A Convenient Fluorescent-labeled Assay for *in vitro* Measurement of DNA Mismatch Repair Activity

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Objective The assay of DNA mismatch repair (MMR) activity can be used as a biomarker for environmental condition detection and human disease diagnosis. Radioactive ^{32}P-endlabeled DNA containing mismatch is extensively used as the substrate for MMR activity analyses. The aim of the present study is to develop a simple non-radioactive, but equally specific and sensitive method for the MMR activity assay. **Methods** A fluorescent label was chosen to replace the radioactive isotope label. Sensitive evaluation of the fluorescent label was carried out for the first time, and then the fluorescent label was compared with the isotope label in the MMR activity and DNA binding assays. **Result** LOD (limit of detection) of the fluorescent label was about 0.1 fmol and the relative signal strength displayed a pretty good linear relationship. Moreover, the fluorescent label method has equivalent sensitivity and performance as compared with the classical radioactive method in experiments. **Conclusion** In light of the sensitivity, reproducibility, safety, rapidity and long lifespan of the fluorescent label, this improved method can be applied to evaluation of biologic and toxic effects of environmental pollutants on man and other forms of life.

Key words: Fluorescent label; MMR activity; EMSA; TDG

INTRODUCTION

With fast economic development and industrialization, a wide range of synthetic chemicals have been produced and distributed in our living environment. A number of factors, including radiations[1], heavy metals[2], polycyclic aromatic hydrocarbon (PAH)[3] and other genotoxic chemicals[4-5] can affect the genetic material DNA of organisms directly or indirectly. *In vivo*, hydrolytic reactions that can cause mismatch occur at a significant rate and include the deamination of DNA bases with exocylic amino groups, i.e. cytosine (C) and 5-methylcytosine (5-meC), adenine (A) and guanine (G)[6]; ROS (reactive oxygen species) is constantly produced from mitochondrial respiration, inflammation, biotransformation and other metabolic processes[7]. These exogenous and endogenous factors frequently lead to various types of DNA damage/mismatch and further serious diseases in human beings. Fortunately, all organisms have evolved repair enzymes that will repair these damaged/mismatched DNA bases. DNA repair plays a major role in suppressing the rate of accumulation of mutations and avoiding the process of carcinogenesis[8]. Although detection of DNA damage can be used as an effective and powerful biomarker for monitoring and recognizing the genotoxicity of pollutants in environment[8-11], the capability of DNA damage/mismatch repair is more informative for human disease diagnosis and prevention[12]. The change in expression of MMR genes has been used as a sensitive biomarker for oxidative DNA damage[13-14]. The MMR activity should serve as a better biomarker for biological status of the body.

The single cell gel electrophoresis assay (Comet assay) is a technique for detection of DNA breakage damage or repair [15-16]. However, for the analysis of the DNA MMR activity, nuclear extracts or purified proteins, as a routine approach, are incubated with DNA substrates containing mismatches *in vitro*, and the ratio of cleavage products to intact DNA

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substrates is used to evaluate the MMR activity[10, 14, 17-20]. DNA substrates which are end-labeled with radioactive ^{32}P are a standard approach in these experiments[17-19]. An isotope label is sensitive, reliable and practicable; nevertheless, it requires special or strict laboratory procedures to handle hazardous radioisotopes. Moreover, attenuation of isotope is inevitable, which may possibly influence experimental repeatability; as a result, the radio-labeled method requires frequent isotope re-purchasing, substrate re-labeling and radioactive waste disposal. The aim of the present study is to develop a simple non-radioactive, but equally specific and sensitive method for the MMR activity assay. We chose a fluorescent label as a substitute for an isotope label to assay the DNA MMR activity. G:T mismatch arising from deamination of 5-methylcytosine by spontaneous hydrolysis is a common type of DNA mismatch, and thymine DNA glycosylase (TDG) is an important protein to repair G:T mismatch. Therefore, we conducted DNA MMR activity experiments with G:T mismatch and TDG, and found that the fluorescent label, while having equal sensitivity and reproducibility as the isotope label, was superior to the latter for its safety, convenience, rapidity and friendliness to environment.

MATERIALS AND METHODS

Expression and Purification of TDG Fusion Protein

Two primers, TDG-*Nhe*I and TDG-*Sal* I (Table 1), were designed to amplify human TDG in order to construct pET23a-TDG. The PCR reaction conditions: 1×Pyrobest buffer, 40 nmol dNTPs, 20 pmol of each primer, 20 ng human cDNA template and 1.5 U pyrobest DNA polymerase (TaKaRa, Japan) in a final volume of 50 μL. The PCR production was digested by using *Nhe*I/*Sal*I and inserted into the *Nhe*I and *Xho*I (the same sticky ends produced with *Sal*I and *Xho*I digestion) fragment of pET23a vector (Novagen, USA), and then the positive clone was confirmed by sequence. The constructed plasmid was transfected into *Escherichia coli* BL21 (DE3) cells (Novagen, USA). Expression and purification of TDG was performed as described[17].

MMR Activity Assay in Vitro

The DNA substrate for the MMR activity assay is a 44-mer duplex containing a G/T mismatch (Table 1). ^{32}P-end-labeled and X-rhodamine (ROX)-end-labeled DNA substrates were used to assess the MMR activity. The oligonucleotide strand containing mispaired T (T-44, Table 1) was labeled at the 5′ end with [γ-^{32}P]ATP by T4 Polynucleotide Kinase (TaKaRa, Japan) according to instructions, while during oligonucleotide synthesis, ROX was directly covalently linked to the 5′ end of oligonucleotide. The labeled strands were annealed with G-44 (Table 1) in annealing buffer (10 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA, 100 mmol/L NaCl) at 90 ℃ for 5 min and then cooled gradually to room temperature over 2 h to form heteroduplexes. For the fluorescent label, the DNA fragments should be protected from exposure to light.

The MMR activity assay was performed as described by GUAN[19]. The difference between the radio- or fluorescence- labeling methodologies was that xylene cyanol and bromophenol blue should be excluded from loading buffer for fluorescence-labeled probes. After separation on 14% polyacrylamide sequencing gel containing 7 mol/L urea, the ^{32}P images were viewed on a phosphorimader and quantified by using Tyhoon 9 410 workstation (Amersham, USA), while the fluorescent image was acquired directly from the polyacrylamide gel by using Tyhoon 9410 workstation with emission filter 610BP30 and 532 nm excitation. Considering the fact that the isotope label is a mature method, the assay with the isotope label was carried out only once, while assays with the fluorescent label were repeatedly performed at least for 3 times.

DNA Binding Assay of TDG

The binding of TDG and mismatch oligonucleotides was assayed by the Electromobility

TABLE 1

Oligonucleotides Used in This Study

Name	Sequence	Purpose
TDG-*Nhe*I	5′-ctagctagcatggaagcggagaacgcgggca-3′	Primers for pET23a-TDG
TDG-*Sal*I	5′-acgcgtcgacagcatggctttcttcttcctg-3′	
T-44	5′-aattgggctcctcgaggaattTgccttctgcaggcatgccccgg-3′	Sense
T-44-Rox	5′-Rox-aattgggctcctcgaggaattTgccttctgcaggcatgccccgg-3′	Sense
G-44	5′-ccggggcatgcctgcagaaggcGaattcctcgaggagcccaatt-3′	Antisense

Shift Analysis (EMSA). The EMSA assay was performed in a 20 μL system containing 10 mmol/L Tris-HCl pH7.5, 0.5 mmol/L EDTA, 0.5 mmol/L DTT, 1 mmol/L $MgCl_2$, 1.25 μg/μL BSA, 50 mmol/L NaCl, 4% Glycerol, 0.5 μg poly (dI-dC): poly (dI-dC), purified TDG and DNA substrates. DNA substrates were end-labeled with [γ-^{32}P] ATP or ROX. Binding reactions were kept on ice for 1h and then the 15 μL complexes were separated on 4.5% native polyacrylamide gels. The gel images were detected and recorded with Tyhoon 9 410 workstation as above.

RESULTS

Fluorescent Label Method Shows Excellent Detection Sensitivity

The aim of the present study is to develop a simple non-radioactive, but equally specific and sensitive method for assaying the MMR activity and DNA binding. We chose a fluorescent label to replace the isotope label, and we carried out for the first time sensitive evaluation by gradient dilution to confirm ROX labeling sensitivity. The results showed that its LOD (limit of detection) was about 0.1 fmol and it could be clearly detected at about 1.2 fmol (Fig. 1A). Moreover, the relative signal strength displayed a pretty well linear relationship within the range of detection with ROX-end-labeled DNA (Fig. 1B).

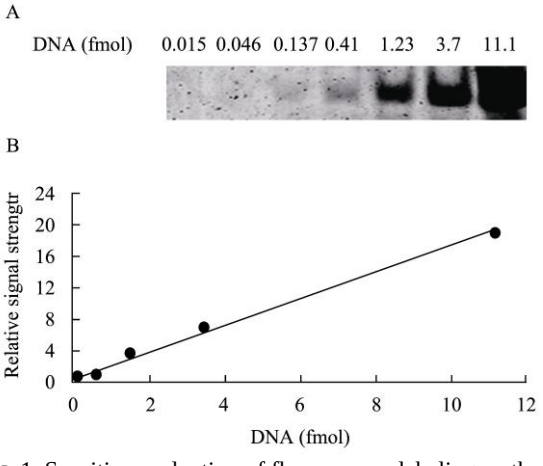

FIG. 1. Sensitive evaluation of fluorescence labeling method. A. ROX-end-labeled oligonucleotide was gradiently diluted and directly loaded onto 4.5% non-denaturing polyacrylamide gels (PAGE); B. Quantitative analysis of PAGE results in 2A.

Fluorescent Label Achieves the Same Effect as Isotope Label in both MMR Activity and DNA Binding Assays

The classical method for the MMR activity assay *in vitro* involves end-labeling of the substrate with isotope. Nuclear extracts or purified proteins are incubated with isotope-end-labeled DNA substrates containing mismatch at 37 ℃ to produce abasic sites (AP sites), and then the mixture is incubated with 0.1 N NaOH at 90 ℃ for 30 min to break the DNA strand containing AP sites. The cleavage products are separated from intact DNA substrates by using the polyacrylamide DNA sequencing gel. By detecting the ratio of cleavage products to intact DNA substrates, it is easy to determine the MMR activity.

In order to evaluate the feasibility of the fluorescent label in the MMR activity assay and DNA binding, we first purified TDG-His fusion protein. As N-terminus of TDG is the DNA binding domain[18], in order to avoid interrupting the DNA binding ability, we used pET23a vector to purify TDG-His protein so that a 6×His tag was placed at the C-termius of TDG. A denaturing SDS-polyacrylamide gel analysis of the purified protein with Coomassie Brillant Blue staining showed a unique band with the expected molecular weight (Fig. 2).

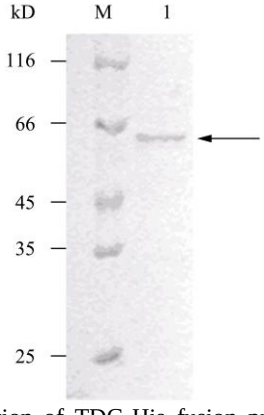

FIG. 2. Purification of TDG-His fusion protein. Lane M, Marker, Lane 1, TDG-His fusion protein.

ROX-end-labeled substrate and ^{32}P-end-labeled substrate were used to assay the MMR activity in vitro. Compared to the ^{32}P label (Fig. 3A & 3C), ROX label worked excellently with the same sensitivity and performance (Fig. 3B & 3D). To confirm the potential application of the fluorescence labeling method for the analysis of DNA binding, this method was further expanded to the Electromobility Shift Analysis (EMSA) experiment. The DNA binding assay of purified TDG was performed by using the ^{32}P-end-labeled or ROX-end-labeled DNA substrate. Both of the labels showed the homogenous DNA-binding band without any background (Fig. 4A & 4B). These results mean that the fluorescence labeling method could be applied to the analysis of the MMR activity or DNA binding.

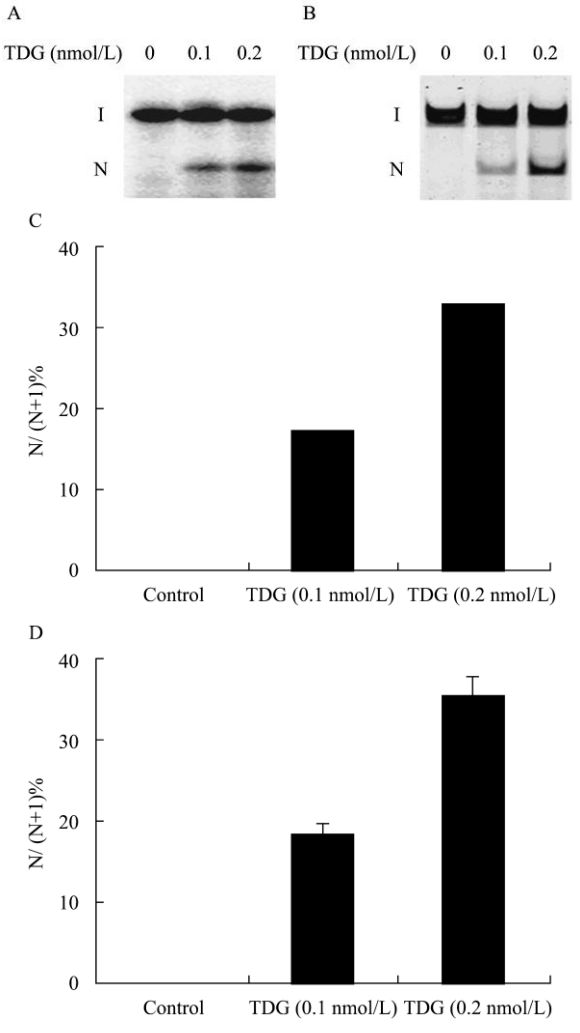

FIG. 3. TDG activity assay in vitro using ^{32}P-end-labeled or ROX-end-labeled DNA substrates. A. TDG activity assay using ^{32}P-end-labeled DNA substrate; B.TDG activity assay using ROX-end-labeled DNA substrate. Each lane contains 3 fmol DNA substrate in 3A and 3B. I and N mark the intact DNA substrate (I) and the cleavage product (N), respectively; C&D. Quantitative analysis of TDG activity assay in 3A and 3B.

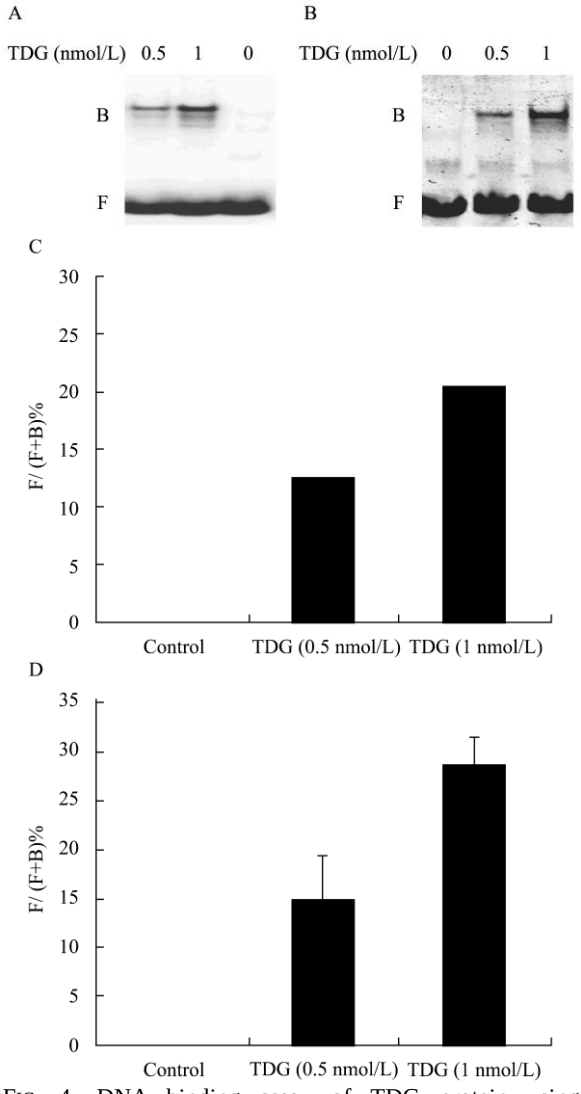

FIG. 4. DNA binding assay of TDG protein using ^{32}P-end-labeled and ROX-end-labeled DNA substrates. A. ^{32}P-end-labeled DNA binding assay of TDG protein; B. ROX-end-labeled DNA binding assay of TDG protein. Each lane contains 3 fmol of ROX-end-labeled DNA substrate in 4A and 4B. B and F mark the binding DNA substrate (B) and the free probe (F), respectively; C&D. Quantitative analysis of DNA binding assay of TDG protein in 4A and 4B.

DISCUSSION

Environmental pollution would affect people's health and lead to genetic changes in organisms, and people around the world are now increasingly concerned with the environment. Biomarker is the most direct and conspicuous evidence to detect environmental pollution. The assay of DNA damage is a good biomarker that has been used, and the capability of DNA damage/mismatch repair is an excellent indicator for human disease diagnosis and generate DNA damage in mammalian cells. DNA damage includes DNA breakages, mismatches, chemical modifications of the bases or sugars and inter- or intrastrand cross-links[21-24]. To deal with DNA lesions, a multitude of repair systems have evolved, including BER, ligation, NER (nucleotide excision repair) and MMR, which have overlapping specificity and may interact or function as back-up systems[25]. There is compelling evidence demonstrating that defects in DNA repair will cause high predisposition to several hereditary cancers[12, 25-27].

Therefore, variations in DNA repair are likely to play an important role in determining cancer risk. Biomonitoring organisms have long been used as a means of warning people to take necessary prevention[12].

DNA breakage and repair can be assessed by the comet assay, but for the analysis of the DNA MMR activity, the radioactive label may pose a potential risk in its extensive use. Consequently, it is of prime importance to avoid radioactive hazards of isotope by looking for a more rapid and safe method to assess the MMR activity. To this end, in the present study we chose a fluorescent label as a substitute for the isotope label. The fluorescent label shows the same sensitivity as the isotope label and its relative signal strength exhibits a good linear relationship within the range of detection. These characters are excellent and sufficient for application.

Both fluorescence-labeled and isotope-labeled substrates were used for the MMR activity assay and the results of the assay by using the two substrates were of similar quality, reproducibility, and sensitivity (Fig. 3A & 3B). However, it took 1-2 days for the isotope labeling method to assay the TDG activity from reaction labeling to final result acquisition, whereas it took only 2-3 h when the fluorescence labeling method was used. The fluorescence labeling method could save the time of reaction labeling and avoid exposure to phosphorimaging. Being harmless and free from radio-waste disposal, it is convenient and rapid in operation. Moreover, in the process of fluorescence labeling, no attenuation or decay took place, and it was stable in terms of the repeatability of the assays. The fluorescence labeling method was further applied to the EMSA experiment to assay DNA binding, suggesting that the fluorescent label and isotope label were comparable to supershift experiments.

In conclusion, we developed a better method to assay the MMR activity *in vitro* with a fluorescent label instead of an isotope label. In light of the safety, rapidity and long lifespan of a fluorescent label, this improved method can be applied to evaluation of biologic and toxic effects of environmental pollutants on man and other forms of life.

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