

Bmi1a Gene Expression changes in Gamma-Irradiated Zebrafish Embryos

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Abstract: *Bmi1* is an oncogene, which occurs in almost all human cancer and 50% of all tumors exhibit variations in their gene expression level. To further assessment of *bmi1* gene pathway involving in cancer, we have exploited the zebrafish which is a powerful vertebrate model system used to analyze the expression level during embryogenesis.

Embryos were collected and treated with different doses of gamma radiation, for further observation. Zebrafish embryos were exposed to low dose rate of external gamma radiation from 5 to 25 Gy (with 5 interval dose rate) over a short period at 3hrs post fertilization with the objective of testing the appropriateness of 25 Gy guidelines as suggested by OECD. Total mRNA was isolated and cDNA was amplified with random primers by using Reverse Transcriptase PCR kit. Expression of two genes; BMI 1a & INK4A were quantified using specific Real Time PCR primers (q-PCR-Syber Green). qPCR showed decreased level at 10 Gy dose, increased expression level at 5 & 10 Gy irradiated embryos. Our present study examines different dose of radiation that enhances the embryonic resistance to particular dose in which *bmi1* expression level decreased when compared to control, 5 and 15 Gy. Lethality, mortality and morphological abnormalities were compared with *bmi1* expression level. Hence the present attempt to identify those radiosensitive pathways in the zebrafish would certainly help to choose the target genes.

Key words: BMI-1 - B – cell Moloney murine leukemia virus insertion region, CDKN2A - Cyclin-dependent kinase inhibitor 2A, PCR - Polymerase chain reaction, hpf - Hour post fertilization, Gy – Gray (radiation unit)

I. Introduction

Embryogenesis is a particularly radiosensitive stage of the vertebrate life cycle, and zebrafish embryos are ideal for evaluating genotoxic stress as well as radiation-related studies [27,13]. Zebrafish is a common freshwater aquarium fish belonging to the cyprinid family of teleost group. The Latin name for zebrafish is *Danio rerio*. It has short life cycle, transparent embryo, larvae, external development and reaching sexual maturity by three months. In the laboratory, zebrafish have a maximal recorded life-span of 5½ years, though an average of 3½ years has been reported [7]. The adult zebrafish can produce hundreds of offsprings per week and it is particularly notable for its regenerative ability to change their fins, skin and heart during larval stages. The development of the zebrafish is very similar to the embryogenesis of higher vertebrates, including that of humans. But unlike mammals, zebrafish develop from a fertilised egg to an adult outside the female in a transparent egg. This makes it possible to observe developing embryos in their natural environment. Moreover, the embryos themselves are transparent during the first few days of their lives. And the OECD guidelines (no.212; 210) currently propose that the early life stages of fish could be used as an experimental model to assess toxic effect. At the cellular level, some authors have assessed the effects of cell irradiation on survival (embryonic ZEB-J2 zebrafish cells) [21] or DNA damage (mutant R1C1 medaka; zebrafish cells and embryos) [10, 1]. Radiation – induced untargeted germline mutations have also been observed in the medaka for chronic exposure (dose rate of 68 mGy/d for 45-153 d) and for acute irradiations (dose ranging from 2- 10 Gy). Some studies relate to the transcriptional genomic instability by studying mutations in germ cells of medaka [28].

Functional motifs in oncogenes and tumor suppressor genes tend to be highly conserved among vertebrate species. Therefore the study of oncogenes and tumor suppressor genes in zebrafish will likely provide valuable insights into molecular mechanisms of tumorigenesis and genetic susceptibility to tumors in humans [5, 24].

BMI1 is a stem cell gene as defined by the fact that its deficiency leads to compromised adult stem cell function [19]. At first, it has been identified in the early 1990s as a component of a key insertion/activation region of the Moloney murine leukemia virus [2]. BMI1 encodes a 37 kDa polycomb group protein (PcG) from chromosome 10p11.23 in humans and 2 A3 in mice. This protein complex modulates chromatin structure and thereby regulates the transcription of a number of important genes, including the *Ink4a* locus which encodes two important tumor suppressor proteins; p16^{Ink4a} and p14^{Arf} [8, 22]. There are increasing evidences that

deregulated expression of PcG proteins contribute to cancer development. Aberrant over expression of PcG proteins, in particular BMI1 is associated with a number of human malignancies [16]. The zebrafish genome-sequencing project has been completed by Wellcome Trust Sanger Institute recently. Tubingen zebrafish reference strain was extensively used to identify mutations affecting embryogenesis.

II. Materials And Methods

Embryo Collection

Embryos were obtained from spawning 30 genitors (sex ratio: 2 female: 1 male fish). Each group of adult fish was placed in specific spawning aquaria to avoid egg predation. After spawning, egg viability was confirmed when the blastula stage (3 hpf) was reached. Embryos were then counted and randomly dispatched in each experimental unit. In each experimental unit, 50 embryos were collected in a petridish filled with 5ml of E3 medium. Five replicates per condition were then placed in the radiation chamber except control. Control and irradiated embryos were maintained under room temperature.

Exposure to Radiation

Cs-137 source from CERCA-LEA (Framatome ANP, Pierrelatte, France) and Gamma-rays were emitted from either a solution of Cs-137 (20 or 200 MBq) or solid ¹³⁷Cs line source (1.85 GBq). Nominal dose rates were 5, 10, 15, 20 and 25 Gy. Dose rates were measured using thermoluminescent dosimeter and represented on average 91% of the nominal values. The control incubation chamber was kept in a separate room. To maintain the radiation exposure duration, the effects-analysis was performed out of the incubation chamber which were carried-out in less than 1 h.

Endpoint Selection

Measured test responses included embryos and larval mortalities, hatching success and morphological development. Additionally, tail detachment, heartbeat, spontaneous movement and oedema were followed as described by Kimmel et al. (1996) [3]. These main endpoints were scored every hour upto 48 hrs after exposure.

Embryos RT-PCR Gene Expression

Normal developments of 50 embryos were maintained as control, which could be used for further analysis of developmental abnormalities measurements both morphologically and genetically. From treated embryos and untreated embryos, 10 embryos at each dose were used to isolate the total DNA using ALL PREP DNA/ RNA/protein Quiagen Kit. For isolation of *bmi1* and *ink4a* primer designed for specific sequence (Invitrogen) were *bmi1a* primer forward sequence 5'-3' AAAGCGACTCAGCCAGTGAT and reverse sequence GTCGATTTGGGCTTGTCATT with amplicon size of 159 between 60.2 and 59.94 melting temperature. *Ink4a/cdk2a* primer for forward sequence 5'-3' TTGGGCCAACCATGTATTTT and reverse primer CTTGCCCTGGTAATCAGCAT with amplicon size of 178 at melting point of 60.05-60 were analyzed using qPCR.

III. Results

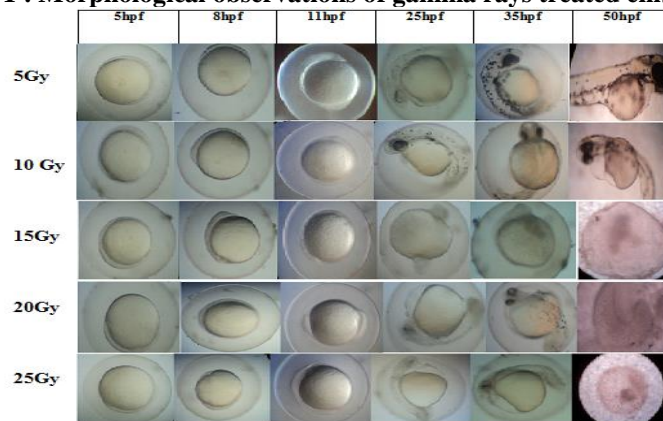
Egg production and collection

Transparent embryos were collected from single cell stage and clearly observed through microscope. Collected embryos were transferred to the clean glass petridish filled with E3 medium. Normal embryos treated with E3 medium as internal control which was closely compared with normal breeding tank water containing embryos.

Morphological observations of gamma rays treated embryos of zebrafish:

Treated embryos were observed at every one hour under compound microscope at 40X and morphological defects were identified. For the different dose experiments, 50 embryos per dose were separated and transferred to new plates with E3 media marked as A, B, C, D and E. where A, B, C, D, E were treated at 5Gy, 10Gy, 15Gy, 20Gy and 25Gy respectively. F was kept as control. No changes were observed in the morphology of embryos up to 11hour post fertilization (hpf) time in all embryos which were treated with different dose. The morphological abnormalities of embryos were observed at 25hpf in 10Gy, 15Gy, 20Gy and E 25Gy treated embryos. Minute level of abnormalities was observed in 5Gy treated embryos at 25hpf time.

Fig. 1 : Morphological observations of gamma rays treated embryos



After gamma radiation treatments the death rate of the embryos were examined. The death rate of embryos was directly proportional to gamma radiation dose. Besides, the gamma radiation dose was increased. The death rate of embryos also increased E, 25Gy of gamma radiation leading to increase of embryos death rate up to 100% within 36 hpf time. In D (20Gy) of gamma radiation 100% of embryos death was observed at 38 hpf time. Similarly, 100% death rate of embryos was observed at 50hpf time in C (15Gy) gamma radiation. 100% of embryos death was observed at 72 hpf of A (5Gy) and B (10Gy) gamma radiation. Low level of embryo death was observed in control (non-treated embryos) (14% of death at 72 hpf) (Fig.1).

Gene expression studies:

Treated and stored embryos were taken for gene expression level analysis by qRT-PCR with control (non-treated embryos). *BMI1a* and *inka/CDKN2A* expressions were analyzed. *BMI1a* gene expression was observed in different doses of gamma radiation. Three treated samples and one control sample were analyzed. High level of *BMI1a* gene expression was observed in B (15Gy) sample. There was no expression of *BMI1a* gene in F (non- treated) embryos (Fig.2).

Fig. 2 : Bmi1a gene expression

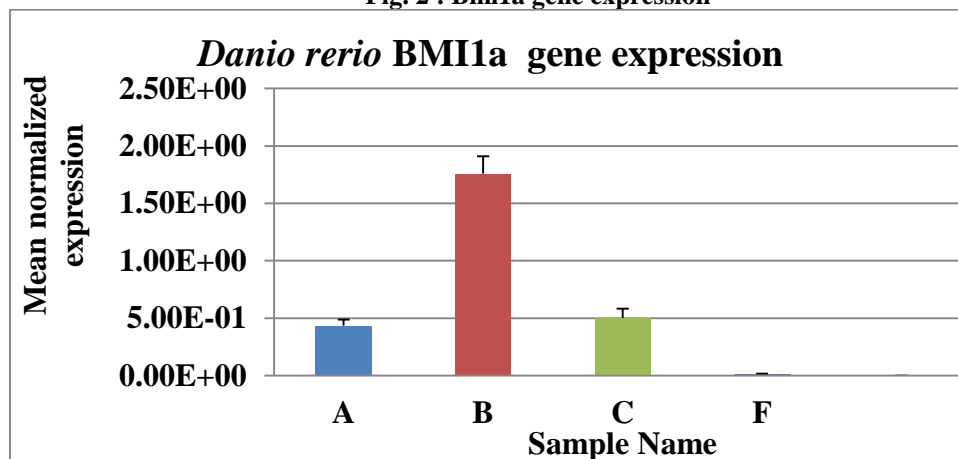
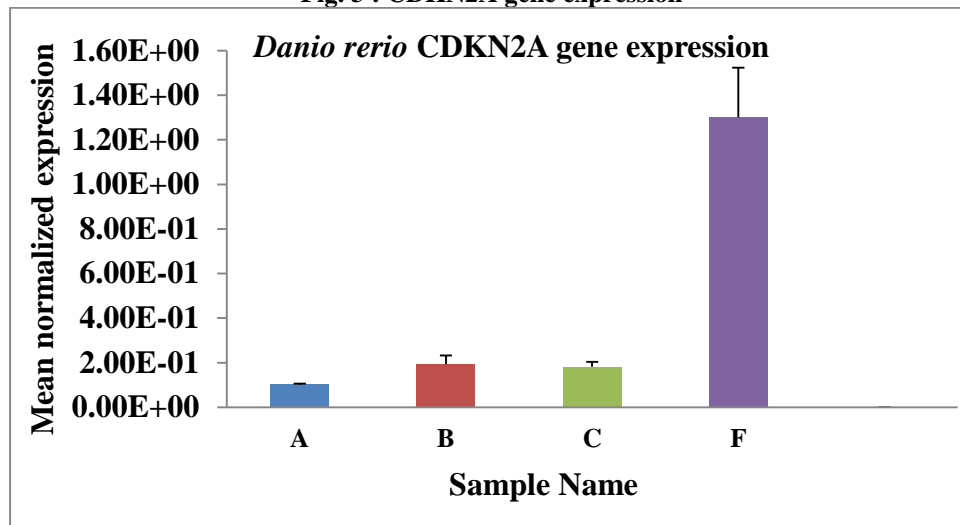


Fig. 3 : CDKN2A gene expression



CDKN2A/ink4a gene expression was observed in different doses of gamma radiation. Three treated samples and one control sample were analyzed. CDKN2A/ink4a gene expression was high in control (Fig.3). In A, B, C, almost similar level of expression was observed, comparing to control, A, B, C, had showed low level of expression.

Z-beta actin, CDKN2A and BMI-1a genes were amplified in qPCR. The amplicons were compared with marker when Z-beta actin (230 bp) was used as a control, CDKN2A (178 bp) and BMI-1a (159 bp) were observed in electrophoresis gel.

IV. Discussion

Zebrafish have provided many advantages over mouse models to study cancer self-renewal. For example, large numbers of zebrafish could be housed in a relatively small space, and husbandry costs are 20 times less when compared to mice. To discover novel radio protective genes using the zebrafish genetic model, it was sought to identify an obvious bright-field phenotype in embryos that distinguished different levels of apoptotic response to gamma rays. High doses of gamma rays, such as 25 Gy, were administered to a transparent zebrafish embryo at 24 hours post fertilization (hpf) that caused extensive apoptosis in neural tissue resulting in the accumulation of opaque tissue in the head. This phenotype was very consistent and readily observable by bright-field microscopy by six hours PF.

Radiated embryos were observed under compound microscope at 40X. Morphological defects were identified when compared to control. No changes were observed in the morphology of embryos up to 11 hour post fertilization (hpf) time in all embryos after treatment. The morphological abnormalities of embryos were observed at 25hpf in 10Gy, 15Gy, 20Gy and E 25Gy treated embryos. Abnormalities were observed in 5Gy treated embryos at 25hpf time. In our present study, eggs used in these experiments were aged at 3 hpf. They were maintained in the E3 medium, which was changed daily. They were kept at 28-30 °C, with a photoperiod of 14:10-h light:dark. For chronic exposure, five eggs were placed in glass petridish and five replicate petridish were used by condition. For acute exposure, 50 eggs were randomly distributed individually into each compartment of a Petri dish with two replicates per dose. The stages of embryo development were observed daily under a binocular microscope by observing the following specific endpoints: blastula (3 hpf), gastrula (4 hpf), segmentation (10–24 hpf) and pharyngeal (24–48 hpf). At 48 hpf the embryos started to hatch and the number of hatched eggs were recorded until 50 hpf (Organisation for Economic Co-operation and Development, Test Method 212). The hatching rate was determined as a percentage of hatched eggs from live eggs at a given time. The median hatching time (HT50), which represented the time necessary for 50% of the eggs to hatch, was then calculated.

Gamma-ray mutagenesis had particularly been used in screening for morphological defects to identify potentially interesting mutants that affect zebrafish embryogenesis [15]. The same results were observed after gamma radiation treatments and the death rate of the embryos were examined. The gamma radiation dose was directly proportional to death rate of embryos. The high dosage of gamma radiation led to apoptotic cell death in zebrafish. The similar results were observed that 25Gy of gamma radiation led to the increase of embryos death rate up to 100% within 36 hpf time. In D (20Gy) of gamma radiation 100% of embryos death was observed at 38 hpf time. Similarly, 100% death rate of embryos was observed at 50hpf time in C (15Gy) gamma radiation.

100% of embryos death was observed at 72 hpf of A (5Gy) and B (10Gy) gamma radiation. Low level of embryo death was observed in control (14% of death at 72 hpf).

Treated and stored embryos were taken for gene expression level analysis by qPCR with control. BMI1a and CDKN2A expressions were analyzed. BMI1a was a member of the Polycomb group (PcG) of genes, which had an essential role in embryogenesis and regulation of the cell cycle and lymphopoiesis. PcG genes were responsible for preservation of gene silencing, and were therefore essential for upholding cell identity. *Bmi-1*-deficient mice died by the time they had reached early adulthood with growth retardation, sign of hematopoietic failure, and neurological abnormalities. Consistent with this, *Bmi-1* was required for the postnatal maintenance of hematopoietic stem cells and neural stem cells from the central and peripheral nervous systems, but had little effect on their frequency in fetal tissues. BMI1a did not generically regulate proliferation as it was not required for the proliferation of at least some restricted neuronal or glial progenitors. BMI1a gene expression was observed at different doses of gamma radiation exposed to embryos of zebrafish. High level of BMI1a gene expression was observed in B (15Gy) sample when compared to control. It was confirmed by analyzing the gene expression using qPCR. Clinically, a growing list of human malignancies had been shown to express high levels of BMI1 including lymphoma, acute myeloid leukemia, colorectal carcinoma, liver carcinoma, non-small cell lung cancer, breast carcinoma, prostate cancer, head and neck cancer, medullo blastoma, and glioblastoma[9, 11, 17, 18 & 23]. Importantly, the elevated levels of BMI1 had been shown to have prognostic relevance in a number of tumor types. Chowdhury and co-workers [4] assessed the prognostic value of high BMI1 protein expression in 64 acute myeloid leukemia patients. The result also indicated that gamma radiation had induced the overexpression of BMI1a gene which caused the tumor in zebrafish embryo.

Jacobs [12] reported that the INK4a-ARF tumour suppressor locus was a critical downstream target of BMI1a. Specifically, BMI1a acted as a negative regulator of the INK4a-ARF locus, which encoded the two tumour suppressors p16INK4a and p14ARF (=human p19ARF). p16INK4a inhibited cell cycle progression by inhibiting cyclin D1-dependent kinases and thereby prevented the phosphorylation of the tumour suppressor Rb [26] whereas p14ARF prevented the degradation and inactivation of the tumour suppressor p53 by binding to mdm-2[20, 28, 25]. In the present study, CDKN2A (INK4a) gene expression was observed at different doses of gamma radiation. Three treated samples and one control sample were analyzed. The 10 Gy of gamma treated embryos showed high level of CDKN2A gene expression by qPCR. Threshold of IR exposure existed between 8 and 15 Gy in wild-type embryos that gave rise to the obvious opaque neural tissue phenotype[6]. The treated zebrafish embryos with different levels of gamma rays showed the opaque tissue in the head which was not observed using doses less than or equal to 8 Gy. Because, any mutation that inactivated a radioprotective gene could sensitize the embryonic neural tissue such that exposure of embryos to 8 Gy would cause a phenotype reminiscent of 15 Gy. Based on this logic, we performed a recessive genetic screen using 10 Gy IR and identified a number of mutations that sensitized embryos to IR. The tumor suppressor gene (CDKN2A) had been mutated in zebrafish and it is limited to the few well studied classic examples. Mouse knockout was generated and human hereditary diseases were known. Mouse *apc* ($\Delta 716$) knockouts were embryonic lethal in the homozygous state, similar to what they found in zebrafish [29].

The results obtained on acute gamma radiation suggested a dose dependent correlation between DNA damage accumulation and abnormalities in embryo development that led to apoptotic cell death in radiated zebrafish embryos due to abnormal expression of BMI1a and CDKN2A gene. ``

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