAL40922	ZDB-GENE-030902-4	F — AAGACAAGGGTTTAAACCTGCCCTAC R — CTCCTTTCTTTGACTTGGCAATGTGC	63	35
ADA2-2	XP_687719	ZDB-GENE-041210-77	F — CTGAAGATGAAGGAAATCACCCCTTTCACC R — TGTCTTCATAAAGCTCTTTCAAACCCCTGG	54	35
ADAL	NP_001028916	ZDB-GENE-050913-145	F — TCATTCAAGAGTTTTCGCGCAGATGG R — TTGGCTTTCTGAAGTGCAGCGAGC	61	35
β -actin	AAC13314	ZDB-GENE-000329-1	F — GTCCCTGTACGCCTCTGGTCCG R — GCCGGACTCATCGTACTCTCTG	54	35

^a The same primers amplified a truncated ADA2-1 splice isoform (ADA2-1/T).

(ANOVA), followed by Tukey HSD test as post-hoc. P values ≤ 0.05 were considered significant.

Results

Identification of zebrafish ADA orthologous genes

Proteins of *H. sapiens*, *M. musculus*, *G. gallus* and *X. laevis* were retrieved from GenBank and used as queries for identification of zebrafish ADA-related orthologous genes. These organisms presented similar sequences to each one of ADA members except *M. musculus*, which had no similar protein to ADA2. The searches resulted in ADA1 (AAH76532), ADA2-1 (AAL40922), ADA2-2 (XP_687719) and ADAL (NP_001028916) similar deduced amino acid sequences in zebrafish. In order to confirm the sequences identity and to achieve the information about the current data annotation, these sequences were compared with zebrafish protein database at ZFIN (Table 1).

The phylogenetic tree was constructed using Neighbor-Joining method and proportional (p) distance (Fig. 1). Three well-resolved terminal clades supported by high bootstrap values were identified. The first clade grouped the AAH76532 zebrafish sequence with all other ADA1 sequences, whereas NP_001028916 was included in ADAL clade. The AAL40922 and XP_687719 sequences were consistently grouped in the ADA2 clade. The tree topology strongly suggests homologous functions on zebrafish genome.

The protein characteristics of zebrafish ADA1, ADAL, ADA2-1, and ADA2-2 were investigated (Fig. 2). The deduced amino acid sequences alignment showed the eight conserved domains among the three ADA proteins subfamilies and the ADA2-1 and ADA2-2 signal peptides, as described previously (Maier et al., 2005). Furthermore, it was possible to verify that

ADA1 and ADAL sequences presented only one potential *N*-glycosylation site, whereas ADA2-1 and ADA2-2 presented five and four sites, respectively. High score putative phosphorylation residues were also investigated using NetPhosk, a kinase-specific prediction of protein phosphorylation sites tool (Blom et al., 2004). The results identified two potential phosphorylation sites for ADA1: Ser40 residue for Protein Kinase A (PKA) and the Thr285 for Protein Kinase C (PKC). The ADAL sequence analysis revealed a putative phosphorylation site at Thr153 residue for PKC. ADA2-1 had the Thr132, Thr284, Ser479 and Ser480 residues as potential PKC targets, whereas ADA2-2 sequence presented Ser60 and Ser165 residues for PKA and Ser291 for PKC. Considering the conserved cysteine residues, it was possible to identify four residues for ADA2-1 and ADA2-2 and only one conserved cysteine residue for ADA1 and ADAL amino acid sequences. Neither hydrophobic transmembrane domains nor potential N-terminal acetylation sites (Kiemer et al., 2005) were identified for zebrafish ADA1, ADAL, ADA2-1 and ADA2-2 proteins.

Gene expression pattern of ADA members in zebrafish

The gene expression pattern of ADA-related genes was investigated in six different tissues of zebrafish. After dissection of brain, gills, heart, liver, skeletal muscle and kidney, the total RNA was isolated. Semi-quantitative RT-PCR experiments were performed and the relative abundance of *ADA1*, *ADAL*, *ADA2-1* and *ADA2-2* mRNA versus β -actin was determined (Table 2 and Fig. 3). The relative amount of *ADA1* transcripts was similar in all tissues studied (Fig. 3A). *ADAL* transcripts were more abundant in the liver and kidney, whereas a lower relative expression of this gene was detected in the heart (Fig. 3B). A product of 440 bp was obtained by RT-PCR, which corresponded to *ADA2-1*, and a 554 bp fragment, with distinct band

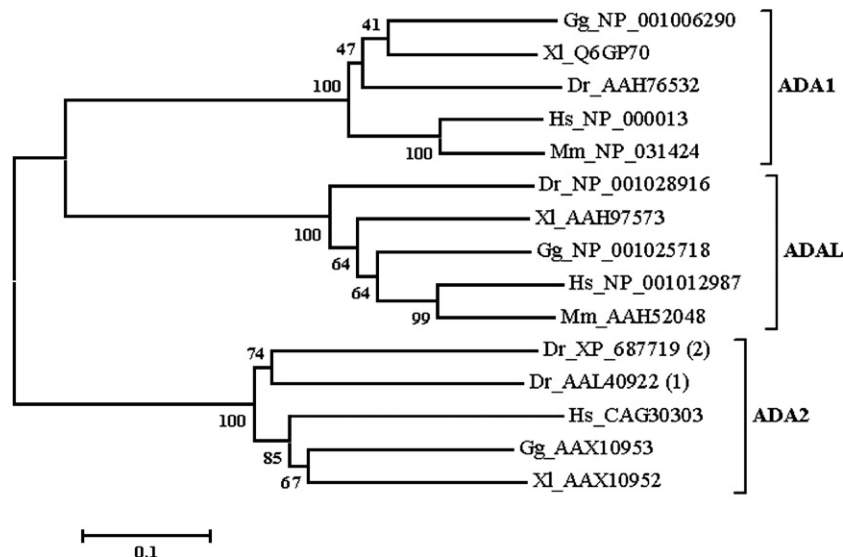


Fig. 1. Phylogenetic analysis of ADA-related family members. The deduced amino acid sequences were aligned with ClustalX program and the phylogenetic tree was constructed using Neighbor-Joining method, proportional (p) distance with MEGA 2.1 program. The phylogenetic tree grouped consistently (Dr) *Danio rerio*, (Xl) *Xenopus laevis*, (Gg) *Gallus gallus*, (Mm) *Mus musculus* and (Hs) *Homo sapiens* ADA1, ADAL and ADA2 orthologous sequences.

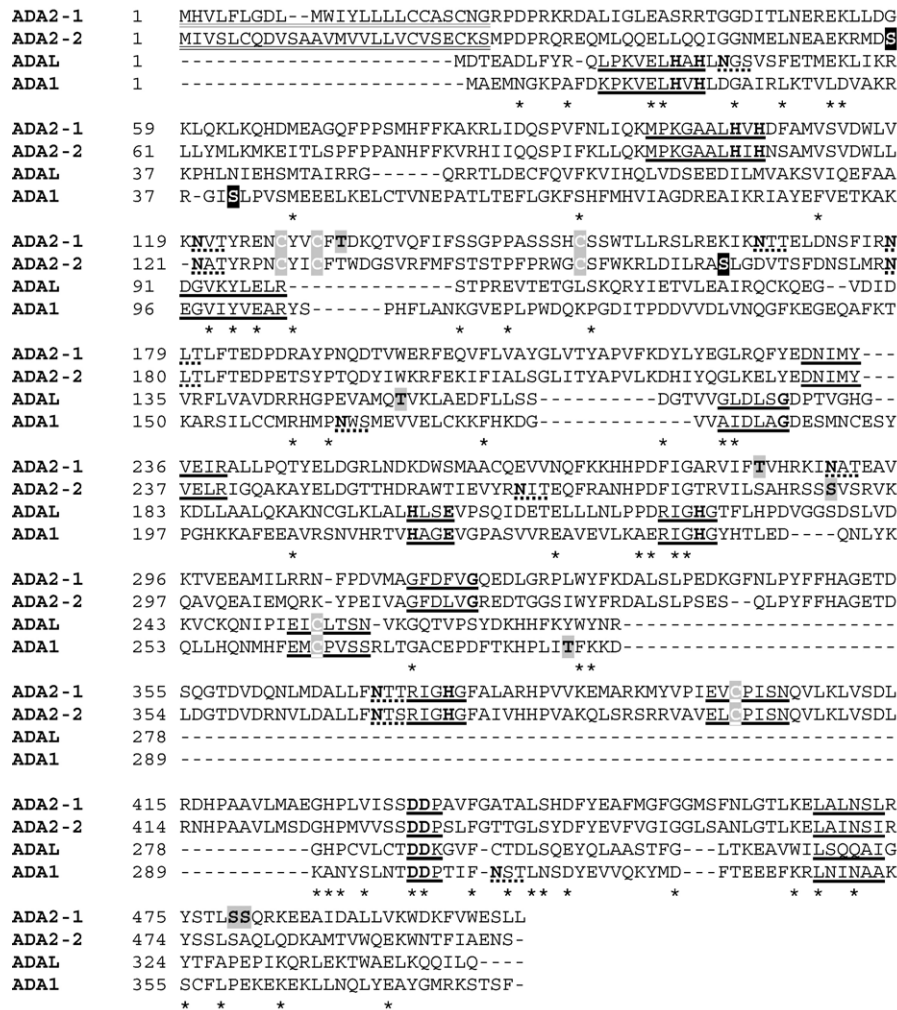


Fig. 2. Deduced amino acid sequences alignment of zebrafish ADA members. The eight conserved domains among the ADA subfamilies are underlined and the eight amino acids important for ADA activity in these domains are bold face. The signal peptide from the two ADA2 orthologous are double underlined, potential N-glycosylation sites are shown by dotted lines and the conserved cysteine residues from ADA1/ADAL and ADA2-1/ADA2-2 are indicated by white letters in gray boxes. Putative PKA phosphorylation sites are marked in black boxes, whereas PKC residues targets are demonstrated in gray boxes. The similarities among at least three different sequences are indicated by the asterisks.

intensities, was also amplified with the *ADA2-1* (Fig. 3C) even when PCR was carried out using high stringency conditions. The brain, gills and liver were the tissues that presented a higher expression of *ADA2-1*. A similar level of expression was evidenced in the muscle and kidney and this gene was less expressed in the heart. Although *ADA-2-2* gene expression could be detected only when the enhancer betain and Taq

Platinum were added to PCR mixture, this gene had a similar expression pattern in all tissues studied (Fig. 3D).

ADA2-1 alternative splicing in zebrafish

In order to understand the significance of the 554 bp fragment, the nucleotide sequence of *ADA2-1* (AF384217) was

Table 2
Relative mRNA expression of ADA-related genes in zebrafish tissues

Genes	Optical densitometry (O.D.): ADA-related genes versus β -actin (mean±S.E.)					
	Brain	Gills	Heart	Liver	Muscle	Kidney
<i>ADA1</i>	0.70±0.08	0.65±0.02	0.69±0.02	0.67±0.01	0.71±0.04	0.60±0.10
<i>ADAL</i>	0.72±0.03	0.70±0.05	0.63±0.03 ^d	0.91±0.12 ^c	0.76±0.02	0.90±0.05
<i>ADA2-1/T</i>	1.02±0.06 ^{c,d,e,f}	0.92±0.04 ^{c,f}	0.52±0.06 ^{a,b}	0.63±0.07 ^a	0.68±0.08 ^a	0.51±0.09 ^{a,b}
<i>ADA2-1</i>	1.00±0.03 ^{c,c}	0.93±0.02 ^c	0.51±0.07 ^{a,b,d}	1.00±0.05 ^{c,e}	0.71±0.10 ^{a,d}	0.79±0.07
<i>ADA2-2</i>	0.85±0.08	0.93±0.04	0.89±0.003	0.86±0.06	0.83±0.03	0.87±0.07

The results were analyzed by ANOVA followed by Tukey HSD test as post-hoc, considering $P \leq 0.05$ as significant. The relative amount of mRNA levels was significantly different from ^abrain, ^bgills, ^cheart, ^dliver, ^emuscle and ^fkidney.

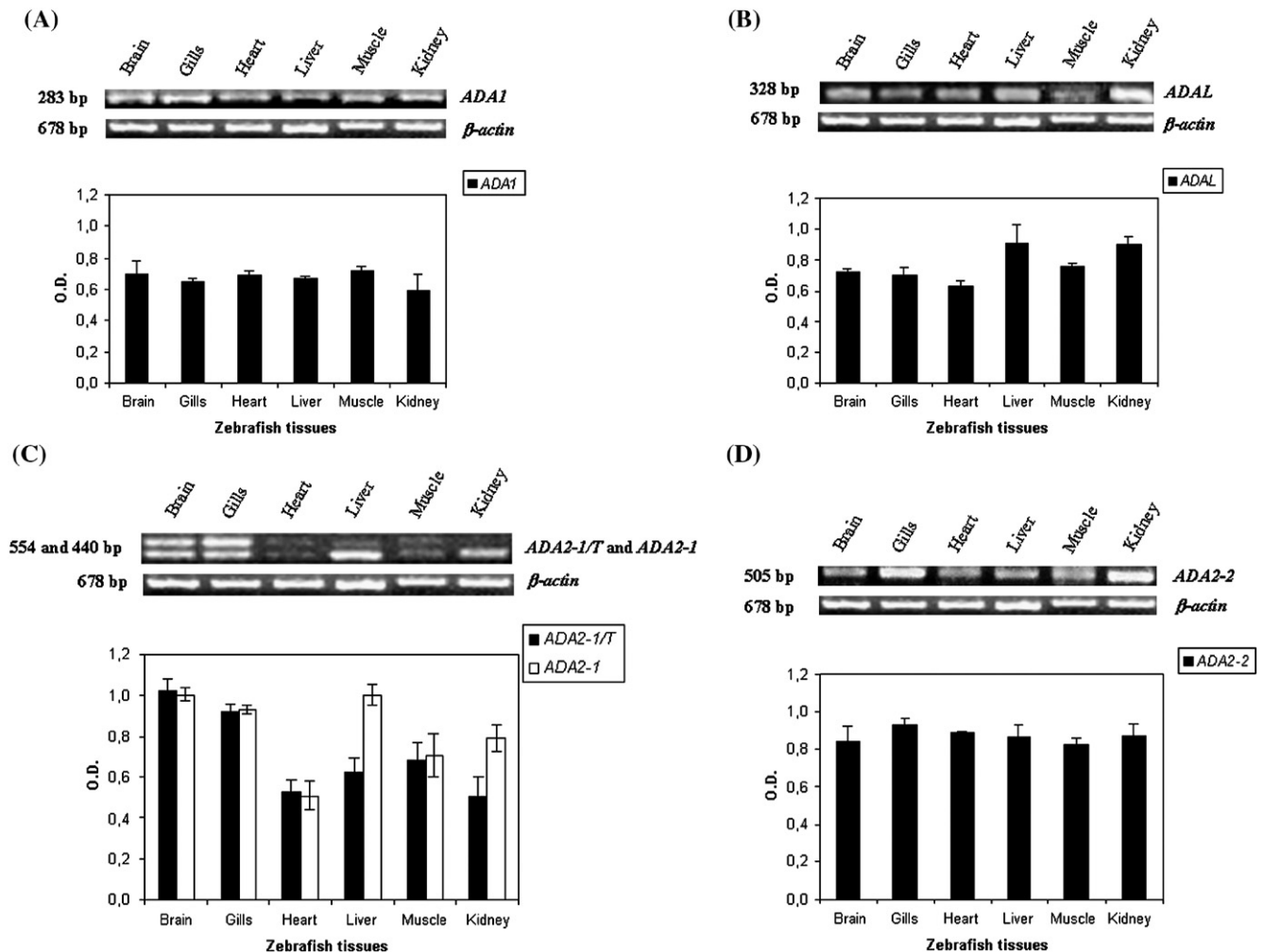


Fig. 3. Expression pattern of *ADA1* (A), *ADAL* (B), *ADA2-1* and *ADA2-1/T* (C) and *ADA2-2* (D) in zebrafish brain, gills, heart, liver, skeletal muscle and kidney. The amplifications resulted in a single product, except *ADA2-1*. The results were expressed as optical density (O.D.) of the ADA-related genes versus β -actin expression (mean \pm S.E.) of four independent replicate RT-PCR experiments.

compared with zebrafish genome and a high score with a genomic clone (BX004976) was observed. The comparison of interprimers region between the EST and genomic deduced amino acid sequences allowed the identification of four exons and three introns. The fragment length of the four exons was 440 bp, which was expected on *ADA2-1* gel analysis. However, the first intron of the interprimers sequence had a length of 114 bp indicating that the other fragment of 554 bp corresponded to a zebrafish *ADA2-1* alternative splicing (Fig. 4A). PCR products were sequenced and the results corroborated with the alternative splicing event. In addition, this isoform of *ADA2-1* in zebrafish is a transcript that is not completely translated, providing a truncated alternative splice isoform (*ADA2-1/T*) due to the appearance of a stop codon localized in the intron sequence (Fig. 4B). This transcript has only four of the eight ADA subfamily conserved domains and it is expressed differently than *ADA2-1* completely processed in the liver and kidney (Fig. 3C). The relative amount of mRNA levels of this gene was higher in the brain and gills. An intermediary expression pattern has been observed in the liver and muscle and a lower expression was identified in the heart and kidney.

Discussion

Adenosine deamination is an important mechanism able to control adenosine signaling in different tissues. In this study, a phylogenetic analysis confirmed the presence of distinct ADA-related genes in zebrafish. The deduced amino acid sequences alignment demonstrated the eight conserved domains among the different ADA enzymes with the catalytically important residues for ADA activity, which have already been demonstrated for these ADA members in several organisms (Maier et al., 2005). Furthermore, both zebrafish ADA2 orthologous sequences shared a signal peptide, demonstrating their potential role to cleave extracellular adenosine in zebrafish tissues. The alignment also demonstrated three conserved putative *N*-glycosylation sites for ADA2-1 and ADA2-2 and two ADA2-1 high score phosphorylation sites for Ser479 and Ser480 residues, suggesting that these regions might be important for posttranslational modifications.

The results demonstrated that *ADA1*, *ADA2* and *ADAL* are expressed in all tissues studied. Semi-quantitative RT-PCR assays have shown that the relative gene expression of *ADA1*

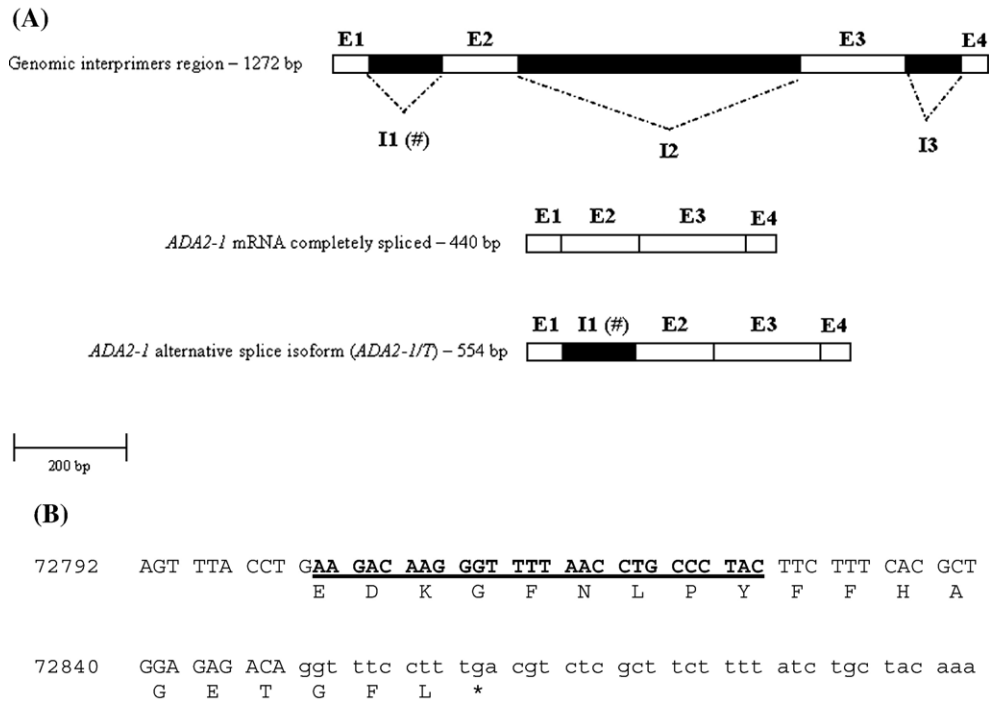


Fig. 4. Alternative splice isoform of *ADA2-1* in zebrafish and the terminal region of *ADA2-1/T*. (A) The genomic BX004976 interprimers region with E (exons) and I (introns), the *ADA2-1* mRNA completely processed and the alternative splice isoform (*ADA2-1/T*) in zebrafish are showed. The intron not spliced in *ADA2-1/T* is represented by the symbol (#). (B) Nucleotide and deduced amino acid sequences from *ADA2-1/T*. The primer forward sequence is bold face and underlined, the nucleotides from intron sequence are represented in lower case and the stop codon is marked by an asterisk.

was similar in all tissues analyzed, whereas *ADAL* mRNA levels were more abundant in the liver and kidney and less expressed in the heart. The two *ADA2* orthologous presented some interesting differences. While *ADA2-1* was easily detected in our experiments, being higher expressed in the brain, gills and liver, the *ADA2-2* products were obtained only when the RT-PCR mixtures were carried out with the enhancer betain. Therefore, even with a similar relative expression pattern of *ADA2-2*, it is plausible to hypothesize that this *ADA2* form might be less expressed when compared to its paralogous *ADA2-1*. In relation to *ADA1*, the expression pattern in murine had already been determined in different tissues and elevated levels of the specific activity of this enzyme appeared to correlate directly with steady state levels of *ADA* mRNA (Chinsky et al., 1989). Thus, the presence of distinct *ADA* members and the differences in the relative amount of mRNA observed may suggest a distinct functional role for *ADA1*, *ADA2* and *ADAL* among zebrafish tissues.

The nucleoside adenosine is a molecule that plays several roles in different tissues. In CNS, adenosine acts as a neuromodulator, controlling the excitatory and inhibitory synapses (Fredholm et al., 2005). Evidence showed that adenosine contributes to insulin-stimulated muscle glucose transport by activating the A_1 receptor (Thong et al., 2007) and plays a role in muscle vasodilatation acting on extraluminal A_{2A} receptors (Marshall, 2007). Moreover, it mediates vasoconstriction of afferent arterioles through A_1 activation, increasing intracellular calcium concentration in mouse kidney (Hansen et al., 2007). It has been suggested that adenosine increases in

the arterio-venous circulation in the gill during hypoxia, leading to an increase of blood supply to heart and gill tissue in *Hemiscyllium ocellatum* shark (Stenslokken et al., 2004). In the liver, it was demonstrated that this nucleoside reversibly inhibits Ca^{2+} fluxes and chemotaxis of hepatic stellate cells and upregulates TGF- β and collagen I mRNA, influencing the cell migration and differentiation at sites of injury (Hashmi et al., 2007). Considering the wide spectrum of actions induced by adenosine, it is possible to suggest that the transcriptional differences observed among *ADA* subfamilies in zebrafish could be important to regulate the adenosinergic signaling in each one of these tissues.

Until the present moment, there is no data about the cellular localization of *ADAL* and whether it participates in the adenosine catabolism. The lack of evidence about *ADAL* functionality in mammals does not signify that it is not physiologically important. In this sense, the existence of *ADAL* leads to the hypothesis that *ADA* function might be compartmentalized, spatially and temporally for various tissues (Maier et al., 2005). Although the adenosine deaminase activity of *ADAL* still remains unclear, we have demonstrated that *ADAL* had distinct levels of mRNA transcripts in zebrafish and thus, it is possible to suggest that this *ADA* member might be expressed and consequently play some physiological function in other organisms.

ADA2 has been identified as a novel family of growth factors (ADGFs) in various organisms including the mollusk *Aplysia californica* (Akmal et al., 2004) and *Drosophila melanogaster* (Maier et al., 2001). The extracellular adenosine deamination

promoted by secreted ADA2 is required for their mitogenic function as growth factor (Zurovec et al., 2002). Previous study demonstrated the presence of six different ADGF genes in *Drosophila*, which were expressed differently during the developmental stages (Maier et al., 2001). Recent evidence showed the molecular characterization of *M. brassicae* ADGF and its role to stimulate cell proliferation by regulating the level of inosine (Zhang and Takeda, 2007). Furthermore, transgenic mice over-expressing *CECR1* adenosine deaminase in the heart presented high rate of phenotypic defects associated to embryonic and neonatal lethality (Riazi et al., 2005). The identification of two different ADA2 sequences (paralogous) was reported in zebrafish (Maier et al., 2005), but no studies were conducted so far. Our results have shown a lower mRNA transcript level of *ADA2-1* in the heart when compared to other tissues studied, which could be important for the cardiovascular homeostasis and wild-type phenotype. Although there is no evidence about ADA2 growth factor activity in zebrafish, the differences observed in *ADA2* expression could be important for the cell proliferation mediated by adenosine depletion among tissues.

In the present study, we obtained two different *ADA2-1* transcripts in zebrafish: an expected fragment of 440 bp, which corresponded to the mRNA completely spliced, and an alternative splice isoform with 554 bp. This fragment had the inclusion of the first intron of the genomic interprimers sequence and, interestingly, after deduced amino acids sequence analysis, it was observed that the *ADA2-1* isoform in zebrafish encodes a truncated protein (*ADA2-1/T*). The function of *ADA2-1/T* in zebrafish is uncertain. Since the deduced amino acids sequence of *ADA2-1/T* did not present four ADA conserved domains, which include the His238, Asp295 and Asp296 residues important for ADA activity, the adenosine deamination promoted by this enzyme could be altered. Considering that an alternative splicing was detected for *ADA2-1* gene, it is not possible to exclude the existence of other *ADA1*, *ADAL* and *ADA2-2* isoforms in zebrafish.

The process named alternative splicing is a tightly regulated post-transcriptional event, responsible to create a diverse array of mRNA from a single pre-mRNA (Lopez, 1998). The intron excision or retention is often differently regulated and may generate several mRNA variants, which encode distinct proteins (Wang et al., 2006). It was demonstrated that the “adenosine deaminase which acts on RNA” (ADAR) transcripts have distinct alternative splicing patterns in mammals and zebrafish (Slavov and Gardiner, 2002). Splicing variants may also result in truncated isoforms. For example, studies have shown that fibronectin (Liu et al., 2003) serine racemase (Konno, 2003), transposase (Gueguen et al., 2006) and TRKB receptors (Haapasalo et al., 2002) may be expressed as truncated isoforms, playing several biological functions. As truncated proteins seem to exert a complex regulatory effect, it is not possible, *a priori*, to determine whether *ADA2-1/T* is at least functionally active. However, the differential expression pattern of *ADA2-1/T* in zebrafish tissues leads to hypothesize that it may have some physiological importance in this vertebrate.

This is the first study to report a differential mRNA expression pattern of ADA-related genes in zebrafish. Like

humans, zebrafish has three distinct members of ADA (*ADA1*, *ADA2* and *ADAL*), with two different *ADA2* forms (*ADA2-1* and *ADA2-2*). Furthermore, the alternative splicing of *ADA2-1* translates to a truncated isoform suggesting that the adenosine deamination depends on the complexity of ADA-related enzyme activities associated with gene expression. Therefore, the differential expression pattern of ADA-related genes family suggests a fine-tuning regulation for adenosine deaminase activity in zebrafish adenosinergic system.

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