Inhibition of Nonsense-mediated Messenger RNA Decay in Clinical Samples Facilitates Detection of Human MSH2 Mutations with an in Vivo Fusion Protein Assay and Conventional Techniques

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ABSTRACT

Germ-line mutations in the human MSH2 (hMSH2) gene account for about 40% of known defects in kindreds with hereditary nonpolyposis colon cancer. We describe a simple fusion protein assay for detection of hMSH2 nonsense mutations in yeast. Detection of nonsense mutations with this assay is severely compromised in many cases by nonsense-mediated mRNA decay, a physiological process that destabilizes the mutant RNA. Triggering of nonsense-mediated decay requires mRNA scanning by the ribosome to detect the stop codon. We show that treatment of cells with the translation inhibitor puromycin suppresses nonsense-mediated decay and facilitates the detection of nonsense mutations in clinical samples by cDNA sequencing, in vitro protein truncation tests, and the yeast fusion protein assay. Given the prevalence of chain-terminating mutations in human disease genes, puromycin treatment of blood samples should improve the signal-to-noise ratio and hence the sensitivity of many RNA-based diagnostic tests. Paradoxically, the yeast hMSH2::ADE2 fusion protein assay also detects some in-frame mutations, presumably through an effect on the folding of the fusion protein.

INTRODUCTION

HNPCC is caused by germ-line mutations in at least four DNA mismatch repair genes (hMSH2, hMLH1, hPMS1, and hPMS2; Ref. 1). Defects in these genes impair postreplicative DNA mismatch repair, leading to a genome-wide increase in the rate of insertion, deletion, and point mutation (2). At-risk individuals have much to gain from genetic screening because frequent colonoscopic examination allows early detection and curative removal of premalignant polyps (3). Even if screening is limited to hMSH2, which is activated by premature stop codons, frameshifts, or aberrant splicing in 40% of known defects in kindreds with hereditary nonpolyposis colon cancer, the costs of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Sections 1734 solely to indicate this fact. 1 This work was supported by the Swiss National Science Foundation and Swiss Cancer League. 2 To whom requests for reprints should be addressed. Phone: 41-21-692-5889; Fax: 41-21-652-6933; E-mail: Richard.Iggo@isrec.unil.ch. 3 The abbreviations used are: HNPCC, hereditary nonpolyposis colon cancer; h, human; RT, reverse transcription. 4 See the Human Genome Mutation Database at the Institute of Medical Genetics in Cardiff. Up-to-date listings of confirmed hMSH2 mutations can be found at http://www.cf.ac.uk/ucwmc/hgmd/0.html. Search term: MSH2.

MATERIALS AND METHODS

Plasmids. The hMSH2::ADE2 expression vector (pCA57) was derived from pLS210 (9) by insertion of a hMSH2 cDNA (E3; Ref. 11) at the BamHI site, insertion of a CEN/ARS fragment (12) at the Clal site, and replacement of the p53 binding sites with CYC1 upstream activating sequences from pLG312 (13). The hMSH2 stop codon in the hMSH2::ADE2 fusion gene was deleted by PCR mutagenesis. pCA28 was made by ligation of a BglII linker between the hMSH2 NaeI and EcoNI sites in pCA57. The vectors for the split assay (pCA175, 176, and 177) were made from pCA57 by PCR mutagenesis. To prepare linear vector for gap repair, pCA28 was cut with BglII, blunted with Klenow, and treated with calf intestinal phosphatase; pCA175, 176, and 177 were cut with PvuII and treated with calf intestinal phosphatase. The vector with the frameshift at codon 243 was made by blunting the hMSH2 AplI site in pCA57.

Cell Lines and Blood Samples. Lymphocytes were fractionated from whole blood by Ficoll density gradient centrifugation (leucoprep, Becton Dickinson), washed with PBS, and frozen in 500 μl of RNA lysis buffer (500 mm LiCl, 1% LiDS, 100 mm Tris, pH 8, 10 mm EDTA, and 5 mm DTT). Puromycin treatment, freshly drawn heparinized whole blood was incubated for 6 h at 37°C on a rotating wheel in the presence of 200 μg/ml puromycin. Lymphocytes were then harvested as above. For inhibition of nonsense-mediated decay in lymphoblastoid cell lines (14, 15), cells were grown for 12 h in the presence of 200 μg/ml puromycin and then harvested in lysis buffer. Lymphoblastoid cell lines (numbers 434, 1383, 1587, and 1097) derived from peripheral blood lymphocytes from HNPCC patients have previously been described (14, 15). Patient 1097 has a 2-bp deletion at codon 129 (CTC CAG to TCA G); patient 1383 has a 3-bp deletion at codon 596 (CTC AAT OAT to TCA G); patient 1587 has a nonsense mutation at codon 383 (COA to TCA G); patient 1097 has a 2-bp deletion at codon 129 (CTC CAG to TCA G); patient 1383 has a 3-bp deletion at codon 596 (CTC AAT OAT to TCA G); patient 1587 has a nonsense mutation at codon 383 (CGA to TGA). Patient 434 has a germ-line MLH1 mutation.

RNA Extraction and RT-PCR. mRNA was purified using oligo(dT)-coated magnetic beads (Dynabeads mRNA direct kit, Dynal AS) and RT-PCR was performed as described previously (16), using a hMSH2-specific dodecamer (TTACAGACAAAT) for cDNA synthesis and primers P10 (TTT-TCTTCAACCGAGGATGAGGAGGTTTCS, where s represents a phosphothioate linkage) and P21 (TTCAATCTCCTCTATTTTCTGTTTCTAC) for gap repair into pCA175; P22 (CGACGCTGCAAGCCCTTAAATCCTTTTTCGAGTTTGGGTTTSC, which s represents a phosphothioate linkage) and P23 (AGGAGTTAAATGCAATTTCATCTTGAAGACTTSC) for pCA175; and P24 (TCAGCGCTATTGGAGACCAATGCGACACTSC) and P11 (ATTACCTTACATTCTAGGAGGTTTTS, which s represents a phosphothioate linkage) for pCA177.

Yeast Techniques. Gap repair and color assays were performed as described previously for p53 (16) using strain yj1B122 (mata ade2–1 his3–11,15 leu2–3,112 trpl–1 ura3–1 can1–100), a haploid derivative of yj1B12 (17). For Western blotting, yeast extracts containing the fusion plasmids were probed with a mouse monoclonal antibody (GB12, Oncogene Science) directed...
against the amino-terminal region of hMSH2. Plasmids were sequenced to confirm that they contained the stated mutations.

**In Vivo Translation.** Full-length hMSH2 cDNA (P10–P11 RT-PCR product) was reamplified with a T7-tailed primer (18), and 35S-labeled protein was synthesized in a coupled transcription-translation reaction (TNT system, PROMEGA; Ref. 19).

DNA Sequencing. Plasmids were rescued from yeast as described previously (16). To identify the source of background in the assay, plasmids were rescued from 12 mutant colonies from a normal individual and 11 colonies from the patient with a codon 596 deletion. To identify clonal mutations in positive cases, plasmids from six colonies should be analyzed. DNA sequencing was performed on a Li-Cor 4000L automated sequencer using IRD41-labeled primers and ThermoSequenase (Amersham Corp.).

**RESULTS**

**In Vivo Detection of Stop Codons.** The overall outline of the assay is shown in Fig. 1. Ade2+ cells grown on medium deficient in adenine turn red because of the accumulation of an intermediate in adenine metabolism. To allow detection of hMSH2 nonsense mutations, hMSH2 cDNA is fused in-frame with the Ade2 gene. Despite the presence of the 934-amino acid hMSH2 extension at its amino terminus, the Ade2p protein remains biochemically active, and strains containing the fusion protein form white colonies. If the hMSH2 cDNA contains a nonsense mutation, the Ade2 part of the fusion is not translated, and the yeast form red colonies. To test clinical samples, unpurified hMSH2 RT-PCR products and linear vector are cotransformed into an ade2- yeast strain, where they regenerate circular plasmid molecules through homologous recombination. Recombinants are selected on plates lacking uracil, and each colony contains the progeny of a single allele because the vector has a centromere and a replication origin. In the region of the gap in the plasmid (codons 70–888), all mutations in the PCR product are copied into the circularized plasmid. In the region of overlap between the linearized vector and the PCR product, the recombinant product can be derived from either the vector or the PCR product. Thus, although the entire open reading frame is screened, mutations in the overlap region will give an abnormally low percentage of red colonies. This is particularly true of mutations at the extreme ends of the PCR products, although in the analogous region tested with the yeast p53 functional assay, mutations have been detected in tumors even in the first codon following the primer (20). 5

To demonstrate that the assay can detect nonsense mutations in vivo, a plasmid with a frameshift at codon 243 (243fs) was constructed. Transformation with circular plasmid encoding wild-type hMSH2::ADE2 gave 100% white colonies, whereas plasmid encoding the 243fs mutant gave 100% red colonies (Fig. 1). Yeast were then transformed with linearized vector and wild-type or 243fs mutant hMSH2 PCR-amplified from plasmid: the mutant gave 100% red colonies, whereas the wild type gave 4% red colonies, a background due mainly to PCR mutations. For heterozygotes to be correctly scored, it is important that the transformation efficiency of plasmids encoding wild-type and mutant hMSH2::ADE2 fusion genes should be the same; this was tested and found to be the case (data not shown). We conclude that the Ade2 fusion protein gap repair strategy is a valid approach for detecting chain-terminating mutations in hMSH2 in vivo.

Analysis of RNA from Cell Lines and Blood. The background with the assay shown in Fig. 1 is about 20% red colonies with RNA extracted from wild-type cell lines and blood; sequencing of plasmids rescued from yeast showed that half of the red colonies contain hMSH2 point mutations, and the remainder contain hMSH2 splice variants. The former are caused by RNA polymerase II, reverse transcriptase, and Pfu polymerase errors. The latter were seen in all cell lines and blood samples tested irrespective of hMSH2 status. To reduce the effective background, the assay was modified to permit screening of the cDNA in three separate parts (Fig. 2): the background with this split assay is under 10% per fragment tested (Table 1). An additional advantage of the split assay is that the hMSH2 mRNA acts as its own control for mRNA quality and PCR artifacts: germ-line point mutations score as positive with only one reporter, whereas PCR mutations and PCR deletions due to RNA degradation are positive with all three.

Translation Inhibition in Clinical Samples Facilitates Detection of Stop Codons. Initial tests with samples from HNPCC patients revealed that the yeast assay was able to detect a frameshift at codon 853 but failed to detect a frameshift at codon 129 or a stop mutation at codon 383 in RNA from either lymphoblastoid cell lines or blood. This pattern, detection of distal but not proximal mutations, is consistent with destabilization of the mutant RNAs by nonsense-mediated mRNA decay (21). Detection of stop codons in vivo requires mRNA scanning by the ribosome, and drugs that inhibit translation prevent nonsense-mediated mRNA decay (22). HNPCC lymphoblastoid cell lines were therefore treated with the antibiotic puromycin, and the yeast assay was repeated. The percentage of red colonies increased significantly for the two samples containing a nonsense mutation (samples 1097 and 1587; Table 1), but remained at background levels for the wild-type sample (sample 434; Table 1). Thus, treatment of cell lines with puromycin increases the signal-to-noise ratio and hence facilitates detection of nonsense mutations with the yeast assay.

To confirm that puromycin acts at the level of RNA abundance, the RT-PCR product used for the yeast assay was sequenced directly. Fig.

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5 R. Iggo, unpublished data.
Fig. 2. Schematic diagrams showing the original version (a) and split version (b) of the hMSH2 assay. Using the split assay, splice variants, artifactual deletions, and PCR mutations give red or pink colonies with all three reporters, whereas clonal mutations are positive with only one. c, split assay testing of whole blood treated with puromycin for 6 h. Top panels, wild-type hMSH2; bottom panels, mutant hMSH2 with a frameshift at codon 129. The frameshift gave 48% red colonies with the 5' reporter (pCA175).

3A shows the result for the amino acid 383 stop codon: in the top panel, the mutant RNA is barely detectable, whereas after treatment with puromycin (bottom panel), the mutant and wild-type peaks are of equal intensity. Nonsense-mediated decay affects all cDNA-based strategies for detection of mutations. Fig. 3B shows that puromycin treatment increases the intensity of the mutant band relative to the wild-type band in standard protein truncation tests (compare Lane 3 with Lane 4, and Lane 5 with Lane 6).

Because the intended use of the yeast assay is detection of germ-line hMSH2 mutations in blood samples, we tested whether incubation of whole blood for 6 h at 37°C in the presence of puromycin can be used to inhibit nonsense-mediated decay in clinical samples. This procedure is simple enough for use in routine clinical practice. Untreated and puromycin-treated blood from the patient with the codon 129 frameshift gave 18 and 48% red colonies, respectively (Table 1 and Fig. 2B). Thus, treatment of whole blood with puromycin increases the signal-to-noise ratio and facilitates detection of nonsense mutations by yeast assay in RNA from peripheral blood lymphocytes.

Detection of In-Frame Mutations. A stop codon assay should not detect in-frame mutations, but paradoxically the yeast hMSH2::ADE2 assay can detect some mutations that do not interrupt the hMSH2 open reading frame. Thus, blood with a Gly to Asp missense hMSH2 mutation (GGT to GAT) at codon 674 gave 41% pink colonies, a lymphoblastoid cell line with a deletion of codon 596 gave 38% pink colonies (the background is <2% pink colonies per fragment tested), and the homozygous in-frame deletion of exons 4–8 in LoVo cells (18) gave 100% red colonies. One possible explanation is that mutant fusion proteins are unstable, but Western blotting showed this not to be the case (Fig. 4). The strain in Fig. 4, Lane 3 contains a fusion protein with a single amino acid deletion; that in Lane 5 contains a

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Table 1 Split assay testing plasmid, lymphoblastoid cell lines, and blood samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample type</th>
<th>hMSH2 status</th>
<th>Region tested</th>
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<th>Plus puromycin, % red</th>
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<td>3'</td>
<td>1</td>
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a Heterozygous mutations: 129fs, 2-bp deletion at codon 129 (TCT CAG to TCA G); 383stop, nonsense mutation at codon 383 (CGA to TGA).
b pCA175, 176, and 177 test the 5', middle, and 3' parts of the hMSH2 open reading frame, respectively (see Fig. 2).
c Gap repair with PCR-amplified cDNA derived from a wild type hMSH2 plasmid.
d —, not applicable.

Discussion

Yeast functional assays are an attractive approach for identification of mutations in human disease genes. In practice, however, the criteria for a true functional assay are rarely met: the yeast model should exactly mimic the human function, and only mutations that interfere with that function in tumors should do so in yeast. Unlike the p53 functional assay (8, 9, 23), the hMSH2 yeast assay described here does not test the critical biological function targeted in tumors, so it cannot be used as a single, definitive test for hMSH2 mutations. Instead, it is a cheap, simple prescreening test that can be used as an alternative to in vitro protein truncation tests and structure-based techniques like single-strand conformation polymorphism. The yeast assay should be less prey to minor changes in assay conditions than single-strand conformation polymorphism (24), and it should score silent mutations correctly. It is simpler to interpret than in vitro protein truncation tests, where in practice the mutant band can become lost among incomplete and reinitiated products, and it can detect some in-frame mutations, including single amino acid deletions and missense mutations.

Fig. 3. a, sequence chromatogram before and after treatment of lymphoblastoid cells with puromycin. Arrow, heterozygous peak (C to T mutation). Lymphoblastoid cell line 1587 (amino acid 383 stop codon mutation) was grown for 12 h in the presence or absence of puromycin. b, in vitro protein truncation tests before and after treatment of lymphoblastoid cell lines with puromycin. Lanes 1, 3, and 5, minus puromycin; Lanes 2, 4, and 6, plus puromycin. Lanes 1 and 2, wild-type hMSH2 (sample 434); Lanes 3 and 4, hMSH2 frameshifted at amino acid 129 (sample 1097); Lanes 5 and 6, hMSH2 amino acid 383 stop codon mutation (sample 1587). Numbers at left, markers (molecular weight in thousands).

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mRNA is frequently used for genetic testing because entire genes can often be screened in a single RT-PCR step. We have shown that nonsense-mediated mRNA decay is a major obstacle to detection of mutations in hMSH2, particularly when they occur early in the message. Many other tumor suppressor genes are inactivated by nonsense mutations, including MLH1, PMS2, APC, BRCA1, BRCA2, and ATM (5, 25–43). One of the few tumor suppressor genes rarely inactivated in this way is the p53 gene, a fact that greatly simplified the development of a yeast functional assay for p53 mutations (23).

Nonsense-mediated mRNA decay is a physiological process found in both prokaryotes and eukaryotes (10). It protects the cell against potential dominant negative effects of truncated proteins by converting nonsense alleles into null alleles. Degradation is triggered by decapping, which exposes the RNA to 5'→3' exonucleolytic attack (44). The fact that premature stop codons in the final one-third of the mRNA are not detected led to the proposal that mRNAs contain nonsense codon-dependent destabilizing signals ("downstream elements"), which are inactivated when the ribosome translates past stabilizing signals in the final one-third of the message (21). Detection of premature stop codons and consequent decapping require ongoing translation and can thus be prevented by translation inhibitors (10). We have shown that puromycin, a tRNA analogue causing chain termination (45), can block nonsense-mediated decay in blood samples and cell lines. Treatment of clinical samples with translation inhibitors should be seriously considered for all mutation detection assays using RNA.

By testing the hMSH2 mRNA in three parts it is possible to reduce the background to 5–10% per fragment tested. Experience with the p53 assay suggests that this level of background is compatible with sensitive detection of mutations in blood samples and tumors (16, 20). Compared with the p53 assay, there is a much larger contribution of alternative splicing to the hMSH2 background. This includes skipping of exon 5, which has previously been reported as a mechanism of germ-line hMSH2 inactivation (18). One potential disadvantage of testing the hMSH2 cDNA in three parts is that deletions spanning more than one fragment will fail to amplify. To avoid missing such mutants, the size of a single PCR fragment spanning the entire open reading frame should always be checked. In addition to apparently normal patterns of alternative splicing, careless sample handling can lead to important biological and technical artifacts. Splicing errors, such as intron 9 retention in the p53 cDNA (46), are probably caused by a decrease in the fidelity of splice site selection as lymphocytes lose viability; they are commonly seen when blood samples are delayed in transit. Technical failures resulting in RNA degradation give rise to random intragenic deletions by PCR splicing of fragmented cDNA (16). Insertions and deletions detected at the cDNA level should thus always be confirmed at the genomic DNA level before concluding that a particular alteration is linked to disease susceptibility.

Unlike in vitro protein truncation tests, the yeast assay can detect some in-frame mutations. In the absence of extensive linkage data, it can be difficult or impossible to determine whether a sequence change is a polymorphism or a tumorigenic mutation. Insofar as in-frame mutations are detected because they affect the folding of the fusion protein in yeast, one can infer that they are likely to have an impact on protein function in human cells. This may be a useful guide to the likely significance of a particular sequence change, although we have not been able to test this point directly because of the paucity of known polymorphisms in hMSH2. The extent to which in-frame mutations can be detected with the yeast assay will only become clear with time, but there are theoretical grounds to suspect that a significant number of missense mutations will be detected. The fraction of DNA molecules mutated in a PCR reaction depends on the length of the PCR product, the number of doublings, and the error rate of the polymerase. For hMSH2 in the conditions described, this theoretical mutation rate is 20%, close to the observed background in the assay when the entire cDNA is tested in one step (Fig. 1). Because around half of the red or pink colonies contain alternatively spliced hMSH2 cDNAs, and most of the remainder contain in-frame mutations, by implication perhaps half of all missense mutations will be detectable with the yeast assay. The biochemical effects on mismatch repair of the codon 596 deletion (sample 1383) and the amino acid 674 glycine-to-arginine mutation are unknown, but we predict that both will alter the folding of the mutant hMSH2 protein. Western blots of the 129f mutant give a band that could plausibly be explained by reinitiation of translation after the stop codon (data not shown), but because this mutant gives red colonies, the mutant protein presumably folds incorrectly. If generally true of reinitiated fusion proteins, this phenomenon should reduce the number of false negatives caused by reinitiation.

Novel approaches frequently shed new light on biological problems, and they can complement existing techniques when they have very different strengths and weaknesses. Thus, the p53 functional assay revealed that the overwhelming effect of p53 mutations is to inactivate p53 as a transcription factor (47), and the p53 assay is up to 2-fold more sensitive than conventional techniques for detection of mutations in some tumors (16, 20). The hMSH2 stop codon assay has highlighted the importance of nonsense-mediated mRNA decay in RNA-based diagnosis, and when coupled with use of puromycin, it should facilitate the screening of HNPCC patients for hMSH2 mutations.

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