Novel approaches in evaluation of pathogenicity of single-base exonic germline changes involving the mismatch repair genes *MLH1* and *MSH2* in diagnostics of Lynch syndrome

Minireview

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Germline defects in the DNA mismatch repair genes *MLH1* and *MSH2* are the major cause of hereditary nonpolyposis colon cancer (HNPCC), also called Lynch syndrome. Detection of inherited pathogenic change in their DNA sequence in HNPCC families allows for identification of asymptomatic individuals who require appropriate medical surveillance. However, evaluation of clinical significance of identified DNA alteration is not always straight-forward and some changes maybe classified incorrectly depending on the method used. The aim of this review is to summarize rationale, practice and pitfalls in the characterization of substitutions localized in the exons and outline new experimental and *in silico* approaches used to determine mutation consequence. Our survey of variants identified in *MLH1* and *MSH2* genes which were confirmed to cause splicing defect but often appear characterized as missense, nonsense or silent mutations in various databases and publications as well as a list of true missense mutations may serve as a valuable aid for laboratories providing HNPCC diagnosis.

Key words: MLH1, *MSH2*, HNPCC, MMR, mutation analysis, in silico assays

According to the new catalogue of *MMR* gene variants [http://www.med.mun.ca/MMRvariants], the *MLH1* and *MSH2* account of 80% from more than 1,500 different changes that have been identified altogether in the four major *MMR* genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*) associated with HNPCC [1]. These include largely single nucleotide substitutions in highly conserved intron-splice dinucleotide sequences, small insertions/deletions within exons or large rearrangements involving a whole or several exons. Assessment of pathogenicity of any DNA change is crucial for correct prediction of cancer risk in HNPCC-suspected individuals and their families. Approximately 40% of changes of *MLH1* or *MSH2* gene represent single nucleotide substitutions in coding regions [http://www.med.mun.ca/MMRvariants/statistics.aspx] and these particularly represent a challenge for the molecular geneticists, as well as for genetic counselors considering their clinical significance.

The most sensitive technique currently used for the detection of germline mutations is exon by exon sequencing of the gene using genomic DNA as a template for analysis. Consequently, the rationale for characterization of any single nucleotide substitution in the coding region of the disease-associated genes is initially based on the knowledge of genetic code. Accordingly, the change may theoretically result in an amino acid substitution (missense mutation), creation of a termination codon (nonsense mutation) or the substitution does change the base but the coding amino acid remains unchanged due to redundancy in genetic code (silent mutation). However, during the long-term genetic testing of various genes in humans it has become evident that solely DNA sequence derived consequence of mutation may be misleading and not reflect real biochemical consequences for some alterations [2–4]. Already in 1999, Nystrom-Lahti et al. have shown, using RT-PCR analysis, that presumably

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missense and nonsense mutations in codon 659 of *MLH1* gene in fact cause aberrant splicing of messenger RNA [5]. Similarly, Stella et al. reported skipping of exon 12 caused by a nonsense mutation at codon 461 of *MLH1* as confirmed by RT-PCR and *in vitro* exon skipping analysis [6]. Moreover, the silent changes such as alteration G>A at the 3rd position of codon 346 of *MLH1* [p.Gln346Gln] may lead to aberrant splicing as shown by using allele separation technique [7]. Despite these leading cases, albeit rare, the RNA techniques retrograded in the course of huge progress in DNA screening technologies based on automatic DNA sequencers. Withdrawal of RNA techniques was further underlined by the presence of alternatively spliced forms of *MLH1* and *MSH2* mRNAs which confused the analysis of inherited mutations affecting splicing [8–11]. At present, improved RNA-based approaches live their comeback along the attempts to establish various protein functional assays as well as utilize novel *in silico* methods [12–15].

Experimental and *in silico* approaches for RNA analysis

Recently, comprehensive analysis of single-base exonic substitutions with uncertain pathogenicity has been performed for *MLH1* and *MSH2* genes using combination of experimental RNA analyses and prediction programs [16–18]. Two experimental methods for analysis of mRNA splicing, namely reverse transcription with subsequent PCR (RT-PCR) from clinical samples and minigene assay may be used. RT-PCR analysis of patient's RNA reveals the splicing pattern of the gene in natural context *in vivo* and can be performed by different ways. A template for the reverse transcription (cDNA synthesis) represents either mRNA extracted from patient's unstimulated PBLs, phytohemagglutinin- stimulated PBLs or EBV-immortalized lymphoblastoid cell lines. The advantage of latter two mRNA sources lies in the absence of naturally-occuring splicing isoforms which may interfere with identification of mutant mRNA transcript [12, 19]. The gene-specific PCR may be performed either as a second, separate step using total cDNA synthesized previously or in the single-tube reaction, in which only gene-specific cDNA is reverse-transcribed and subsequently PCR amplified. Two-step RT-PCR enables the synthesis of full-length cDNA (about 2300 and 2800 bp for *MLH1* and *MSH2*, respectively) [20], single-step RT-PCR may be used for analysis of partial cDNA encompassing several exons of the gene [21]. The minigene assay examines the splicing efficiency of an individual exon in the cell lines transfected with chimeric minigene. It represents an alternative approach to compare splicing of the variant and wild-type allele in the cases when endogenous RNA from affected individuals is not available. However, it is performed in an artificial *in vivo* environment and might not reveal the mutations that affect splicing only in the context of the full-length mRNA [22]. Minigene assay is rather demanding and consists of several steps: 1. preparation of the minigene construct, which contains genomic segment including examined exon and its flanking regions, 2. transient transfection into a cell line (e.g. HeLa, COS etc.), 3. RNA extraction and RT-PCR analysis.

RT-PCR analysis of *MLH1* and *MSH2* using endogenous RNA from patients may encounter evaluation problems due to the naturally-occurring alternative splicing. Number of studies addressed this issue and reported multiple splicing isoforms for both *MLH1* and *MSH2* [19, 20, 23–28]. However, in the case of the alterations located in the alternatively spliced exons, it is complicated to distinguish, whether and how the substitution influences the splicing pattern. For example, the substitutions at nucleotide 883 of exon 10 in *MLH1* (c.883A>C and c.883A>G) have been described to have different consequences. Zavodna et al. [21] have detected skipping of exon 10, in contrast with absent expression from variant allele found by Goldschmidt et al. [29]. The comparison of RT-PCR experimental setup has shown that Zavodna et al. have analyzed partial cDNA encompassing exons 5 to 11, while Goldschmidt et al. had used primers localized in exons 10 and 11, respec-

Table 1. *In silico* tools used to evaluate the effect of exonic DNA substitutions on RNA and protein level

Evaluated effect	Software tool	Website	Reference*
Splice site disruption and/or creation	NNSPLICE	www.fruitfly.org/seq_tools/splice.html	[42]
	SpliceSiteFinder	www.genet.sickkids.on.ca/~ali/splicesitefinder.html	[43]
	GENSCAN	http://genes.mit.edu/GENSCAN.html	[18]
	Information analysis	https://splice.cmh.edu	
ESEs/ESSs disruption and/or creation	ESEfinder	http://rulai.cshl.edu/tools/ESE2	[16]
	RescueESE	http://genes.mit.edu/burgelab/rescue-ese	[17]
	PESX	http://cubweb.biology.columbia.edu/pesx	[18]
Change in the protein structure and/or function	SIFT	http://blocks.fhcrc.org/sift/SIFT.html	[44]
	PolyPhen	http://coot.embl.de/PolyPhen	[41]
	Align-GVGD	http://agvgd.iarc.fr	[45]

* The references in which *MLH1* and/or *MSH2* gene changes have been evaluated

tively [21, 29]. It is very likely, that RT-PCR design of Goldschmidt et al. could not detect skipping of exon 10 and putative lack of expression from c.883G-carrying allele represents a false-negative result. Thus, for proper assignment of pathogenic consequences, a careful design of cDNA analysis and detailed knowledge of the alternative splicing profile is essential.

Currently available software tools for predicting disruption/creation of splice sites or splicing enhancers/silencers are listed in Table 1. Studies comparing the predictive efficiency of software tools with experimental results have shown that predictions do not always correspond with an evident defect in the splicing pattern, i.e. are false negative or vice versa. According to our literature/database search and own experience, the substitutions affecting exonic part of the donor (last three nucleotides of an exon) or rarely acceptor (first nucleotide in the exon) are nearly always causing an aberrant splicing, usually exon skipping. In these cases it seems to be redundant to use *in silico* analysis, however it may be useful for predicting aberrant consequences other than exon skip-

Table 2. Exonic changes in the *MLH1* gene affecting splicing

Exon	Substitution	Theoretically deduced consequence	Experimentally proved consequence	In (I) or Out (O) frame	Reference
2	c.122A>G	p.Asp41Gly	del of exon 2	O	[42]
6	c.544A>G	p.Arg182Gly	del of exon 6	O	[16]
8	c.677G>A	p.Arg226Gln	del of exon 8	O	[42]
10	c.842C>T	p.Ala281Val	del of exon 10[1]	O	[18]
	c.882C>T	p.Leu294Leu	del of exon 10	O	[16]
	c.883A>C	p.Ser295Arg	del of exon 10	O	[21]
	A>G	p.Ser295Gly	del of exon 10	O	[46]
11	c.1038G>C	p.Gln346His	ins 59 bp of intron 11	O	[17]
	G>A	p.Gln346Gln	ins 59 bp of intron 11	O	[7]
12	c.1381A>T	p.Lys461X	del of exon 12[1]	O	[6]
14	c.1666A>G	p.Ser556Gly	del of exon 14	O	[47]
	c.1667G>T	p.Ser556Ile	ins 88 bp of intron 14	O	[42]
15	c.1731G>A	p.Ser577Ser	del of exon 15	O	[17]
17	c.1975C>T	p.Arg659X	del of exon 17[1]	I	[5]
	c.1976G>C	p.Arg659Pro	del of exon 17[1]	I	[5]
	c.1988A>G	p.Glu663Gly	del of exon 17	I	[48]
	c.1989G>T	p.Glu663Asp	del of exon 17	I	[16]
18	c.2103G>C	p.Gln701His	del of exon 18	I	[17]

[1]due to exonic splicing enhancer (ESE) inactivation, del- deletion, ins- insertion

Table 3. Exonic changes in the *MSH2* gene affecting splicing

Exon	Substitution	Theoretically deduced consequence	Experimentally proved consequence	In (I) or Out (O) frame	Reference
5	c.806C>T	p.Ser269Leu	del of exon 5[1]	I	[18]
	c.815C>T	p.Ala272Val	del of exon 5[1]	I	[18]
	c.942G>A	p.Gln314Gln	del of exon 5	I	[49]
	c.1275A>G	p.Glu425Glu	del 48 bp of exon 7	I	[17]
8	c.1355A>T	p.Glu452Val	del 33 bp of exon 8	I	[50]
10	c.1516G>T	p.Asp506Tyr	del of exon 10[1]	O	[18]
	c.1600C>T	p.Arg534Cys	del of exon 10[1]	O	[18]
	c.1660A>G	p.Ser554Gly	del of exon 10	O	[46]
	c.1661G>C	p.Ser554Thr	del of exon 10	O	[51]
12	c.1915C>T	p.His639Tyr	del 92 bp of exon 12	O	[52]

[1]due to exonic splicing enhancer (ESE) inactivation, del- deletion, ins- insertion

Table 4. Missense changes affecting MLH1 protein expression and function

Exon	Nucleotide substitution	Aminoacid change	Experimental characterization[1]				First reference
			Expression	*In vitro* MMR	PMS2 interaction	Nuclear localization	
1	c.83C>T	p.Pro28Leu	D	D	N	D	[53]
2	c.189C>A	p.Asp63Glu	D	D	n.a.	D	[39]
	c.199G>A	p.Gly67Arg	D	D	N	D	[54]
3	c.229T>C	p.Cys77Arg	D	D	N	D	[55]
	c.238T>G	p.Phe80Val	N	D	N	N	[56]
	c.250A>G	p.Lys84Glu	N	D	N	D	[57]
4	c.320T>G	p.Ile107Arg	D	D	N	D	[58]
	c.350C>T	p.Thr117Met	N	D	n.a.	n.a.	[59]
6	c.464T>G	p.Leu155Arg	D	D	N	D	[60]
7	c.554T>G	p.Val185Gly	D	D	n.a.	D	[8]
9	c.731G>A	p.Gly244Asp	D	D	n.a.	n.a.	[61]
	c.739T>C	p.Ser247Pro	D	D	N	D	[60]
11	c.986A>C	p.His329Pro	D	N	N	D	[62]
14	c.1649T>C	p.Leu550Pro	D	N	N	D	[60]
16	c.1766C>A	p.Ala589Asp	D	N	N	D	[60]
	c.1853A>C	p.Lys618Thr	N	N	N	D	[63]
17	c.1942C>T	p.Pro648Ser	D	N	N	D	[64]
	c.1943C>T	p.Pro648Leu					[56]
	c.1961C>T	p.Pro654Leu	D	N	N	D	[60]
	c.1976G>C	p.Arg659Pro[2]	N	D	D	D	[5]

[1]Raevaara et al., 2005, Trojan et al., 2002 / 1[36], [39], [2]causes aberrant splicing (see Table 2), D – decreased (expression or nuclear localization) or deficient (*in vitro* MMR), N – normal, n.a. – not available

Table 5. Missense changes affecting MSH2 protein expression and function

Exon	Nucleotide substitution	Aminoacid change	Experimental characterization[1]			First reference
			Expression	*In vitro* MMR	MSH6 interaction	
1	c.97A>C	p.Thr33Pro	N	D	N	[65]
3	c.482T>A	p.Val161Asp	N	D	N	[56]
	c.484G>A	p.Gly162Arg	N	D	N	[66]
	c.490G>A	p.Gly164Arg	N	D	N	[60]
	c.518T>C	p.Leu173Pro	N	D	N	[16]
	c.560T>C	p.Leu187Pro	N	D	N	[67]
6	c.998G>A	p.Cys333Tyr	N	D	N	[68]
12	c.1807G>A	p.Asp603Asn	N	D	N	[69]
	c.1906G>C	p.Ala636Pro	N	D	N	[70]
13	c.2090G>T	p.Cys697Phe	N	D	N	[53]
14	c.2245G>A	p.Glu749Lys	N	D	N	[16]

[1] Ollila et al., 2006 / 1[40], D – decreased (expression) or deficient (*in vitro* MMR), N – normal

ping, e.g. creation of an alternative splice site. Substitutions localized anywhere in the exon may disrupt the function of exonic splicing enhancer (ESE) or exonic splicing silencer (ESS). However, computational algorithms identifying disruption/creation of an ESE/ESS are most sensitive when putative mutation falls in short, weakly defined exon (i.e. alternatively spliced) and is localized not far from the splicing site [2, 18]. Thus, for pathogenicity assessment of

nucleotide substitutions, the programs predicting aberrant splicing should be considered only as an adjunct tool.

Exonic changes in the *MLH1* and *MSH2* genes affecting splicing

Tables 2 and 3 summarize exonic substitutions of the *MLH1* and *MSH2* genes, respectively, which were characterized as missense, nonsense or silent mutations previously but more detailed investigations of these changes have shown that they cause an aberrant pre-mRNA splicing. Such changes are often located near the 5'- splice site/donor (i.e. within last three nucleotides of the exon). Moreover, the ESEs and ESSs already mentioned above are now becoming increasingly recognized in *MLH1* and *MSH2* [16, 18, 30] and single nucleotide changes in their sequence may result in disruption and/or creation of a new splice-site. According to the experimental data of Lastella et al., the exonic substitutions altering splicing are clustering preferentially in exons subjected to naturally-occurring alternative splicing or showing suboptimal splice site scores by prediction softwares, i.e. in exons 10 and 17 of *MLH1*, and exons 5 and 10 of *MSH2* [18].

Exonic splicing mutations most often result in exon skipping, in- or out-of-frame nucleotide deletion and subsequently synthesis of a polypeptide with internally deleted amino acids or terminally truncated protein, respectively. The use of alternative splice sites, either cryptic or created directly by the mutation within the exon or flanking introns is also possible but infrequent. *De novo* created splice sites usually result in the deletions of exonic nucleotides or insertions of intronic parts into the coding sequence.

Experimental and *in silico* approaches for mutant protein analysis

A single amino acid change does not necessarily result in a dysfunctional protein which occurs usually when normal and mutated amino acids have similar physical and/or chemical properties. Other changes can give rise to nonfunctional, dominant negative, unstable or only partly active proteins. The complexity of MMR pathway offers various scenarios including inactivation of enzymatic activity (ATP binding or hydrolysis), defective protein-DNA binding (recognition of mismatch), defective protein-protein interaction (heterodimers formation), changes in expression of MMR proteins (stochiometry of MMR complexes), altered stability of proteins, and defective subcellular localization of MMR proteins.

The experimental evaluation of mutated MLH1/MSH2 proteins is not easy but represents most accurate assessment of functional consequences of putative true missense substitutions (i.e. for which aberrant splicing has been excluded). Several expression systems have been established for the functional analysis of the variant MLH1 and MSH2. Most of these assays have been performed in the different strains of *S. cerevisiae* and are based on the homology of amino acid sequences between the human and yeast MMR proteins. In some studies, yeast *mlh1* and *msh2* mutations equivalent to the known missense alterations in HNPCC patients have been generated and tested for their mismatch repair complementation ability in MMR-deficient strains [13, 31, 32]. Other authors have utilized interference between human MLH1 and MMR-proficient *S. cerevisiae* [14, 33]. Overexpression of the normal human MLH1 caused a competitive inhibition of yeast mlh1, hybrid interaction of MLH1 with yeast pms1 and a final defect in the yeast MMR. In contrast, the expression of a pathogenic human alteration (i.e. mutant MLH1 which could not interact with yeast pms1) paradoxically did not decrease the MMR efficiency. Both analytical approaches require that the missense mutations affected evolutionary conserved amino acid. An alternative approach which enables functional analysis of non-conserved amino acid substitutions represents the fusion gene constructs encoding hybrid human-yeast proteins [34, 35]. Other studies have used human cell lines (293T embryonic kidney fibroblasts, HCT116 and LoVo colon adenocarcinoma) or insect cells (*S. frugiperda*) to produce MLH1 or MSH2 variants [36–38]. In addition to the performance in the *in vitro* MMR assay they investigated also protein stability as well as the formation and subcellular localization of the variant heterodimers [39, 40].

As for the theoretical evaluation of DNA changes on protein level, the use of evolutionary conservation in sequence between human MMR proteins and their homologues in other species maybe effective but is limited to amino acids of conserved protein segments. Therefore, in the last decade several *in silico* methods for complex protein modeling and prediction of the impact of amino acid substitutions on the protein structure and/or function have been developed (Table 1). Besides the comparison of homologous proteins and the assessment of physical/chemical changes, these programs are mapping amino acid substitutions onto the 3D crystallographic polypeptide structures and evaluating multiple parameters of normal and altered proteins. Chan et al. [41] have compared three computational algorithms (SIFT, PolyPhen and A-GVGD, Table 1) in predicting the consequences of more than 40 missense variants in *MLH1* and *MSH2*, which were validated as either neutral or deleterious by functional data published previously [39, 40]. These methods are based on the combination of comparative sequence (evolutionary conservation) and protein structure (amino acid change) analysis and have correctly predicted impact of 70-90% of the *MLH1* and *MSH2* variants. Authors have found that prediction accuracy improves when the variant occurs at an amino acid that is absolutely conserved or when all three methods agree that it is deleterious. In conclusion, pathogenicity evaluation of DNA substitutions on protein level by prediction programs is demanding and may be not always in agreement with experimental results of functional protein analyses.

Missense changes affecting MLH1 and MSH2 protein expression/stability and function

Because of their experimental complexity, the functional protein analyses are currently not applicable in routine clinical laboratories. However, there is an ever-increasing number of tested missense alterations in various experimental studies. Tables 4 and 5 summarize missense *MLH1* and *MSH2* substitutions, which are very likely deleterious in HNPCC patients according to the results of functional protein studies in eukaryotic cell lines. Most of the true missense mutations in *MLH1* have affected quantity, MMR capability and subcellular localization of the MLH1 protein. Several alterations caused decreased MLH1 expression and aberrant nuclear localization without impairing MMR function. It is noteworthy that the substitution p.Phe80Val was clearly pathogenic only in the MMR assay and p.Lys618Thr affected only the subcellular protein localization and the functional protein defect related to p.Arg659Pro change may be additional to the aberrant mRNA splicing (Table 2). Interestingly, all the *MSH2* missense mutations showed severe defect in MMR efficiency, without changes in expression/stability and interaction properties of the corresponding proteins.

Conclusion

In general, it is often unclear whether a single-base exonic substitution in a gene is a disease-causing mutation or an allelic variant not associated with disease. The evidence has accumulated that assessment of pathogenicity of these changes can not be simply derived from DNA sequence. When evaluating such germline alterations identified in HNPCC patients during mutational screening, it is best when clinical data (segregation of the mutation with HNPCC-associated cancers in the family) and analysis of tumors (immunohistochemical assay for MMR protein expression) are complemented by *in silico* and experimental methods evaluating the consequences of the mutation on RNA and protein level. This combined approach is important both for correct ascertainment of cancer risk in the HNPCC family and for the understanding of the particular *MMR* gene function and regulation.

According to our opinion the optimal sequence for pathogenicity assessment of missense and silent DNA substitutions should prefer experimental over *in silico* approaches, as well as less demanding analyses of RNA splicing over more complicated assays of protein expression/function. Nonsense alterations may be invariably regarded as pathogenic, because they result either in the creation of premature termination codon leading to a truncated protein, or rarely the splicing aberration. Regarding missense and silent changes, the localization of the substitution alone may be very informative and determine further evaluation steps. When located in the exonic part of the donor, the alteration can be expected to disrupt splicing and then RNA analysis should be performed only for confirmation. For other localizations, *in silico* RNA and/or protein algorithms may be used optionally, however, we recommend to proceed preferentially with experimental RNA analysis. Detection of the normal splicing pattern implies that protein defect is still possible. It should be first verified in the literature, whether the substitution has been subjected to some functional assay previously. If not, the collaboration with the laboratory performing functional analyses would be desired.

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