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RESEARCH ARTICLE

Systematic mRNA Analysis for the Effect of *MLH1* and *MSH2* Missense and Silent Mutations on Aberrant Splicing

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A substantial proportion of *MLH1* and *MSH2* gene mutations in hereditary nonpolyposis colon cancer syndrome (HNPCC) families are characterized by nucleotide substitutions, either within the coding sequence (missense or silent mutations) or in introns. The question of whether these mutations affect the normal function of encoding mismatch DNA repair proteins and thus lead to the predisposition to cancer is determinant in genetic testing. Recent studies have suggested that some nucleotide substitutions can induce aberrant splicing by disrupting *cis*-transcription elements such as exonic enhancers (ESEs). ESE disruption has been proposed to be the mechanism that underlies the presumed pathological missense mutations identified in HNPCC families. To investigate the prevalence of aberrant splicing resulting from nucleotide substitutions, and its relevance to predicted ESEs, we conducted a systematic RNA screening of a series of 60 patients who carried unrelated exonic or intronic mutations in *MLH1* or *MSH2* genes. Aberrant splicing was found in 15 cases, five of which were associated with exonic mutations. We evaluated the link between those splicing mutations and predicted putative ESEs by using the computational tools ESEfinder and RESCUE-ESE. Our study shows that the algorithm-based ESE prediction cannot be definitely correlated to experimental observations from RNA screening. By using minigene constructs and in vitro transcription assay, we demonstrated that nucleotide substitutions are the direct cause of the splicing defect. This is the first systematic screening for the effect of missense and silent mutations on splicing in HNPCC patients. The pathogenic splicing mutations identified in this study will contribute to the assessment of "unclassified variants" in genetic counseling. Our results also suggest that one must use caution when determining the pathogenic effect of a missense or silent mutation using ESE prediction algorithms. Analysis at the RNA level is therefore necessary. Hum Mutat 27(2), 145–154, 2006.

KEY WORDS: hereditary nonpolyposis colon cancer; HNPCC; missense mutations; aberrant splicing; nucleotide substitutions; exon skipping; *MLH1*; *MSH2*

INTRODUCTION

Germline mutations in the DNA mismatch repair (MMR) system lead to hereditary nonpolyposis colon cancer (HNPCC, MIM# 114500), a common cancer syndrome characterized by an increased risk of early onset of colon cancer frequently associated with extracolonic cancers, especially endometrium, gastric, and small bowel cancers. Mutations that have been shown to be involved in the disease affect at least six MMR genes: *MLH1*, *MSH2*, *MSH6*, *PMS2*, *MLH3*, and *EXO1*. Of these, *MLH1* (MIM# 120436) and *MSH2* (MIM# 609309) clearly play the most important role, since the two genes are the causative factor in more than 70% of HNPCC families. The *MSH6* gene is estimated to be involved in about 10% of these families. The role of other genes in HNPCC has not been well established, either because of the very low mutation frequency observed in affected families (*PMS2*), or because of the large number of "unclassified" variants whose functional significance have not been definitely established (*MLH3*, *EXO1*) [Wang et al., 1999; Liu et al., 2001b, 2003;

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Wu et al., 2001a, b]. The pathogenic mutations most frequently found in *MLH1* and *MSH2* genes are characterized by premature disruption of the coding sequence by nonsense or frameshift mutations or gross genomic deletions. The transcripts generated by mutant (Mt) alleles are often unstable, and are rapidly degraded by a cellular self-protection system known as "nonsense-mediated RNA decay" (NMD) [Hentze and Kulozik, 1999]. However, a considerable proportion of exonic nucleotide substitutions (known as "missense mutations" when they induce amino acid replacement, or "silent mutations" when the residue remains intact) has been routinely detected in those genes. According to our previous mutation screening studies in HNPCC, as well as data obtained by others, as many as 30% of mutations for *MLH1*, 25% for *MSH2*, and more than 50% for *MSH6*, *MLH3*, and *EXO1* are of the missense type (Wang et al., unpublished data; Mutation Database: www.insight-group.org/) [Wu et al., 2001b; Liu et al., 2003]. Determining their pathogenic effect is crucial in HNPCC genetic counseling. Unfortunately, many of those exonic substitutions remain "unclassified" because their pathogenic effect has not been clearly defined.

Growing evidence suggests that exonic substitution mutations can be deleterious by affecting normal pre-mRNA splicing. Aberrant splicing, such as exon skipping, has been detected in a number of disease-causing genes, including cancer predisposition genes such as *ATM*, *BRCA1*, *BRCA2*, *NF1*, and *APC*, as well as *MLH1* [Liu et al., 2001a; Teraoka et al., 1999; Mazoyer et al., 1998; Fackenthal et al., 2002; Ars et al., 2000; Zatkova et al., 2004; Aretz et al., 2004; Stella et al., 2001, McVety et al., 2005]. The underlying mechanism is attributed to the disruption of one of the *cis*-transcription elements known as exonic splicing enhancers (ESEs), either by the localization of the mutation within a putative ESE or by in vitro splicing assay. ESEs are 6–8 nucleotide motifs that are recognized by the SR proteins, a family of splicing factors that contain RNA-recognition motifs and, in their c-terminal domain, a region highly enriched in Arg/Ser dipeptides (the RS domain). The binding of SR proteins to ESE motifs promotes exon definition by stimulating the recruitment of spliceosome proteins [for review see Cartegni et al., 2002]. One representative approach for identifying sequences with enhancer activity is functional systematic evolution of ligands by exponential enrichment (SELEX). This method is based on a "minigene" system in which a natural ESE is replaced by a random sequence, combined with in vitro splicing in S100 extract complemented with specific SR proteins. A score matrix is then generated based on consensus sequences defined for each SR protein tested, and is further used to predict the location of putative SR protein-specific ESEs in exonic sequences [Liu et al., 1998, 2000]. A set of degenerated and partially overlapping ESE motifs identified using this approach has enabled the generation of a web-based ESE prediction tool termed ESEfinder (http://rulai.cshl.edu/tools/ESE). Correlations between the disruption of a predicted ESE and exon skipping have been demonstrated in several genes [Liu et al., 2001a, Fackenthal et al., 2002; Cartegni and Krainer, 2002]. Using the ESEfinder algorithm, Gorlov et al. [2003] found that *MLH1* and *MSH2* missense mutations that were identified in HNPCC patients and presumed to be deleterious were more frequently located within the predicted ESE loci in these two genes compared to randomly distributed neutral polymorphisms. They hypothesized that splicing defects due to the disruption of putative ESEs were responsible for the pathogenic effect of those missense mutations. However, no experimental data are available to confirm this hypothesis. More recently, another statistical and computational method for predicting ESE, named relative enhancer and silencer classification by unanimous enrichment (RESCUE-ESE; http://genes.mit.edu/burgelab/rescue-ese), was developed. It identifies hexanucleotides as putative ESEs because they are significantly enriched in human exons or they are located with a significant frequency in exons with weak (nonconsensus) splice sites [Fairbrother et al., 2002]. This online ESE tool can be used to predict splicing phenotypes by identifying sequence changes that disrupt or alter predicted ESEs.

To get an overall view of the role of aberrant splicing in HNPCC, its association with nucleotide substitutions, and its correlation with predicted ESEs, we conducted a systematic study on endogenous mRNA from a total of 45 carriers of exonic nucleotide substitutions, and 15 carriers of intronic mutations in *MLH1* and *MSH2* genes. Aberrant splicing was found in five patients who carried exonic mutations and 10 who harbored intronic mutations. The majority of the exon skipping-associated mutations were located in the vicinity of highly conserved consensus splice sites.

MATERIALS AND METHODS

Patients

Blood samples from HNPCC families were collected through genetic consultation after informed consent was obtained [Wang et al., 1999], and then subjected to germline mutation screening of *MLH1* (NM_000249 for cDNA and NC_000003 for genomic DNA) and *MSH2* (NM_000251 for cDNA and NC_000002 for genomic DNA) genes using genomic DNA sequencing [Wang et al., 1999].

RNA Isolation and RT-PCR

For each sample included in this study a lymphoblastoid cell line was established from frozen lymphocytes by EBV virus immortalization and maintained in RPMI 1640 medium supplemented with 20% fetal bovine serum. Six hours before harvesting, translation inhibitor puromycin (Sigma; www.sigma-aldrich.com) was administered to the culture medium at a concentration of 200 μg/ml. The total RNA of each cell line was isolated using an RNeasy Mini Kit (Qiagen; www.qiagen.com) following the manufacturer's instruction. Then 3 μg of total RNA were used to synthesize complementary DNA (cDNA) using a first strand cDNA synthesis kit (Amersham Pharmacia Biotech; www.amershambiosciences.com). RT-PCR was performed to amplify *MLH1* and *MSH2* coding sequences with specific primers (35 cycles). The primer sequences and PCR conditions used for each fragment are available upon request.

Detection of Aberrant Splicing

RT-PCR products were separated by 1–2% agarose gel electrophoresis for approximately 4–5 hr. The gels were checked on a UV illuminator and photographed at least three times during the migration, generally at 1 and 3 hr after the beginning and at the end of the migration, in order to detect abnormal fragments of different sizes. Abnormal RT-PCR products were isolated from the gel and purified with a Qiaquick gel extraction kit (Qiagen) followed by direct sequencing using ABI 377 or ABI 3100 automated sequencers (Applied Biosystems; www.appliedbiosystems.com) and a PRISM terminator reaction kit (Applied Biosystems). When abnormal fragments were too close to the normal transcript to be isolated from the gel, the total RT-PCR product was inserted into pGEM-T Easy Vector (Promega; www.promega.com) before the clones containing abnormal-sized RT-PCR fragments were sequenced.

Minigene Construction and Transient Transfection

The genomic DNA of exons of interest and flanking introns was PCR amplified from mutation-carrying lymphoblastoid cell lines with Ampli Taq DNA polymerase (Applied Biosystems) or Platinum Taq DNA Polymerase High Fidelity (Invitrogen; www.invitrogen.com) following protocols supplied by the manufacturer (primers are available upon request). After purification with Qiaquick PCR purification Kit (Qiagen), the PCR-amplified DNA fragments were inserted into the mammalian expression vector pTARGET (Promega) using a Rapid DNA ligation kit (Roche), and then digested with appropriate restriction endonucleases to determine the orientation of each insert. Right-oriented clones were then sequenced to distinguish between wild-type (WT) and Mt constructs. Then 3–6 μg of plasmid DNA from each construct were independently transfected in COS-7 cells (monkey kidney cells) using ExGen 500 in vitro transfection reagent (Fermentas; www.fermentas.com). Subsequently 3 μg of total RNA isolated 48 hr after transfection were reverse-transcribed as described above and used as a template to amplify transcripts expressed from WT or Mt constructs with exonic primers. The resulting transcripts were analyzed by agarose gel electrophoresis and verified by sequencing.

RESULTS

Forty-five unrelated HNPCC patients who carried different exonic variants, including a single nucleotide substitution and in-frame deletion of three nucleotides (n = 26 for *MLH1*; n = 19 for *MSH2*), were screened for abnormal splicing regardless of their predicted ESE status. Since intronic sequences can also induce aberrant splicing by affecting 5′ or 3′ splice sites (5′ss or 3′ss), the branch site, or the 3′ polypyrimidine tract, we included 15 intronic mutations susceptible to affect those sites. Variants previously described as common polymorphisms were not included. The lymphoblastoid cell lines were established for each case and all samples were treated with the protein synthesis inhibitor puromycin prior to analysis in order to prevent the degradation of mRNA by NMD. The mRNA of each mutation carrier was RT-amplified by at least one set of RT-PCR primers covering several flanking exons. Previously reported [Genuardi et al., 1998] alternative spliced isoforms of *MLH1* or *MSH2* transcripts were observed as extra bands on electrophoresis gels, in both mutation-carrying samples and control cells that did not contain detectable mutations. An additional isoform of *MLH1* characterized by a deletion of five bases at the 3′ end of exon 3 (c.302_306delGTGAG) was detected (Fig. 1A).

As summarized in Table 1, of the 45 exonic and 15 intronic mutations tested, a total of 15 aberrant splicing mutations (five associated to exonic mutations, and 10 associated to intronic mutations) were detected. All intronic splicing mutations were associated to exon skipping, with the exception of a mutation at c.1276+2 (*MSH2*). Besides the skipping of the entire exon 7, this mutation led to the loss of the last 48 bp of the exon due to the activation of a cryptic splice site (Fig. 1). Four exonic mutations in *MLH1* (c.544A>G, c.882C>T, c.1731G>A, and c.1989G>T) were associated to exon skipping, resulting in the skipping of exons 6, 10, 15, and 17, respectively (Fig. 1, Table 1). All four mutations occurred within three nucleotides from the 3′ end of the respective exon. Of note, the C-to-T transition at c.882 and the G-to-A transition at c.1731 were classified as "silent mutations," since original amino acids would not be replaced when translated from the mutated codons. One of the 19 *MSH2* exonic mutations, the C-to-T transition at c.1915, was associated to aberrant splicing. Although it was not splice-site-related, the mutation changed the WT sequence AGGC to AGGT. As a consequence, this newly created AGGT sequence was used as an ectopic splice site leading to the skipping of the last 92 bp of coding sequences of exon 12. The skipping of exon 9/10, exon 17 of *MLH1*, and exon 5 of *MSH2* was previously reported and identified as alternative transcripts produced in normal cells [Genuardi at al., 1998]. However, we observed a high-level expression of exon-lacking transcripts in mutation-carrying samples compared to a very low level (nearly undetectable) in control cells. Figure 2 shows the RT-PCR profile displaying the skipping of exon 9/10 (*MLH1*) and exon 5 (*MSH2*). The alternatively transcribed isoform was only detectable in mutation carriers, consistent with a finding by Froggatt et al. [1995]. No aberrant splicing was detected in the remaining 40 exonic mutation-carrying samples. To ascertain that the absence of aberrant splicing was not due to the nonexpression of Mt alleles, RT-PCR products from the negative samples were sequenced. Heterozygous mutations were detected at expected positions in all cases, confirming the normal expression of both WT and Mt alleles.

To determine whether aberrant splicing could be the result of the disruption of a predicted ESE, we tried to correlate the observed data to the predicted ESE status of each missense mutation by using two currently available computerized methods: ESEfinder and RESCUE-ESE. ESEfinder provides a score matrix based on the frequencies of the individual nucleotides at each position of the motif sequences specifically recognized by four SR proteins: SF2/ASF, SC35, SRp40, and SRp55 [Cartegni et al., 2003]. The RESCUE-ESE algorithm predicts whether a given hexamer can act as a candidate ESE and evaluates the consequences, such as disruption or alteration of a putative ESE by a mutation in those hexamers [Fairbrother et al., 2002, 2004]. As summarized in Tables 1 and 2, of 45 exonic variants analyzed, 27 were predicted to lie within putative ESEs by ESEfinder (19 and eight in *MLH1* and *MSH2*, respectively) with a score value above threshold (positive score) for at least one SR protein. Eighteen (67%) displayed a positive score for more than one SR protein. Prediction with RESCUE was more stringent, since only 13 variants were shown to lie within putative ESEs (five and eight in *MLH1* and *MSH2*, respectively) with seven being part of different (up to five) candidate hexamers. Of note, only four variants were located in a sequence recognized as a putative ESE by both methods. All four *MLH1* variants associated to aberrant splicing were putative ESEs predicted by ESEfinder. In contrast, no aberrant splicing was associated to putative ESEs predicted by RESCUE-ESE. To assess whether aberrant splicing could be the consequence of the disruption or alteration of those putative ESEs, we evaluated the relevant scores after nucleotide substitution using both methods. As shown in Table 3, no change was predicted with RESCUE-ESE. ESEfinder predicted that the G-to-A transition at c.1731 reduced the positive score obtained for SC35 (2.8) to below the threshold. Regarding the variant c.1989G>T, positive scores for SF2/ASF and SC35 were decreased to a level that was still above the threshold (4.3 → 2.3 for SF2/ASF, and 4.5 → 2.4 for SC35). However, regarding c.544A>G, the A-to-G transition changed the scores for SF2/ASF and SRp40 from above (3.6 and 2.9, respectively) to below the threshold, while it also produced a new positive score for another SR protein, SC35 (2.4). A similar situation was observed in c.882C>T, where the positive scores for SF2/ASF, SRp44, and SRp55 (4.5, 4.9, and 3.2, respectively) were reduced to below thresholds but a new positive score appeared for SC35 (2.6), implying that the Mt

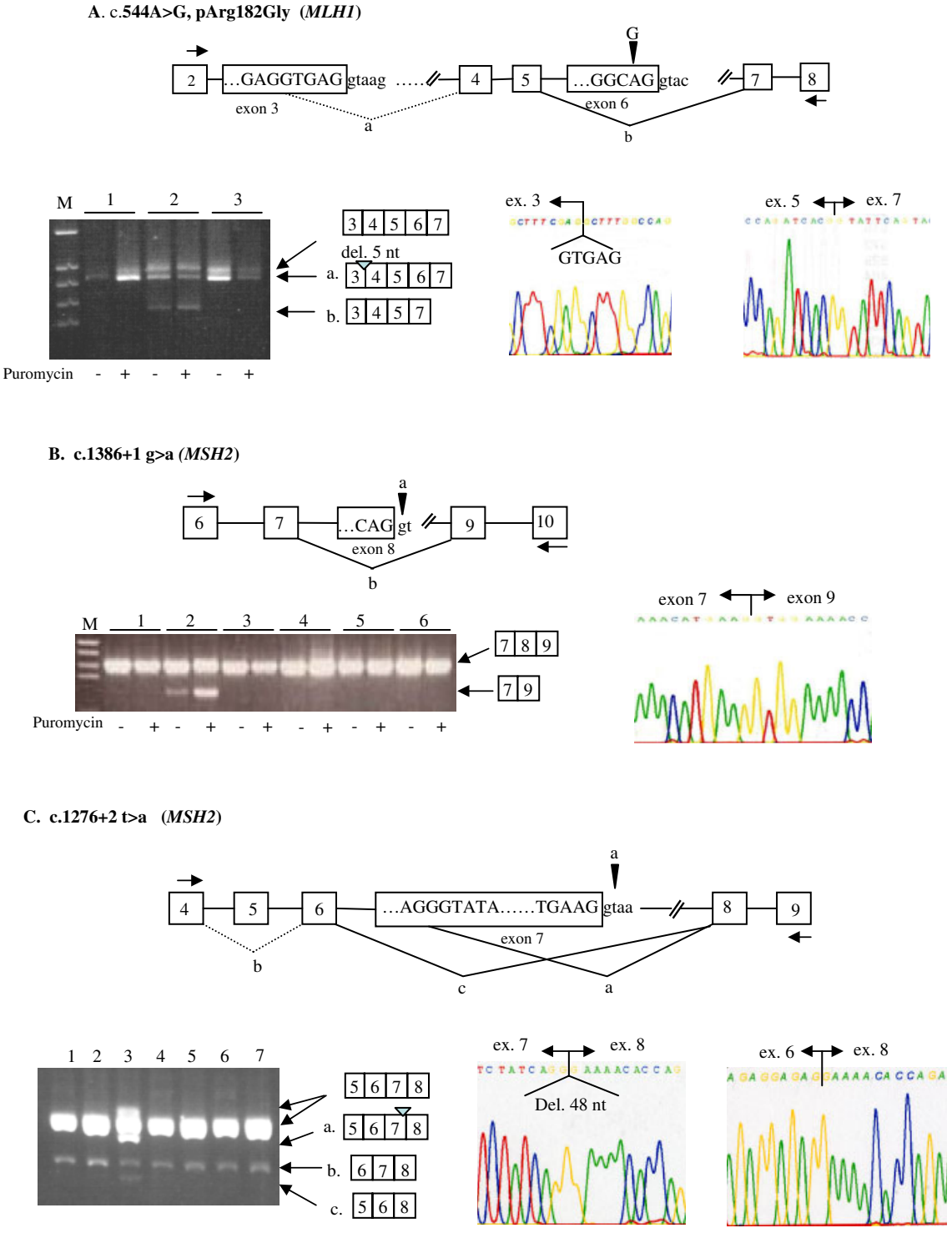

FIGURE 1. **Examples of aberrant splicing detected by RNA screening. Schematic diagrams represent fragments analyzed by RT-PCR using the primers indicated as arrows and the resulting aberrant splicing and/or alternative splicing. Open boxes with numbers denote individual exons that are separated by introns indicated as horizontal lines. Mutations and surrounding sequences from which an aberrant or an alternative splicing originated are indicated. Results of electrophoretic analyses are shown using pGEM as the molecular weight marker (M). A:** Analysis of exonic mutation c.544A>G (*MLH1*). Skipping of exon 6 was detected in the mutation-carrying sample (sample 2) in cells with or without treatment with puromycin, as confirmed by sequencing. This aberrant splicing is absent from the control (sample 1) and from sample 3 harboring variant c.2T>G. Another isoform of the transcript was detected in all analyzed samples that differed from the constitutive one by the deletion of 5 bp at the end of exon 3, as evidenced by sequencing (middle panel). **B:** Analysis of intronic mutation at position c.1386+1 (*MSH2*), which revealed the skipping of exon 8 (sample 2) evidenced by sequencing. The level of the aberrantly spliced transcript was shown to be higher in cells treated with puromycin. **C:** Aberrant splicing revealed in the sample carrying intronic mutation c.1276+2 (*MSH2*). In addition to the previously reported alternative splicing characterized by the skipping of exon 5, two aberrant transcript forms were observed in mutation-harboring sample 3: one due to the deletion of the last 48 bp of exon 7, and one caused by skipping of the entire exon 7, as shown by sequencing analysis. Sequence references: *MLH1*, NM_000249, and NC_000003; *MSH2*, NM_000251, and NC_000002. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE 1. Nucleotide Substitutions and Aberrant Splicing in MLH1 and MSH2 Genes

Familly code	Gene	Mutation description	Nt change and surrounding sequences	Scores with ESEfinder (SF2/ASF/SC35/SRp40/SRp55)[a]	No. of candidate ESE (RESCUE-ESE)	Aberrant splicing
Exonic nucleotide substitution						
6004	MLH1	c.2T>G, pMet1Arg	AaATG > aaAGG	–	No	Not detected
0013	MLH1	c.91_92GC>TG, pAla31Cys	ATGCTA–ATTGTA	–/2.7/3/–	No	Not detected
0114	MLH1	c.146T>A, pVal49Glu	AGTGA > AGAGA	–	No	Not detected
7149	MLH1	c.199G>T, pGly67Trp	CCGGG > CCTGG	4.3/–/–/–	No	Not detected
0H04	MLH1	c.199G>A, pGly67Arg	CCGGG > CCAGG	4.3/–/–/–	No	Not detected
L0334	MLH1	c.229T>C, pCys77Arg	TATGT > TACGT	–	No	Not detected
8H04	MLH1	c.304G>A, pGlu102Lys	GTGAG > GTAAG	2.7/–/–/–	No	Not detected
6058	MLH1	c.318C>A, pSer106Arg	AGCAT > AGAAT	–/4/–/3.3	No	Not detected
6147	MLH1	c.338T>A, pVal113Asp	TGTTA > TGATA	2.6/2.7/2.8/–	No	Not detected
6070	MLH1	c.350C>T, pThr117Met	AACGA > AATGA	–/–/4.6/–	4	Not detected
7G02	MLH1	c.375A>G, pAla125Ala	GCATA > GCGTA	–/–/5/5.2	No	Not detected
5069	MLH1	c.544A>G, pArg182Gly	CAGgt > CGGgt	3.6/–/2.9/–	No	Exon 6 skipping
L0224	MLH1	c.637G>A, pVal213Met	CCGTG > CCATG	2.9/–/2.7/–	No	Not detected
B0018	MLH1	c.739T>C, pSer247Pro	TATCC > TACCC	–	1	Not detected
L0346	MLH1	c.882C>T, pLeu294Leu	CTCAGgt > CTTAGgt	4.5/–/4.9/3.2	No	Exon 10 skipping
0013	MLH1	c.1360G>C, pGly454Arg	AGGGG > AGCGG	2.9/–/3.7/–	No	Not detected
LYTF1	MLH1	c.1652A>C, pAsn551Thr	CAACA > CACCA	–/–/3.9/–	No	Not detected
L0324	MLH1	c.1731G>A, pSer577Ser	TCGgt > TCAgt	–/2.8/–/–	No	Exon 15 skipping
8D09	MLH1	c.1757C>A, pAla586Asp	TGCCA > TGACA	–	No	Not detected
4097	MLH1	c.1852_1853AA>GC, pLys618Ala	AGAAGG > AGGCGG	–	4	Not detected
7006	MLH1	c1852_1854del3, pLys618del	AGAAGGC > AG…GC	–	4	Not detected
6099	MLH1	c.1959G>T, pLeu653Leu	CTGCC > CTTCC	2.1/–/–/3.5	No	Not detected
1046	MLH1	c.1989G>T, pGlu663Asp	GAGgt > GATgt	4.3/4.5/–/–	No	Exon 17 skipping
B9908	MLH1	c.2059C>T, pArg687Trp	TCCGG > TCTGG	2.6/–/–/–	No	Not detected
9074	MLH1	c.2146G>A, pVal716Met	CTGTG > CTATG	2.2/3.8/–/3.4	1	Not detected
7H03	MLH1	c.2263A>T, pArg755Trp	AGAGG > AGTGG	3.8/–/3.8/–	No	Not detected
0013	MSH2	c.97A>G, pThr33Ala	CCACA > CCGCA	3.1/2.6–4.5/2.8–3.6/–	No	Not detected
1G12	MSH2	c.128A>G, pTyr43Cys	CTATA > CTGTA	–/4.7/3/–	No	Not detected
L2186	MSH2	c.380A>G, p.Asn127Ser	CAATC > CAGTC	–	No	Not detected
JCS0316	MSH2	c.518T>C, pLeu173Pro	ACTAG > ACCAG	–	1	Not detected
6031	MSH2	c.560T>G, pLeu187Arg	TCTTG > TCGTG	–/–/2.9/–	No	Not detected
3152	MSH2	c.573C>T, pLeu191Leu	CTCAT > CTTAT	–	No	Not detected
2H05	MSH2	c.728G>A, pArg243Gln	CCGGT > CCAGT	2.1–2.6/3/2.7/–	No	Not detected
6113	MSH2	c.965G>A, pGly322Asp	TGGCT > TGACT	–/3.4/5.7/–	No	Not detected
L972	MSH2	c.1022T>C, pLeu341Pro	ACTTG > ACCTG	–	1	Not detected
8H04	MSH2	c.1168C>T, pLeu390Phe	GACTT > GATTT	2.1/–/2.9/–	No	Not detected
B9814	MSH2	c.1642G>T, pGly548Cys	ATGGT > ATTGT	–/3/–/–	1	Not detected
3019	MSH2	c.1666T>C, pLeu556Leu	AATTG > AACTG	–	No	Not detected
8H02	MSH2	c.1681G>A, pGlu561Lys	ATGAA > ATAAA	–	4	Not detected
1H14	MSH2	c.1737A>G, pLys579Lys	AAAGA > AAGGA	–	2	Not detected
2H04	MSH2	c.1786_1788del3, pAsn596del	TCAATGA > TC…GA	–	1	Not detected
B9707	MSH2	c.1881A>C, pLys627Asn	AAAGG > AACGG	2.1/–/2.9/–	5	Not detected
EL022	MSH2	c.1915C>T, pHis639Tyr	AGGCAT > AGGTAT	–	No	c.1914_2005del92
6156	MSH2	c.2089T>C, pCys697Arg	CATGT > CACGT	–	No	Not detected
0112	MSH2	c.2245G>A, pGlu749Lys	ATGAA > ATAAA	–	2	Not detected
Intronic nucleotide substitution						
L0014	MLH1	c.454–2a>g	cagGTG > cggGTG			Exon 6 skipping
L0341	MLH1	c.790+1g>a	ACCgta > ACCata			Exon 9–10 skipping
L045	MLH1	c.791–5t>g	tttagAT > gttagAT			Exon 10 skipping
S0311	MLH1	c.791–23delg	ctggtt > ctgtt			Not detected
7072	MLH1	c.1558+14g>a	gctgtg > gctgag			Not detected
6001	MLH1	c.1668–19a>g	gtcact > gtcgct			Not detected
S0314	MLH1	c.1732–9t>c	gcttct > gctcct			Not detected
3024	MLH1	c.1990–16_–2del15	…ccagGTG > …ttggGTG			Exon 18 skipping
EL023	MSH2	c.366+1g>t	AAGgt > AAGtt			Exon 2 skipping
L014	MSH2	c.793–2a>c	tagGTT > tcgGTT			Exon 5 skipping
5073	MSH2	c.942+3a>t	CAGgta > CAGgtt			Exon 5 skipping
4070	MSH2	c.1076+1g>a	GAGgt > GAGat			Exon 6 skipping
5H007	MSH2	c.1276+2t>a	AAGgt > AAGga			c.1228_1276del48 Exon 7 skipping
1034	MSH2	c.1386+1g>a	CAGgt > CAGat			Exon 8 skipping
0012	MSH2	c.2005+8_+9insa	aaacct > aaaacct			Not detected

[a]Only the score above the threshold was indicated. –: score below the threshold either for one of specific SR protein when others are positive or for all four SR proteins when there are no positive scores recorded for a given sequence (Thresholds: SF2/ASF: 1.956; SC35: 2.383; SRp40: 2.67; and SRp55: 2.67)(ESEfinder). Sequence references: MLH1, NM_000249, and NC_000003; MSH2, NM_000251, and NC_000002.

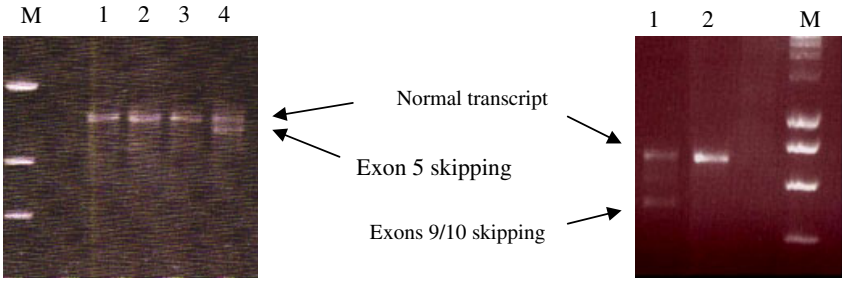

FIGURE 2. **Alternatively skipped exons in missense mutation carriers. RT-PCR products were separated on agarose gel. Additional shortened bands are shown in carriers of missense mutation c.790+1g>a (lane 1: right panel) and c.942+3a>t (lane 4: left panel), due to the skipping of exons 9–10 (*MLH1*) and 5 (*MSH2*), respectively. In other samples including control cells (lane 1: left panel; lane 2: right panel), only the constitutive transcript was detectable. M: molecular weight marker.**

TABLE 2. **Correlation Between Predicted ESEs and Aberrant Splicing**

	Number of variants predicted to lie within a putative ESE[a]			Number of variants not predicted to lie within a putative ESE[b]		
Number of variants	ESEfinder	RESCUE	Both	ESEfinder	RESCUE	Both
With aberrant splicing	4	0	0	1	5	1
Without aberrant splicing	23	13	4	17	27	8
Total	27	13	4	18	32	9

[a]Variants whose score value is above to threshold with any of the 4 SR proteins (see footnote for Table 1); or they match candidate ESEs using RESCUE-ESE (RESCUE), or predicted as a putative ESE by both methods (Both).
[b]For variants whose score value is below to threshold, or does not match any candidate hexamers (RESCUE) or with no indication as an ESE by both methods.

allele can still be considered as a putative ESE. Thus, no clear relation between the disruption of a putative ESE and altered splicing can be established from these data. Among the remaining variants not recognized as putative ESEs (18 by ESEfinder and 32 by RESCUE), one aberrant splicing in *MSH2* gene was observed: c.1915C>T associated to the deletion of the last 92 bp of exon 12.

To further investigate whether the aberrant splicing we detected was directly induced by the nucleotide substitution, and whether a "theoretical" ESE could induce in vitro an aberrant splicing that may have been missed by mRNA screening, we carried out an in vitro splicing analysis using "minigene" constructs. We first analyzed two aberrant splicing-associated cell lines carrying variants c.882C>T in *MLH1* and c.1915C>T in *MSH2*. The minigenes were constructed by inserting genomic DNA segments containing the affected exon and its flanking intron and exon sequences into a mammalian expression vector (Fig. 3). The constructs were then transfected transiently into COS-7 cells. Using sequencing, we confirmed the presence of the exonic mutation as the unique genomic alteration in the Mt allele-derived minigene, and the absence of mutation in the WT allele-derived minigene. RT-PCR was then performed using mRNA isolated from transfected cells. The expression of endogenous *MLH1* from COS-7 cells did not interfere with the detection of predominantly expressed transcripts from exogenous minigenes, since in both cases only aberrantly spliced transcripts were generated in cells transfected with Mt alleles (Fig. 3), as confirmed by sequencing. Constitutive transcripts were detected in cells transfected with the WT allele. Unexpectedly, in the case of variant c.882C>T, RT-PCR revealed the as yet unexplainable presence of a weak amount of aberrant splicing in cells transfected with the WT allele. The possibility of a potential alternative splicing switch in an in vitro system is raised. From this result we can conclude that the aberrant splicing found in RNA screening was indeed induced by the corresponding exonic mutations. Furthermore, we analyzed three variants that were predicted as putative ESEs but were not associated with aberrant splicing in endogenous RNA screening. Minigenes with insertion of genomic DNA encompassing intron 16 to 3′ NTR of *MLH1* were generated (Fig. 3), which made it possible to analyze variants c.1959G>T and c.2059C>T. Both were predicted to disrupt a putative ESE since the positive score for more than one SR protein was decreased to below the threshold. This system also allowed us to analyze c.2146G>A, which was predicted as a putative ESE by both ESEfinder and RESCUE algorithms. For c.2146G>A, although the G-to-A transition led to the decrease of positive scores for SF2/ASF (2.2) and SRp55 (3.4) to below the threshold, the score for SC35 remained nearly unchanged (3.8–3.4). RESCUE-ESE predicted that the nucleotide substitution would destroy the existing ESE but endow two new hexamers with the property of ESE. No aberrant splicing was revealed by in vitro expression in cells transfected with the Mt allele. To ascertain that the constitutive transcript was produced from the Mt allele rather than from endogenous expression of COS-7 cells, we carried out sequencing and confirmed the presence of mutations in the observed transcripts. Nevertheless, a faint additional band was observed in all transfected cells, regardless of the type of mutations or whether a WT or Mt allele was involved (Fig. 3). Sequencing showed that it corresponded to a transcript lacking exons 17 and 18. Since it was absent from control lymphocytes, it was likely an artifact of in vitro transcription from this minigene construct. Thus, our results showed that none of the putative ESEs could be linked to aberrant splicing, even in an in vitro system. However, the absence of aberrant splicing in variant c.2146G>A may be explained by the fact that the mutation led to

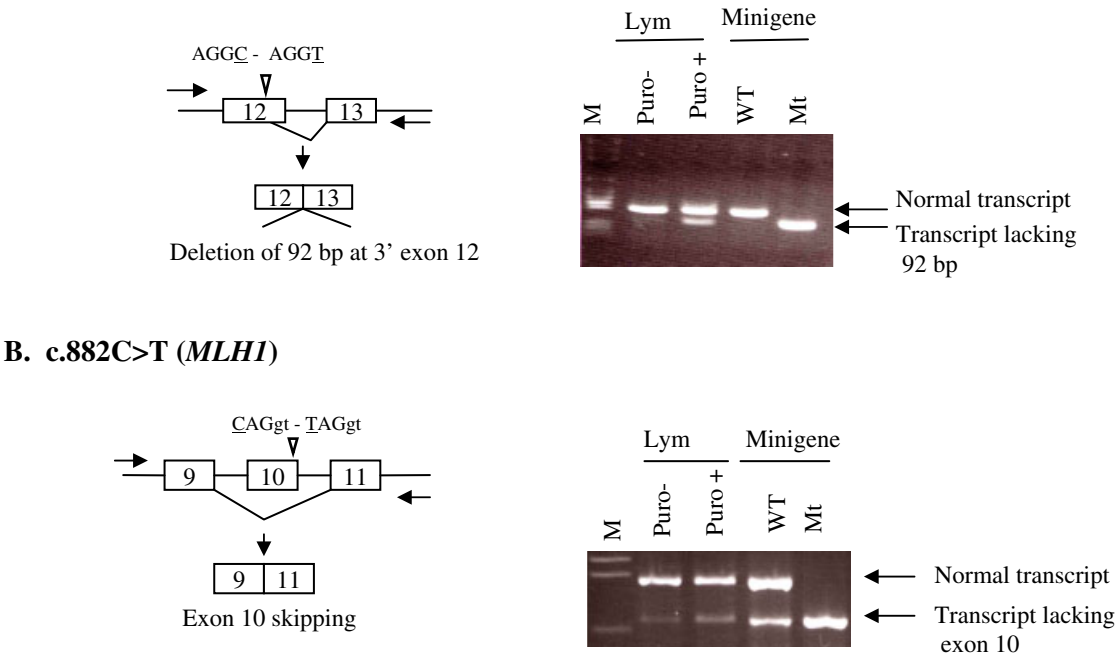

FIGURE 3. **Minigene transient transfection assay for exonic missense mutations: (A) c.1915C>T in *MSH2*, (B) c.882C>T in *MLH1*, and (C) c.1959G>T, c.2059C>T, and c.2146G>T in *MLH1*. Schematic representations show the DNA fragments used for the construction of minigenes, amplified with primers indicated as arrows. Individual exons are represented as open boxes with the corresponding exon number inside. The position of the mutation and surrounding sequences, and the consequent splicing observed in the assay are indicated. The right panels show 2% agarose gel electrophoresis of RT-PCR products. RT-PCR products from WT allele or Mt allele derived minigenes were compared with those from lymphocyte cells (Lym) from the same patient, treated or not treated with puromycin (puro+ or puro–, respectively). M denotes the molecular weight marker (pGEM) used in this assay.**

the generation of new motifs capable of being recognized by other members of the SR protein family or new hexamers possessing exonic enhancer activity.

DISCUSSION

Nucleotide substitution-induced missense, silent, or nonsense mutations can result in defective splicing during pre-mRNA processing by affecting splicing regulatory elements, such as ESEs, or other elements, such as exonic splicing silencers (ESSs) or the recently identified composite exonic regulatory element of splicing (CERES) [Caputi et al., 2002; Kashima and Manley, 2003; Pagani et al., 2003a,b; Sironi et al., 2004]. This finding is particularly important for genetic counseling because it can help investigators determine the inactivating property of a nucleotide substitution in cancer predisposition genes. We conducted a systematic screening for aberrant splicing in *MLH1* and *MSH2* genes in a total of 60 unrelated HNPCC patients who carried different exonic or intronic nucleotide substitutions, using an RT-PCR-based analysis of endogenous RNA obtained from the lymphoblastoid cell line established from each patient treated with puromycin. A new isoform of the transcript that differs from the normal one by the deletion of 5 bp was revealed. The presence of this isoform in

TABLE 3. ESE Score Change in Cases With Aberrant Splicing in *MLH1*

Case with aberrant splicing	ESEfinder score[a] SF2/ASF	SC35	SRp40	SRp55	RESCUE-ESE prediction
c.544A>G, pArg182Gly					
Wild type	3.6	–	2.9	–	No
Mutant	–	2.4	–	–	No
c.882C>T, pLeu294Leu					
Wild type	4.5	–	4.9	3.2	No
Mutant	2.2	2.6	–	–	No
c.1731G>A, pSer577Ser					
Wild type	–	2.8	–	–	No
Mutant	–	–	–	–	No
c.1989G>T, pGlu663Asp					
Wild type	4.3	4.5	–	–	No
Mutant	2.3	2.4	–	–	No

[a]Only the scores above the threshold (see Table 1) are indicated. Scores below threshold are indicated as –.

several samples, including normal cells, suggests that this is an alternative splicing. The abundant amount of this isoform revealed by RT-PCR, and its resistance to NMD (Fig. 1) may suggest a functional role. However, further studies are needed to determine its relevant function in DNA repair. The detection of a deletion as small as five nucleotides suggests that our detection system has satisfactory sensitivity, although the analysis of some regions of both genes required the use of several sets of RT amplification primers because of the presence of complex patterns of alternative splicing. Nevertheless, we cannot exclude the possibility that some complex aberrant splicings, such as the retention of large introns or the duplication of several exons, might have been missed because they made the transcripts too large to be amplified by the conventional amplification methods used.

This approach enabled the detection of a total of 15 aberrant splicings that were believed to be disease-causing, as evidenced primarily by the absence of skipping of constitutive exons (2, 6, 10, 15, and 18 of *MLH1*, and 4, 6, 7, and 8 of *MSH2*) in normal control cells. Aberrantly spliced transcripts frequently result in the disruption of the reading frame, leading to the generation of premature stop codons (exons 2, 6, 10, and 15 of *MLH1*, and exons 6, 7, and 8 of *MSH2*, as well as deletion of coding sequences in exons 7 and 12 of *MSH2*). Such transcripts are then degraded at least partially by NMD, as evidenced in this study by a less abundant amount of abnormal transcripts compared to samples treated with puromycin. Deletion of exons 17 and 18 of *MLH1* and exon 5 of *MSH2* causes in-frame loss of respectively 31, 38, and 50 amino acids of encoded proteins. These transcripts may also escape NMD because of the lack of a premature termination codon. The defective function of the peptide lacking exon 17 has been shown in vitro [Nyström-Lahti et al., 1999]. Transcripts lacking exon 9/10, exon 17 in *MLH*, and exons 5 and 13 in *MSH2* were previously reported as alternative transcripts [Genuardi et al., 1998]. However, the ratio of consecutively and alternatively transcribed isoforms was altered in mutation-carrying samples, with a reduced level of consecutive transcripts but a high level of alternative isoforms. Although the natural function of those alternative isoforms is unknown, such a ratio alteration can be disease-related, as previously demonstrated in the disease-causing genes *GH-1*, *WT1*, *MAP*, and *CFTR* [reviewed in Cooper and Mattox, 1997; Faustino and Cooper, 2003]. The pathogenic effect of aberrant splicing is further supported by cosegregation analyses in families in which samples from other affected members were available. Splicing mutations associated to the skipping of exons 6, 17, and 18 of *MLH1*, and exon 8 of *MSH2* were detected in all affected members tested. In family 3024, mutation c.1990-16_-2del, leading to the in-frame deletion of exon 18, was detected in all three affected members who developed early-onset multiple primary HNPCC component tumors. Moreover, mutations c.942+3a>t (*MSH2*) and c.1731G>A (*MLH1*) appear to be recurrent in HNPCC [Froggatt et al., 1995; Kohonen-Corish et al., 1996; Wang et al., 1997, 1999; Viel et al., 1997] (see also the online Mutation Database: www.insight-group.org), which suggests that they play an important role in the disease. Our study also demonstrated, by in vitro expression essay, that the aberrant transcripts were indeed induced by mutations detected in the patients. The pathogenicity of these nucleotide substitution mutations can thus be established.

Given that systematic RNA analysis cannot be included as a routine screening method in genetic testing because of the difficulties frequently encountered in isolating good-quality RNA from fresh blood samples, or establishing lymphoblastoid cell lines from patients carrying missense or silent mutations, the ability to predict defective transcriptions induced by a given mutation would be of great benefit. The hypothesis regarding disruption of a putative ESE was examined with the use of existing computerized ESE prediction algorithms. Four out of five exonic substitutions resulting in aberrant splicing did take place in ESEs predicted by ESEfinder. However, all of the four exonic mutations are located at or close to 5′ss. This raises a question as to whether the aberrant splicing is the consequence of the disruption of an ESE or the failure of 5′ss. Highly conserved exonic splicing sequences, especially positions 1 and 2 before the end of the exons, are essential for base-pairing with the spliceosome element snRNA U1 [reviewed in Ast, 2004]. Three out of four aberrantly splicing-related mutations affected those positions, with the exception of c.882C>T, which affected position 3 before the end of exon 10. Of note, another variant that affected the same position in exon 3 of *MLH1* (c.304G>A) was not associated to aberrant splicing. A computational exonic splicing prediction program (www.fruitfly.org/seq_tools/splice.html) was further used to evaluate the consequence of mutations affecting splicing sites. It revealed that the mutations c.544A>G and c.1989G>T lead a great decrease in the predictive score (0.66–0.4 and 0.91–0.4, respectively), suggesting a loss of the splicing-site recognition property. Nevertheless, a high predictive score is maintained for mutations c.882C>T and c.1731G>A (0.81 and 0.93, respectively). Taken together, the aberrant splicing caused by c.882C>T and

c.1731G>A are more likely due to the disruption of ESE, consistent with the decrease in the positive ESE predictive score for those mutations (particularly c.1731G>A).

Overall, our analysis of the potential correlation between aberrant splicing and prediction of ESE by ESEfinder revealed a sensitivity of 80% and a specificity of 42%. The prediction power of RESCUE-ESE was weaker in this study, since none of the aberrant splicing-related variants were predicted to lie within a putative ESE by this method. Further analyses on a larger number of samples are needed to evaluate its sensitivity and specificity. An excess number of predicted ESEs was not associated with aberrant splicing. This may be explained by the presence of partially redundant or multiple ESEs in nearly every exon. It is possible that not all of the ESEs affected by a mutation analyzed in this study were functional. Another explanation would be that functional ESEs seem to be cell type specific [Slaugenhaupt et al., 2001]. Some mutations may disrupt a functional ESE and induce defective splicing and subsequent loss of function of the Mt allele in target cells, such as digestive epithelial cells or endometrial cells, rather than in the lymphoblastoid cells examined here. Studies of transcripts from tumor cells might help to clarify this issue. It is also important to point out that one aberrant splicing-related variant, c.1915C>T (*MSH2*), was not predicted as a putative ESE by any of the two algorithms. The discrepancy between splicing defect and ESE prediction was also observed in other studies [Campos et al., 2003; McVety et al., 2005]. In summary, it is clear that splicing defects can be caused not only by the disruption of ESEs, but also by the alterations of other regulatory elements or an unbalanced effect between positive and negative elements. Investigators must use caution when evaluating such missense or silent mutations in genetic testing, especially when using in silico prediction. RNA analysis is required to confirm the effect of such mutations on splicing.

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REFERENCES

Aretz S, Uhlhaas S, Sun Y, Pagenstecher C, Mangold E, Caspari R, Möslein G, Schulmann K, Propping P, Friedl W. 2004. Familial adenomatous polyposis: aberrant splicing due to missense or silent mutations in the APC gene. Hum Mut 24:370–380.

Ars E, Serra E, Garcia J, Kruyer H, Gaona A, Lazaro C, Estivill X. 2000. Mutations affecting mRNA splicing are the most common molecular defects in patients with neurofibromatosis type 1. Hum Mol Genet 9:237–247.

Ast G. 2004. How did alternative splicing evolve? Nat Rev Genet 5:773–782.

Campos B, Diez O, Domènech M, Baena M, Balmana J, Sanz J, Ramirez A, Alonso C, Baiget M. 2003. RNA analysis of eight BRCA1 and BRCA2 unclassified variants identified in breast/ovarian cancer families from Spain. Hum Mut 22:337.

Caputi M, Kendzior RJ Jr, Beemon KL. 2002. A nonsense mutation in the fibrillin-1 gene of a Marfan syndrome patient induces NMD and disrupts an exonic splicing enhancer. Genes Dev 16:1754–1759.

Cartegni L, Krainer AR. 2002. Disruption of an SF2/ASF-dependent exonic splicing enhancer in SMN2 causes spinal muscular atrophy in the absence of SMN1. Nat Genet 30: 377–384.

Cartegni L, Chew SL, Krainer AR. 2002. Listening to silence and understanding nonsense: exonic mutations that affect splicing. Nat Rev Genet 3:285–298.

Cartegni L, Wang J, Zhu Z, Zhang MQ, Krainer AR. 2003. ESEfinder: a Web resource to identify exonic splicing enhancers. Nucleic Acids Res 31:3568–3571.

Cooper TA, Mattox W. 1997. The regulation of splice-site selection, and its role in human disease. Am J Hum Genet 61: 259–266.

Fackenthal JD, Cartegni L, Krainer AR, Olopade OI. 2002. BRCA2 T2722R is a deleterious allele that causes exon skipping. Am J Hum Genet 71:625–631.

Fairbrother WG, Yeh RF, Sharp PA, Burge CB. 2002. Predictive identification of exonic splicing enhancers in human genes. Science 297:1007–1013.

Fairbrother WG, Yeo GM, Yeh RF, Goldstein P, Mawson M, Sharp PA, Burge CB. 2004. RESCUE-ESE identifies candidate exonic splicing enhancers in vertebrate exons. Nucleic Acids Res 32: W187–190.

Faustino NA, Cooper TA. 2003. Pre-mRNA splicing and human disease. Genes Dev 17:419–437.

Froggatt NJ, Joyce JA, Davies R, Gareth D, Evans R, Ponder BA, Barton DE, Maher ER. 1995. A frequent hMSH2 mutation in hereditary non-polyposis colon cancer syndrome. Lancet 18: 345–727.

Genuardi M, Viel A, Bonora D, Capozzi E, Bellacosa A, Leonardi F, Valle R, Ventura A, Pedroni M, Boiocchi M, Neri G. 1998. Characterization of MLH1 and MSH2 alternative splicing and its relevance to molecular testing of colorectal cancer susceptibility. Hum Genet 102:15–20.

Gorlov IP, Gorlova OY, Frazier ML, Amos CI. 2003. Missense mutations in hMLH1 and hMSH2 are associated with exonic splicing enhancers. Am J Hum Genet 73:1157–1161.

Hentze MW, Kulozik AE. 1999. A perfect message: RNA surveillance and nonsense-mediated decay. Cell 96:307–310.

Kashima T, Manley JL. 2003. A negative element in SMN2 exon 7 inhibits splicing in spinal muscular atrophy. Nat Genet 34: 460–463.

Kohonen-Corish M, Ross VL, Doe WF, Kool DA, Edkins E, Faragher I, Wijnen J, Khan PM, Macrae F, St John DJB. 1996. RNA-based mutation screening in hereditary nonpolyposis colorectal cancer. Am J Hum Genet 59:818–824.

Liu HX, Zhang M, Krainer AR. 1998. Identification of functional exonic splicing enhancer motifs recognized by individual SR proteins. Genes Dev 12:1998–2012.

Liu HX, Chew SL, Cartegni L, Zhang MQ, Krainer AR. 2000. Exonic splicing enhancer motif recognized by human SC35 under splicing conditions. Mol Cell Biol 20:1063–1071.

Liu HX, Cartegni L, Zhang MQ, Krainer AR. 2001a. A mechanism for exon skipping caused by nonsense or missense mutations in BRCA1 and other genes. Nat Genet 27:55–58.

Liu T, Yan H, Kuismanen S, Percesepe A, Bisgaard ML, Pedroni M, Benatti P, Kinzler KW, Vogelstein B, Ponz de Leon M, Peltomäki P, Lindblom A. 2001b. The role of hPMS1 and hPMS2 in predisposing to colorectal cancer. Cancer Res 61:7798–7802.

Liu HX, Zhou XL, Liu T, Werelius B, Lindmark G, Dahl N, Lindblom A. 2003. The role of hMLH3 in familial colorectal cancer. Cancer Res 63:1894–1899.

Mazoyer S, Puget N, Perrin-Vidoz L, Lynch HT, Serova-Sinilnikova OM, Lenoir GM. 1998. A BRCA1 nonsense mutation causes exon skipping. Am J Hum Genet 62:713–715.

McVety S, Li L, Gordon PH, Chong G, Foulkes WD. 2005. Disruption of an exon splicing enhancer in exon 3 of MLH1 is the cause of HNPCC in a Quebec family. J Med Genet (published online 27 May 2005).

Nyström-Lahti M, Holmberg M, Fidalgo P, Salovaara R, de la Chapelle A, Jiricny J, Peltomäki P. 1999. Missense and nonsense mutations in codon 659 of MLH1 cause aberrant splicing of messenger RNA in HNPCC kindreds. Genes Chromosomes Cancer 26:372–375.

Pagani F, Buratti E, Stuani C, Baralle FE. 2003a. Missense, nonsense and neutral mutations define juxtaposed regulatory elements of splicing in cystic fibrosis transmembrane regular exon 9. J Biol Chem 278:26580–26588.

Pagani F, Stuani C, Kanavakis E, Efthymiadou A, Doudounakis S, Casals T, Baralle F. 2003b. New type of disease causing mutations: the example of the composite exonic regulatory elements of splicing in CFTR exon 12. Hum Mol Genet 12: 1111–1120.

Sironi M, Menozzi G, Riva L, Cagliani R, Comi GP, Bresolin N, Giorda R, Pozzoli U. 2004. Silencer elements as possible inhibitors of pseudoexon splicing. Nucleic Acids Res 32: 1783–1791.

Slaugenhaupt SA, Blumenfeld A, Gill SP, Leyne M, Mull J, Cuajungco MP, Liebert CB, Chadwick B, Idelson M, Reznik L, Robbins C, Makalowsa I, Brownstein M, Krappmann D, Scheidereit C, Maayan C, Axelrod FB, Gusella JF. 2001. Tissue-specific expression of a splicing mutation in the IKBKAP gene causes familial dysautonomia. Am J Hum Genet 68: 598–605.

Stella A, Wagner A, Shito K, Lipkin M, Watson P, Guanti G, Lynch HT, Fodde R, Liu B. 2001. A nonsense mutation in MLH1 causes exon skipping in three unrelated HNPCC families. Cancer Res 61:7020–7024.

Teraoka SN, Telatar M, Becker-Catania S, Liang T, Önengüt S, Tolun A, Chessa L, Sanal Ö, Bernatowska E, Gatti RA, Concannon P. 1999. Splicing defects in the ataxia-telangiectasia gene, ATM: underlying mutations and consequences. Am J Hum Genet 64:1617–1631.

Viel A, Genuardi M, Capozzi E, Leonardi F, Bellacosa A, Paravatou-Petsotas M, Pomponi MG, Fornasarig M, Pecesepe A, Roncucci L, Tamassia MG, Benatti P, Ponz de Leon M, Valenti A, Covino M, Anti M, Foletto M, Boiocchi M, Neri G. 1997. Characterization of MSH2 and MLH1 mutations in Italian families with hereditary nonpolyposis colorectal cancer. Genes Chromosomes Cancer 18:8–18.

Wang Q, Desseigne F, Lasset C, Saurin JC, Navarro C, Yagci T, Keser I, Bagci H, Luleci G, Gelen T, Chayvialle JA, Puisieux A, Ozturk M. 1997. Germline hMSH2 and hMLH1 gene mutations in incomplete HNPCC families. Int J Cancer 73:831–836.

Wang Q, Lasset C, Desseigne F, Saurin JC, Maugard C, Navarro C, Ruano E, Descos L, Trillet-Lenoir V, Bosset JF, Puisieux A. 1999. Prevalence of germline mutations of hMLH1, hMSH2, hPMS1, hPMS2 and hMSH6 genes in 75 French kindreds with nonpolyposis colorectal cancer. Hum Genet 105:79–85.

Wu Y, Berends MJW, Sijmons RH, Mensink RG, Verlind E, Kooi KA, van der Sluis T, Kempinga C, van der Zee AGJ, Hollema H, Buys CHCM, Kleibeuker JH, Hofstra RMW. 2001a. A role for MLH3 in hereditary nonpolyposis colorectal cancer. Nat Genet 29:137–138.

Wu Y, Berends MJW, Post JG, Mensink RGJ, Verlind E, van der Sluis T, Kempinga C, Sijmons RH, van der Zee AGJ, Hollema H, Lkeibeuker JH, Buys CHCM, Hofstra RMW. 2001b. Germline mutations of EXO1 gene in patients with hereditary nonpolyposis colorectal cancer (HNPCC) and atypical HNPCC forms. Gastroenterology 120:1580–1587.

Zatkova A, Messiaen L, Vandenbroucke I, Wieser R, Fonatsch C, Krainer AR, Wimmer K. 2004. Disruption of exonic splicing enhancer elements is the principal cause of exon skipping associated with seven nonsense or missense alleles of NF1. Hum Mut 24:491–501.