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Identification of Putative Pre-B Cell Leukaemia Transcription Factor 1 Gene by Differential Display: A Novel Fish mRNA Expressed Upon Cadmium Exposure

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ABSTRACT

Pre-B cell leukaemia transcription factor (Pbx1) is a member of a ubiquitous class of homeodomain (HOX) proteins. Subsequent studies have shown that Pbx1 is widely expressed and acts as a co-factor for a variety of HOX proteins. This also means that Pbx1 is a one of the markedly and specifically suppressed gene in Promyelocytic leukaemia zinc finger (PLZF) which has been characterized as one of the regulated genes in cancer. In addition, PLZF is also a transcription repressor which suppresses the transcription of genes such as c-myc, cyclin A2 and HoxD11. In this study, the use of differential display reverse transcriptase polymerase chain reaction was reported to identify a novel hybrid tilapia mRNA sequence which is highly homologous to Pbx1 gene. For this purpose, hybrid tilapia Pbx1 was cloned. Then, a specific primer for the hybrid tilapia was designed for the Pbx1 mRNA measurements using the real-time PCR. The hybrid tilapia was exposed to 0.469, 0.938, 1.875 and 2.813 mg/l cadmium (Cd) to determine the relationship between Pbx1 mRNA expression levels. The cloned Pbx1, consisting of 343 bp encoding a protein of 53 amino acids, showed higher than 60% identity with the deduced amino acid sequence. Pbx1 mRNA expression and Cd accumulation appeared to be dose-responsive following cadmium treatment. Based on these results, the Pbx1 mRNA expression levels could be used as a bio-indicator to monitor the carcinogenic level of Cd in biological samples. The study is currently in progress to obtain the full gene sequence of Pbx1 using RACE-PCR.

Keywords: **Hybrid tilapia, cadmium, cancer, bioindicator, mRNA differential display**

INTRODUCTION

Over the past decades, the contamination of cadmium (Cd) has increased drastically. By the early 1990s, the world- wide annual release of Cd reached 22000 tons, which was largely found in water and soil (Liu *et al.*, 2007). Cadmium is a member of the IIb group in the periodic table of elements and one of the most toxic heavy metals able to produce genotoxic and mutagenic events at high concentrations (Waalkes, 2003). Moreover, Cd has been classified as a human carcinogen by International Agency for Research on Cancer. When Cd enters into the aquatic environment, it poses a serious threat to the living organisms as it has been found to be carcinogenic and mutagenic (Carginale *et al.*, 2002). In fish, Cd can damage gills because chloride cells are the primary targets of waterborne Cd and this metal decreases the activity of gills Ca2+-ATPase, which leads to fish hypocalcemia (Wong and Wong, 2000). In addition, Cd has adverse effects on growth, reproduction, respiratory functions and osmoregulation (Wu *et al.*, 2007). The first response to the carcinogenic

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of Cd is the regulation of genes which includes immediate early response genes (IEGs), stress response genes (metallothionein genes, heat-shock genes, genes controlling glutathione and related proteins), transcription factors, translation factors and miscellaneous genes (Waisberg *et al.*, 2003). Various approaches have been employed to identify such genes, including mutant screening (Price *et al.*, 1998), micro arrays (Akhtar *et al.*, 2002; Panda *et al.*, 2002) and differential display (Carginale *et al.*, 2002; Basile *et al.*, 2005). Differential display is a sensitive mRNA screening technique which enables the comparison of reverse transcribed and arbitrarily amplified cDNAs from two or more cell or tissue types. The isolated cDNAs can be identified by sequencing and interrogation of databases; subsequently, they can be cloned and used as probes for further analysis.

This study reports on the use of differential display, in the identification of a novel hybrid tilapia mRNA sequence which is highly homologous to Pre-B cell leukaemia transcription factor (Pbx1) and its expression level using real time PCR analysis. In particular, Pbx1 has been reported as IEG and belongs to the group of non-Hoxhomeodomain transcription factors which significantly contribute to embryonic differentiation and organogenesis. During this process, Pbx1 allows homeodomain proteins such as Hox (1), engrailed (2), MyoD (3), Meis (4), or Pad (5) to bind to DNA with higher affinity and specificity. Complexes of Pbx1/Hox can act as transcriptional activators or as repressors, depending on the recruitment of other transcription factors and modulation through extra cellular signals. Pbx1 is one of the markedly and specifically suppressed genes in Promyelocytic leukaemia zinc finger (PLZF) which has been characterized as one of the regulated genes in cancer. Similarly, PLZF is also a transcription repressor which suppresses the transcription of genes such as c-myc, cyclin A2, and HoxD11.

MATERIALS AND METHODS

Fish and Experiment Design

Immature hybrid tilapia (*Oreochromis niloticus* x *O. aureus*) (15 g) were purchased from a local supplier. Fish were then transferred to flow through the system, and 30 days prior exposure for acclimatization to laboratory conditions. Acute bioassay procedure was based on the standard methods (APHA-AWWA-WPCF, 1998). The experiment was run for 96 hours and repeated for three times. Acute toxicity effect of Cd on hybrid tilapia was determined using the Finney's Probit Analysis LC_{50} Determination Method (Finney, 1971). The computer analysis was also carried with LC_{50} 1.00 software developed by EPA (1999). Based on this, the test result of the LC_{50} of Cd 96 h was indicated as 4.832 mg/l. Tilapia fingerlings were tested against lower concentrations of cadmium based on the data obtained from the acute toxicity test. Fish were exposed to 10, 20, 40 and 60% of the 96 hours LC_{50} for 7 days.

Total RNA Extraction, Removal of Genomic DNA Contamination

Gills of hybrid tilapia were ground into fine powder, under liquid nitrogen (5 ml), using pestle and mortar. The total RNA was isolated from 100 mg of this material using the RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. Genomic DNA contamination was removed from the total RNA by a treatment of 10 µg RNA with 5U Dnase I from Promega. A quality control of the total RNA was done by the standard PCR amplification using differential display primer.

Differential Display

Differential display was performed using the Gene Fishing ™PCR Kit (Seegene Inc., Korea), according to the supplier's protocol. The DNA free total RNA (3µg) from gills, were reversely transcribed (RT) with oligo-DT primer (dT-ACP1: 5`-CTGTGAATGCTGCGACTACGATX XXXX$(T)_{18}$-3`). The reaction contained 5 x RT buffer (25 mM Tris-Cl pH 8, 35 mM KCl, 1.5 mM $MgCl_2$, 5 mM DTT), 2 mM dNTPs, 40 U RNase Inhibitor and 200 U of MMLV reverse transcriptase. The RT reactions were incubated in 42°C for 90 min, 94°C for 2 min and chilled on ice for 2 min. As for the PCR amplification, each reaction mixture of 20 µl contained 5 µl of first-strand cDNA (50 ng), 10 µl of 2 x See Amp™ ACP Master Mix, 1 µl of 10 µM dT-ACP2 (5`-CTGTGAATGCTGCGA CTACGATXXXXX$(T)_{15}$-3`) and 2 µl of 5 µM arbitrary primer. The PCR program consisted of 1 cycle of 94°C for 5 min, 50°C for 3 min, 72°C for 1 min; 40 cycles of 94°C for 40 s, 65°C for 40 s. 72°C for 40 s and followed by a final elongation step at 72°C for 5 min.

Re–amplification, Purification and Cloning of PCR Product

Differentially expressed bands were excised. The DNA was recovered and re-amplified into a 40 µl reaction. All the reaction conditions were identical to the ones described above for the PCR amplification. The re-amplification cDNA was purified using Gel Purification Mini Kit (Favorgen, Biotech Corp, Taiwan) and cloned into pGEM-T vector (Promega). Specific primers were designed from this clone for total quantification using real time PCR.

Quantification Real Time PCR

The differential expressed gene (DEG) transcripts were quantified by real-time quantitative PCR using Mini Opticon Real Time PCR machine (BioRad) and SYBR Green Fluorescein mix per manufacturer's specification (Qiagen). The quantification was based on a 210-bp amplicon generated using the specified primer set (forward primer: 5`-CGACGATGATGAAGTGGATG-3` and reverse primer: 5`-TCAGCCTTGGTGGTAGTGGT-3`) of the DEG. The reaction mixture of 25 µl was set using the following concentrations: 100 nM each forward and reverse primers, 1x SYBR Green Fluorescein mix and 50 ng of cDNA. The reaction conditions were 94°C for 5 min for 1 cycle; 94°C for 30 s, 55°C for 30 s, 72°C for 1 min; and melting at 60-90°C for 15 s. The number of cDNA transcripts was determined using the standard curves generated with references plasmid of DEG.

Statistical Analysis

Data are presented as the mean ± SD. Results were analyzed using the one way ANOVA with Tukey`s multiple comparison. The statistical significance was accepted at a level of $p< 0.05$.

RESULTS

Identification of the Cd Induced Gene by Differential Display

Messenger RNA (mRNA) expression patterns of the control and cadmium-treated hybrid tilapia were compared in order to identify the genes whose transcription were up-regulated or down regulated by cadmium. To prevent isolation of 'false positive', all amplification experiments were performed on two different dilutions of each cDNA sample. Only cDNA bands, whose levels of expression were affected by cadmium in both dilutions, were selected for further analysis. *Fig. 1* shows a representative differential display gel. The cDNA band (a33.9) was excised and cloned. The cloned size of the cDNA band was 343 bp and renamed as pA33.9 for further analysis.

Nucleotide Sequencing, Homology Searching and Classification of the Isolated Gene

The DEG (pA33.9) was completely sequenced and registered at genbank (Genbank accession no: EU717966). The nucleotide sequence was then analyzed, by searching for protein homologies against the GenBank database, using the BLASTX programme and the results are summarized in *Fig. 2.* The clone showed

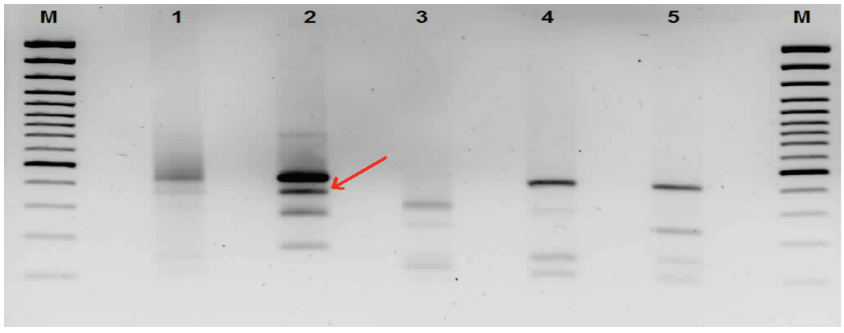

Fig. 1: Differential display of mRNAs from normal and exposed hybrid tilapia to Cd. N = O mg/l; 1 = 0.469 mg/l; 2 = 0.938 mg/l; 3 = 1.875 mg/l and 4 = 2.813 mg/l. Arrow indicates cDNA band which was only present, specifically at 0.938 mg/l excised and renamed pA33.9 for further analysis

significant amino acid homologies to the known protein (Pre-B cell leukaemia transcription factor interacting protein (Pbx1) in the database. The clone product encodes a 53 amino acids (nt. 3-158) partial sequence, with 93.6% homology to the *Mus muscles*, 80.5% homology to the *Rattus norvegius* and 68% to *Homo sapiens*.

Real-time PCR Analysis

The quantification of transcripts, amplified by real-time RT-PCR in this study, was based on the widely used standard curve method. This method assumed that the efficiency in the amplification of the samples was the same (Livak, 1997). The references plasmid, used to generate the standard curves for quantification of pA33.9 gene transcript, was pGEMT*pA33.9*. The real-time PCR amplification of this reference plasmid was performed together with the samples in the same run and reaction conditions. The Ct values were plotted versus the log of the initial amount of plasmid DNA to give the standard curves, as shown in *Fig. 3*. The correlation coefficient for the standard curves was 0.998 with the PCR reaction efficiency of 1.88. The transcript levels were determined based on the amount of DNA in femtogram (fg) of reference plasmid. *Fig. 4* shows the transcript levels for gene a33.9 at various cadmium exposures. It can be seen that the level of gene a33.9 was approximately 500-fold higher at the 0.938 mg/l as compared to the control. However, the expression level started to decrease at 1.875 mg/l and 2.813 mg/l.

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pA33.9          EVDDFEDFVFGHFFGDKALKKRSRKKEKHSWNPRVVGPREEHSRHPHHYHQG 52
Mus             EVDDFEDFVFGHFFGDKALKKRSRKKEKHSWNPRVVGPREEHSRHPHHYHQG 52
Rattus          EVDDFEDFIFSHFFGDKALKRRSKKKEKQPWNHRAVGPREEHSRHPHHYHQG 52
Homo            EVDDFEDFIFSHFFGDKALKKRSGKKDKHSQSPRAAGPREGHSHSHHHHHRG 52
                ********:*.*********:** **:*:. . *..**** **:  **:*:*
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Fig. 2: Alignment of the partial amino acid translated from pA33.9 (from this study) to a Pre-B cell leukaemia transcription factor interacting protein (Pbx1) from Mus muscles *(GenBank Acc. No: EDL15193),* Rattus norvegius *(GenBank Acc. No: EDM00624) and* Homo sapiens *(Genbank Acc. No: CAI13238). The alignment was performed with Clustal* W program

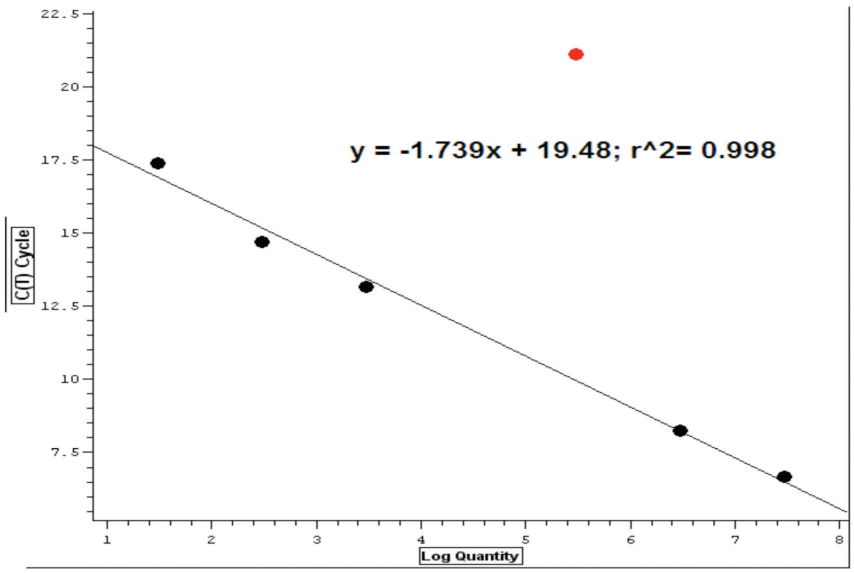

Fig. 3: Standard curves for pGEMTpA33.9. The graph shows the threshold cycle (Ct) plotted against log_2 starting quantity of plasmid defined by copy number. The efficiency of the reaction was 1.88

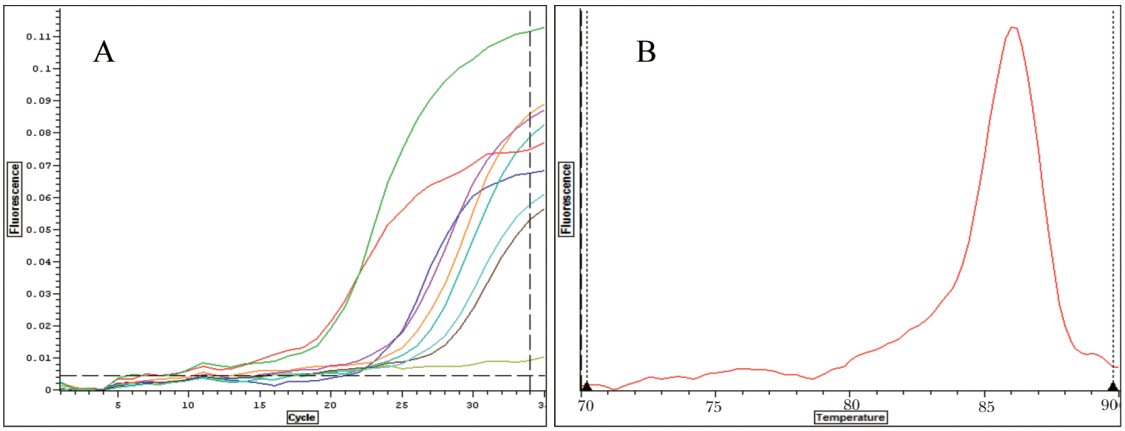

Fig. 4: (A) Fluorescence profiles of the cDNA of a33.9 gene amplicons; (B) Melting curve of the reaction for a33.9 gene

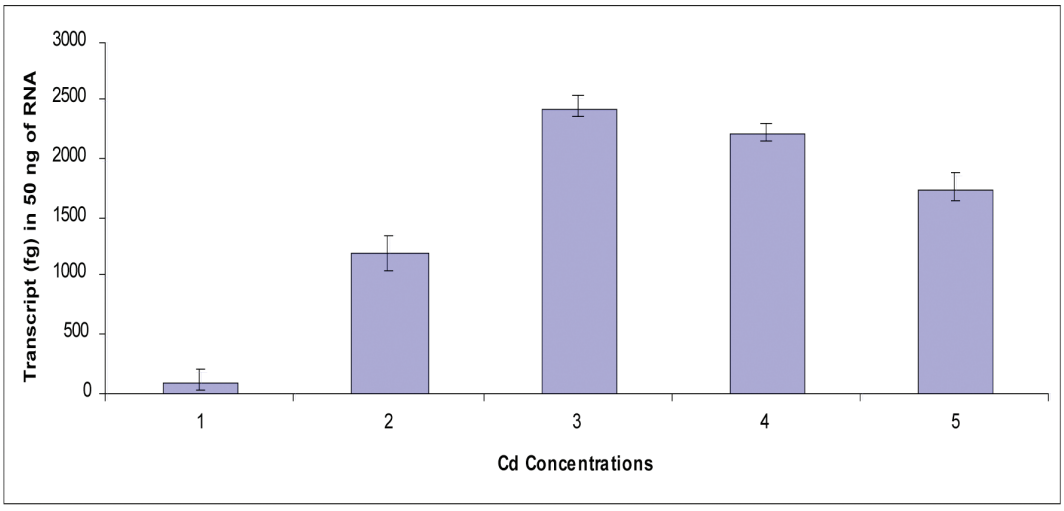

Fig. 4: Expression profiles for gene a33.9 at various cadmium exposure. 1 = O mg/l 2= 0.469 mg/l; 3 = 0.938 mg/l; 4 = 1.875 mg/l and 5 = 2.813 mg/l. All concentrations showed significant difference as compared to control (p<0.05)

DISCUSSION

Finney's Probit Analysis gave 96-h LC_{50} value for the hybrid tilapia exposed to different Cd concentrations as 4.832 mg/l. The results of the present study are within the range of the results reported by the following researchers. For instance, the LC_{50} values of cadmium on rainbow trout (*Oncorhynchus mykiss)* for 24, 48, 72 and 96 h were found to be 7.76, 1.95, 0.5, and 0.45mg/l, respectively (Oryan and Nejatkhah, 1997). In addition, Chambers (1995) investigated the effect of acute cadmium toxicity on marron, *Cherax tenuimanus* (Smith, 1912); the author found 96-h LC_{50} value as 17.9 (13.4–23.9) mg/l. Furthermore Muley *et al.* (2000) reported the 96-h LC_{50} value of cadmium on *C. carpio* as 121.8 ppm. The 96-h LC_{50} values of cadmium on *Salmo gairdneri* and *Xenopus laevis* larvae were reported to be between 80 and 100mg/l (Woodal *et al.*, 1988). According to Wright (2001) there are many factors involve in determining the 96-h LC_{50} and these include temperature, pH, alkalinity, water hardness, dissolved oxygen, total organic carbon, etc.

Differential display is a useful method used to identify genes; the expression of which may be up- or down-regulated in one sample relative to another. Following its introduction by Liang and Pardee (1992), the methodology has been widely used and improved. In this study, a significant expression of Pbx1 was observed from 0.938 mg/l of fish exposed to Cd toxicity. The over expression of PBx1 is a result of carcinogenic effect of Cd. However, the expression of Pbx1 started to decrease at 1.875 mg/l of Cd exposure onwards. This might due to the presence of detoxification genes such as heat shock proteins, metallothioneins and oxidative stress response gene. For example, metallothioneins sequester cadmium, with high affinity, resulted in decreased availability of Cd^{2+} capable of interacting with cellular targets to elicit toxicity, including carcinogenicity (Waalkes, 2003).

Generally, Pbx1 has widely been expressed during embryonic differentiation and organogenesis. However, Kikugawa *et al.* (2006) revealed that Pbx1 was one of the markedly and specifically suppressed genes in PLZF over-expressed DU145 cells. It was analyzed by Western blotting and RT-PCR that Pbx1 was endogenously higher expressed in DU145 cells than in LNCaP cells. Forced expression of androgen receptor in DU145 cells recovered the response of androgen-dependent PLZF expression and subsequent repression of Pbx1 expression. In particular, Pbx1 was found to achieve high-affinity DNA binding when Hox proteins became a partner of hetero-dimerization and the formed Hox-Pbx heterocomplex acted as an activator of transcription. This means, cadmium has the potential to influence the activity of several transcriptional factors leading to deregulation of gene expression. The proto-oncogenes *c-fos* and *c-jun* code for proteins which are the members of the AP-1 element that functions as a transcriptional factor regulation, i.e. the expression of a large number of genes controlling the growth and division of cell (Angel and Karin, 1991). This shows that Cadmium is a powerful inducer of *c-fos* and c-jun and this has been considered as a major mechanism for cadmium-induced cell transformation and tumorigenesis. Several other transcription factors (for example, metal regulatory transcription factor 1 or MTF1, upstream stimulator factor or USF, nuclear factor kB or NF-kB and NF-E2-related factor or NRF2) were activated by exposing them to Cd, resulting in modulation of gene expression (Li *et al.*, 1998; Alam *et al.*, 2000; Smirnova *et al.*, 2000). On the other hand, the exposure to Cd also resulted in the suppression of the DNA binding activities of the transcription factors hypoxia-inducible factor-1 (HIF-1) (Obera *et al.*, 2003) and Sp1 (Watkin *et al.*, 2003). HIF-1 is involved in controlling the expression of the erythropoietin gene, whereas Sp1 plays a key role in cell proliferation and its inactivation leads to cell death.

Cadmium is a complete carcinogen in experimental animals. Among other, Cd stimulates the expression of many genes and changes in these genes may be the precursors of some of the numerous effects reported at higher levels of biological organization. Furthermore, some can be used as a potential biomarker for hazard identification in the risk assessment of eco-toxicological studies as they are very specific to Cd toxicity. The results from this study indicated that the Pbx1 gene could be considered as a biomarker to monitor the carcinogenic level of Cd in the biological samples, especially fish.

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