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Comparative Biochemistry and Physiology, Part C 147 (2008) 299–305
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Modulatory effect of environmental endocrine disruptors on N-*ras* oncogene expression in the hermaphroditic fish, *Kryptolebias marmoratus*

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Received 28 September 2007; received in revised form 24 November 2007; accepted 26 November 2007
Available online 4 December 2007

Abstract

Kryptolebias marmoratus is the only known internally self-fertilizing vertebrate. It shows high susceptibility to many chemical carcinogens and has been proposed as a potential cancer model species alternative to mammals. Since use of this fish species is expected to rise in cancer research, regulation of oncogenes from *K. marmoratus* needs proper understanding. We cloned and deduced full-length sequence of cDNA of N-*ras* oncogene from *K. marmoratus*. Study of expression profile of N-*ras* by using quantitative real-time RT-PCR revealed that brain had the highest level of expression compared to other tissues. Some embryonic stages showed more N-*ras* expression than juveniles and adults. Exposure to two environmental endocrine disrupting chemicals (EDCs), bisphenol A (BPA) and 4-nonylphenyl (NP) caused up-regulation of N-*ras* in gonad, intestine and liver of hermaphrodite *K. marmoratus*. It is suggested that *K. marmoratus* may be a suitable model species for oncogene expression studies. The observed EDC-induced expression of N-*ras* supports the assumption that EDC exposure may predispose the host to the risk of environmental carcinogenesis.

Keywords: Fish; *Kryptolebias marmoratus*; Oncogenes; N-*ras*; Endocrine disrupting chemicals; Environmental carcinogenesis

1. Introduction

Active *ras* oncogenes are frequently detected in human and animal tumors with single point mutations occurring within exons 1 and 2; codons 12, 13 and 61. *Ras* mutations have been observed in fish and mammalian species following exposure to chemical carcinogens (Hendricks et al., 1994; Sills et al., 1999; Rotchell et al., 2001). In fish, *ras* mutations at codon 12 have been observed in diethylnitrosamine (DEN)-induced rainbow trout (*Oncorhynchus mykiss*) hepatic tumors (Hendricks et al., 1994). *Ras* mutations have also been observed in feral fish populations exposed to environmental pollutants. Winter flounder (*Pseudopleuronectes americanus*) and tomcod (*Microgadus tomcod*) exposed to polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) contained single point mutation at codon 12 in liver tumors (Wirgin et al., 1989; McMahon et al., 1990). Two cases of codon 11 mutations were additionally reported in PAH- and PCB-exposed dragonets (*Callionymus lyra*) (Vincent et al., 1998). Recently, *ras* mutation analysis has been performed in pink salmon (*Oncorhynchus gorbuscha*) embryos (Cronin et al., 2002) to confirm the earlier observation of Roy et al. (1999) who had reported Ki-*ras* mutations in pink salmon embryos exposed to weathered Prudhoe Bay oil samples. However, study of Cronin et al. (2002) in field samples revealed that heritable mutations in Ki-*ras* are not induced by *Exxon Valdez* oil spill or oil seeps. This suggests that effect of chemicals on *ras* genes in fish still remains uncertain. Like mammals, the three dominant forms of *ras* in fish include Ki-, Ha-, and N-*ras*, as well as the related family genes (Rotchell et al.,

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doi:10.1016/j.cbpc.2007.11.006

2001). N-*ras* gene which shows mutations in several forms of cancer has been studied in a few fish species (Rotchell et al., 2001). Chemically-induced N-*ras* activation has been reported in mammals (Mangues et al., 1994; Ember et al., 1998; Sills et al., 1999).

Fish have been recognized as potential models for cancer induction and prevention studies. Some fish species such as medaka (*Oryzias latipes*), rainbow trout and zebrafish (*Danio rerio*), have been extensively used in cancer research (William et al., 2003; Grabher and Look, 2006; Beckman, 2007). Using liver cancer oncogenomics, recently Lam and Gong (2006) observed striking molecular similarities between zebrafish and human liver neoplasia. Some of the molecular similarities extended to the progression of liver tumors. In fish, several researchers noted the role of the hot-spot codons (12, 13, and 61) of the Ki-*ras* gene (Chang et al., 1991; Vincent et al., 1998; Franklin et al., 2000; Liu et al., 2003). However, it is still uncertain as to whether the role of *ras*-gene mutation in fish tumor would be the same as in the human cancer.

Recently, endocrine disrupting chemicals (EDCs) have emerged as environmental contaminants of great concern in human and environmental health (Jenssen, 2006; Yang et al., 2006). Role of EDCs in human cancer, especially as developmental exposure has been highlighted in some recent studies (Birnbaum and Fenton, 2003). It has also been observed that pre-exposure to EDCs modulates carcinogenic responses of chemicals (Fukamachi et al., 2004). However, contradictory observation is also reported (Tanaka et al., 2004). Transgenic animals carrying human c-Ha-*ras* oncogenes have shown increased susceptibility to carcinogen challenge (Tsuda et al., 2001). These findings highlight importance of EDC-induced modulation of *ras* oncogene expression in environmental chemical carcinogenesis.

Kryptolebias marmoratus is the only known internally self-fertilizing vertebrate. It has shown high incidence of tumors when exposed to chemical carcinogens and induction of tumor is faster than zebrafish or medaka (Lee et al., in press). In *K. marmoratus* some oncogenes and tumor suppressor gene, *p53* are reported to be modulated by exposure to EDCs (Lee et al., 2006, 2008). A comparison of expressed sequence tags (ESTs) of *K. marmoratus* with EST database of medaka shows that several potential oncogenic pathway genes match between the two species (Lee et al., 2007). These observations establish importance of *K. marmoratus* in cancer research and its usefulness in impact assessment of environmental EDC exposure.

Here we report on molecular cloning and sequence analysis of N-*ras* oncogene in *K. marmoratus*, its tissue distribution in adult fish and at various stages of development and modulation of expression by environmental endocrine disruptors.

2. Materials and methods

2.1. Fish

K. marmoratus were reared and maintained under the following conditions: water temperature of 25±1 °C, photoperiod of 12 h/12 h light/darkness and 10‰ salinity. They were fed on a diet of *Artemia* nauplii (<24 h after hatching) once a day. In *K. marmoratus* female do not exist. Adult are either hermaphrodites or secondary males. The secondary males develop for the hermaphrodites by the atresia of ovarian tissues.

2.2. cDNA cloning of K. marmoratus N-ras (Km-N-ras)

Total RNA was isolated from *K. marmoratus* embryonic stage 4 (12 day post-fertilization, dpf) by homogenization with TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). The first strand cDNA was synthesized by SuperScript™ III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' protocol described earlier (Lee et al., 2006). To obtain N-*ras* partial sequence, degenerative primers were designed based on highly conserved regions after multiple alignments of full-length N-*ras* cDNAs of other species from the GenBank using ClustalX. The primer information is given in Table 1. PCR was carried out with three different cycles (1 cycle at 98 °C for 4 min; 40 cycles of 98 °C for 25 s, 50 °C for 40 s, and 72 °C for 90 s; 1 cycle at 72 °C for 10 min) using iCycler (Bio-Rad, Hercules, CA, USA). The RT-PCR product was eluted from agarose gel, and then ligated to pCR2.1® vectors (Invitrogen), and sequencing was performed with commercial primers T7 and M13R in both directions. To get full-length *K. marmoratus* N-*ras* cDNA, we used the GeneRacer kit (Invitrogen) according to the manufacturers' instructions and method published elsewhere (Lee et al., 2006). The primers used for this purpose are described in Table 1.

2.3. Genomic DNA amplification

In order to determine the genomic DNA structure of *Km*-N-*ras*, we extracted genomic DNA from whole body of juvenile fish and proceeded for cloning and sequence analysis as reported previously (Lee et al., 2006). The primers used for genomic DNA amplification are shown in Table 1.

2.4. Phylogenetic relationship analysis

For study of phylogenetic relationship of the members of *ras*-gene family, complete *Km*-N-ras amino acid sequence determined in this study and those obtained from the DDBJ/EMBL/GenBank were aligned using the Clustal X 1.83 with pairwise parameter settings as 10 of gap opening, 0.1 of gap extension, and PAM 350 of protein weight matrix. The alignment results were adjusted manually for obvious alignment errors. Positions that could be unambiguously aligned were used in the analysis. This resulted in 174 sites out of the 251 alignment positions for the subsequent analysis. The Neighbor-Joining (NJ) analysis was conducted with the alignment dataset and using the Dayhoff PAM model of amino acid substitution (Dayhoff et al., 1978). An unrooted tree was predicted using MEGA 3.1 (Kumar et al., 2004).

2.5. Tissue distribution of N-ras

Km-N-*ras* mRNA expression was studied in seven tissues (brain, eye, gonad, intestine, liver, muscle and skin) of adult hermaphroditic fish. Total RNA was isolated using the standard

Table 1
Detail of primers used in this study during various PCR amplification steps

Gene	Oligo name	PCR step	Sequence (5′→3′)	Primer position
Km-N-ras	F	cDNA amplification	CAA CAA CAG CAA ATC CTT CGC C	Degenerate
	R	cDNA amplification	TCT GGT TTT GGC AGA GGT TTC T	Degenerate
	5GSP1	5′-RACE	TTG CTC TTC TTG CTC TT	498–519
	5GSP2	5′-RACE	GTT GCT GCG GTT GGT CTC TTT G	523–539
	5GSP3	5′-RACE	TTT GGC AGA AGT CTC GAC GAA	421–441
	3GSP1	3′-RACE	AAG TCG TTT GAG GAC GTG CAC C	262–283
	3GSP2	3′-RACE	GCT ACG GCG TAC CGT TCG TA	407–426
	RT-F	Real-time RT-PCR	CGA GGA AGA AAT CTC AGG CTA A	162–183
	RT-R	Real-time RT-PCR	CCA GCT TGT ACT CGG TCA TAC	243–266
	Genomic-F	Genomic DNA amplification	GGC TGG AGG TGT GGG GAA GAG	30–50
	Genomic-R	Genomic DNA amplification	CTA TAG TAT GGT GCA GCG TCT C	543–564
Km-β-actin	RT-F	Real-time RT-PCR	CTT GCG GAA TCC ACG AGA CC	812–831
	RT-R	Real-time RT-PCR	CCA GGG CTG TGA TCT CCT TCT G	940–960

F = forward, R = reverse, RT = real-time primers, GSP = gene specific primer.

procedure and the relative expression level of *Km*-N-*ras* was compared using real-time RT-PCR with SYBR® Green (Molecular Probe, Invitrogen) using the primers as described in Table 1. Amplification and detection of SYBR® Green were performed with a real-time MyiQ cycler (Bio-Rad). The *K. marmoratus β-actin* gene was used as a housekeeping reference gene to normalize expression levels between samples. All the data (in triplicate) were expressed relative to *β-actin* to normalize for any difference in reverse transcriptase efficiency. Fold change for the relative gene expression to control was determined by the $2^{-\triangle\triangle Ct}$ method of Giulietti et al. (2001).

2.6. N-ras expression at different developmental stages

Km-N-*ras* mRNA expression was studied at different stages of development of *K. marmoratus* which included embryonic stages 1–5, juvenile stage 1 (1.5 cm), juvenile stage 2 (2.0 cm), adult hermaphrodite and adult secondary male. The expression was studied using the real-time RT-PCR method described above.

2.7. EDC exposure

Effect of EDC exposure on *Km*-N-*ras* expression was studied in adult hermaphroditic fish. Fish ($n=10$ each group) were exposed to bisphenol A, BPA (600 µg/L) and 4-nonylphenol, NP (300 µg/L) in the tank water for 96 h. Both the EDCs were procured from Sigma (MO, USA) and stock solutions were made in dimethyl sulfoxide (DMSO, Sigma). Control group fish were exposed to an equal concentration of DMSO. These concentrations of EDCs have previously been standardized in case of expression of Ki-*ras*' Ha-*ras* and other genes in *K. marmoratus* (Lee et al., 2006, 2007). After exposure, brain, gonad, intestine, and liver tissues were collected and *Km*-N-*ras* mRNA expression was studied using real-time RT-PCR as described above.

2.8. Statistical analysis

All the data expressed as means±S.E. were arcsine transformed before statistical analysis. The significance of difference of data between control and exposed groups was analyzed using Student *t*-test. $p<0.5$ was considered significant.

3. Results and discussion

3.1. Km-N-ras gene

The full-length *K. marmoratus* (*Km*)-N-*ras* cDNA was 1694 bp consisting of an open reading frame (ORF) of 564 bp encoding putative protein of 187 aa (amino acids) (Fig. 1A, GenBank accession No. EU162748). Theoretical p*I* and calculated molecular weight of the deduced protein were 6.75 and 21.4 kDa, respectively. When compared with the amino acid sequence length of other species such as zebrafish (NM_131145) 188 aa, *Xenopus* (NM_001090869) 189 aa, mouse (NP_035067) 193 aa and human (NM_002524) 189 aa, *Km*-N-*ras* (187 aa) showed a similar range. The *Km*-N-*ras* gene was composed of 4 exons which are the same as zebrafish and human orthologues. When gene size of ORF region (exons 1–4) among the above species was compared, *Km*-N-*ras* gene of 3.4 kb was found to be smaller than zebrafish (6.6 kb) and human (7.6 kb) (Fig. 1B).

The ras proteins which are associated with the inner face of the plasma membrane play an important role in the regulation of signaling pathways that control cellular responses such as proliferation, survival and differentiation (Campbell and Der, 2004; Schubbert et al., 2007). A ras protein classically works like a molecular off/on switch; inactive when bound to guanosine diphosphate (GDP) and active when bound to guanosine triphosphate, GTP (Schubbert et al., 2007). Furthermore, *ras* genes and their products show a high degree of nucleotide and amino acid sequence similarities suggesting that they are evolutionary conserved and play an important role in normal cellular functions. Our analysis showed that *Km*-N-ras had sequence identity in range 79–81% with that of other species described above. When compared with other ras proteins of *K. marmoratus* (Lee et al., 2006), *Km*-N-ras shared 79% identity with *Km*-Ki-ras-4A (AY886901), 80% identity with *Km*-Ki-ras-4B (AY886900) and 81% identity with *Km*-Ha-ras (DQ078265) (data not shown). This implies that *Km*-N-*ras* gene may be third *ras* gene distinct from the other *ras* genes reported from *K. marmoratus*.

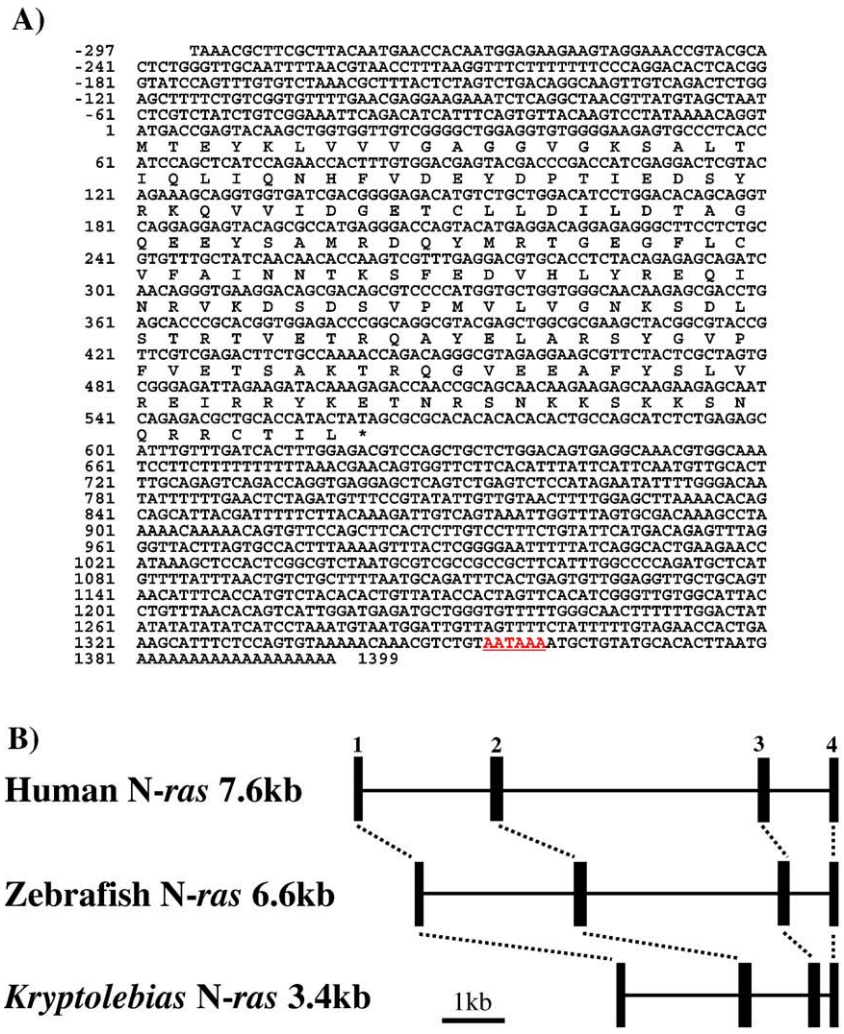

Fig. 1. A) Full-length cDNA sequence and deduced amino acid sequence of *Kryptolebias marmoratus* N-*ras* gene. The poly(A) signal sequence (AATAAA) is underlined. GenBank accession No. EU162748. B) Comparison of genomic structure of *K. marmoratus* N-*ras* gene with human (NC_000001) and zebrafish (NC_007119) N-*ras* genes retrieved from GenBank. The 5′- and 3′-UTR regions are excluded.

The deduced *Km*-N-ras amino acid sequence was aligned with those of other species retrieved from GenBank such as zebrafish (*D. rerio*, NM_131145), African clawed frog (*Xenopus laevis*, NM_001090869), mouse (*Mus musculus*, NP_035067), and human (*Homo sapiens*, NM_002524) (Fig. 2A). The C-terminal sequences between 166 aa and 187 aa were hypervariable region (HVR, Fig. 2A). Homology of HVR among the four *K. marmoratus* ras proteins was 18–28%, while that of N-ras proteins between *K. marmoratus* and other species was 16–20%. These HVR sequences are involved in the membrane trafficking of ras protein by giving membrane-anchoring capacity on ras (Choy et al., 1999). It has also been suggested that specific ras proteins are processed differently and therefore follow distinct trafficking pathways (Mor and Philips, 2006; Schubbert et al., 2007).

3.2. Molecular phylogeny

The branch pattern analysis of NJ tree (Fig. 2B) showed that individual *ras* gene was separated into four distinct clades according to the gene type rather than to species. Of these clades, R-*ras* was found to be highly diverse than Ha- and Ki-*ras*, based on their branch lengths. Overall, *ras* gene of fish, including that of *K. marmoratus*, might have no relationship with those of mouse and human, while they are close to each other in each gene cluster, as judged by the branch topology.

3.3. Tissue distribution and developmental stage-specific distribution

The *Km*-N-*ras* transcripts were ubiquitously distributed in all the tissues. However, expression in brain was greater than other tissues (Fig. 3A). Ki-*ras* expression has also been observed in all the tissues examined in *K. marmoratus* (Lee et al., 2006). However, level of expression differed tissue-wise. Leon et al. (1987) observed ubiquitous expression of three *ras* genes in mouse. They also reported tissue-specific differences in the level of expression. The difference in case of N-*ras* expression was more remarkable as compared to other *ras* genes. Besides tissue-specific distribution, we observed that at some stages of development N-*ras* expression was more pronounced. The *Km*-

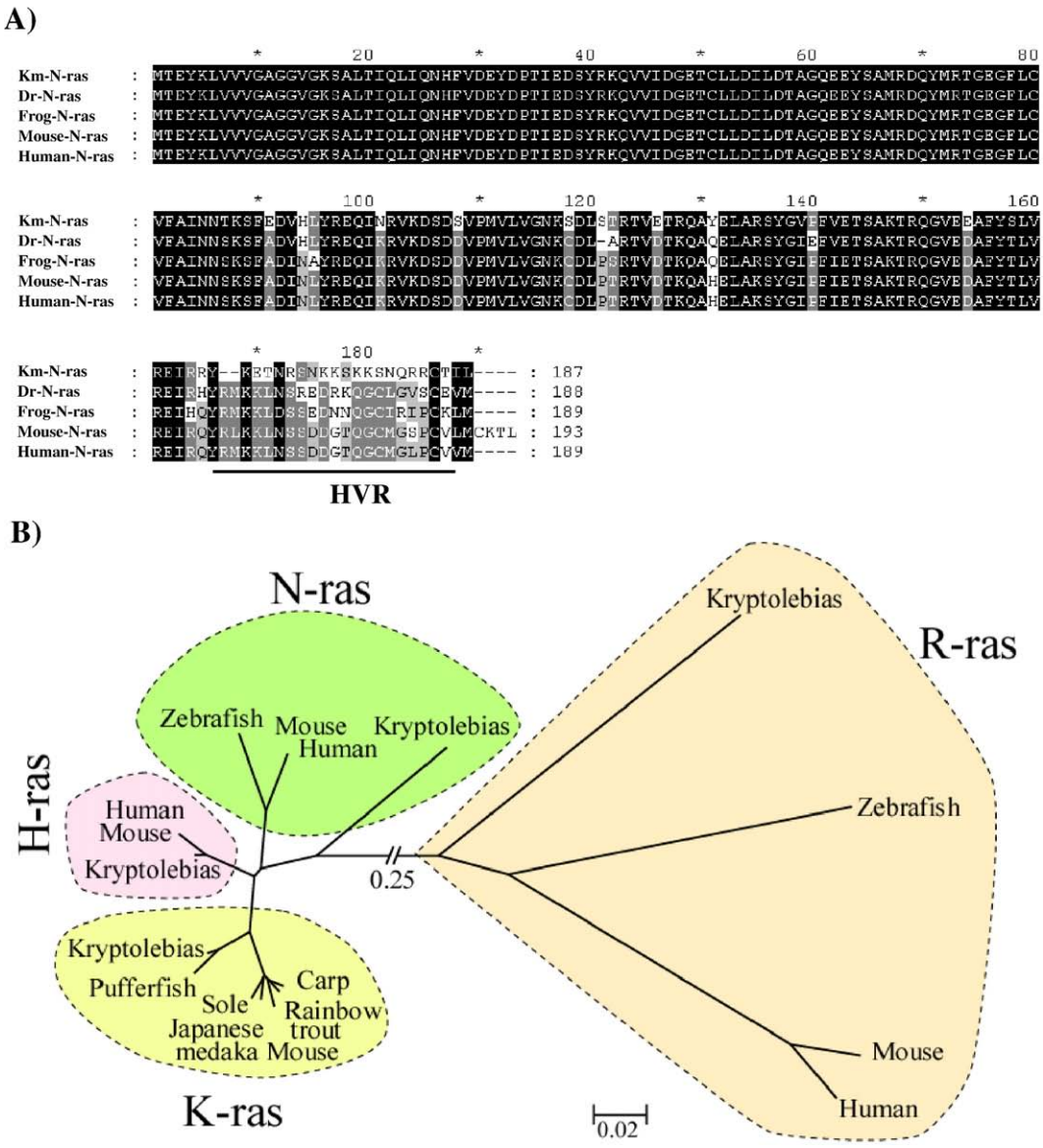

Fig. 2. A) Multiple alignments for amino acid sequence of *Kryptolebias marmoratus* (*Km*) N-*ras* with those of other vertebrates i.e. Dr, zebrafish (*Danio rerio*, NM_131145); frog, African claw frog (*Xenopus laevis*, NM_001090869); mouse (*Mus musculus*, NP_035067); human (*Homo sapiens*, NM_002524). The black shade shows conserved residues. Position 166 aa to 187 aa is hypervariable region (HVR). B) Unrooted phylogenetic tree constructed from amino acid sequences of *ras*-gene family, including H-, K-, N-, R-*ras*, revealed from fish, mouse and human. Branch lengths are proportional to the scale given. The four types of *ras*-gene family were included and their amino acid sequences were as follow: H-*ras*: AAY82451 (*Kryptolebias*), NP_005334 (human), AAH61885 (mouse); K-*ras*: AAW78852 (*Kryptolebias*), AAD10839 (common carp, *Cyprinus carpio*), NP_067259 (mouse), O42277 (Japanese medaka, *Oryzias latipes*), AAC25633 (English sole, *Parophrys vetula*), A54321 (rainbow trout, *Oncorhynchus mykiss*), CAG03433 (pufferfish, *Tetraodon nigroviridis*); N-*ras*: EU162748 (*Kryptolebias*), CAJ18567 (mouse), P01111 (human), AAB40625 (zebrafish); R-*ras* (*Kryptolebias*), AAA40038 (mouse), AAA60256 (human), NP_001005931 (zebrafish).

N-*ras* mRNA expression highly increased from embryonic stage 2 (4 dpf) and during entire embryonic stage (up to 12 dpf), gradually decreased at juvenile stage 1 (1.5 cm), and then recovered from juvenile stage 2 (2.0 cm) to adult stage (Fig. 3B). Hermaphrodites and secondary males showed no significant difference in expression levels (Fig. 3B). Cheng et al. (1997) have reported that zebrafish N-*ras* was highly expressed at early embryonic stages and then gradually there was decline in expression at 48 h after fertilization. In case of mouse also, Leon et al. (1987) observed developmental stage-specific expression of *ras*-gene transcripts. It is interesting that although all three *ras* genes encode for the p21 proteins, the expression level varies for each gene in different tissues signifying their functional differences. The presence of transcripts at embryonic stage is supported by the findings in mouse showing that cellular oncogenes, including *ras* are involved in normal cellular proliferation and differentiation during embryonic and fetal development (Slamon and Cline 1984).

3.4. EDC-induced expression of N-ras gene

N-*ras* gene expression was significantly modulated by EDCs in different tissues of *K. marmoratus* at the concentrations used in this study. In previous studies BPA and NP at the same

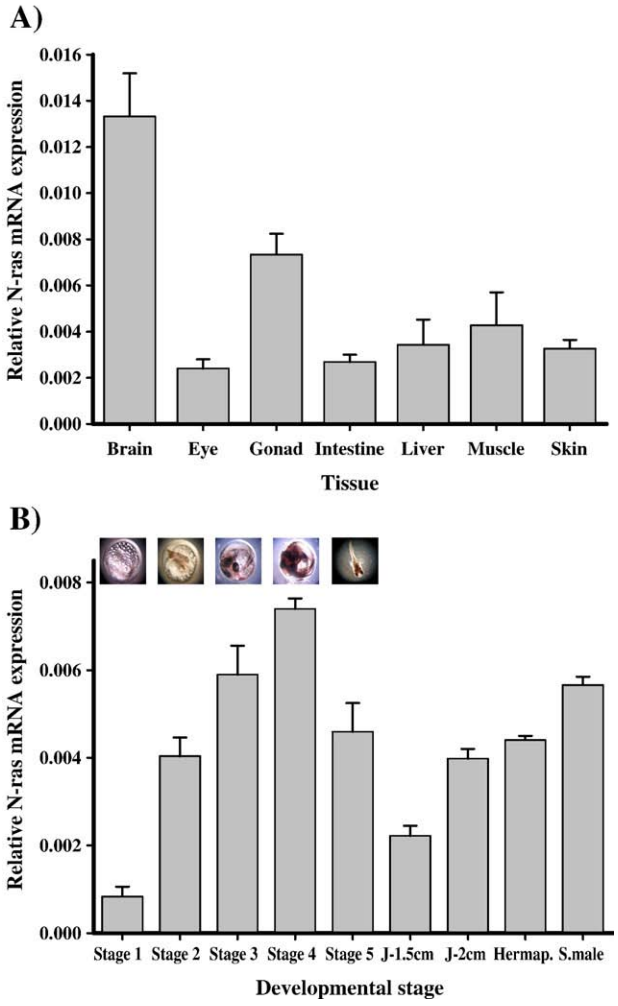

Fig. 3. A) Tissue distribution of N-*ras* gene in *Kryptolebias marmoratus* hermaphrodite fish. B) Expression of *K. marmoratus* N-*ras* gene at different developmental stages. Five embryonic stages represent stage 1 = 2 day post-fertilization (dpf), stage 2 = 4 dpf, stage 3 = 9 dpf, stage 4 = 12 dpf and stage 5 = 4 h after hatching, two juvenile stages are J 1.5 cm and J 2.0 cm depending on their length, hermap = hermaphrodite and S. male = secondary male. *Km*-N-*ras* expression is shown as relative to *Km-β-actin* which was used as reference house keeping gene. Data are means±S.E. of three replicates of individual fish.

concentrations caused induction of *ras* genes and tumor suppressor gene *p53* in *K. marmoratus* (Lee et al., 2006, 2008). Both the EDCs caused significant up-regulation of N-*ras* gene expression in all the fish tissues, except in case of the brain where BPA caused slight down-regulation (Fig. 4). However, BPA treatment caused significant ($p<0.5$) up-regulation in gonad and intestine. Chemically-induced mutation, activation and expression of *ras* genes have been reported in mammals and fish species (Hendricks et al., 1994; Sills et al., 1999; Rotchell et al., 2001). Observations on modifying role of EDCs on chemically-induced carcinogenesis are contradictory. While Tanaka et al. (2004) showed that atrazine lacked modifying effect on 7,12-dimethylbenz(α)anthracene (DMBA)-induced ovarian carcinogenesis in rats, Fukamachi et al. (2004) showed that atrazine and nonylphenol have enhancing effect on DMBA-induced mammary tumor development in human c-Ha-*ras* proto-oncogene transgenic rats. Concerning expression of other *ras* genes, Lee et al. (2006) observed that NP (300 μg/L for 96 h) caused a significant over-expression of c-Ki-*ras* (long form) in the liver. However, there was no significant up-regulation of c-Ki-*ras* (short form). In the case of Ha-*ras* gene, EDC caused over-expression in the brain while down-regulation in the gonad. In our previous report on *Km*-Ki-*ras* and *Km*-Ha-*ras*, we found 3 and 12 ERE (estrogen response element) half-site position in promoter regions of these genes, respectively (Lee et al., 2006). This suggests that these *ras* genes could be modulated by xenoestrogenic compounds through ERE sites. Therefore, search for ERE in the promoter region of *Km*-N-*ras* gene may be helpful to determine whether expression of *Km*-N-*ras* gene results from the direct interaction of EDCs.

Major focus of *ras* oncogene research in fish is on understanding the mechanism of their activation during carcinogenesis. However, there is no information available on fish other than *K. marmoratus* to indicate whether fish *ras* genes would be modulated by exposure to EDCs. The findings reported here show that N-*ras* expression was modulated by the EDCs. The concentrations used in this study are higher than those normally encountered in the environment and exposure was given for a short duration. Nevertheless, due to persistent nature of EDCs there may be exposure build-up and some populations may be more susceptible than the others. Therefore, long-term low level exposure studies may provide information on a more realistic scenario.

In conclusion, we cloned the full-length cDNA sequence of *K. marmoratus* N-*ras* oncogene and showed that although it is detectable in all the tissues of fish, level of its expression differed among tissues. *Km*-N-*ras* gene also showed developmental stage-specific variation in level of expression. We observed that environmental endocrine disrupting chemicals

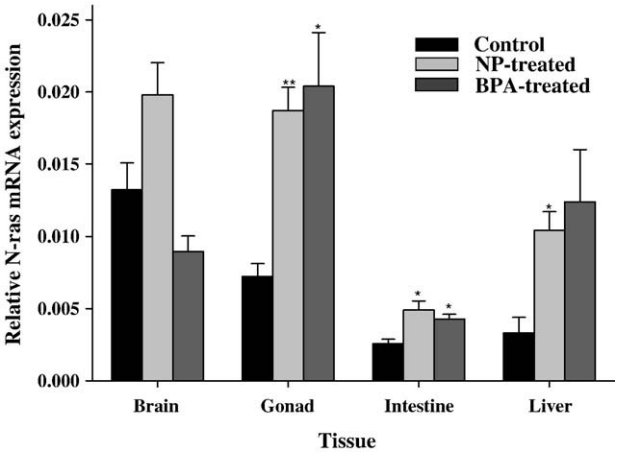

Fig. 4. Expression of *Kryptolebias marmoratus* N-*ras* mRNA after exposure to 4-nonylphenol (300 μg/L) and bisphenol A (600 μg/L) for 96 h in adult hermaphroditic fish. *Km*-N-*ras* expression is shown as relative to *Km-β-actin* which was used as reference house keeping gene. The experiment was performed in triplicate and mean fold change is indicated relative to that of the control group. All data shown as means±S.D. were arcsine transformed and analyzed for statistical significance using Student *t*-test. The asterisk symbol means a statistically significant difference, $p<0.05$ (*) and $p<0.01$ (**) when compared with untreated control group.

up-regulated expression of N-*ras* in most of the tissues. Our study provides evidence that although EDCs as such are not potentially environmental carcinogens, they may predispose the host to environmental carcinogenesis.

Acknowledgements

This work was supported by grants of National Research Lab of KOSEF (2006) and KRF (J01902) funded to Jae-Seong Lee, and also supported by a grant of KOPRI Top Brand (Grant # PE07050) funded to Hong Kum Lee (KORDI, Incheon, South Korea).

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