Allele Separation Facilitates Interpretation of Potential Splicing Alterations and Genomic Rearrangements

Hidewaki Nakagawa, Hai Yan, Janet Lockman, Heather Hampel, Kenneth W. Kinzler, Bert Vogelstein, and Albert de la Chapelle

Abstract

Mutations that alter normal splice patterns and genomic rearrangements are common causes of hereditary diseases including hereditary nonpolyposis colorectal cancer. However, abnormal transcripts can be difficult to detect and interpret because splicing patterns are often heterogeneous even in normal cells. Standard techniques including sequencing and Southern hybridization fail to detect some genomic rearrangements. We show here that separation of alleles in somatic cell hybrids, through "conversion" technology, considerably facilitates the interpretation of abnormal splicing patterns and the detection of genomic rearrangements. We detected novel mutations in MLH1 in each of four hereditary nonpolyposis colorectal cancer patients. The genomic mutations were CAG > CAA predicting G346Q; GAG > AAG predicting E102K; > a>g at nucleotide 1559 –2 at intron 13, and a tandem duplication involving exons 7–12. By separating the two alleles, we showed that one allele produced only abnormal transcript or no transcript whereas the other allele produced only normal transcript. These results allowed pathogenicity to be unambiguously assigned to the mutations and increased the sensitivity of genomic testing.

Introduction

Mutation detection is key to the diagnosis of inherited disorders. In some diseases, searching for one or a small number of recurrent or widespread mutations is sufficient for most diagnostic purposes. For example, in sickle cell anemia most patients have a missense founder mutation of the β-globin gene (1), and in achondroplasia almost all patients have a recurrent missense mutation of the fibroblast growth factor receptor-3 gene (2). By contrast, in many diseases the phenotype results from a variable number of different mutations. Moreover, some diseases are genetically heterogeneous in that mutations in more than one gene can cause the phenotype. For instance, in HNPCC (3), germ-line mutations of at least four mismatch repair genes have been implicated (MLH1, MSH2, MSH6, PMS2; Refs. 3 and 4). The total number of different mutations in these genes is presently greater than 300, and the list is rapidly growing (3).

In 20–50% of putative HNPCC families and patients, no mutation is detectable by standard methods (5–7). Many methods have been devised to improve mutation detection (8, 9); however, one class of changes remains particularly difficult to detect and interpret (i.e., rearrangements of the transcripts, many of which lead to the decrease or disappearance of the transcript through NMD; Ref. 10). These mutations mainly consist of sequence changes causing splicing errors or consist of large genomic rearrangements. The clinical significance of abnormal transcripts is particularly difficult to interpret in genes where alternatively spliced isoforms of the transcript occur naturally and frequently. One such gene is MLH1 (11, 12), which shows several alternative splicing patterns. In this study, we show that the separation of alleles in somatic cell hybrids (here referred to as conversion; Ref. 13) considerably facilitates the detection and interpretation of abnormal transcripts.

Materials and Methods

Patients and Samples. Four unrelated patients belonging to HNPCC families fulfilling the Amsterdam criteria (4), each with a microsatellite unstable colorectal carcinoma, were studied. Three of the patients were previously mentioned in the initial report on the conversion technology (13) as: patient 1 (ML), patient 2 (CG), and patient 3 (GS). Patient 4 has not been published previously. For each patient, an EBV-transformed lymphoblastoid cell culture was available as a source of DNA and RNA. In patients 1–3 lymphoblastoid cells were used to produce the fusion clones converted to haploidy, whereas in patient 4 lymphocytes from a fresh blood sample were used for this purpose.

Diploid-to-Haploid Conversion. Haploid-converted clones of patients 1–3 were created by using the conversion technology of Yan et al. (13). Haploid-converted clones from the fourth patient were created by GMP Genetics, Inc. In brief, human fresh lymphocytes or established lymphoblastoid cells were electrofused with a specifically designed mouse cell line (E2). Unfused mouse parental cells were negatively selected by sodium hypoxanthine, aminopterin, and thymidine (HAT; Life Technologies, Inc.), and unfused human lymphocytes were negatively selected by Genetica (Life Technologies, Inc.). Hybrid cells were maintained in DMEM (Life Technologies, Inc.) including 10% FBS, 0.5 mg/ml Geneticin, 1× HAT, and penicillin-streptomycin.

RT-PCR Analysis. RNA was extracted from original lymphoblastoids and converted cells by Trizol according to the manufacturer (Life Technologies, Inc.). Two micrograms of total RNA were treated with Superscript II (Life Technologies, Inc.) to produce cDNA, using random hexamers according to the manufacturer. We designed four sets of primers to amplify the entire coding sequence (a) in a single amplicon and (b) as three overlapping amplicons. These were designed so as not to amplify mouse MLH1. The primers for the amplification of the entire coding sequence were 5′-ggcgcctaatgtctgtgtgg-3′ (Ex1F) and 5′-ccccacgtctataacac-3′ (Ex19R). The primers for the first part of MLH1 were Ex1F and 5′-cttttgctggagctttgta-3′ (Ex9R), for the second part 5′-gagaggtgtatgttctggctg-3′ (Ex8F) and 5′-cgtctttcttctgg-3′ (Ex13R), and for the third part 5′-agaggactctccagcaac-3′ (Ex12F) and Ex19R. Other primers used for amplification are available on request.

Received 4/18/02; accepted 6/24/02.

The costs of publication 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. Section 1734 solely to indicate this fact.

4 Internet address: www.nfht.nl/.

Advances in Brief

Haploid-converted clones of patients 1–3 were created by using the conversion technology of Yan et al. (13). Haploid-converted clones from the fourth patient were created by GMP Genetics, Inc. In brief, human fresh lymphocytes or established lymphoblastoid cells were electrofused with a specifically designed mouse cell line (E2). Unfused mouse parental cells were negatively selected by sodium hypoxanthine, aminopterin, and thymidine (HAT; Life Technologies, Inc.), and unfused human lymphocytes were negatively selected by Genetica (Life Technologies, Inc.). Hybrid cells were maintained in DMEM (Life Technologies, Inc.) including 10% FBS, 0.5 mg/ml Geneticin, 1× HAT, and penicillin-streptomycin.

RT-PCR Analysis. RNA was extracted from original lymphoblastoids and converted cells by Trizol according to the manufacturer (Life Technologies, Inc.). Two micrograms of total RNA were treated with Superscript II (Life Technologies, Inc.) to produce cDNA, using random hexamers according to the manufacturer. We designed four sets of primers to amplify the entire coding sequence (a) in a single amplicon and (b) as three overlapping amplicons. These were designed so as not to amplify mouse MLH1. The primers for the amplification of the entire coding sequence were 5′-ggcgcctaatgtctgtgtgg-3′ (Ex1F) and 5′-ccccacgtctataacac-3′ (Ex19R). The primers for the first part of MLH1 were Ex1F and 5′-cttttgctggagctttgta-3′ (Ex9R), for the second part 5′-gagaggtgtatgttctggctg-3′ (Ex8F) and 5′-cgtctttcttctgg-3′ (Ex13R), and for the third part 5′-agaggactctccagcaac-3′ (Ex12F) and Ex19R. Other primers used for amplification are available on request.

Received 4/18/02; accepted 6/24/02.

The costs of publication 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. Section 1734 solely to indicate this fact.

4 Internet address: www.nfht.nl/.
Long-range RT-PCR was performed by using Expand Long Template PCR system (Roche), and PCR was carried out at 94°C (10 s), 60°C (30 s), and 68°C (2 min) for 10 cycles, and at 94°C (10 s), 60°C (30 s), and 68°C (2 min + cycle elongation for more yields of 20 s for each cycle) for 25 cycles. The PCR products were run on a 1.5% agarose gel to analyze their size differences.

DNA Sequencing. The RT-PCR products were isolated using QIAquick PCR product purification kit (Qiagen). The purified RT-PCR products were sequenced directly by the primer used for PCR, or cloned into TA vector pCR2.1 (Invitrogen). DNA from plasmid clones was extracted by QIAprep Spin Miniprep kit (Qiagen) and sequenced using the ABI sequencing system (Perkin-Elmer Applied Biosystems). Amplification of the exons and exon-intron junctions of MLH1 and sequencing was described previously (14).
Results and Discussion

Genomic Sequencing. Exon-by-exon genomic sequencing including the promoter regions of MLH1 and MSH2 revealed no changes in lymphoblast DNA from cases 2 and 3. In case 1, there was an a to g change at the conservative splice acceptor site of exon 14 of MLH1 (a→g at nucleotide 1559-2). In case 4, there was a G to A change at codon 102 predicting a glutamic acid to lysine, E102K, amino acid substitution of unknown significance.

RT-PCR of Diploid Cell Transcripts. Using RNA from lymphoblastoid cells, in all four cases a normal-sized transcript was seen by RT-PCR; in addition, in cases 1–3, a very faint aberrant band was also seen (Fig. 1A). In case 4, only a normal-sized transcript was seen in the diploid cells (Fig. 1A).

Haploid Conversion Hybrids. In each case hybrid clones were studied for their content of alleles for markers on chromosome 3 (D3S1263, D3S1569, D3S1300, and D3S1601). On the basis of data not shown, we selected two clones having one copy of one of the chromosomes 3, and two clones having the other copy of chromosome 3. In Fig. 1, these clones are referred to as W for wild type and M for presumed mutated allele, respectively. RT-PCR and sequencing of cDNA and genomic DNA from these clones revealed the nature of the mutation in each case as:

In case 1 (Fig. 1), RT-PCR demonstrated only normal transcript in the W clones and only a smaller than normal transcript in the M clones; this transcript was of extremely weak abundance, apparently as a consequence of NMD. Sequencing revealed that this transcript lacked exons 14 and 15. The a→g change at nucleotide 1559-2 seen previously in heterozygous form in lymphoblastoid cell DNA occurred in the M clones but not in the W clones. Thus, this change apparently caused the splicing error. In case 2 (Fig. 1), RT-PCR showed a normal product in W clones but a larger product in M clones. By sequencing, a 59-bp insertion emanating from the 5’ end of intron 11 was seen in this transcript. Sequencing of the M clones revealed a G→A change in the last nucleotide of exon 11 that had not been noticed in the sequencing of lymphocyte DNA. This change obviously pathogenic. In contrast, by demonstrating that the abnormal transcript emanates from one of the alleles as shown in all four cases here, and that it is of greatly diminished quantity, as shown in 2 cases here, a decisive, deleterious role is likely.

Exonic splicing enhancers and silencers have been proposed to explain how some missense or silent mutations can affect the splicing pattern and result in the inactivation of the protein (15, 16). In this study, we demonstrated two missense and one silent mutation of MLH1 affecting its splicing pattern. This approach could be generally applied to the analysis of presumptive splicing mutations and complicated genomic rearrangements in any gene, as long as that gene continues to be expressed in somatic cell hybrids. Such studies usually allow pathogenicity to be assessed and, thus, increase the sensitivity of mutation testing for inherited diseases (17).

Acknowledgments

We thank Tamara Hemingway and Getachew Boru for assistance with cell culture and DNA preparation and Barbara Fesch for preparation of this manuscript.

References

Allele Separation Facilitates Interpretation of Potential Splicing Alterations and Genomic Rearrangements

Hidewaki Nakagawa, Hai Yan, Janet Lockman, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/16/4579

Cited articles
This article cites 17 articles, 4 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/16/4579.full.html#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/62/16/4579.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.