Free Radical Biology and Medicine 69 (2014) 86–95

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

A dual-fluorescent reporter facilitates identification of thiol compounds that suppress microsatellite instability induced by oxidative stress

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ARTICLE INFO

Article history:
Received 20 September 2013
Received in revised form
13 December 2013
Accepted 19 December 2013
Available online 8 January 2014

Keywords:
Colorectal cancer
DNA mismatch repair
Dual-fluorescent reporter
Glutathione
Inflammation
Microsatellite instability
N-acetylcysteine
Oxidative stress
Free radicals

ABSTRACT

The DNA mismatch-repair (MMR) system corrects replicative errors and minimizes mutations that occur at a high rate in microsatellites. Patients with chronic inflammation or inflammation-associated cancer display microsatellite instability (MSI), indicating a possible MMR inactivation. In fact, H_2O_2–generated oxidative stress inactivates the MMR function and increases mutation accumulation in a reporter microsatellite. However, it remains unclear whether MSI induced by oxidative stress is preventable because of the lack of a sufficiently sensitive detection assay. Here, we developed and characterized a dual-fluorescent system, utilizing DsRed harboring the $(CA)_{13}$ microsatellite as a reporter and GFP for normalization, in near-isogenic human colorectal cancer cell lines. Via flow cytometry, this reporter sensitively detected H_2O_2–generated oxidative microsatellite mutations in a dose-dependent manner. The reporter further revealed that glutathione or *N*-acetylcysteine was better than aspirin and ascorbic acid for suppressing oxidative microsatellite mutations. These two thiol compounds also partially suppressed oxidative frameshift mutations in the coding microsatellites of the *hMSH6* and *CHK1* genes based on a fluoresceinated PCR-based assay. MSI suppression by *N*-acetylcysteine appears to be mediated through reduction of oxidative frameshift mutations in the coding microsatellite of *hMSH6* and protection of hMSH6 and other MMR protein levels from being decreased by H_2O_2. Our findings suggest a linkage between oxidative damage, MMR deficiency, and MSI. The two thiol compounds are potentially valuable for preventing inflammation-associated MSI. The dual-fluorescent reporter with improved features will facilitate identification of additional compounds that modulate MSI, which is relevant to cancer initiation and progression.

The DNA mismatch-repair (MMR) system maintains genomic stability by correcting mismatches and deletions/insertions that frequently occur in microsatellites during DNA replication [1]. In the human MMR system, hMutSα (hMSH2 and hMSH6) and hMutSβ (hMSH2 and hMSH3) complexes preferentially recognize base–base mismatches and insertion–deletion loops, respectively, and recruit the hMutLα (hMLH1 and hPMS2) complex to initiate the DNA repair process [1]. MMR also minimizes mutations generated by DNA-damaging agents such as reactive oxygen species (ROS). The MutSα complex recognizes and removes 8-oxoguanine, a major oxidized base that often mispairs with adenine [2,3].

The MMR function can be inactivated by genetic and epigenetic alterations. Germ-line mutations of *hMSH2* or *hMLH1* genes [4,5] occur in Lynch syndrome [6,7], and the *hMLH1* promoter is hypermethylated in 15–25% of sporadic cancers, including colorectal and endometrial tumors [5,8,9]. The MMR function can also be inactivated by oxidative stress in human cells via reduced steady-state levels of MMR proteins such as hMSH6 [10].

Approximately a million microsatellite loci, mostly as $(CA)_n$, are dispersed in the introns and exons of the human genome. Because of the nature of short tandem repeats, microsatellites are prone to replication slippage [1,11]. When MMR is inactivated or deficient, unrepaired replicative errors result in microsatellite instability (MSI). At least 40 genes containing microsatellites in their coding

Abbreviations: BER, base excision repair; EV, electronic volume; GSH, glutathione; HPRT, hypoxanthine–guanine phosphoribosyl transferase; MMR, mismatch repair; MSI, microsatellite instability; NAC, *N*-acetylcysteine; NSAID, nonsteroidal anti-inflammatory drug; 8-oxoG, 8-oxoguanine; ROS, reactive oxygen species; SS, side scatter

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http://dx.doi.org/10.1016/j.freeradbiomed.2013.12.019

regions are frequently mutated in MMR-deficient colorectal tumors [12]. These genes participate in key biological pathways such as DNA repair (e.g., *hMSH3, hMSH6*), apoptosis (e.g., *BAX, caspase 5*), signal transduction (e.g., *TGFβ2R, IGF2R*), and DNA-damage response (e.g., *CHK1, MRE11A*) [12]. MMR deficiency increases frameshift mutations of coding microsatellites leading to the loss of protein functions [13]. These secondary mutations are believed to contribute to cancer pathogenesis and drug resistance [8,14].

Oxidative stress occurs in patients with chronic and cancer-associated inflammation [15] when ROS exceed the capacity of the antioxidant defense system [16]. Inflammation-associated oxidative stress has been linked to microsatellite instability. In 13–50% of ulcerative colitis patients, MSI is found in colonic mucosa negative for dysplasia [17–20]. MSI is also detected in patients with other inflammatory diseases such as rheumatoid arthritis and pancreatitis [21,22]. Because viral infection generates oxidative stress, MSI has been reported in patients with *Helicobacter pylori* infection and in human PH5CH8 hepatocytes challenged with hepatitis C virus core protein [23–25]. Moreover, MSI is detected in ulcerative colitis-associated colon cancer and T-lymphocyte-infiltrated colon cancer [26–30].

Previously we reported that hydrogen peroxide (H_2O_2)-generated oxidative stress inactivates the MMR function [10] while increasing frameshift mutations of a reporter microsatellite [31]. However, it is largely unknown whether inflammation-associated MSI is preventable because of the lack of a sufficiently sensitive detection assay. In this study, we therefore developed and characterized a dual-fluorescent reporter in a human colorectal cancer cell model, consisting of MMR-deficient HCT116 and MMR-proficient HCT116+ch3 cell lines [32]. This reporter was used to facilitate identification of compounds that suppress MSI induced by oxidative stress.

Materials and methods

Chemicals and reagents

H_2O_2, methotrexate, L-ascorbic acid, *N*-acetyl-L-cysteine (NAC), L-glutathione (GSH; reduced form), and acetylsalicylic acid (aspirin) were from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium with F-12 nutrient mixture (DMEM/F-12) and fetal bovine serum (FBS) were from Hyclone (Logan, UT, USA). Trypsin, L-glutamine, G418, hygromycin, and Lipofectamine 2000 were from Invitrogen (Grand Island, NY, USA). Restriction enzymes were from New England Biolabs (Ipswich, MA, USA) and EasyPure Genomic DNA mini-kit was from Bioman Scientific (Taipei, Taiwan). Oligonucleotides and primers were synthesized by Invitrogen.

Construction of dual-fluorescent reporters

The pDsRed-IRES-GFP dual-fluorescent vector (GenBank Accession No. JX088702) was generated by inserting DsRed cDNA amplified from the pDsRed1-N1 plasmid (Clontech, Mountain View, CA, USA) into pIRES-hrGFP-1a (Stratagene, La Jolla, CA, USA) via SacII and NotI sites. In pDsRed-IRES-GFP, oligonucleotides containing ATG-$(CA)_{13}$ or ATG-$(N)_{16}$ (Table 1) were inserted upstream of the start codon of DsRed via SacI and AgeI sites, resulting in p$(CA)_{13}$DsRed-IRES-GFP (GenBank Accession No. JX088703) and p$(N)_{16}$DsRed-IRES-GFP (GenBank Accession No. JX088704). The hygromycin resistance

Table 1
Oligonucleotides and primers used in this study.

Oligo	GDB No.	Sequence	Size of PCR product	Microsatellite
Oligos for cloning				
ATG-$(CA)_{13}$		5′-CTGgagctc**ATG**CACACACACACACACACACACACAGTACGCGTACCGGTCGC		$(CA)_{13}$
ATG-$(N)_{16}$		5′-CTGgagctc**ATG**GATATCATTACTAGTAACCGGTCGC		
Primers for PCR and DNA sequencing				
DsRed-F		5′-GTTTTGGCAGTACATCAATGG		
DsRed-R		5′-GTCCTTATCATCGTCGTCTT		
Primers for the fluoresceinated PCR-based assay				
BAT25-F	9834508	5′-(***Hex**)TCGCCTCCAAGAATGTAAGT	124 bp	TTTT.T.TTTT.$(T)_7$.A$(T)_{25}$
BAT25-R		5′-TCTGCATTTTAACTATGCCTC		
BAT26-F	9834505	5′-(***Tet**)TGACTACTTTTGACTTCAGCC	122 bp	$(T)_5$…$(A)_{26}$
BAT26-R		5′-AACCATTCAACATTTTTAACCC		
D17S250-F	177030	5′-(***Fam**)GGAAGAATCAAATAGACAAT	150 bp	$(TA)_7$…$(CA)_{24}$
D17S250-R		5′-GCTGGCCATATATATATTTAAACC		
D2S123-F	187953	5′-(***Tet**)AAACAGGATGCCTGCCTTTA	220 bp	$(CA)_{13}$TA(CA)(T/GA)$_7$
D2S123-R		5′-GGACTTTCCACCTATGGGAC		
D5S346-F	181171	5′-(***Fam**)ACTCACTCTAGTGATAAATCG	120 bp	$(CA)_{26}$
D5S346-R		5′-AGCAGATAAGACAGTATTACTAGTT		
$(CA)_{13}$-F		5′-(***Tet**)CGCCAAGCTCGAAATTAACCCTCACT	164 bp	$(CA)_{13}$
$(CA)_{13}$-R		3′-GTGCCCTCCATGCGCACCTT		
hMSH3-F	641986	5′-(***Fam**)AGATGTGAATCCCCTAATCAAGC	153 bp	$(A)_8$
hMSH3-R		5′-ACTCCCACAATGCCAATAAAAAT		
BAX-F	22808	5′-(***Fam**)ATCCAGGATCGAGCAGGGCG	94 bp	$(G)_8$
BAX-R		5′-ACTCGCTCAGCTTCTTGGTG		
Caspase-5-F	3908461	5′-(***Hex**)CAGAGTTATGTCTTAGGTGAAGG	141 bp	$(A)_{10}$
Caspase-5-R		5′-ACCATGAAGAACATCTTTGCCCAG		
CHK1-F	9834732	5′-(***Fam**)CTCGCTGGAGAATTGCCA	93 bp	$(A)_9$
CHK1-R		5′-TTTCCAAGGGTTGAGGTA		
TGFB2R-F	224909	5′-(***Tet**)GCTGCTTCTCCAAAGTGCAT	149 bp	$(A)_{10}$
TGFB2R-R		5′-CAGATCTCAGGTCCCACACC		
hMSH6-F	632803	5′-(***Hex**)GGGTGATGGTCCTATGTGTC	94 bp	$(G)_8$
hMSH6-R		5′-CGTAATGCAAGGATGGCGT		

For cloning, only sense strands of ATG$(CA)_{13}$ and ATG$(N)_{16}$ oligonucleotides are shown, in which the SacI site is underlined and in lower case, the AgeI site is underlined, the new start codon of DsRed is in boldface. Forward and reverse primers are indicated by F and R. respectively. Asterisk indicates a specific fluorescent dye that end-labeled each forward primer, including fluorescein-CE phosphoramidite (Fam), hexachlorofluorescein-CE phosphoramidite (Hex), and tetrachlorofluorescein-CE phosphoramidite (Tet).