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In vivo DNA mismatch repair measurement in zebrafish embryos and its use in screening of environmental carcinogens

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HIGHLIGHTS

- We developed an *in vivo* DNA mismatch repair (MMR) measurement assay in zebrafish embryos.
- This assay involves microinjection of homo- and heteroduplex EGFP plasmids into zebrafish embryos.
- This novel assay was validated with embryos from the MMR-deficient *mlh1* mutant fish.
- We successfully applied this assay for detecting environmental chemicals with carcinogenic effect.
- This novel assay can be used for screening of environmental carcinogens.

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ABSTRACT

Impairment of DNA mismatch repair (MMR) function leads to the development and progression of certain cancers. Many environmental contaminants can target DNA MMR system. Currently, measurement of MMR activity is limited to *in vitro* or *in vivo* methods at the cell line level, and reports on measurement of MMR activity at the live organism level are lacking. Here, we report an efficient method to measure DNA MMR activity in zebrafish embryos. A G-T mismatch was introduced into enhanced green fluorescent protein (EGFP) gene. Repair of the G-T mismatch to G-C in the heteroduplex plasmid generates a functional EGFP expression. The heteroduplex plasmid and a similarly constructed homoduplex plasmid were injected in parallel into the same batch of embryos at 1-cell stage and EGFP expression in EGFP positive embryos was quantified at 24 h after injection. MMR efficiency was calculated as the total fluorescence intensity of embryos injected with the heteroduplex construct divided by that of embryos injected with the homoduplex construct. Our results showed 73% reduction of MMR activity in embryos derived from MMR-deficient *mlh1* mutant fish (positive control) when compared with embryos from MMR-competent wild type AB line fish, indicating feasibility of *in vivo* MMR activity measurement in zebrafish embryos. We further applied this novel assay for measurement of MMR efficiency in embryos exposed to environmental chemicals such as cadmium chloride ($CdCl_2$), benzo[a]pyrene (BaP), and perfluorooctanesulphonic acid (PFOS) from 6 hpf to 24 hpf. We observed significant reductions of MMR efficiency in embryos exposed to 0.1 μM $CdCl_2$ (52%) and 0.5 μM BaP (34%), but no effect in embryos exposed to PFOS. Our study for the first time provides a model system for *in vivo* measurement of DNA MMR activity at the organism level, which has important implications in risk assessment of various environmental carcinogens.

Introduction

The DNA mismatch repair (MMR) system widely exists in organisms from bacteria to mammals. It undertakes to correct single-strand DNA damages such as mismatch and insertion or deletion loops (IDLs) generated during DNA replication [1]. Besides MMR, single-strand DNA damages can also be repaired by other excision repair mechanisms such as the base excision repair (BER)

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and the nucleotide excision repair (NER). Although studies have shown that mismatches or IDLs can be repaired by BER or NER in MMR-deficient model system [2,3], these types of single-strand DNA damages are usually more efficiently repaired by MMR system because the nucleotides are not damaged or altered. In contrast, BER generally repairs damaged bases [4] and NER usually repairs bulky, helix-distorting damage such as pyrimidine dimerization caused by UV light [5]. MMR is a complex process in living organisms. In prokaryotes, especially in *Escherichia coli* [6,7], base mismatch or IDL mutations in DNA strands will be recognized by protein MutS, binding MutS to DNA mismatch site will lead to recruitment of other repair proteins, including MutL and MutH, further trigger a serial of enzymatic reaction resulting in repairing mismatch and IDL mutation in genomic DNA. MMR system in human appears more complicated than in *E. coli* [8,9]. Multiplex homologs of MutS and MutL have been identified and cloned in human. Three important MutS homologs are MSH2, MSH6 and MSH3. MSH2 can heterodimerize with either MSH6 or MSH3, forming two different protein complexes, called MutSα or MutSβ, respectively [10–12]. MutSα complex can recognize single base–base mispairs and IDL of 1–2 nucleotides whereas MutSβ complex primarily targets large IDL containing multiple extra nucleotides [9]. Several MutL homologs including MLH1, PMS1, PMS2 and MLH3 have been shown to form heterodimers, such as MLH1–PMS2 (MutLα), MLH1-PMS1 (MutLβ) and MLH1–MLH3 [13–15]. These MutL complexes may function through interacting with MSH2-containing complexes bound to mispaired bases [9], and induce MMR initiation and help to complete mismatch repair. MMR defects have been detected in many patients with colorectal [16], breast [17], lung [18], prostate [19], and gastric cancers [20]. Thus, it plays an important role in enhancing the fidelity of DNA replication and maintaining genome stability.

Currently, DNA MMR activity can be measured either *in vitro* or *in vivo*. The *in vitro* method involves incubating the cell-free extracts [21–24] or nuclear/cytoplasmic extracts with mismatched DNA substrates such as mismatched M13mp2 phage DNA, and subsequent measurement of MMR activity directly by endonuclease restriction analyses and/or DNA sequencing, or indirectly by further transforming MMR^- bacteria with repaired phage DNA mixture. The traditional *in vivo* method involves first introducing mismatch in heteroduplex DNA such as plasmids and viral DNA into cell and later on retrieving this introduced DNA for analysis [25,26]. However, the retrieval process is usually laborious and tedious. A more advanced *in vivo* method involves the introduction of a mismatch within the enhanced green fluorescent protein gene (EGFP) construct, allowing direct measurement of EGFP expression and fluorescent intensity by flow cytometer [27]. In our laboratory, we have recently improved the sensitivity and efficiency of this EGFP-based *in vivo* DNA MMR activity assay [28], and have also applied this assay for effective measurement of MMR efficiency in breast cancer cells exposed to environmental carcinogens [29]. However, all these *in vitro* and *in vivo* methods are limited to MMR quantification at the cellular level using cell line models. It is now widely recognized that the cell line model has the limitation of being too simple to mimic the complexity of live organisms, and thus DNA MMR activity at organism level can better predict the true effect of carcinogens on the MMR system. At present, *in vivo* measurement of MMR activity in live organisms has not been achieved.

Zebrafish, as a newly emerging animal model, has been widely used in various research fields including developmental biology [30,31], toxicology [31–33], drug discovery [34], human disease [35], and DNA damage and repair [36]. In comparison with rodent models, the zebrafish model has many advantages such as small size, high fecundity, and rapid embryonic development [31]. In particular, zebrafish exhibit external embryonic development, which allows *in vivo* manipulation of developing embryos (*e.g.*, microinjection). Further, embryonic zebrafish are optically transparent during early developmental stages, which allows for direct visualization of EGFP expression if any. In addition, expression of genes involved in MMR such as *msh6*, *msh2*, and *mlh1* has been detected in zebrafish embryos as early as 12 h post fertilization [37,38]. These unique advantages associated with embryonic zebrafish make it a perfect model for an EGFP-based *in vivo* MMR assay in live organisms. Our specific aims are to (1) establish an EGFP-based method for measurement of DNA MMR activity in live zebrafish embryos and (2) to validate the feasibility of this method to be used in risk assessment of various environmental contaminants. In particular, we first optimized the microinjection method of introducing the homo- and heteroduplex plasmids into the zebrafish embryos, and then validate this method by comparing MMR activity in embryos from MMR deficient *mlh1* mutant zebrafish with embryos from the wide type zebrafish, and finally we applied this assay to detect MMR efficiency in embryos exposed to various environmental carcinogens.

Materials and methods

Two strains of zebrafish were used in this study: wild-type AB line and *mlh1* mutant line. *Mlh1* mutant strain was produced by ENU mutagenesis [39]. There is a premature stop codon at the exon 10 of *mlh1* gene in this strain. Therefore, the *mlh1* mutant strain cannot express Mlh1 protein, and has reduced DNA MMR efficiency. We thus use this strain as our positive control. We obtained $mlh1^{+/-}$ embryos from Prof. Edwin Cuppen at the University of Utrecht (Netherlands) and screened homozygous mutant zebrafish by sequencing according to a previous report [39]. Because male homozygous mutant zebrafish is sterile, we use female homozygous mutant zebrafish crossing with male heterozygous mutant zebrafish to produce embryos. All fish were raised and kept at standard laboratory conditions of 28 °C with a 14:10 light/dark photoperiod in a recirculation system according to standard zebrafish breeding protocols [40]. Water supplied to the system was filtered by reverse osmosis (pH 7.0–7.5). Instant Ocean® salt was added to the water to raise the conductivity to 450–1000 μS/cm. The adult fish were fed twice daily with live artemia (Jiahong Feed Co., Tianjin, China) and dry flake diet (Zeigler, Aquatic Habitats, Apopka Florida). Fish care was in accordance with the approved Institutional Animal Care and Use Committee protocols at Wenzhou Medical University, China.

Preparation of nicked homoduplex and nicked heteroduplex

Nicked homoduplex and heteroduplex plasmids were prepared based on our previous method [28] with minor modification (see Fig. S1 for diagram). In brief, pGEM5Z(+)–EGFP (also named p111) was used to generate single-strand DNA (ssDNA) containing a coding strand of EGFP with a helper phage according to manufacturer's instruction. The ssDNA was then annealed with restriction enzyme MluI linearized p111 in 1.5-fold excess molar ratio to generate a homoduplex plasmid with a nick in the EGFP template strand located 688 bp 3′ upstream from start codon. To generate a nicked heteroduplex, we first introduce a premature stop codon (TGG58-TAG) to the EGFP gene of the p111 (termed as p189), and subsequently anneal the ssDNA from p111 with MluI-linearized p189. Thus this heteroduplex plasmid will have a G-T mismatch and a nick located 864 bp 3′ upstream from mismatch. A G-T mismatch has been proven by earlier studies as an optimal substrate for the MMR system [41–43], and a nick is introduced to serve as strand discrimination signal [41]. After annealing, the mixture was subjected to plasmid-safe ATP-dependent DNase (PSAD) digestion from Epicenter to eliminate redundant ssDNA and lin-