

## R659X mutation in the *MLH1* gene in hereditary non-polyposis colorectal cancer (HNPCC) in an Indian extended family

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**Background & objective:** Hereditary non-polyposis colorectal cancer (HNPCC or Lynch syndrome), is a genetically heterogeneous disorder that is believed to account for 2–10 per cent of all the colorectal cancer cases. The disease follows autosomal dominant inheritance pattern with high penetrance (85%) and younger age of onset when compared to patients with sporadic tumours. HNPCC is associated with germ-line mutations in the DNA mismatch repair (MMR) genes namely *MLH1*, *MSH2*, *MSH6*, and *PMS2*. The present study was aimed at analyzing mismatch repair gene(s) in an extended Indian family satisfying the Amsterdam criteria, and extending the analysis to general population to estimate frequency of the mutations/polymorphisms observed.

**Methods:** A total 12 members of the HNPCC family were studied for genetic investigation. Ethnically matched 250 normal individuals were also included as controls to study the observed mutations/polymorphisms at population level.

**Results:** The analysis resulted in identification of a 1975C>T mutation in exon 17, resulting in substitution of arginine residue with stop codon at codon 659. 655A>G substitution was also observed, resulting in replacement of isoleucine with valine at codon 219. Similar analysis on 250 ethnically matched control subjects revealed complete absence of R659X mutation, while I219V variant was found in 9.8 per cent of the controls.

**Interpretation & conclusion:** R659X mutation correlates with disease phenotype, and 655A>G locus is highly polymorphic. Our study suggested that R659X substitution was prime cause for the disease phenotype in this family. I219V substitution is a polymorphism having no association with the disease onset or segregation. The family members harbouring this mutation were advised to be under regular medical surveillance.

**Key words** Colorectal cancer - gastric cancer - HNPCC - Lynch syndrome - *MLH1* gene

Hereditary colorectal cancers can be subdivided into the polyposis and the nonpolyposis syndromes, depending upon presence and frequency of polyps in the large intestine. Only a small proportion of colorectal

malignancies are caused by the polyposis syndromes, of which familial adenomatous polyposis (FAP) is the most frequent. Hereditary non-polyposis colorectal cancer (HNPCC), also named as Lynch syndrome, on

the other hand, is a genetically heterogeneous disorder that is believed to account for 2-10 per cent of all cases of colorectal cancer<sup>1</sup>. The disease follows autosomal dominant inheritance pattern with high penetrance (85%) and manifests at younger age when compared to patients with sporadic tumours. This disorder is characterized by a smaller number of colorectal polyps, synchronous and metachronous colorectal cancers, and cancers of other organs.

Surprisingly, in HNPCC cancer may originate in the extracolonic organs despite no manifestation in the colon. The predominant extracolonic organ affected is the endometrium in women<sup>2</sup>, however, stomach, small bowel, hepatobiliary tract, kidney, ureter and ovarian cancers may cluster in HNPCC families. These cancers most commonly occur approximately 20 yr earlier than in the general population. The International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (ICG-HNPCC) defined the Amsterdam Criteria for identification of the HNPCC kindreds<sup>3</sup>, that is: (i) at least three relatives should have histologically verified colorectal cancer, one of them should be a first degree relative to the other two; (ii) at least two successive generations should be affected; and (iii) in one of the relatives, colorectal cancer should be diagnosed under 50 yr of age.

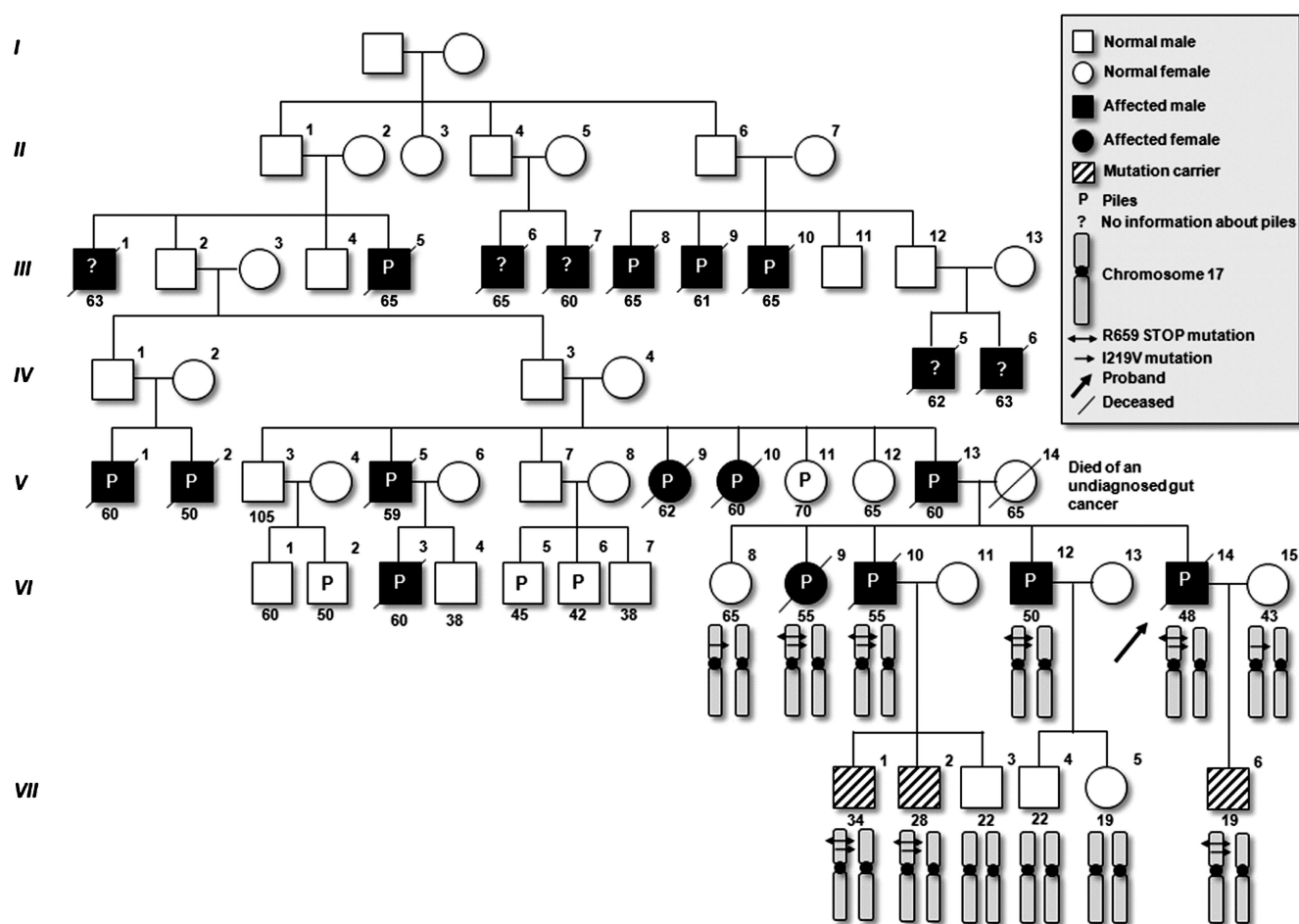
HNPCC is associated with germ-line mutations in DNA mismatch repair (MMR) genes namely *MLH1*, *MSH2*, *MSH6*, and *PMS2*<sup>4-7</sup>. Currently, more than 300 different mutations have been described in these genes, which account for approximately 500 HNPCC kindreds in the world<sup>8,9</sup>. According to the International Society for Gastrointestinal Hereditary Tumors database, mutations in the *MLH1* and *MSH2* genes account for approximately 50 and 40 per cent of the HNPCC cases, respectively<sup>10</sup>. Mutations in the *MSH6* and *PMS2* genes comprise remaining 10 per cent. Therefore, studies on new cases primarily target analysis of the *MLH1* and *MSH2* genes<sup>11-13</sup>. Despite substantial advances in our understanding of HNPCC, much remains to be learned about the correlations between the genetic changes and clinical features of the disease. Description of novel or recurrent alterations of the *MMR* genes in clinically well-defined HNPCC families has the potential to reveal new properties of the disease, mechanisms of pathogenicity and links to new biological pathways. Understanding of the mechanisms of disease initiation and proliferation may help in designing preventive and therapeutic strategies in the future. The present study was undertaken to screen a large Indian HNPCC family,

following the Amsterdam criteria, for mutations in the DNA mismatch repair genes followed by analysis of the observed mutations/polymorphisms in general population.

### Material & Methods

**Subjects and clinical history:** The study was conducted between January and May 2007 at the Centre for Cellular and Molecular Biology (CCMB), Hyderabad. The study protocol was approved by the Institutional Ethics Committee. The present family was selected on the basis of availability of detailed family history and the patients were selected upon their visit to the clinic or on the basis of available medical records for the deceased ones. The type of cancer was identified by histologic examination of the biopsies from the organ affected. The family belonged to a suburban area of Narwana, Haryana, India. The index patient (VI-14) in the pedigree (Fig. 1) reported at the Post Graduate Institute of Medical Sciences (PGIMS), Rohtak, Haryana, with complains of unusual weight loss (10 kg in 2 months), early satiety, loss of appetite and blood in vomiting. The patient had undergone an earlier surgical treatment of piles at the age of 37 yr. Upon recording the family history it was found that two siblings of the proband had died of cancer of small intestine and colon cancer (individuals VI-9 and VI-10 in the pedigree), while the third sibling (individual VI-12 in the pedigree) had undergone an surgery for rectal cancer, and was under medical surveillance for the last two years. One of the proband's siblings was normal (individual VI-8 in the pedigree) without any complications of gastrointestinal tract.

Further history across the previous generations indicated that proband's father also died of cancer of the large intestine, however, the disease could not be characterized because of availability of only limited medical records. The mother of the proband underwent the surgical removal of the small intestine because of cancer but the disease remained uncharacterized. Upon recording the family history of the previous generations it was found that cancer and piles were common problems at least for the last five generations. The origin of cancer showed a characteristic anatomic shift with cancers of brain, lung, small intestine, right colon, rectum and anus reported in the same family. Individuals in the youngest generation (VII) were too young for the disease to manifest; hence relatively less medical history of these individuals was available in comparison to the individuals in other generations.



**Fig. 1.** Medical history of the selected family. The individuals genotyped are shown along with the genotypes. The history of generations I, II, III and IV are based on the memory of the family members.

**Clinical examination:** In the initial stages of clinical course, the proband (individual VI-14 in Fig. 1) was advised to undergo ultrasound, computer tomography (CT) scan, complete haematological examination and necessary biochemical tests to detect any malignancy with gastrointestinal tract or the mesenteric region. Elevated levels of alpha foetoprotein along with the lymph node masses indicated possibility of lymphoma.

**Histopathologic examination:** To confirm the presence of lymphoma, abdominal part was examined by surgical opening of the retroperitoneal area. The biopsies were taken from different regions of the stomach for histopathologic analysis.

**Genetic analysis:** Peripheral blood samples (5 ml) were collected from the proband, his siblings and the next generation (Fig. 1) with their informed written consent. Blood samples from a total of 250 normal males with

age between 25 and 60 yr with similar ethnic and geographical backgrounds were collected to screen for the underlying mutation in the general population. DNA was isolated from blood samples using the protocol described earlier<sup>14</sup>. Primers for the *MLH1* gene were designed using the GeneTool software<sup>15</sup> (Table I). The primers covered the complete coding region along with the splice signal sequences for each exon. The smaller exons of this gene were combined together for amplification and subsequent sequencing reactions. The primers were synthesized using ABI 394 DNA/RNA synthesizer (Applied Biosystems, USA). Most of the primers were standardized to amplify under similar PCR conditions (Table I). Exonic regions of the above gene were amplified under PCR conditions, 94.0 °C for 5 min, 30 cycles of 94.0 °C for 45 sec, 55-63 °C for 45 sec and 72 °C for 1 min, final extension at 72 °C for 10 min. Amplicons were directly sequenced using Big-Dye chain terminator cycle sequencing protocol

**Table I.** Primer sequences for *MLH1* gene

Primer name	Sequence	PCR conditions (Annealing temperature, °C)
MLH1-E1	AGCCGCTTCAGGGAGGGACG	63
MLH1-E2	CCGTTGTGGGCATGCGCTGTAC	59
MLH1-E3	GAGGCACTATTGTTTGTATTGGAG	55
MLH1-E4	AAAAATCCCATCTGCAAAAGCCTAG	62
MLH1-E5	CCAAGAAAAAGAAAAAAACTC	63
MLH1-E6	CCATCAAGAAACATATATCAG	63
MLH1-E7, 8	CAACATGTCATCAAAGCAAGTG	63
MLH1-E9	GGGCAGAAATTACATCAGCAGC	63
MLH1-E10	GCCTGGGAAACAAGAGCAAAAC	61
MLH1-E11	CATGCCACAAAAGCCAATAGTC	63
MLH1-E12	GGCCCCAGTCAGTGCTTAGAAC	63
MLH1-E13	TTGGCTAGAGTCACTTTGTTTGTCT	63
MLH1-E14	AAAAGGGGGCTCTGACATCTAG	63
MLH1-E15	AACACATGATTACGCCACAG	63
MLH1-E16	CCAAGTCTTCGGGGCCCTCATTTAC	63
MLH1-E17	CATGCTTCCCAATAAAACCAAAC	63
MLH1-E18	GGAAAGTGGCGACAGGTAAAGGTG	63
MLH1-E19	GGAAACTGTGCCTTGTACCTGTA	63
MLH1-E20	CTGTGTCATCTGGCCTCAAATCTTC	63
MLH1-E21	TGGAGGCAAAAGTGAGGAAGTGAG	63
MLH1-E22	GCTTCTTTCTTAGTACTGCTCCATT	63
MLH1-E23	GCACCATTCAGAGGGCAAGTCAG	63
MLH1-E24	GCACAGGGGTTCATTACAGC	63
MLH1-E25	GTGGGTTAGTAAAGGAAGAGGAGC	63
MLH1-E26	CGTTTTCACAGGAGGCTCAATTCAG	63
MLH1-E27	CCAAAGCCTGTGCCCTCCCAAC	63
MLH1-E28	CTGGTTGAAGACGTTGGGAATC	63
MLH1-E29	GCCCGCCTCAGCCTCCCAAGTG	63
MLH1-E30	TGGGGTATATGGGGGATGAGTG	63
MLH1-E31	AGCATCTCAGCCTTCTTCTTCAG	63
MLH1-E32	TGCCCAGCCCAATCAAGTAAC	63
MLH1-E33	CCGAAATTTTAGAGATGGGCAAG	63
MLH1-E34	TCCAGACCCAGTGCACATCCATC	63
MLH1-E35	GGCAACAAAGCAAGACTCCG	63

on ABI 3730 DNA analyzer (Applied Biosystems, USA)<sup>16</sup>.

## Results

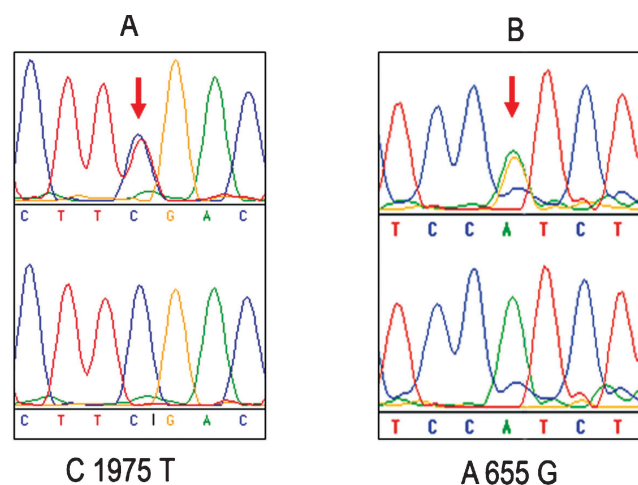
**Clinical findings:** Ultrasound report of the proband (individual VI-14 in Fig. 1) showed diaphragm, liver, gall bladder, pancreas, spleen, kidneys, urinary bladder and prostate to be normal. CT scan reports of the proband indicated presence of multiple lymph node masses in the retroperitoneal area of stomach while all other organs (gall bladder, pancreas, gut, kidney and liver) were apparently normal. Haematological analyses showed all the parameters (various blood cell types) to be in normal range. Biochemical analyses showed high levels of alpha-fetoprotein at 12.3 IU/ml (normal male range: 0.5-5.5 IU/ml, normal female range: 0.56-2.64 IU/ml), indicating cancerous growth.

All other biochemical parameters such as blood sugar, serum creatinine and blood urea were normal. Surgical exploration of the retroperitoneum indicated metastatic proliferation of lymph node masses. Histopathological examination of the tissue biopsy confirmed the presence of metastatic undifferentiated carcinoma. Clinical details of family members is shown in Table II.

**Genetic analysis:** Direct sequencing of the PCR products resulted in identification of a heterozygous 1975C>T mutation (R659X) in exon 17 among all affected individuals (individual analyzed shown in Fig. 2 with number of mutations). Normal sibling of the proband did not carry this mutation. The mutation resulted in replacement of an arginine residue at 659<sup>th</sup> codon with a stop codon, and was consistent with segregation of the disorder in the family. Introduction of stop codon and consistent inheritance of the mutation with the disorder

**Table II.** Clinical details of members

Indi- vidual	Age at diagnosis, yr	Age at death, yr	Piles	Site of cancer	Any other information
III-1	62	63	?	Right colon	---
III-5	65	65	Yes	Right colon	The individual also had brain tumor
III-6	65	65	?	Right colon	This individual also had cancerous growth in lung
III-7	60	60	?	Rectum	---
III-8	64	65	Yes	Right colon	---
III-9	60	61	Yes	Rectum	---
III-10	65	65	Yes	Left colon	---
IV-5	61	62	?	Right colon	---
IV-6	63	63	?	Right colon	---
V-1	60	60	Yes	Rectum	---
V-2	50	50	Yes	Right colon	---
V-5	58	59	Yes	Right colon	---
V-9	62	62	Yes	Right colon	---
V-10	60	60	Yes	Rectum	---
V-13	59	60	Yes	Large intestine	The location of cancer site could not be ascertained
VI-3	60	60	Yes	Anus	---
VI-9	54	55	Yes	Small intestine	---
VI-10	55	55	Yes	Right colon	---
VI-12	46	---	Yes	Rectum	Surgery done at diagnosis and the individual is healthy now
VI-14	48	48	Yes	Abdomen	---
VII-1	---	---	Yes	---	---
VII-2	---	---	Yes	---	---
VII-6	---	---	---	---	---



**Fig. 2.** Electropherograms of the mutations observed. Panel A: 1975C>T substitution. Panel B: 655A>G substitution. Arrow (→) shows substitution.

indicated its pathogenic nature. Similar analyses on 250 control samples showed complete absence of the above mutation in normal population, indicating its deleterious nature. Another heterozygous 655A>G substitution in exon 8 was observed in this family, resulting in replacement of isoleucine with valine at codon 219. In contrast to 1975C>T mutation, this mutation was present in normal sibling of the proband as well. The mutation was inherited to the next generation but the segregation pattern was not linked with the disorder in this family. Analysis on 250 control samples showed that Ile219Val substitution existed in normal population at a ratio of approximately 90:10 (A:G), indicating its polymorphic nature. The mutation existed exclusively in the family members who had a mutation at 659<sup>th</sup> position as well, in addition to its presence in normal sibling of the proband. All the individuals with normal sequence at 1975<sup>th</sup> nucleotide position were negative for this mutation as well, which indicated that the two mutations were in linkage disequilibrium. Sequence analysis of the remaining exons of the *MLH1* gene did not reveal any known or novel mutation/polymorphism.

### Discussion

In the present study, a family of HNPCC was analysed for the *MLH1* gene involved in mismatch repair (MMR) of the DNA. The analysis resulted in the identification of 655A>G substitution in exon 8 (I219V) and 1975C>T mutation (R659X) in exon 17 in all the affected members of the family.

The mutation 1975C>T in exon 17 introduced stop codon leading to truncation of the protein

synthesis beyond this point. Consistent inheritance pattern of the mutation with disease phenotype and its absence in 250 control samples (500 chromosomes), indicated its pathogenic nature. The mutation has been earlier reported in HNPCC patients in various studies<sup>17-19</sup>. In addition to truncation of the protein at 659<sup>th</sup> codon, this mutation has been earlier shown to result in aberrant splicing of mRNA leading to formation of the RNA molecules lacking exon 17<sup>20</sup>. It has been suggested in an earlier study that the presence of nonsense or missense mutations at codon 659 of *MLH1* gene can result in aberrant splicing of *MLH1* mRNA resulting in skipping of exon 17. Both, the protein with truncation at 659<sup>th</sup> codon (resulting from insertion of stop codon) and the protein lacking internal 31 amino acids (through skipping of exon 17), have been shown to be inactive in mismatch repair<sup>20</sup>.

Another substitution 655A>G observed in this family resulted in substitution of isoleucine with valine at codon 219. The substitution did not seem to be pathogenic given replacement of an aliphatic amino acid (isoleucine) with a similar amino acid (valine). This was further supported by inconsistent association of the mutation with disease phenotype. Nonpathogenic nature of this mutation was further confirmed by the presence of this mutation in normal Indian population at a frequency of 9.89 per cent. This polymorphism has been reported earlier at a rate of 31-80 per cent in different populations<sup>17,21,22</sup>. Although this polymorphism has been shown to be functionally inert<sup>23-26</sup>, some earlier reports have suggested functional relevance associated with this polymorphism<sup>27</sup>. Analysis of the microsatellites in close proximity of this polymorphism has shown that G/G or G/A but not A/A genotypes avoid unequal crossing over in close proximity of this polymorphism<sup>27</sup>. Difference in the rate of crossing over near this polymorphism might result in different deleterious effects resulting from inter-repeat crossovers. The later phenomenon may affect the age of disease onset depending upon the frequency of DNA defects, but it needs functional proof before it can be accepted.

In comparison to other mutation sites reported in exon 17, mutations at codon 659 had been observed in HNPCC patients more frequently<sup>10</sup>. Mutations at codon 659 have been reported in at least seven different studies on patients with different ethnic backgrounds, of which, 1975C>T was the underlying mutation in at least three different studies on American, Scottish and Finnish patients<sup>10</sup>. It is known that a significant fraction

of the families with mutations in mismatch repair genes are due to founder mutations in Finnish populations<sup>28</sup> but the scenario in Indian subcontinent is still beyond understanding. In our study arginine residue at 659<sup>th</sup> codon was in linkage disequilibrium with isoleucine residue at 219<sup>th</sup> codon in most of the individuals, except normal sibling of the proband (individual VI-8 in Fig. 1). It can be speculated that R659X mutation originated on the chromosome carrying I219V substitution on the paternal side, since then the two mutations are in strong linkage disequilibrium in this family. I219V substitution in normal sib of the proband (individual VI-8 in Fig. 1) might have been contributed from the maternal side (individual V-14 in Fig. 1) with a normal chromosome from the paternal side (individual V-13 in Fig. 1). R659X mutation probably originated long back in the paternal lineage and was segregating for at least last five generations, resulting in a high frequency of morbidity and mortality in this family.

HNPCC is well known for anatomic shift in the origin of cancer. Classically, colon cancer was believed to be a disease of left or the distal colon<sup>29</sup>; however, incidence of the right sided or proximal colon cancer has been increasing in North America, Europe and Asian countries<sup>30</sup>. Endometrial cancer in the females heads the list of other organs affected with renal cancer, bladder cancer, prostate cancer, hepatic cancer, gastric cancer and gall bladder cancer being the other common cancers in HNPCC families. The possibility of other cancers in these patients not only increases complexity of the disease but also raises doubts about usefulness of routine screening procedures of lower gastrointestinal tract by colonoscopy or upper gastrointestinal tract by endoscopy for the individuals at high risk. The family under study presented a typical example of anatomic shift in disease manifestation. We observed that one of the proband's sibling (individual VI-10 in Fig. 1) developed cancer of left colon, the other (individual VI-12 in Fig. 1) developed anal cancer, while the proband suffered from gastric cancer. According to medical history of the family, even lung cancer and brain tumours have been reported in earlier generations on the paternal side of the proband. However, lung and brain tumours have not been included in the list of extracolonic cancers, which warrants further exploration in more patients.

Although genetic basis of the disease is well established, underlying factors for anatomic shift are not well understood. It may be possible that certain mutations more often associate with anatomic shift in the disease than others. Exploration of more

and more mutations along with functional analysis and clinical characterization of the disease in large families (involving multiple affected individuals) may help understand genetic basis of anatomic shift. It is also possible that mutations in the DNA mismatch repair genes are accompanied by mutations in other interacting genes, which may increase relative susceptibility of certain organs to undergo malignant growth. Investigation of more genes interacting with the DNA mismatch repair genes may ultimately give some insights into the mechanism of anatomic shift.

As per the revised Bethesda guidelines for HNPCC mutation screening<sup>31</sup>, microsatellite instability is a useful tool to further select the patients for *hMLH1* and *hMSH2* mutation screening. Out of the two best candidate genes for further analyses, earlier studies have described that *MLH1* mutations are more common in Asian patients<sup>32,33</sup> than mutations in other mismatch repair genes.

In conclusion, our observation of mutations in *MLH1* gene suggests that it is worthwhile to first sequence the *MLH1* gene at least in the Asian colorectal cancer patients and proceed to other genes later. Identification of the underlying mutation helped in providing genetic counselling to this family. Individuals harbouring the mutation were advised to be under regular medical surveillance by undergoing colonoscopy and endoscopy once every two years after attaining the age of 25 yr. Female individuals were additionally suggested to undergo screening for endometrial and ovarian cancer as the disease shows anatomic shift.

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