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Original Article

Confirmation of damaging effect of *MSH2* c.2634+1G>C mutation on splicing, its classification and implications for counseling

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ABSTRACT

Introduction: Lynch syndrome (LS) is predisposing mainly to colorectal and endometrial carcinomas, but also to urinary tract cancers. LS association with upper urinary tract carcinomas is known, but its association with bladder cancer is not so clear. Confirmation of pathogenicity of detected mutations in LS-associated genes is required for adequate counseling.
Material and Methods: Tested young female has family history of two early colorectal and two bladder carcinomas. NGS sequencing revealed *MSH2* splice site mutation c.2634+1G>C, which was confirmed by Sanger sequencing. *MSH2* cDNA part containing potential splicing change was sequenced. *in silico* softwares were used to predict the effect of detected mutation on splicing and protein structure. ACMG Guidelines were used for mutation classification.
Results: *in silico* softwares predict damaging effect of detected mutation on splicing and loss of protein-binding domains. cDNA sequencing confirmed this mutation causes exon 15 excision. ACMG Guidelines classify this mutation as Pathogenic.
Discussion: *MSH2* c.2634+1G>C mutation was not reported previously as LS associated. We confirmed its damaging effect on splicing. *in silico* tools predict consequent loss of protein domains implicating disrupted protein function. Our results suggest that this mutation should be classified as Pathogenic, and indicate inclusion of bladder cancer in LS cancer spectrum.

Introduction

Lynch Syndrome (LS) is a hereditary cancer syndrome which is characterized by the most frequent cancer localizations in colorectum and endometrium. Aside from familial aggregation of cancer, the striking hallmarks of this hereditary cancer syndrome are early diagnosis of these two types of carcinomas (<50 years old) [1]. In addition, Lynch syndrome is associated with other cancer localizations, less frequently than colorectal and endometrial, but more frequent in LS compared to general population [2]. Such associated cancers are cancers of urinary tract [2–5], ovarian cancer [2–5], other parts of digestive tract (gastric [2–6], small bowel [4,5], hepatobiliary tract [3,5]), brain tumors [3,5,7], etc.

According to IARC Global Cancer Observatory (http://gco.iarc.fr/), in general population, bladder cancer is more frequent than upper urinary tract (kidney, renal pelvis and ureter) carcinomas. In LS however, the situation seems to be the other way around [8,9]. The association of LS with upper urinary tract carcinomas has been known for more than two decades [10]. However, the association of LS with bladder cancer has not been so clear and available results are controversial [11,12]. Some results indicate that bladder cancer should be included in the LS associated cancers spectrum [13,14].

LS is cancer susceptibility syndrome associated with germline mutations in one (or more) of the genes involved in mismatch repair (MMR): *MLH1, MSH2, MSH6* and *PMS2*, as well as with deletions in *EpCAM* gene, which lead to loss of *MSH2* expression [12]. Mutations in these genes disrupt mismatch repair, leading to genome instability and faster cancer progression. Therefore, individuals with inherited mutations in these genes are more susceptible to cancer development and often develop cancer earlier compared to general population.

Around 90% of LS cases are associated with *MLH1* and *MSH2* mutations, while approximately 10% of LS cases harbor *MSH6* and *PMS2* mutations [15]. Results of some studies suggest that the risk

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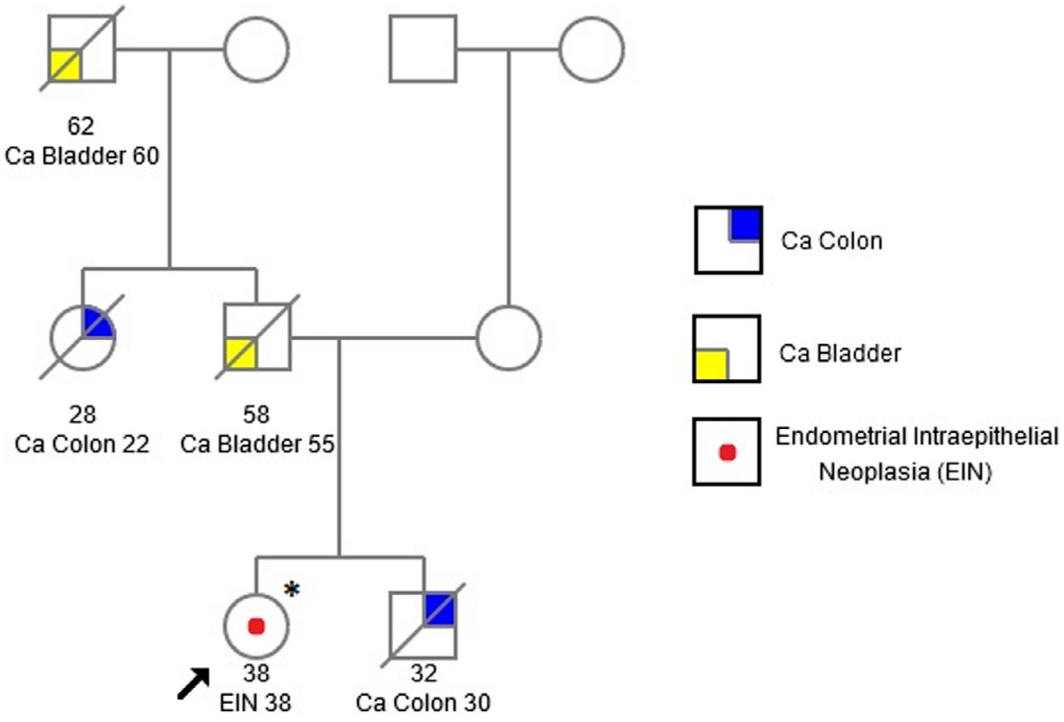

Fig. 1. Proband's pedigree. Proband is marked with an arrow. Asterix (*) marks mutation carrier (*MSH2* c.2634+1G>C).

for LS associated cancers other than colorectal cancer is highest in *MSH2* mutation carriers, especially for urinary tract cancers [8,16]. In addition, urinary tract cancers are the most frequent primary cancers after colorectal cancer, for both male and female LS patients, particularly among *MSH2* and *MLH1* mutation carriers [8,14].

In the era of genetic testing as everyday routine practice, it became evident that the same genetic variants are being classified differently by different laboratories. The introduction of high throughput sequencing and gene panel testing further deepened this problem and created the need for the guidelines for universal classification of detected genetic variants. Therefore, American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology developed such guidelines [17], with detailed criteria and method for genetic variant classification, and cautions one has to be aware of when using these Guidelines.

In the everyday course of Lynch syndrome mutation detection in families that fulfill the criteria for testing (Amsterdam II criteria [18] and Revised Bethesda criteria [19]), we detected mutation c.2634+1G>C in *MSH2* gene that has not been previously reported in scientific literature as associated with colorectal cancer. Mutations in the same position but substitutions with different nucleotides (c.2634+1G>T and c.2634+1G>A, rs267608019) have been reported as Likely Pathogenic or Pathogenic, because the latter one has been shown to disrupt mRNA splicing [20]. We assumed the same effect of our novel detected mutation and aimed our investigation at confirming its deleterious effect through splicing disruption. We used various *in silico* softwares to predict mutation effect on mRNA splicing and consequently on protein structure and function. In addition, in this manuscript we describe the application of ACMG Guidelines for genetic variant classification to the classification of detected mutation, as well as its consequences to counseling.

Materials and methods

Our proband was young female (age 38), who at the moment of testing already had developed endometrial intraepithelial neoplasia (EIN). Her family history includes two early colorectal cancer cases: her brother developed colorectal cancer when he was 30 years old, and her father's sister when she was 22 years old. In addition, on the same side of the family there were two bladder cancer cases: her father developed bladder carcinoma when he was 55, and her paternal grandfather when he was 60 years old (Fig. 1). All affected family members passed away and were not available for testing.

Proband was interviewed prior to testing and signed Informed Consent approved by Ethics Committee of the Institute for Oncology and Radiology of Serbia.

DNA was extracted from peripheral blood using salting-out method. The coding sequences and exon/intron boundaries of 96 genes were amplified and prepared for sequencing using Nextera DNA Library Preparation Kit and TruSight® Cancer Panel (Illumina, San Diego, USA) according to manufacturer's instructions and sequenced on Illumina MiSeq Sequencing System (Illumina, San Diego, USA). MiSeq Reporter Software 2.5.1 was used for secondary data analysis and base calling, while Illumina Variant Interpreter software was used for variant classification. Detected splice site mutation was confirmed by Sanger sequencing, using BigDye Cycle Sequencing Kit v3.1 and sequenced on ABI 3130 genetic analyzer.

in silico predictions of detected mutation on splicing were performed using online available tools: Mutation Taster (http://www.mutationtaster.org/), FATHMM (http://fathmm.biocompute.org.uk), Human Splicing Finder (http://www.umd.be/HSF/), FSPLICE (http://www.softberry.com/berry.phtml?topic=fsplice&group=programs&subgroup=gfind) and SROOGLE (http://sroogle.tau.ac.il/).

For the determination of the effect of detected mutation on splicing, we sequenced mRNA isolated from proband's peripheral blood cells using TRI Reagent (Sigma, St. Louis, USA). Reverse transcription of 2 µg of isolated mRNA into cDNA was performed using High Capacity cDNA Reverse Transcription Kit (AppliedBiosystems, Foster City, USA) according to manufacturer's instructions. Part of *MSH2* mRNA containing segment with potential splicing change was amplified using forward primer

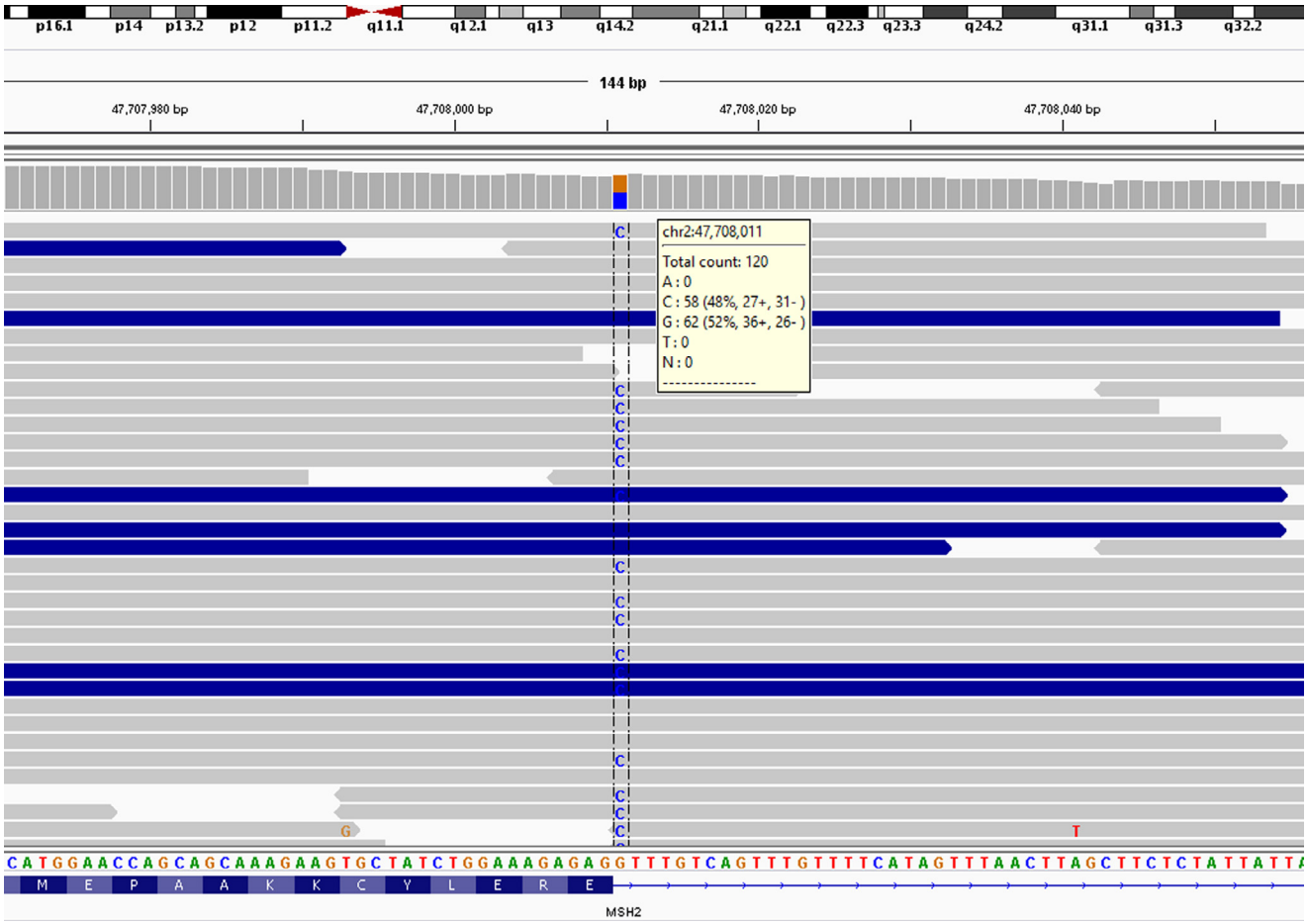

Fig. 2. The result of NGS sequencing, visualized in IGV software, showing heterozygous mutation at exon 15-intron 15 splice site.

5′-TTGGCCAATCAGATACCAACTG-3′ (binds within exon 14) and reverse primer 5′-ACTTTATTGTGATGTTTTCTTCTGAC-3′ (binds within exon 16) [21]. PCR was performed using following conditions: 94 °C for 5 min, 20 cycles of 94 °C for 30 s, 65 °C for 45 s with the temperature decreasing by 0.5 °C in each cycle, and 72 °C for 45 s. This touchdown PCR was followed by 20 cycles of 94 °C for 30 s, 57 °C for 45 s, and 72 °C for 45 s, and final extension on 72 °C for 10 min. PCR products were separated using gel electrophoresis on 2% agarose gel, 80 V for 25 min. Products of different sizes (wt and mutated allele) were separately cut from the agarose gel and prepared for Sanger sequencing using BigDye Cycle Sequencing Kit v3.1 and sequenced on ABI 3130 genetic analyzer.

For prediction of consequences of disrupted splicing on protein structure and function we used Open ProteinPredict (https://open.predictprotein.org/) and Pfam (http://pfam.xfam.org/).

Results

Results from NGS sequencing of our proband's DNA revealed heterozygous splice site mutation in *MSH2* gene (OMIM 609309), c.2634+1G>C (NM_000251.2) (Fig. 2), which was confirmed by Sanger sequencing (Fig. 3). Variant Interpreter software classified this variant as Class 4 – Likely Pathogenic. Other Lynch syndrome predisposing genes (*MLH1, MSH6, PMS2,* and *EPCAM*), as well as other genes covered by TruSight Cancer Panel associated with colorectal cancer (*APC, MUTYH, ATM, BLM, BMPR1A, BRCA1, BRCA2, CHEK2, PTEN, SMAD4, STK11, TP53*), did not contain any other exonic or splice site mutations of Classes 3, 4 or 5.

All *in silico* softwares used to predict the effect of detected splice site mutation (Mutation Taster, FATHMM, Human Splicing Finder, FSPLICE and SROOGLE) predicted disrupted splice site and consequently disrupted splicing.

In order to investigate the effect of detected mutation on splicing, we isolated mRNA and converted it to cDNA. PCR amplification of cDNA using primers encompassing predicted splice site change revealed two products of different sizes (Fig. 4). The sizes of these two PCR products corresponded to expected length of products we calculated based on the position of primers: 179 bp from mutated allele and 355 bp from wt allele. Sanger sequencing of two PCR products confirmed that the product amplified from wt allele contains exon 15 (Fig. 5A), while shorter product amplified from mutated allele does not contain entire exon 15 (Fig. 5B). This confirms that detected mutation affects mRNA splicing, leading to changes in protein sequence. The excision of exon 15 leads to change in amino acids at the positions 820 and 821 and creation of novel STOP codon at the position 822 (p.Gly820Alafs*3). The protein product from mutated allele is thus more than 100 amino acids shorter than wt one (934 amino acids). This huge protein change suggests consequences to its MMR function. In order to assess whether this truncation could affect MSH2 protein domains and function we used *in silico* softwares Open ProteinPredict and Pfam. These softwares predict the loss of important protein binding domains and the part of ATPase domain, which implicate disrupted protein function.

Based on results described here and the application of ACMG Guidelines, c.2634+1G>C mutation should be classified as Class 5 – Pathogenic.

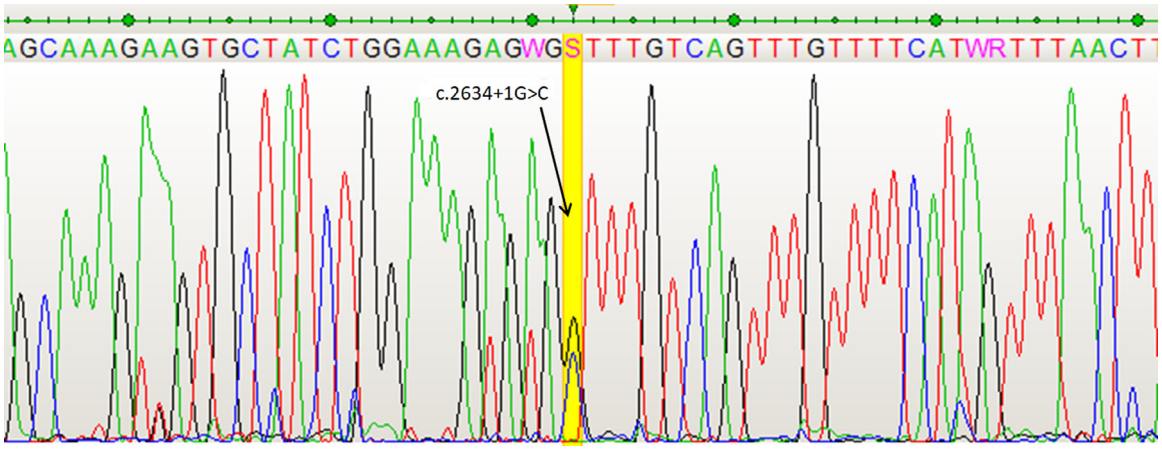

Fig. 3. *MSH2* c.2634+1G>C confirmed by Sanger sequencing of 3′ end of *MSH2* exon 15 of proband's gDNA.

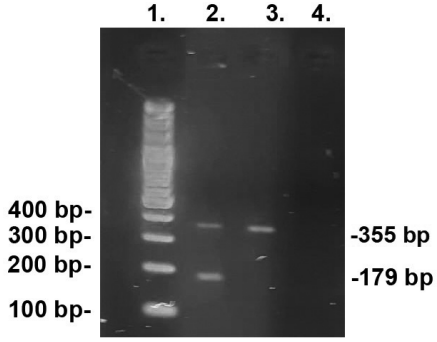

Fig. 4. Agarose gel electrophoresis of PCR products derived from amplification of affected parts of *MSH2* gene. 1. 100 bp ladder. 2. cDNA amplificates from our proband who is heterozygous for *MSH2* c.2634+1G>C. Higher (longer) band derives from wt allele, while lower (shorter) band derives from mutated allele. 3. cDNA amplificates from non-mutated control – sample of a patient previously sequenced and confirmed of being wt at *MSH2* c.2634+1 position. 4. Non-template control.

Discussion

Lynch syndrome, in addition to colorectal and endometrial cancer, predisposes to other cancers, such as urinary track cancers [2]. In contrast to upper urinary tract cancers, which have been associated with Lynch syndrome for years, data for bladder cancer remain unclear. There are studies which showed increased risk for bladder cancer in hereditary colorectal cancer families [22], significantly higher risk for bladder cancer among *MSH2* compared to *MLH1* and *MSH6* mutation carriers [13,16], as well as high frequency of *MSH2* mutations in large number of bladder cancer cases [14]. All of these data suggest that bladder cancer should be included as one of the cancers within Lynch syndrome cancer spectrum.

In order to resolve whether the *MSH2* mutation we detected could be the reason for numerous cancer cases in our proband's family history, we needed to confirm its pathogenicity. Scientific literature data and mutation databases provided limited data. Mutation in *MSH2* gene we detected, c.2634+1G>C, is reported in COSMIC database in one case of thyroid cancer (https://cancer.sanger.ac.uk/cosmic/mutation/overview?id=7339730). However, it has not been reported in association with LS or colorectal cancer in COSMIC or other mutation databases (InSiGHT (http://insight-database.org/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar), Ensembl (http://www.ensembl.org), UMD (http://www.umd.be/MSH2/)), nor in scientific literature.

Since the substitution in the same position but with different nucleotide has been previously shown to disrupt splicing leading to exon 15 excision [20], we assumed the same effect for the mutation we detected. Online *in silico* prediction models Mutation Taster, FATHMM, Human Splicing Finder, FSPLICE and SROOGLE confirm this assumption.

PCR amplification of region of interest from proband's cDNA confirmed NGS results which showed that our proband is heterozygous for the *MSH2* mutation c.2634+1G>C (Fig. 4). Separate Sanger sequencing of both PCR products confirmed that the sequence of shorted PCR product is missing entire exon 15 (Fig. 5A). This confirms *in silico* predictions that this mutation disrupts splice site, causing the synthesis of significantly shorter protein. Such shorter protein most probably has disrupted function, since available data suggest it lacks part of ATPase domain [23] and domain for interaction with other MMR proteins [24] at its 3′ end (http://www.umd.be/MSH2/4DACTION/WWS11; http://atlasgeneticsoncology.org/Genes/GC_MSH2.html).

In order to explore the effect of exon 15 excision on protein structure we used available *in silico* softwares. Open ProteinPredict software, that identifies protein-protein interaction sites and protein-DNA binding sites, predicted the loss of protein binding domains at the 3′ of the MSH2 mutated protein (Fig. 6). In the wt sequence, numerous protein binding sites are located downstream from position 800 (Fig. 6A). Due to the creation of STOP codon at the position 822, mutated sequence lacks mentioned numerous protein binding sites at its 3′ end (Fig. 6B), thus suggesting affected protein function.

in silico software Pfam identifies protein domains by the comparison of given protein sequence to its large protein sequence database [25]. The analyses of wt and mutated protein sequence using this software showed that mutated protein, in comparison to wt sequence, has truncated MutS_V domain (Fig. 7A and B). This part of the protein has ATPase activity and binds other MMR proteins, which are crucial for proper MSH2 activity. Consequently, this suggests that protein lacking 3′ part of this important domain has disrupted protein function.

All these analyses described above suggest defective *MSH2* function due to the splice site mutation we detected, which may lead to suboptimal function of its protein product within the MMR machinery, predisposing to cancer.

Based on previously reported substitutions at the same position, the mutation we detected could be classified as Likely Pathogenic or Pathogenic. In order to resolve which of these two classifications is more adequate, we applied ACMG Guidelines. The analyses showed that this mutation meets PVS1 criteria, as it is a canonical +1 splice site mutation. In addition, ACMG Guidelines

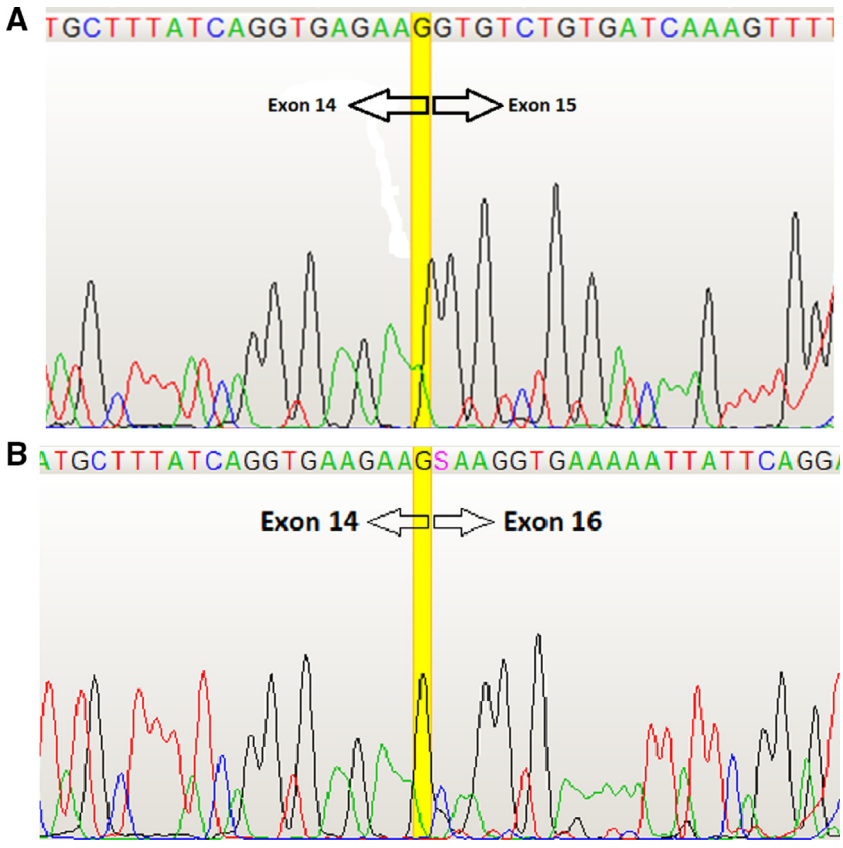

Fig. 5. Sanger sequence of part of *MSH2* cDNA affected by splice site mutation c.2634+1G>C. A. wt allele shows normal mRNA splicing. B. Mutated allele shows exon 15 skipping.

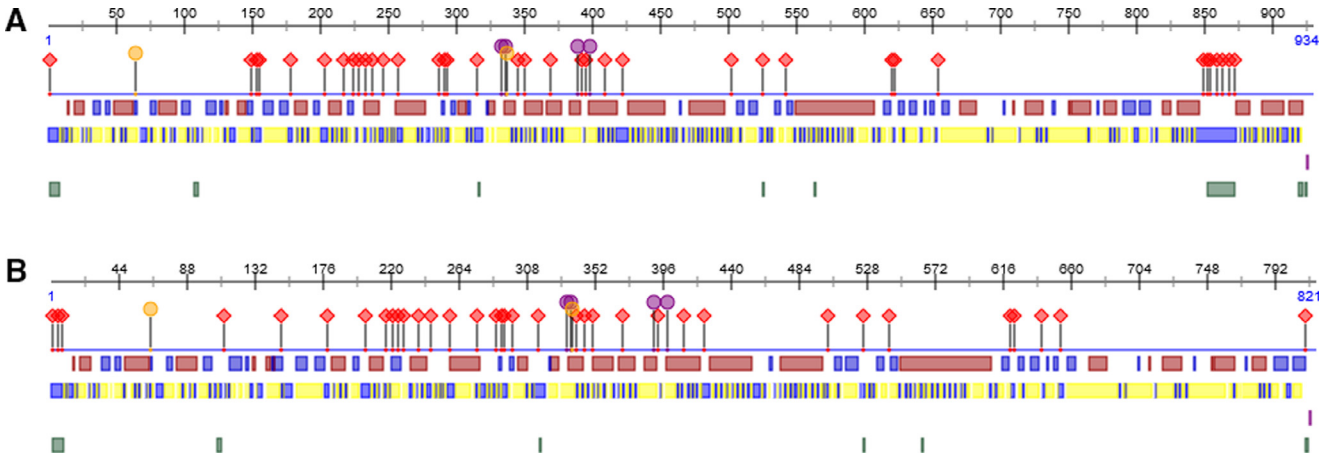

Fig. 6. Results of protein sequence analysis by Open Protein Predict. A. wt protein sequence, showing numerous protein binding residues at 3′ end of the sequence. B. Mutated protein sequence lacks numerous protein binding residues due to protein truncation as a consequence of exon 15 excision.

Fig. 7. Results of protein sequence analysis by Pfam. A. wt sequence with its domains. B. Mutated protein shows truncated MutS_V domain.

provide certain caution principles for each criterion. Thus, this mutation does not meet caution criteria for PVS1: homozygous (null) MSH2 mutations are pathogenic [26], and there are known pathogenic mutations reported in mentioned databases that are further downstream from mutation we detected. By describing damaging effect of this mutation *in vitro*, as described in this paper, we showed that it also meets PS3 criteria. Guidelines' rules for combining criteria for classification conclude that these two criteria classify a variant as Class 5 – Pathogenic. In the light of these results, we propose that the other two substitutions at the same position should be reclassified as Class 5 – Pathogenic, as they meet the same criteria, especially c.2634+1G>A, which has been shown to disrupt splicing [20] and thus has the same effect on the protein structure and function as described above.

The certainty of damaging effect of detected mutation is very important because it affects genetic counseling approach. The importance of confirmation of damaging effect of detected mutation is even more obvious knowing that information of carrier status of damaging mutation may cause serious distress and anxiety and lead to important and drastic health-related decisions, such as undergoing prophylactic surgery. Therefore, before reporting on detected mutation, all reasonable efforts to discern its effect on protein structure and/or function have to be made.

Genetic test results interpretation and counseling should be performed not only in the light of detected genetic mutations, but also in the light of proband's personal and family history. The striking family history of cancer cases described here, with two very early colorectal cancer cases in two close relatives and two bladder cancer cases on the same side of the family, and confirmed damaging effect of the detected mutation in our proband, confirms the presence of Lynch syndrome in this family. Other affected members were not available for testing, but their early onset of disease suggests their mutation carrier status.

For colorectal cancer, colonoscopy every 1 to 2 years is recommended (NCCN Guidelines, https://www.nccn.org/professionals/physician_gls/pdf/genetics_colon.pdf). According to current European Guidelines for endometrial cancer, women carriers of mutations associated with LS are considered as high risk group, who should undergo annual hysteroscopy and endometrial sampling starting at the age of 35, and prophylactic surgery (hysterectomy and bilateral salpingo-oophorectomy) should be discussed at the age of 40 [27].

Thus, our proband was informed of her carrier status due to confirmed damaging effect of detected mutation and consequently elevated cancer risk. She was presented with options of frequent clinical surveillance for early colorectal and endometrial cancer detection, especially due to the fact that she already developed endometrial premalignant lesions. She was also informed of the option of prophylactic surgery procedures for cancer risk reduction.

In addition to endometrial and colorectal cancer, she might also have elevated risk for developing bladder cancer, due to her family history. Therefore, she was informed of the necessity of frequent clinical check-ups also for bladder cancer. There are no widely accepted recommendations for bladder cancer screening in individuals with Lynch syndrome, although intensive screening of high risk individuals, particularly those with family history of urinary tract cancers or *MSH2* mutations is recommended [28]. Frequent urinalyses and urinary cytology are recommended, as default screening method for LS individuals [29], and our proband was counseled in this respect.

Although limited, our results presented in this paper (confirmed *MSH2* mutation pathogenicity and family history of bladder cancer in proband's family) go along with the previous reports suggesting that bladder cancer should be included in LS cancer spectrum [13,14] and that therefore LS mutation carriers with bladder cancer cases in family history should be counseled in this respect.

In this paper we describe the procedure of confirmation of damaging effect of detected c.2634+1G>C *MSH2* mutation on splicing, both by cDNA sequencing and using *in silico* prediction tools. In addition, we conclude that this mutation, based on our results described here and according to ACMG Guidelines, should be classified as Class 5 – Pathogenic, suggesting re-classification of other nucleotide substitutions on the same position from Class 4 to Class 5. The certainty of pathogenicity of detected mutation is very important for genetic test results interpretation and counseling. Additionally, the data presented in this paper support previous suggestions that bladder cancer should be regarded as one of the associated cancers in Lynch syndrome, and increased risk for bladder cancer should be taken into account during counseling of individuals at risk.

Declaration of Competing Interest

Authors declare no conflict of interest.

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