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Original Paper

Transcripts with Splicings of Exons 15 and 16 of the *hMLH1* Gene in Normal Lymphocytes: Implications in RNA-based Mutation Screening of Hereditary Non-polyposis Colorectal Cancer

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Germline mutations of the *hMLH1* gene are estimated to account for a large fraction of kindreds affected by hereditary non-polyposis colorectal cancer (HNPCC). In a significant number of cases, *hMLH1* mutations result in the expression of truncated proteins. We report here two novel alternatively spliced forms of *hMLH1* mRNA in normal lymphocytes. One of these novel isoforms lacks the coding region of the gene between codons 557 and 578, corresponding to the entire exon 15. The deletion introduces a frameshift that results in a premature stop signal. The other isoform is characterised by an in-frame deletion spanning codons 578–632, corresponding to loss of the entire exon 16. Further studies are necessary to establish the biological significance of these alternative splicings. The presence of alternatively spliced *hMLH1* transcripts that mimic pathogenic mutations should be taken into account in the mutational screening of the *hMLH1* gene by reverse transcription–polymerase chain reaction methodologies.

Key words: HNPCC, *hMLH1* gene, alternative splicing
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INTRODUCTION

HEREDITARY NON-POLYPOSIS colorectal cancer (HNPCC) is one of the most frequent autosomal dominant cancer susceptibility syndromes and is estimated to account for 1–5% of all colon cancers [1]. HNPCC is characterised by early onset colorectal cancer associated with an increased risk of extracolonic tumours including carcinomas of the endometrium, ovary, stomach, small intestine, as well as other neoplasms [1]. HNPCC is due to mutations occurring in one of at least four genes responsible for postreplication DNA mismatch repair, designated *hMSH2*, *hMLH1*, *hPMS1* and *hPMS2* [2–6], that are homologues of the prokaryotic *MutS* and *MutL* genes [7, 8]. Linkage studies have demonstrated that most cases of HNPCC depend upon germline mutations of either *hMSH2* or *hMLH1* [4]. The *hMLH1* gene, located on chromosome 3p21, is composed of 19 exons [5] and is predicted to encode a protein of 756 amino acids [9]. Exons 1–7 contain a region that is highly conserved in the *hMLH1* and *PMS1* genes of yeast [10]. Alternatively spliced forms of *hMLH1* mRNA, lacking exons 9, 10 or 11 and combinations of these exons, have already been described in normal cells from different tissues [11].

We report here two novel alternatively spliced forms of *hMLH1* gene mRNA, involving exon 15 in one case and exon 16 in the other case, that are co-expressed in normal lymphocytes. These alternatively spliced forms mimic transcripts resulting from pathogenic *hMLH1* germline mutations and have obvious implications for the mutational analysis of HNPCC patients by reverse transcription–polymerase chain reaction (RT–PCR)-based methodologies.

MATERIALS AND METHODS

Genomic and complementary DNA preparation

Whole fresh blood from 14 healthy donors and from 10 unrelated colorectal cancer patients, that fulfilled the criteria for HNPCC [12], was collected for *hMLH1* analysis. Genomic

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DNA (gDNA) was isolated from whole fresh blood using the QIAmp Blood Kit 50 (Qiagen Inc., Chatswort, California, U.S.A.), according to the manufacturer's specifications. Total cellular RNA was isolated from fresh blood following the acid guanidinium isothiocyanate–phenol–chloroform extraction method [13]. Complementary DNA (cDNA) was prepared by incubating DNase-treated total RNA (5 μg) with 0.5 units of amplification enhancer and 300 units of Superscript II reverse transcriptase (Gibco-BRL, Gaithersburg, Maryland, U.S.A.) in the presence of random hexamers.

Exon amplifications and sequencing

For the analysis of exons 15 and 16 of the *hMLH1* gene we utilised a two-step protocol, consisting of an external PCR, followed by a nested internal PCR, that used a 1:10 000 final dilution of the primary PCR product as the template. Primers used for external and internal nested PCRs were 1608S (GAGATGTTGCATAACCACTC) and 2015A (AAGAATGAAGATAGGCAGTCC) (external pairs); 1687S (TTCTCAACACCACCAAGC) and 1972A (TCAATCAGAAGGGGTAATCC) (internal pairs). The external PCR was performed in 10 μl of a mixture containing 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 mM each deoxynucleotide triphosphate, 10 pmol of each primer, 0.1 mg of cDNA, and 0.3 units of Taq polymerase (Perkin Elmer, Norwalk, Connecticutt, U.S.A.). Samples were denatured at 94°C for 5 min and processed through 30 temperature cycles, consisting of 1 min at 94°C, 90 sec at 58°C and 90 sec at 72°C, with a final elongation step at 72°C for 10 min. One microlitre of the external PCR product, diluted 1:1000, was used as the DNA template in a 10 μl reaction containing the internal pair of primers. PCR products were analysed by 2% agarose gel electrophoresis and were subsequently radioactively labelled and run on a 6% denaturing acrylamide gel as described previously [14]. Prior to sequence analysis, RT–PCR products were separated by electrophoresis on a sequencing gel apparatus. The bands were cut out and eluted overnight at 37°C in 100 μl of water. One microlitre of each eluted band was then amplified (10 PCR cycles) in a 10 μl reaction containing internal primers and the PCR products were sequenced as described previously [15]. To exclude *Alu*-mediated recombinations, gDNAs spanning the region from intron 15 to exon 17 were amplified in a single amplification reaction using primers and conditions kindly provided by Dr Peltomäki [16]. Amplification products were analysed by 2% agarose gel electrophoresis. RNA samples prepared from lymphoblastoid cell lines were not available for this study.

Estimation of hMLH1 *transcripts*

To estimate the relative amounts of the different *hMLH1* transcripts, we performed a radioactive nested PCR. The radioactive nested PCR was as described elsewhere [14], except that the concentrations of deoxyguanosine 5′-triphosphate (dGTP), deoxythymidine 5′-triphosphate (dTTP) and deoxycytidine 5′-triphosphate (dCTP) were 14 mM, while deoxyadenosine 5′-triphosphate (dATP) was 3.5 mM and 2 μCi of [α-^{35}S] dATP at 1000 Ci/mmol were used. To verify the influence of the number of cycles on the intensity of the amplification products, we performed in parallel internal nested labelled PCRs at 10, 13 and 16 amplification cycles, with an annealing temperature of 58°C. Labelled PCR products were electrophoresed for 2 h through a 35×45 cm nondenaturing 6% polyacrylamide gel at 8W constant power and 24°C in a buffer containing 45 mM Tris-borate and 1 mM ethylenediamine tetraacetic acid (EDTA). Gels were analysed using a Bio-Rad molecular imager system (Hercules, California, U.S.A.). Experiments were repeated using three independent DNA and RNA extractions.

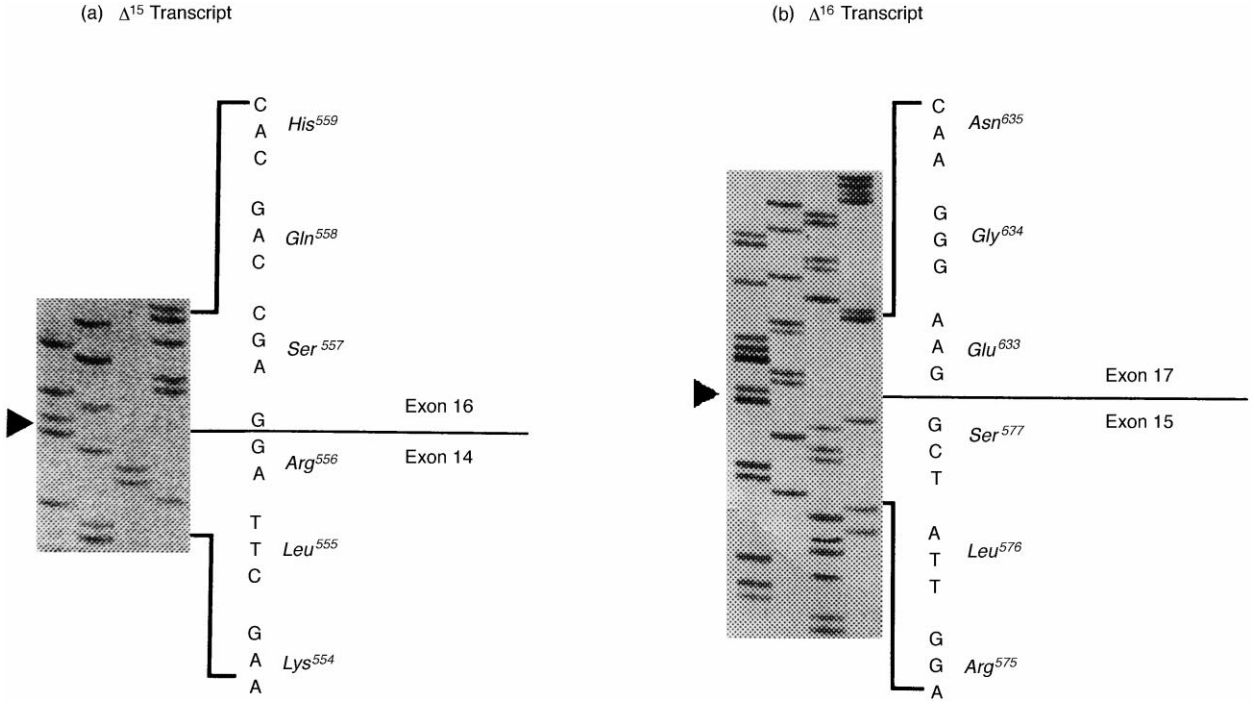

Figure 1. Sequencing analysis of transcripts bearing deletions of exons 15 and 16 of the *hMLH1* gene. The sequence in (a) shows the out-of-frame deletion spanning codons 557–578, corresponding to the complete loss of exon 15 (Δ^{15} transcript). The sequence in (b) shows the in-frame deletion spanning codons 578–632, corresponding to the complete loss of exon 16 (Δ^{16} transcript).

RESULTS

To investigate *hMLH1* gene alterations, we analysed total RNA from lymphocytes of HNPCC patients and normal subjects. Besides the full length transcript, in most samples, agarose gel electrophoresis of nested RT–PCR products spanning codons 557–632 demonstrated the presence of two additional transcripts of lower molecular weight.

Sequence analysis demonstrated that the two novel transcripts presented extensive deletions. One deleted transcript was characterised by an out-of-frame deletion of 64 bp spanning codons 557–578, corresponding to the complete loss of exon 15 (Δ^{15} transcript, Figure 1a). The loss of exon 15 determines a frameshift that is predicted to introduce a novel peptide of 13 amino acids, followed by premature termination. The other deleted transcript was characterised by an in-frame deletion of 165 bp, spanning codons 578–632, corresponding to the complete loss of exon 16 (Δ^{16} transcript, Figure 1b).

The possibility that the deleted transcripts could have resulted from PCR false priming or other artefacts was excluded, since triplicate experiments based on independent RNA extractions and cDNA preparations yielded consistent results. Furthermore, external and internal primers used for cDNA amplification were positioned in exons 14 and 17 and were not complementary to splicing sites. Data were confirmed by sequence analysis of the sense and antisense cDNA strands.

To verify if the exon deletions observed in our samples were caused by genomic *hMLH1* alterations, we amplified regions of *hMLH1* genomic DNA that included the splicing sites of exons 15 and 16. Single strand conformation polymorphism (SSCP) analysis of both exons, including donor/acceptor splicing sites, performed as described elsewhere for gDNA [14], did not demonstrate germline mutations responsible for these deletions (data not shown). Nyström-Lahti and colleagues described a deletion of exon 16 of *hMLH1* due to intronic *Alu*–mediated recombination in Finnish HNPCC patients of common ancestral origin [16]. To exclude the presence of genomic recombinations between intron 15 and 16 *Alu* sequences, we amplified gDNA samples of our cases positive for the Δ^{16} transcript using primers designed to detect the founding *Alu*-mediated Finnish mutation, kindly provided by Dr Peltomäki. The analysis did not reveal bands of molecular weight lower than the full length.

We extended the analysis to peripheral blood leucocytes from 14 normal subjects, all of which were positive for the Δ^{15} and Δ^{16} transcripts. We estimated the relative amounts of the full length and deleted transcripts in normal lymphocytes by radioactive nested PCR.

Radioactively amplified products were electrophoresed on a non-denaturing polyacrylamide gel and the relative expression of the three transcripts was assessed using a Bio-Rad molecular imager system (Figure 2). There was marked variability in the expression of the full length and shorter transcripts in peripheral blood leucocytes of normal subjects and HNPCC patients. There was a marked variability in the expression levels of the three transcripts, both among healthy donors (Figure 2b) and among HNPCC patients and even in the same subject in different experiments (data not shown). These results were in agreement with those reported by Charbonnier and associates for alternative *hMLH1* transcripts characterised by exon 9, 10 or 11 deletions [11]. However, compared with the Δ^{15} and Δ^{16} transcripts, the full length transcript resulted expressed at the highest relative

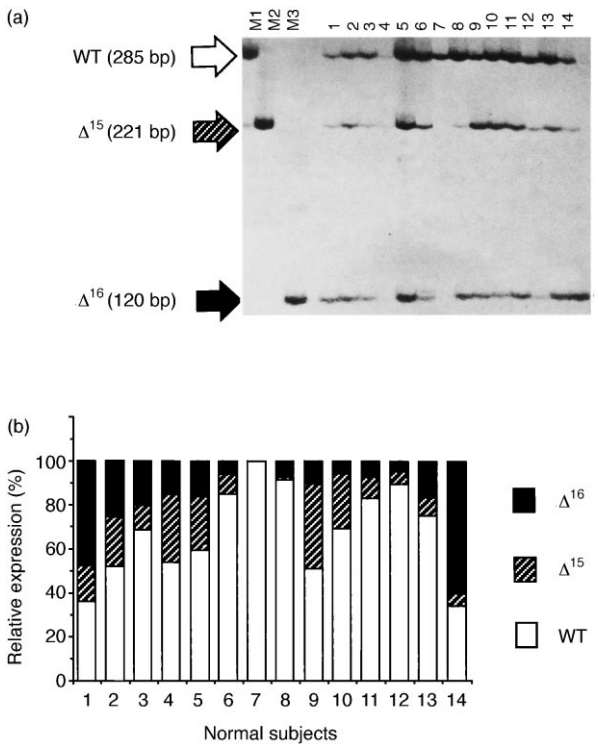

Figure 2. (a) illustrates the autoradiography of reverse transcription–polymerase chain reaction (RT–PCR) products of the *hMLH1* gene obtained using primers 1687S and 1972A, positioned on exons 14 and 17, respectively. The full length (WT), Δ^{15} and Δ^{16} transcripts were resolved by non-denaturing polyacrylamide gel electrophoresis. Lanes M1, M2 and M3 are molecular markers, obtained after elution, PCR amplification and sequencing of bands cut out from previous experiments. Lanes 1–14 correspond to cDNA samples of normal unrelated subjects. Size indications (number of base pairs), are reported in parentheses. Similar results were obtained using cDNA samples from hereditary non-polyposis colorectal cancer (HNPCC) patients. (b) illustrates the relative intensifies of the WT and alternatively spliced isoforms, estimated by contact exposure to a Bio-Rad molecular imager system.

level in most samples (Figure 2b). The average intensities of the full length and of the Δ^{15} and Δ^{16} transcripts were 67, 16 and 17%, respectively. The relative expression of the three transcripts did not significantly vary in repeated experiments performed in parallel at 40, 43 and 46 amplification cycles (30 external and 10, 13 and 16 internal nested PCRs).

DISCUSSION

We have demonstrated the existence in normal lymphocytes of novel alternatively spliced *hMLH1* transcripts characterised by the deletion of exons 15 or 16. These novel *hMLH1* isoforms appear to occur as common variants in the normal population and may have no implications in predisposing to HNPCC. Our results complement those of Charbonnier and associates [11], who described in lymphocytes from healthy donors alternative *hMLH1* transcripts characterised by deletions of exons 9, 10, 11 or their associations, and those of Kohonen-Corish and colleagues who found shortened RT–PCR *hMLH1* fragments, unrelated to hereditary defects, in RNA samples from peripheral blood lymphocytes and, to a lesser extent, lymphoblastoid cell lines of HNPCC patients [17]. The expression in normal leucocytes of transcripts that could mimic pathogenic *hMLH1* mutations,

such as those due to genomic deletions of exon 15 or 16 [9, 16], needs to be considered when designing strategies for mutational analysis of the *hMLH1* gene.

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