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Identification and preliminary function study of *Xenopus laevis* DRR1 gene

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Abstract

Xenopus laevis has recently been determined as a novel study platform of gene function. In this study, we cloned *Xenopus* DRR1 (xDRR1), which is homologous to human down-regulated in renal carcinoma (DRR1) gene. Bioinformatics analysis for DRR1 indicated that xDRR1 shared 74% identity with human DRR1 and 66% with mouse DRR1, and the phlogenetic tree of DRR1 protein was summarized. The xDRR1 gene locates in nuclei determined by transfecting A549 cells with the recombinant plasmid pEGFP-N1/xDRR1. RT-PCR analysis revealed that xDRR1 gene was expressed in all stages of early embryo development and all kinds of detected tissues, and whole-mount in situ hybridization showed xDRR1 was mainly present along ectoderm and mesoderm. Furthermore, xDRR1 expression could suppress A549 cell growth by transfecting with plasmid pcDNA3.1(+)/xDRR1. xDRR1 probably plays important roles involving in cell growth regulation and *Xenopus* embryo development.

Keywords: *Xenopus laevis*; *Xenopus* down-regulated in renal cell carcinoma (xDRR1); Eukaryotic expression; Embryo development

Human down-regulated in renal cell carcinoma (DRR1) gene, which was a putative tumor suppressor gene, locates in the chromosomal 3p21.1 [1]. Its expression was dramatically decreased or even lost in a number of different cancer cell lines and primary tumors, such as renal cell carcinoma (RCC), small cell lung cancer (SCLC), non-small cell lung cancer (NSCLC), and ovarian cancer (OC). Transfection with wild-type DRR1 can suppress cell proliferation in RCCs and/or other cancer cell lines [1,2]. Though DRR1 gene has a potential role in the development of several cancers, little is known about its biological roles.

Xenopus laevis has recently been determined as a novel model organism for gene function studies [3] due to the following reasons: (1) its genes have high homology with human corresponding genes; (2) *Xenopus* oocytes and eggs are large and easy to manipulate; (3) the embryos develop rapidly for easily observing its phenotype, etc. Based on these advantages, we focused on human homologous DRR1 gene in *X. laevis* in order to get some clues for its function.

To understand the biological function of DRR1 gene in development and cell growth, we first cloned the homologous genes of DRR1 from *X. laevis* based on DRR1 bioinformatics analysis. Furthermore, we paid our attention to the preliminary functional annotation of *X. laevis* DRR1 (xDRR1), including as gene expression profiles in developing embryo and adult organs, protein sequence characterization, subcellular localization, and transfection analysis *in vitro*. Our primary studies on xDRR1 showed that xDRR1 locates in nuclei, and it may play important roles in embryo development.

Materials and methods

RNA extraction. Total RNA was prepared from 0.1 g of *X. laevis* oocytes. Briefly, the oocytes were pulverized by grinding in liquid nitrogen.

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One milliliter of TRIzol (Invitrogen) was added to mix and incubated for 10 min, then mixed with 0.2 ml of chloroform. The sample was centrifuged at 12,000 rpm for 10 min at 4 °C. The aqueous phase was precipitated by mixing with equal volume of isopropyl alcohol. After centrifugation, the pellet was washed in 75% ethanol, dried, and redissolved in RNase-free water. RNA sample was subjected to electrophoresis in 1.5% denaturing agarose gels to make certain that it was complete. RNA concentration was determined spectrophotometrically. The total RNA sample was treated with RNase-free DNase I for 30 min at room temperature to eliminate contaminating DNA and then followed by heating for 10 min at 65 °C to inactivate the DNase I.

*RT-PCR amplification for xDRR*1 *cDNA.* According to human DRR1 protein sequence (GenBank protein_id NP_009108), we found a hypothetical protein from *X. laevis* (GenBank protein_id AAH82866) using blastp in NCBI. The corresponding mRNA sequence (GenBank Accession No. BC082866) was found out to clone the CDS region. Here we named the gene as xDRR1. The primers used were 5′-ATACTCGAG AAATCCGCTTCTATG-3′ (forward) and 5′-ATAGGATCCAACCCCA CCCTCTC-3′ (reverse), and its PCR fragment was inserted into the expression vector pEGFP-N1. Another amplified PCR fragment was cloned into expression vector pcDNA3.1(+), using the following primers, 5′-ATAGGATCCCTGGTTAAATCCGCTTCTATG-3′ (forward) and 5′-ATACTCGAGTTACCCCACCCTCTCCTC-3′ (reverse). Sequences of the forward and reverse primers contained BamHI or XhoI restriction enzyme site (underlined), respectively. RT-PCR amplification was performed using the following program: reverse transcription at 50 °C for 30 min, initial PCR activation step at 94 °C for 2 min, 30 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and a final extension cycle of 72 °C for 5 min.

Construction of recombinant plasmids. The two amplified cDNA fragments, with the expression vector pEGFP-N1 (Clonetech) and pcDNA3.1(+) (Invitrogen), were doubly digested with restriction enzymes BamHI and XhoI, respectively. The digested products were separated on 0.8% agarose gels and DNA bands were extracted. The digested xDRR1 cDNA and the corresponding linear vector were ligated overnight at 16 °C and transformed into *E. coli* DH5α-competent cells. Preparation and transformation of competent *E. coli* DH5α were performed according to standard procedures [4]. The transformation mixture was plated on LB agar plates containing 50 μg/ml kanamycin or 100 μg/ml ampicillin. The plates were incubated for 12–16 °h at 37 °C. The desired recombinant plasmids pEGFP-N1/xDRR1 and pcDNA3.1(+)/xDRR1 were, respectively, confirmed by restriction enzyme digestion with BamHI/XhoI and DNA sequencing.

Bioinformatics analysis. Homology search for DRR1 protein was carried out among *Homo sapiens* and other species using the ClustalW program [5], and phlogenetic analysis of DRR1 proteins was performed using the MEGA3.1 software.

Expressing profiles of xDRR1 gene in embryo and tissues. Total RNA was extracted according to the method described above from developing embryos of various stages or specific organs of an adult frog. *Xenopus* eggs were fertilized in vitro, dejellied, cultured using standard methods [6], and staged according to the normal table of development [7]. The primers used were 5′-GCCCAAGAAACTCCTTAATCC-3′ (forward) and 5′-TTACC CCACCCTCTCCTCAC-3′ (reverse) for xDRR1, and 5′-ACGTCAATG ATGGAGTGTATGG-3′ (forward) and 5′-CAGTACAGACAGCAG GTGCAAG-3′ (reverse) for ornithine decarboxylase (ODC). The ODC was used as a control for RT-PCR under the same conditions. RT-PCR amplification was carried out using the following program: reverse transcription at 50 °C for 30 min, initial PCR activation step at 94 °C for 2 min, 30 cycles at 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, and a final extension cycle of 72 °C for 5 min. The relative expression quantities of xDRR1 and ODC in cells were calculated using Quantity One v4.6.2 (Bio-Rad) software on gel grayscale.

In situ hybridization. Whole-mount in situ hybridization was performed with digoxigenin (DIG)-labeled probes as described [8]. The probes against xDRR1 were generated by linearizing the pGEM-T/xDRR1 construct with BamHI and transcribing it with SP6 RNA polymerase from DIG Northern Starter Kit (Roche). Hybridization was detected with an alkaline phosphatase-coupled anti-DIG antibody and visualized using BM purple (Roche).

Cellular localization. The recombinant vector pEGFP-N1/xDRR1 expressed a fusion protein containing an EGFP epitope and 146 amino acids of xDRR1. The vectors with/without the insert were used to transfect tumor cell line A549. The cells were plated and cultured in RPMI1640 medium with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's recommendation. After 24 h transfection, the cells were observed under fluorescence microscope.

xDDR1 effects on cell growth. For translation in vitro, xDRR1 cDNA was directionally cloned into the expression vector pcDNA3.1(+). A549 cells were transfected by Lipofectamine 2000 reagent using the vectors with/without the insert in a 96-well format. Each transfection was repeated using 6 wells. After transfection for 48 h, cell viability was determined using One Solution Cell Proliferation Assay Kit (Promega) according to the manufacturer's recommendation.

Data analysis. Statistical differences were assessed using one factor analysis of variance (ANOVA) followed in some cases by Dunnett's test.

Results and discussion

Xenopus DRR1 is the ortholog of mammalian DRR1

It proved that homologous genes share a high sequence identity and similarity [9]. The xDRR1 gene encodes a protein consisting of 146 amino acids, and homology search showed that xDRR1 is the orthologous protein of human DRR1 (Fig. 1A). The two proteins share a conserved coiled domain and a signal peptide. The coiled-coil-containing structure suggests that they may interact with other proteins or DNA to regulate gene transcription and signal transduction [10].

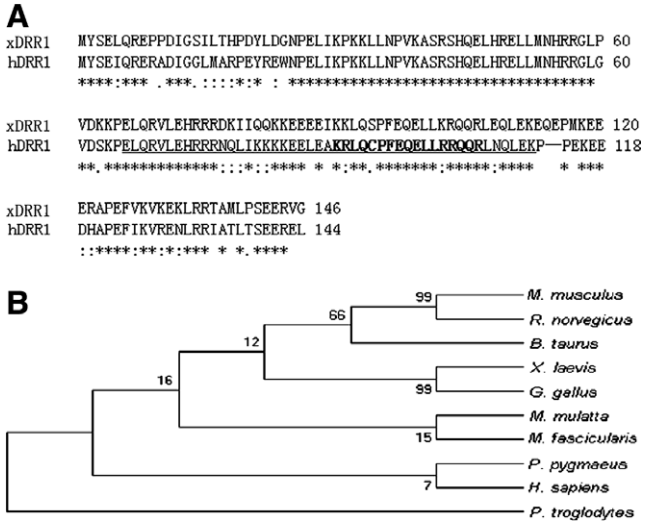

Fig. 1. Comparison of DRR1 proteins from *X. laevis* (GenBank protein_id: AAH82866) with those of other species. (A) xDRR1 and hDRR1 proteins (GenBank protein_id: NP_009108) are homologous proteins. There is a coiled domain (underlined) and a signal peptide (bold) in hDRR1 protein and xDRR1. Asterisks (*) indicate amino acid residues that are conserved across species. Colons (:) indicate strong similarity between protein xDRR1 and hDRR1. Dots (.) indicate weak similarity. (B) Phlogenetic tree of the DRR1 protein.

Comparing the sequence of xDRR1 protein with those of other species including *Gallus gallus* (GenBank protein_id: XP_425164), *Macaca mulatta* (GenBank protein_id: XP_001095875), *Pongo pygmaeus* (protein_id: CAI29620), *Homo sapiens* (protein_id: NP_009108), *Macaca fascicularis* (protein_id: BAE88114), *Bos taurus* (protein_id: NP_001076957), *Rattus norvegicus* (protein_id: NP_001020300), *Mus musculus* (protein_id: NP_899010), and *Pan troglodytes* (protein_id: XP_001174304), the identity is, respectively, 75%, 74%, 74%, 74%, 70%, 70%, 68%, 66%, and 56%. This confirms that their genes share a common ancestor. Based on the homology analysis, the phlogenetic tree of the DRR1 protein was summarized as Fig. 1B.

Cloning xDRR1 cDNA and recombinant plasmid identification

To obtain xDRR1 cDNA, RT-PCR was carried out with total RNA from *X. laevis* oocytes as the template using its forward and reverse primers containing restriction enzyme sites BamHI/XhoI or XhoI/BamHI. Two fragments were obtained, which were consistent with the xDRR1 cDNA of 441 bp in length.

DRR1 cDNA products were, respectively, inserted into the sites of BamHI and XhoI in the expression plasmid pEGFP-N1 or pcDNA3.1(+). The recombinant plasmids pEGFP-N1/xDRR1 and pcDNA3.1(+)/xDRR1 were confirmed correctly by restriction endonuclease digestion with BamHI/XhoI and DNA sequencing (data not shown).

xDRR1 expressing profiles in embryo development and tissues

To analyze the expression pattern of DRR1 in *X. laevis*, total RNA was isolated from different stages of embryo development and organs, and used as template for RT-PCR. PCR products of the expected size were obtained not only from embryos of stage 1, 6, 10, 13, 17, 22, 28, and 35 cells (Fig. 2A), but also from heart, liver, spleen, lung, kidney, brain, eye, and testis tissues (Fig. 2B) through analysis by relative quantitative program (Fig. 2C, D). The expression level of DRR1 gene increases gradually with embryo development in the early stages (Fig. 2A, C). Compared with other tissues, a relatively higher expression level of xDRR1 mRNA was found in brain and heart (Fig. 2D), which indicates that xDRR1 plays pivotal roles in *Xenopus* brain and heart development. The extensive temporal and tissue expression profiles of xDRR1 gene suggested that it may play an important role in *Xenopus* embryo development.

Whole-mount in situ hybridization showed that xDRR1 was mainly present along ectoderm and mesoderm (Fig. 2E). In later embryogenesis, xDRR1 can be detected in the head, heart, and somites in tail bud embryos (Fig. 2E, right), this was consistent with higher xDRR1 mRNA expression in brain and heart (Fig. 2D). Therefore, we speculate xDRR1 may relate with brain and/or heart development of *X. laevis*. In the following researches, we will study the phenotype during xDRR1 over-expression or suppressed expression in *Xenopus* embryo to learn about its role in brain and/or heart development.

Cellular localization of xDRR1 protein

As we know, confirming the cellular localization of proteins is particularly helpful for gene functional annotation. There is 74% identity between xDRR1 and human DRR1 protein in amino acid residues, and xDRR1 protein shares a highly conserved coiled domain and signal peptide with human DRR1 protein (Fig. 1A). It has been demonstrated that human DRR1 protein is indeed present in nucleus [1]. Therefore, we inferred that xDRR1 protein probably also located in nucleus. In order to validate our hypothesis, the recombinant eukaryotic expression vector pEGFP-N1/xDRR1 was used to produce the fusion protein containing EGFP epitope and xDRR1 protein. After pEGFP-N1/xDRR1 was transfected into A549 cells for 24 h, the fusion protein expression was detected and counted under fluorescence microscope. About 76% (16/21) of transfected cells showed strong green colors in nuclei (Fig. 3), demonstrating that xDRR1 protein is a nuclear protein.

Cell growth inhibition by xDRR1 in vitro

We have demonstrated that DRR1 expression was undetected in several other tumor cell lines as well as lung cancer cell line A549 (data not shown). At present, we compared cell proliferation after xDRR1 plasmids were transiently transfected into A549 cells for 48 h. The solution of 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was added into transfected A549 cells, then the cells were cultured for another 2 h. The absorbance was detected at 490 nm. Cell growth viability is shown in Fig. 4. Included in this figure are untransfected cells (control) and vector-transfected cells [pcDNA3.1(+)] as well as xDRR1-transfected cells [pcDNA3.1(+)/xDRR1]. Cell proliferation was analyzed by MTS assay. The result showed that transfection of xDRR1 into DRR1-negative cell line definitely resulted in growth suppression of cancer cells (Fig. 4). Re-expression of DRR1, no matter whether it comes from human or *X. laevis*, can inhibit cell growth in vitro, indicating that expression of DRR1 gene correlates definitely with the regulation of cell growth.

Conclusion

DRR1 protein is well conserved across species. Here we identified the DRR1 homolog from *X. laevis* based on human DRR1 protein. xDRR1 protein shows a high identity and similarity to human DRR1, indicating that there are some analogous functions of this protein in *X. laevis* cells. The xDRR1 gene is widely expressed during early

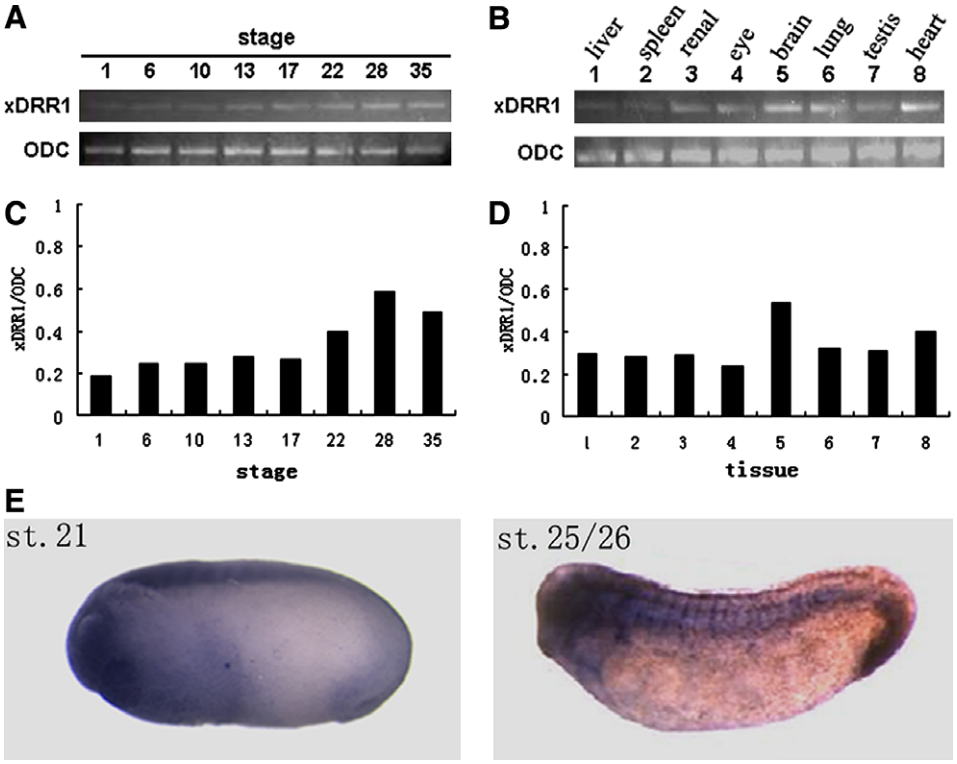

Fig. 2. Expression pattern of xDRR1 gene in developing embryos and tissues from an adult frog. (A) Temporal expression profile of the xDRR1 mRNA detected by RT-PCR. (B) Tissue expression profile of xDRR1. The relative expression ratio of xDRR1 was shown in *Xenopus* different developing stages (C) and several different organs (D). The ODC expression was taken as control. (E) Spatial expression of xDRR1 using whole-mount in situ hybridization analysis. Numbers indicate developmental stage. At stage 21, expression is mainly detectable in neuroectoderm. At stages 25/26, the transcripts are present in brain, heart, notochord, and somites.

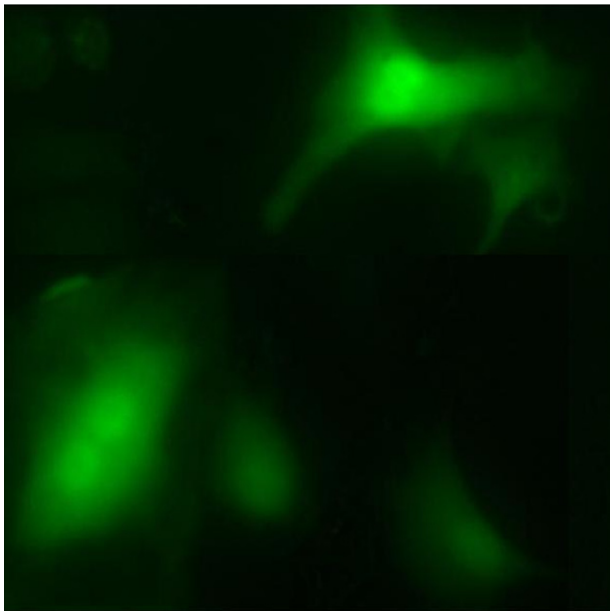

Fig. 3. Cellular localization of the xDRR 1 protein. The A549 cells were transfected with expression vector pEGFP-N1/xDRR1, which expresses a fusion protein consisting of DRR 1 and EGFP epitope, and then detected under fluorescence microscope (×200). Included in the picture are cells which show the fusion protein (strong green) in nuclei. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this article.

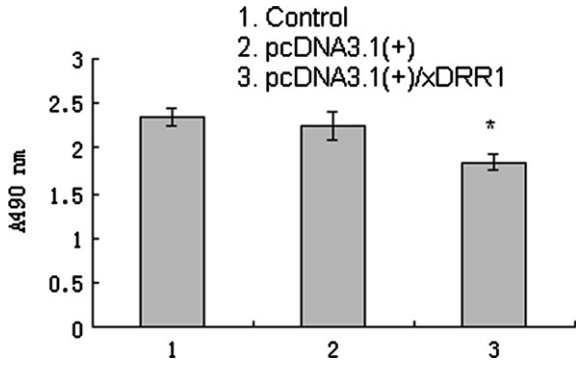

Fig. 4. Suppression of cell growth by re-expression of xDRR1 gene. The xDRR1 plasmids were transfected into A549 cells. At the indicated time points, the cells were subjected to proliferation analysis using MTS and the absorbance detected at 490 nm. The cells transfected with the xDRR 1 plasmids were suppressed. Each bar represents the mean ± standard deviation ($n = 6$). $^{*}P < 0.05$.

embryo development and in various organs. We also proved that xDRR1 is a nuclear protein. The xDRR1 protein, which contains a coiled domain like human DRR1 and can suppress cell proliferation *in vitro*, may interact with other proteins or DNA to regulate gene transcription and signal transduction, and thus it may play important roles in embryo development. It will be of

importance to confirm the relationship between xDRR1 and embryo development and its action mechanism in future studies.

Acknowledgments

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