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Fish Models in Impact Assessment of Carcinogenic Potential of Environmental Chemical Pollutants: An Appraisal of Hermaphroditic Mangrove Killifish *Kryptolebias marmoratus*

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Abstract—Fish models have gained acceptability in toxicological research in a big way. The major stimulus comes from two much-researched fish, medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*). To a lesser extent, rainbow trout (*Oncorhynchus mykiss*) also pioneered research mostly on the liver cancer development and prevention. Recent understanding of genomes of these fish and finding of extensive homology between fish and human genomes have further catapulted their position in toxicology research and search for mechanisms and remedies for human diseases. Induction of cancer in most of the fish is rapid requiring low dose and short duration of exposure. About 20 years back it was demonstrated in *Kryptolebias marmoratus* that a single low dose exposure to carcinogen rapidly induced tumors. *K. marmoratus* is the only known internally self-fertilizing vertebrate. It has shown high sensitivity to a number of carcinogens. Furthermore, recent studies have demonstrated that exposure to endocrine-disrupting chemicals (EDCs) modulated expression of genes critical in tumor development and regulation. For example, exposure to EDCs caused significant increase in *p53* expression within 3 h in juveniles. Similarly, some EDCs also modulated expression of *Ras* oncogenes. It is suggested that *K. marmoratus* can be used in study of environmental carcinogenesis and impact assessment of EDCs and environmental carcinogens.

Keywords: environmental carcinogens, fish model, cancer susceptibility, endocrine-disrupting chemicals

INTRODUCTION

Fish is a popular model for toxicological research, especially for the contaminants which are likely to exert their impact on aquatic ecosystems. A few freshwater and marine fish species have been standardized for toxicity studies and used in regulatory exercises. Among these, some as medaka (*Oryzias latipes*), fathead minnow (*Pimephales promelas*), and zebrafish (*Danio rerio*) are very popular in toxicity studies. For last 10 years or so when there has been emphasis on

development of alternative models for toxicity studies, disease and cancer, fishes have attracted the maximum attention among non-mammalian species. Fish models have been developed for diseases such as diabetes, muscular dystrophy and neurodegenerative disease and to elucidate the molecular mechanisms of mutagenesis and carcinogenesis following exposure to environmental contaminants (Rubinstein, 2003; Jha, 2004, 2008; Lieschke and Currie, 2007). Physiological process such as aging could also be studied in fish (Gerhard, 2007). Such researches are not only contributing to our knowledge of disease mechanisms but also to drug development. Fish has also been used as a model for cancer research (Bailey *et al*., 1996; Amatruda *et al*., 2002; William *et al*., 2003; Yee and Pack, 2005; Lam and Gong, 2006). In this regard, the rainbow trout (*Oncorhynchus mykiss*) has been employed as a model for study of carcinogenicity of several food and environmental contaminants (Bailey *et al*., 1996; William *et al*., 2003). The embryonic exposure to aflatoxin B_1 (a toxin produced by fungus *Aspergillus flavus* on foods and feeds), rainbow trout produced a high incidence of hepatocellular carcinomas in adults (Bailey *et al*., 1996). Using rainbow trout tumor model prevention of chemically-induced carcinogenesis by natural products has also been studied (Tilton *et al*., 2007). Toxicogenomics study of transcriptional patterns in aflatoxin B_1-induced hepatocarcinoma in rainbow trout and human hepatocarcinoma showed a high degree of similarity (Tilton *et al*., 2005). Compared to mammals tumor induction in fish appears to be easy. The carcinogen can be exposed through aquarium water and liver tumor induction, in particular, provides best results with most of the carcinogens so far studied. Rainbow trout is a big fish and compared to small fish models such as medaka and zebrafish, genome information is limited. Therefore, small fish species as model for carcinogenesis are favorites.

Small fish model, zebrafish has been used in cancer studies for many years (Stanton, 1965; Amatruda *et al*., 2002). A great deal of zebrafish genomics information is available online through dedicated portals. Similarly, studies using medaka in chemically-induced carcinogenesis have shown promising results (Okihiro and Hinton, 1999; Reddy *et al*., 1999; Liu *et al*., 2003). However, compared to zebrafish, research efforts on medaka are not much intensified as far as its use in cancer research is concerned. In recent years, voluminous gene information has been made available for small fish mangrove rivulin *Kryptolebias marmoratus* (order Cyprinodontiformes, family Rivulidae). *K. marmoratus* is the only known vertebrate with internal self-fertilization (Harrington, 1961). Because of this peculiar reproduction and distinctive habitat, *K. marmoratus* has attracted interest from some research groups for its use in toxicology and carcinogenesis research. Laboratory maintenance of *K. marmoratus* is easy and it is a kind of euryhaline fish able to thrive in a broad salinity range (Lee, J.-S. *et al*., 2008). Additionally, *K. marmoratus* has a long life span and egg production in laboratory is achievable with little efforts. In this paper we briefly appraise the status of *K. marmoratus* in cancer research with focus on its use in environmental carcinogenesis and recent development showing that exposure to endocrine-disrupting chemicals (EDCs) modulate expression of genes critical in tumor development and regulation.

K. MARMORATUS IN CANCER RESEARCH

Usefulness of *K. marmoratus* in cancer research for the first time was highlighted by Koenig and Chasar (1984). By using diethylnitrosamine (DEN) they studied hepatocellular carcinoma development in adult, larvae, and embryos and found that in adults and larvae, incidence of tumor was very high and tumor developed quickly. Later in the same year, Park and Kim (1984) observed that DEN exposure for 2 h was sufficient to induce hepatic neoplasms in *K. marmoratus*. Induction of tumor after exposure to a carcinogen for such a short duration was amazing as in other fish models it takes longer time and sometime repeated exposure is also necessary. In zebrafish about eight week's exposure to DEN is required to induce tumors (Mizgireuv and Revskoy, 2006). Exposure for short-term development duration may be advantageous for researchers. Hepatic tumor induction by butylated hydroxyanisole (BHA) was also reported by Park *et al*. (1990). A single exposure to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) through tank water induced thyroid tumors in *K. marmoratus* (Park *et al*. (1993). Almost all the fish developed tumor within four months. When tumor tissue was grafted in the anterior eye chambers of the fish, most of the grafts developed into tumor mass. Earlier, papillary thyroid tumor induction by *N*-methyl-*N*-nitrosourea (MNU) was reported by Lee *et al*. (2000). About 95% of fish developed tumors after a single exposure to 50 ppm of carcinogen. These studies highlight that *K. marmoratus* shows a good tumor induction response even when exposed to a single carcinogen dose.

K. marmoratus was used for study of oncogenes in biliary and hepatic neoplasms and necrotic and regenerative phases of DEN-toxicity by Goodwin and Grizzle (1994a, b). Thiyagarajah *et al*. (1995) reported on carcinoembryonic antigen (CEA) in DEN-induced liver, gut and biliary neoplasms in *K. marmoratus* suggesting that the response was similar to mammals. These findings strengthen the suggestion that *K. marmoratus* would be a good model for cancer studies. Some key oncogenes (Ha-, Ki-, and N-*ras*, c-*src*, c-*fos*, c-*myc*, *p53*) and a DNA repair gene (O^6-methylguanine-DNA-methyltransferase (O^6-*MT*) were studied in MNU-induced papillary thyroid tumor (Lee *et al*., 2000). Development of chemically-induced tumors and its transplantation as shown in case of thyroid tumor are important features of *K. marmoratus* for its consideration and development as model species for cancer research. Furthermore, recent studies on sequence and expression data of oncogenes (N-*ras* and R-*ras*, in particular) and tumor suppressor gene *p53* have further enriched our knowledge about oncogenic responses in *K. marmoratus*. All these features make use of *K. marmoratus* in experimental cancer research a good choice.

ENDOCRINE-DISRUPTING CHEMICAL EXPOSURE AND ONCOGENE AND TUMOR SUPPRESSOR GENE EXPRESSION

Endocrine disrupting chemicals have emerged as environmental contaminants of great concern (Jenseen, 2006; Yang *et al*., 2006). EDCs not only disrupt natural populations but their impact on human health has also been recognized. Role of EDCs in human cancer, especially as developmental exposure has been highlighted

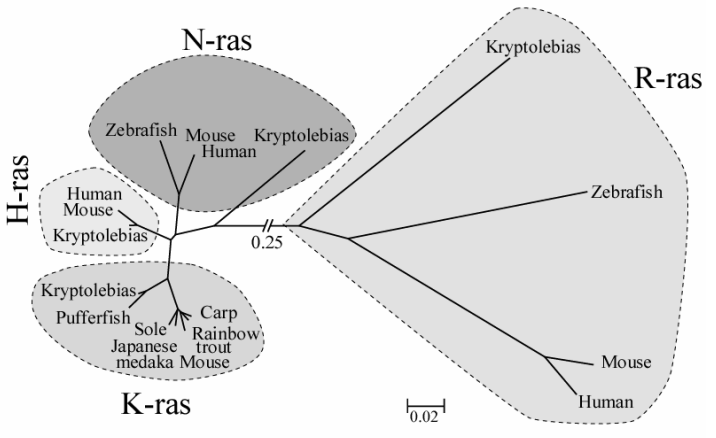

Fig. 1. The unrooted phylogenetic tree based on the amino acid sequences of *ras*-gene family, including H-, K-, N-, R-*ras* (from Lee, Y.-M. *et al*., 2008a).

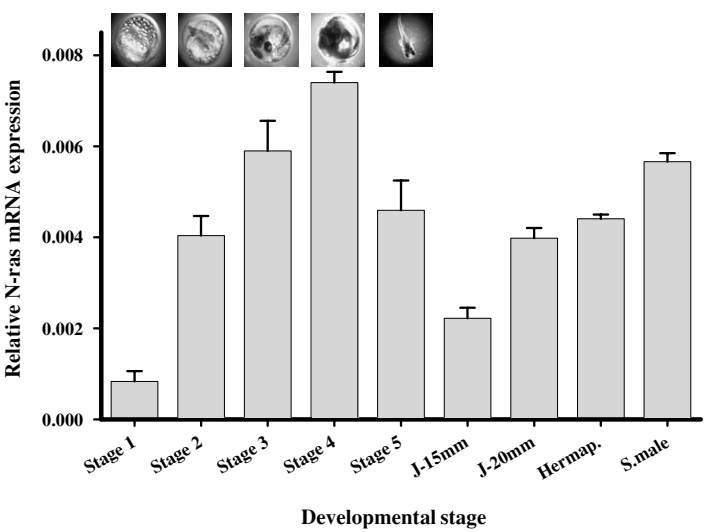

Fig. 2. Expression of *K. marmoratus* N-*ras* gene at different developmental stages. The embryonic stages represent stage 1 = 2 day post-fertilization (dpf), stage 2 = 4 dpf, stage 3 = 9 dpf, stage 4 = 12 dpf and stage 5 = 4 h after hatching, two juvenile stages are J 1.5 cm and J 2.0 cm depending on their length, hermap = hermaphrodite and S. male = secondary male (from Lee, Y.-M. *et al*., 2008a).

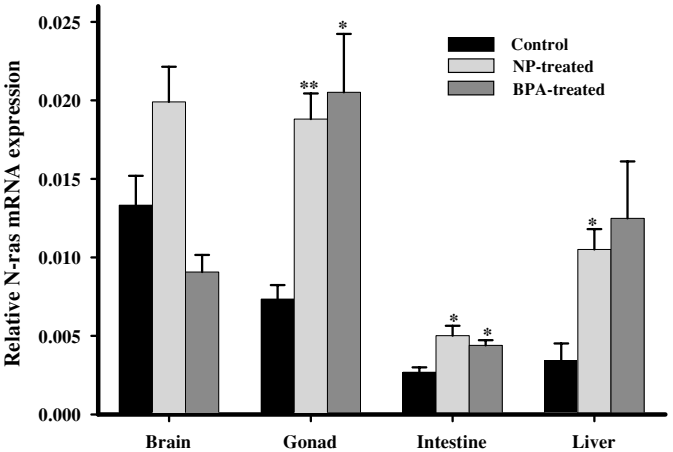

Fig. 3. Expression profile N-*ras* mRNA in different tissues of *K. marmoratus* after exposure to 4-nonylphenol (300 μg/L) and bisphenol A (600 μg/L) for 96 h in adult hermaphroditic fish. The asterisk symbol means a statistically significant difference, $p < 0.05$ (*) and $p < 0.01$ (**) when compared with untreated control group (from Lee, Y.-M. *et al.*, 2008a).

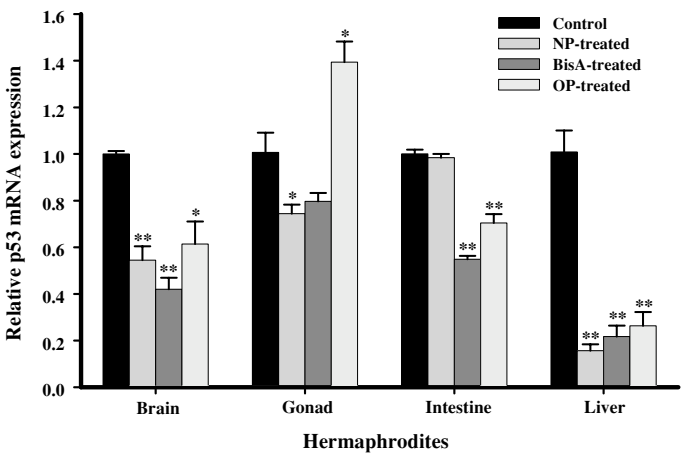

Fig. 4. Expression of *p53* mRNA in different tissues of adult hermaphroditic *K. marmoratus* after exposure to 4-nonylphenol (300 μgL), bisphenolA (600 μgL), and 4-*tert*-octylphenol (300 μgL) for 96 h. Statistically significant differences over control are indicated by $*p < 0.05$ and $**p < 0.01$ (from Lee, Y.-M. *et al.*, 2008b).

in some recent studies (Birnbaum and Fenton, 2003). EDCs may also potentially induce genetic damage and can modulate carcinogenic responses of chemicals (Fukamachi *et al.*, 2004; Jha, 2004, 2008). However, little knowledge exists about likely impact of their exposure on environmental carcinogenicity of

chemical contaminants. Furthermore, there is no empirical model to test the predisposing potential of EDCs. Fish models may be suitable in this regard as fish in its natural habitats are exposed to a variety of toxic chemicals and a substantial proportion of these chemicals could be carcinogenic (Jha, 2004). In a complex environmental condition, a chemical may be influencing the effect of the other by interactions at various levels.

Recently, we have successfully cloned and sequenced various *ras* oncogenes and tumor suppressor gene *p53* from *K. marmoratus* (Lee, Y.-M. *et al*., 2008a, b). Phylogenetic relationships of these genes have also been studied (Fig. 1). Subsequently, expression of genes was studied using quantitative realtime RT-PCR in gender types (hermaphrodites and secondary males), different tissues, different stages of development and fish exposed to selected EDCs (Figs. 2 and 3). N-*ras* expression was highest in the brain and had the highest level of expression compared to other tissues. Furthermore, some embryonic stages showed more N-*ras* expression than juveniles and adults. Exposure to EDCs such as bisphenol A (BPA) and 4-nonylphenyl (NP) caused upregulation of N-*ras* in gonad, intestine and liver of hermaphrodite *K. marmoratus*. Regarding *p53* expression, although basal level of expression of *p53* mRNA was low, all the major tissues showed some level of expression. After exposure to BPA, NP and 4-*tert*-octylphenol (OP), *p53* expression was significantly enhanced within 3 h of exposure in juveniles. However, expression was down-regulated by exposure to most of the EDCs at 96 h in adult fish. The suppressive effect of EDCs was more pronounced in liver as compared to other tissues (Fig. 4). These findings suggest that *p53* gene would be involved in cellular defense mechanism in early stage of exposure to EDCs and long-term exposure may suppress its expression. Since *p53* is key to tumor development at the initial stages, its modulation by EDCs in fish population necessitates its consideration in risk assessment studies. The above findings on oncogenes and tumor suppressor genes demonstrate that *K. marmoratus* could also serve as suitable fish model to study the predisposing effects of EDCs and other persistent environmental pollutants.

RECENT FINDINGS

Our current research efforts are focused on study of pathways of apoptosis, signal transduction, endocrine regulation, DNA repair mechanisms. Recently, we observed that O^6-*MT*, which plays an important role in determining the sensitivity to cancer development, is expressed at low level in juveniles and aged secondary males (Rhee *et al*., unpublished data). This pattern of O^6-*MT* expression is somewhat similar to humans. Therefore, *K. marmoratus* may also play a significant role in assessing the sensitivity to carcinogens and data could be extrapolated to human situations. One of our current focuses is the study of genes involved in circadian rhythms and endocrinological functions. We have successfully sequenced gonadotropin-releasing hormone receptor (*GnRHR*) gene from *K. marmoratus* and studied its expression in normal and EDC-exposed fish (Rhee *et al*., 2008). These studies would overall facilitate a better understanding of chemical carcinogenesis in *K. marmoratus*.

CONCLUSIONS

Although zebrafish has been more extensively used in research on human diseases and cancer, *K. marmoratus* has several advantages and deserves consideration as an alternative to animals or to zebrafish itself. At present we know structures and expression profiles of several genes from *K. marmoratus* critical in carcinogenesis. Since this fish is a hermaphrodite with internal fertilization capabilities, it serves a kind of naturally inbred specimen which are preferred in cancer research. Our studies suggest that in line with other well-established fish models to elucidate impact of environmental contaminants at molecular and cellular level, *K. marmoratus*, a hermaphroditic fish, appears to be an excellent model. It is suitable for study of expression of marker genes following exposure to carcinogens. In addition, this fish species could also be helpful in conducting behavioral studies following exposure to EDCs. It is expected that in future we will be able to know more about *K. marmoratus* genome sequence which will augment its further use in cancer research.

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