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Incidental Finding of Lynch Syndrome Role of Genetic Counselling

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ABSTRACT The most common familial colorectal cancer (CRC) known is Lynch syndrome (LS) which is caused by mutations in DNA mismatch repair (MMR) genes. LS accounts for three to five percent of CRC and increases the risk of several types of cancers such as CRC, endometrial cancer in women, and to a lesser extent in other cancers like small intestine, ovary, stomach, urinary and hepatobiliary. The syndrome follows an autosomal dominant inheritance pattern and the cancers are characterized by their occurrence at an early age, familial involvement and development of metachronous cancers in the same individual. Here, the researchers report two CRC cases diagnosed with strong family history of CRC and various cancers. For one case, microsatellite instability (MSI-H) was confirmed by polymerase chain reaction (PCR) but immunohistochemistry (IHC) showed abnormal staining. Inherited cancer testing using germline multigene mutation analysis using Next Generation Sequencing (NGS) detected heterozygous likely pathogenic (p.Glu102Asp) and pathogenic (p.Ala681Thr) missense mutations in MLH1 gene in both the probands respectively. Post-test genetic counselling recommended other family members for screening and testing for the presence of the same mutation. A pre symptomatic diagnosis was made leading to an adapted surveillance strategy. The case reports illustrated in this paper have also highlighted the importance of genetic counselling and screening to detect an inherited cancer syndrome.

INTRODUCTION

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancy which represents third most common cancer and the fourth leading cause of cancer-related deaths in the world. The burden of CRC is expected to increase by more than 2.2 million new cases and estimated to reach 1.1 million cancer deaths by 2030 (Ferlay et al. 2013). It is most often found in people 50 years or older but the incidence is on the rise in those younger than 50 (Fairley et al. 2006). Disparities in the incidence of CRC and incidence based mortality (IBM) related to age has been studied recently. It has been reported that the increased incidence of CRC is predominantly among individuals under age 50 years (Loomans-Kropp and Umar 2019). There were over 1.8 million new cases and almost 861,000 deaths in 2018 as reported by the World Health Organization GLOBOCAN database. Though it has been thought that CRC is less common in Asian population compared to Western countries, data suggests that there is high incidence rates of CRC in the Asian population (Deng 2017). The most important factor contributing to the rapid increase in CRC incidence has been found to be an increase in the adoption of a Western lifestyle, particularly in dietary habits, increase of aging population, smoking, physical inactivity, and other risk factors (Deng 2017; Onyoh et al. 2019). It has been reported that a large proportion of patients in India develop CRC before the age of 45 years. The propensity of LS-CRC cases ranges from ten to fifteen percent

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(Maharaj et al. 2014). However, overall incidence is comparatively lower than in the West.

The majority of CRC cases can be sporadic caused by environmental and genetic factors and can occur in individuals regardless of any familial susceptibility to the disease. Nevertheless, about five to ten percent of all cases of CRC can be hereditary which are caused due to mutations and defects in certain DNA mismatch repair genes and can be found in individuals even when the predisposing genetic factors causing the disease have not yet been characterized (Giardiello et al. 2001). The presence of large or few numbers of adenomatous polyps or the presence of hamartomatous polyps have been reported to be an important "hallmark" of inherited syndromes that predispose an individual to the risk of CRC. Several CRC syndromes including hereditary nonpolyposis CRC (HNPCC), familial adenomatous polyposis (FAP), MYH-associated polyposis (MAP) and the hamartomatous polyposis syndromes (Peutz–Jeghers, juvenile polyposis, and Cowden disease) are known. Although these are less common, accounting for as many as five percent of all CRC cases (Jang and Chung 2010), major efforts have been put to understand the genetic landscape of such syndromes in the past two decades.

About three to five percent of colon cancer burden arise from Lynch syndrome (LS) or hereditary nonpolyposis colorectal cancer (HNPCC) (Hampel et al. 2008). The lifetime risk of CRC in individuals with LS is fifty two to eighty two percent and that of gastric cancer is six to thirteen percent (Kohlmann and Gruber 2018) and the rise in the incidence of LS in CRC cases has been associated with compromised quality of colonoscopy reporting (Lappalainen et al. 2019). There is also an increased risk of developing other cancer types including small bowel, liver, gallbladder ducts, urinary tract, pancreas, brain and skin. In females, LS confers increased risks of both endometrial (25-60%) and ovarian cancers (4-12%) (Kohlmann and Gruber 2018). CRC associated with LS have been found to manifest at a much younger age. LS is inherited in an autosomal dominant pattern due to germline mutations in DNA mismatch repair (MMR) genes MLH1, MSH2, MSH6 or PMS2 accounting for approximately thirty seven, forty one, thirteen and nine percent respectively (Moreira et al. 2012). The DNA MMR plays an important role in maintaining genetic stability by correcting base-base mismatches and insertion/deletion mispairs during DNA replication. The instability of repetitive DNA sequences called microsatellites is the most recognized molecular events of defective MMR. The resulting mutated phenotype is called microsatellite instability (MSI) which is tested for in the LS spectrum of tumours suspected of harbouring deleterious MMR gene variants (Walcott et al. 2016). Among the MMR genes, the mutations in MLH1 and MSH2 are very common whereas mutations in MSH6 and PMS2 genes are rare respectively (Kohlmann and Gruber 2018).

Individuals with LS do not present clinically with polyposis, but with only a few adenomas similar to sporadic CRC, therefore making it the most challenging hereditary colorectal syndrome to be recognized. Therefore, every patient with CRC should be counselled by the physician or genetic counsellor to understand the detailed family history to establish the diagnosis. A family history suggestive of LS is defined by the Amsterdam I and II criteria (Vasen et al. 1991; Vasen et al. 1999), illustrated in Annexure 1 and a broader set of Bethesda guidelines (Umar et al. 2004) illustrated in Annexure 2. The important criteria for detection of LS are multiple family members affected with CRC or associated extracolonic tumors, young age of onset of colon cancer, or multiple LS-associated cancers in a single individual. In certain cases, fulfilment of Amsterdam criteria is insufficient to confirm the diagnosis of LS, thus warranting the need of molecular testing for the presence of MSI (Jang and Chung 2010). The gold standard for testing MSI indirectly is to analyse MMR protein expression by immunohistochemical (IHC) staining. MSI is also tested directly by fluorescent multiplex polymerase chain reaction (PCR) based amplification of specific microsatellite repeats, or by germline mutation testing from blood or saliva (Buecher et al. 2013). MSI is detected through PCR by fragment analysis (FA) of five conserved satellite regions using a panel of five microsatellite markers BAT-25, BAT-26, D5S346, D2S123 and D17S250 and the differences in tandem repeats are identified by comparing cancer tissue to normal tissue (De la Chapelle and Hampel 2010; Zhang 2008). When the difference

in the loci were >30 percent, it was scored as unstable. MSI-high may indicate that the tumor is more likely to respond favourably to immunotherapies as high MSI correlates to an increased neoantigen burden. There could be many challenges in diagnosing LS in certain cases due to small family size, incomplete information about relatives, lack of awareness and reduced penetrance of a germline mutation in a MMR gene. Therefore, widespread testing of MSI/IHC of all colon tumors has been proposed. MSI is found in >90 percent of colon malignancies in patients with LS (due to germline MMR gene mutation) and in twelve percent of patients with sporadic CRC (due to somatic hypermethylation of the *MLH1* gene) (Aaltonen et al. 1993). MSI is graded as MSI-high if >30 percent of markers are unstable, MSI-low if <30 percent of markers are unstable, and MS-stable when no markers are unstable (Boland et al. 1998). MSI-high is very common in individuals having CRCs in LS. IHC using antibodies to the MMR gene proteins MLH1, MSH2, MSH6, and PMS2 is a very useful technology to evaluate the loss of MMR protein expression and thus identifying patients with LS (Lindor et al. 2002). The pathogenic germline or somatic mutations in specific DNA MMR result in loss or partial production of the MMR protein produced by that gene. In case of abnormal IHC staining, genetic testing using peripheral blood/saliva DNA is recommended (Chang 2006). In an unselected population of 500 CRC who were tested for MSI by IHC, 113 cases showing MSI or abnormal IHC underwent genetic analysis. Germline mutations in MMR genes were found in 18 cases out of which only 13 patients (72%) met the revised Bethesda guidelines. This finding clearly establishes the fact that if the patients are tested based on Bethesda criteria only, about twenty eight percent of cases of LS (Hampel et al. 2008) might be missed out. For families in which tumor tissue is not available, direct germline testing is an option. If the result is positive for a pathogenic mutation, other family members can be tested. Since genetic testing is a complex issue which may have medical, psychological, ethical, social, and legal implications on the individual and the family, genetic counselling, pre- and post-test are an important part of the testing process (ASCO 1996). The pre-test counselling is a nondirective and interactive process where the patient and the family members are given information regarding the benefits, risks and limitations of testing allowing them to take informed decisions to consent for the test (Geller et al. 1997; McKinnon et al. 1997). During the session, the patient's personal and family history is reviewed to check the probability of a hereditary cancer syndrome (Vasen et al. 1991; Vasen et al. 1999; Rodriguez-Bigas et al. 1997; Couch et al. 1997). Multigene germline testing by Next Generation Sequencing (NGS) enables identification of common and rare mutations associated with cancer risk at the time of first presentation and avoids additional testing and counselling. This helps the clinicians to better manage individuals and families who are affected or "at risk" for these inherited disorders and recommend effective screening, treatment options, monitoring and surveillance (Morash et al. 2018). The surveillance measures help in early detection of cancer thus reducing colon cancer mortality. Genetic counselling for patients affected with CRC and their family members play an important role in identifying the "at risk" individuals thus helping the patients and their families with hereditary cancer syndromes.

In this report, the researchers present two interesting cases one showing MSI-H by PCR but abnormal staining of MSI by IHC and the other case where MSI was non-contributory due to depleted tumor tissue on the formalin fixed paraffin embedded (FFPE) block. In both the cases, genetic counselling helped to predict the hereditary syndrome and germline mutation testing detected the prevalence of LS. The findings helped in the risk assessment of family members and management thus highlighting the importance of the test.

Objectives

- To provide genetic counselling and recommend germline mutation analysis for the proband and family members of colorectal cancers (CRC) and to check MSI by PCR and IHC.
- To analyse the presence of hereditary syndromes in CRC based on the test results and explain the surveillance measures.

METHODOLOGY

In this study, two CRC cases have been evaluated for the prevalence of LS through genetic counselling and testing. Tumor-based IHC and MSI along with germline mutation testing by NGS was performed for one case and since the surgical specimen was insufficient for the second case, only mutation testing was carried out. Both the cases were found to have a strong family history of CRC.

Microsatellite Instability Analysis by Immunohistochemistry

IHC was performed on formalin fixed paraffin embedded (FFPE) tissue on Biocare IntelliSite IHC equipment. The IHC markers used for MMR testing were MLH1, MSH2, MSH6 and PMS2 following routine diagnostic procedures (Wagner et al. 2001). The surgical specimen was embedded in paraffin and sections of the tissue were sliced into 3 μm thickness and were mounted on a slide glass. The de-paraffinization of the slide was done using xylene for about 15 minutes followed by dexylene treatment by increasing concentrations of ethanol. Antigen retrieval was done on decloaking chamber using a decloaking solution with pH 6.0 for MLH1, MSH2 and MLH6 and EDTA at pH 9.0 for PMS2 at 110 deg for 30 min. Primary antibody used against each MMR protein were anti-hMLH1 antibody (Clone BC 22, ready to use), anti-hMSH2 antibody (Clone FE11, 1:100 dilution), anti-hMSH6 antibody (Clone BC 19, 1:100 dilution) and anti-hPMS2 antibody (Clone A-16-4, ready to use). All the four antibodies were procured from Biocare medicals, CA, USA. Briefly, slides were incubated with the primary antibody at room temperature for 1 hr. The sections were washed and then incubated for 30 mins with biotinylated secondary antibody (ready-to-use). They were then blocked in peroxidase blocking solution for 7.5 min followed by washing and incubated for 30 mins with ready-to-use streptavidin-conjugated with peroxidase. Sections were washed thrice and incubated for 8 mins with the two-component hydrogen peroxide/diaminobenzidine substrate. Counterstaining of the sections was done with hematoxylin, washed twice and dexylenated before coverslip mounting. Nuclear positivity in any percentage and any intensity in tumor cells is taken as positive. The normal colonic mucosa, lymphoid cells and stromal fibroblasts were used as internal control for validating the assay apart from an external control. Absence of nuclear staining in tumor cells was interpreted as abnormal by the pathologist. A pathological slide scanner (Ultrafast scanner UFF 300, Philips, The Netherlands) was used to capture the histological images.

Microsatellite Instability Analysis by Polymerase Chain Reaction

Since the results from IHC to check the expression of MMR genes was inconclusive, MSI by PCR was recommended by the physician. Formalin fixed paraffin embedded (FFPE) samples with >20 percent tumor content were taken for the assay. Genomic DNA was extracted using commercial kits from FFPE sample (Qiagen, USA) and blood (germline sample) (Genfind, USA). PCR was performed using Promega MSI Analysis System (Version 1.2) for which about 20ng of DNA was used in a total of 10 μL PCR reaction mix. MSI amplification was performed as per manufacturer's protocol for 5 mononucleotide repeat markers (NR-21, BAT-26, BAT-25, NR-24, MONO-27) in tumor and germline sample. PCR products were resolved by capillary electrophoresis on the Genetic Analyzer (3500 DX, Thermo Scientific, USA). The combination of a four-dye fluorescent system allows for simultaneous amplification and separation of different loci in the assay. The markers were analyzed by using GeneMapper v4.1 software (Applied Biosystems, USA). Instability detected in two or more markers was interpreted as MSI-high (MSI-H), detected in only one marker as MSI-low (MSI-L) and no instability at all loci was reported as microsatellite stable (MSS).

Mutation Analysis by Next Generation Sequencing

Approval to perform the genetic testing of the patient's samples for this study was obtained by the Institutional Ethics Committee of Health-Care Global Enterprises, Bangalore. Written informed consent was taken from both the cases of this study. The pre-test counselling was

provided in a non-directive process where the patient and the family members were given information regarding the benefits, risks and limitations of testing allowing them to take informed decisions to consent for the test. After obtaining the consent, germline mutation testing was performed to check if any genes responsible for CRC was altered which also included the MMR genes. The test was performed based on Illumina Trusight Cancer sequencing panel by Nextera technology that includes 94 genes associated with commonly inherited cancers and several rare cancers. Fourteen genes (APC, BMPR1A, CDH1, EPCAM, MLH1, MSH2, MUTYH, PMS2, PTEN, SMAD4, STK11, TP53) associated with CRC and American College of Medical Genetics and Genomics (ACMG) recommended 25 genes for either key or secondary findings were evaluated.

Genomic DNA was isolated from the proband's blood sample using Qiamp Blood DNA mini kit (Qiagen, Germany). The quantification of DNA was done using Qubit Fluorometer and about 50ng was taken for library preparation using Nextera DNA library preparation protocol (Illumina, USA). The genomic DNA was sheared using transposons from which 'tagmented' (fragmented and tagged) DNA was obtained that was converted into adapter tagged indexed libraries. Limited cycles of PCR (AB 19700, Life Technologies) was performed to add specific index sequences or barcodes for multiplexing of samples. About 500 ng of individual libraries were pooled and hybridized to biotin labelled probes that are specific to the targeted regions. The sample pools were enriched by streptavidin beads that bind to the biotinylated probes. The gDNA fragments that were partially enriched were eluted and processed for second hybridization. The quality of the tagged and amplified libraries was checked and after quantification, about 6-10 pM of the pooled library was loaded on the Miseq platform (Illumina, USA) as per manufacturer's instruction. Strand NGS v2.5 and StrandOmics v3.0 (a proprietary clinical genomics interpretation and reporting platform from Strand Life Sciences) was used for the data analysis and interpretation after the completion of sequencing. For interpretation and reporting of sequence variations, the identification and labelling of the variants that had read quality >Q30 and confidence score >50 was done according to ACMG recommended standards (Richards et al. 2008). The identified variants were classified into five categories: 1) pathogenic, 2) likely pathogenic, 3) variant of unknown significance (VUS), 4) likely benign, and 5) benign. SNVs, small indels and copy number analysis was performed in Strand ® NGS. On receiving the test reports, post-test counselling was arranged for the patient and the family members. The results were discussed with the patients and after obtaining the consent to share the results, the family members were counselled to address the further issues based on the test results. Management options available for the patients and a further gene profiling for family members at risk was suggested. Guidelines for early detection and prevention were provided considering the psychological and social impact on the proband and the family members.

Case Report 1

A 54-year old gentleman presented with gastroenteritis and loss of appetite associated with significant weight loss, upper abdominal discomfort and anaemia in Jan 2017. Upper gastrointestinal endoscopy showed the presence of gastric polyps and colonoscopy revealed a transverse colon growth. PET/CT scan demonstrated the transverse colon growth with omental nodules. He underwent extended right hemicolectomy in view of near obstructive lesion. The histopathological examination (HPE) of the specimen indicated high grade mucinous adenocarcinoma of colon and was staged as PT4N2M1 according to the Tumor-Node-Metastasis (TNM) of classification of malignant tumors. The microscopic findings showed cell clusters and cords invading through the muscularia propia into the adjacent pericolic tissue and metastasis of 8/24 lymph nodes. MSI was analysed by IHC on the tissue sample. The molecular analysis of the genes KRAS, NRAS and BRAF were found to be wild type based on which he was treated with FOLFOX6+Cetuximab for 6 months followed by 5FU+Leucovorin+Cetuximab for 3 months. PETCT done in February 2018 showed multiple metabolically active lesions in the liver, transverse mesocolon and gastrosplenic ligaments confirming metastasis (Fig. 1a). The SUV was reported to be 9 as against the previous SUV of 3.6. He received cyberknife guided robotic ra-

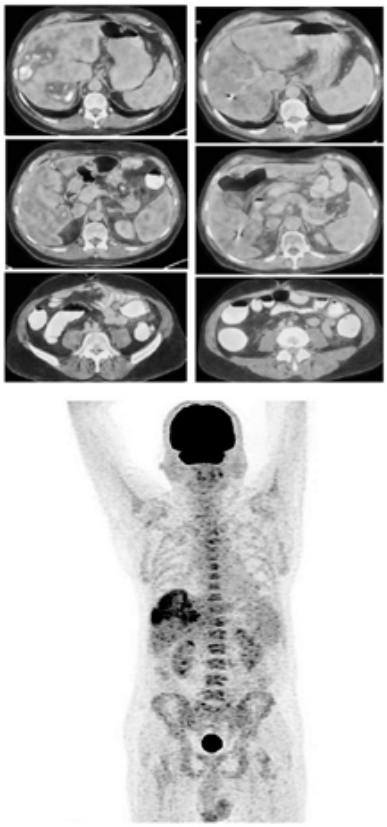

Fig. 1a. Comparitive PET CT Scan of transverse colon. The images to the left showed recurrence in the liver and images to the right show that there was no evidence of anastomotic recurrence and stable necrotic hepatic metastases

diosurgery to the liver lesions at a dose of 30Gy/5# and 12 cycles of bevacizumab + FOLFIRI. Follow up PETCT done in November 2018 showed good response (Fig. 1a) with no evidence of anastomotic recurrence and stable necrotic hepatic metastases with no SUV value as compared to 9 in the previous PET CT. After few months, there was recurrence in the ometum as omental deposit for which he underwent cyberknife guided robotic radiosurgery. At present, the patient is being treated with bevacizumab and capecitabine and is doing well.

The results of IHC showed intact but weak expression of MLH1 (Fig. 1b). In case a tumor sample exhibits abnormal or weak staining in MSI by IHC, genetic testing is the next step and hence mutation analysis was considered. Pre-genetic test counselling disclosed a strong history of Ca Colon, Ca tongue and head and neck cancer in first and second degree relatives for which a pedigree was sketched using scientific symbols. The pedigree suggested the prevalence of Ca Colon in proband's grandmother, father and second sister (Fig. 1c; I-1, II-1, III-2, III-5). The second sister (III-2) was earlier diagnosed with Ca breast in 2003 and with Ca colon in 2017. The eldest sister (III-1) was diagnosed with Ca tongue and her son (IV-1) with head and neck cancer. Based on the clinical presentation and the family history, germline mutation testing by NGS was recommended for the proband and his family members to check the presence of any germline mutation. With informed written consent, 14 genes associated with CRC were checked

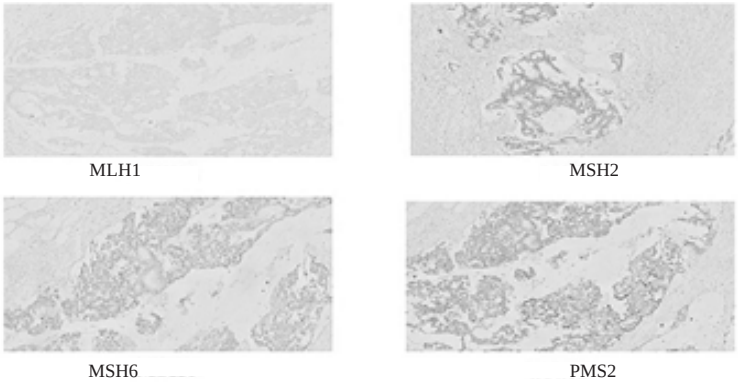

Fig. 1b. Immunohistochemical staining. The sections showed faint staining for MLH1 and positive staining for MSH2, MSH6 and PMS2 (x400)

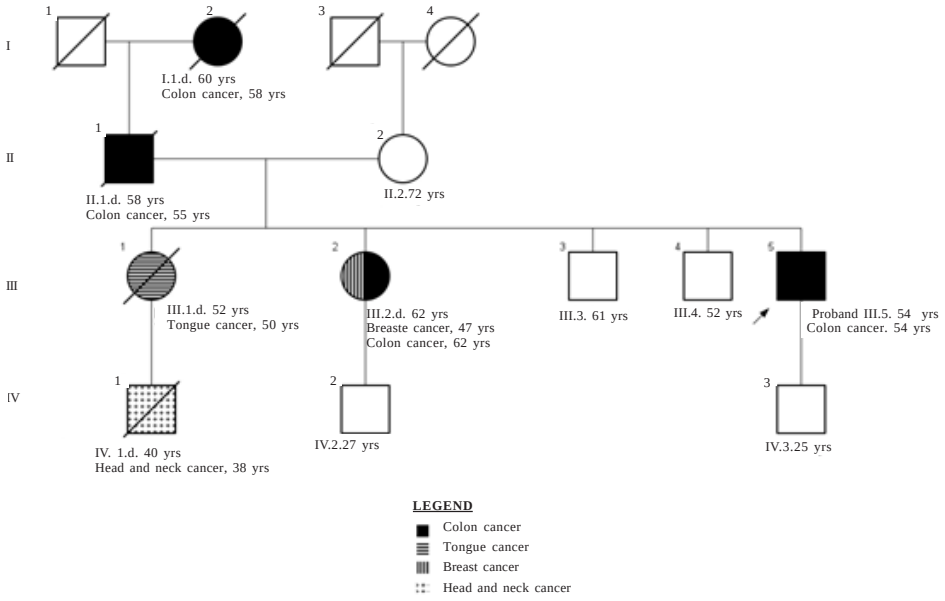

Fig. 1c. Pre-genetic test pedigree chart of case 1 (III-5). Three family members among three generations were affected with Ca Colon (I-2. II-1, III-2)

along with 23 genes recommended by ACMG for either key or incidental findings. Also for additional confirmation, MSI by PCR was performed on the tissue and blood sample. The sample was tested positive by PCR with MSI detected at two or more of the five loci tested.

Case Report 2

A 41 year old male consulted the oncologists in May 2016 with a suspicion of Ca colon. On detailed consultation, the proband was found to have a strong family history of cancer and hence the physician wanted to check for any underlying familial syndrome. Upon recommendation of genetic testing, the patient and the family members were subjected to pre-test counselling and a pedigree (Fig. 2b) was drawn based on the information on the history of cancers in the family. The information revealed a strong predominance of Ca colon in first, second and third degree relatives. The inheritance pattern was observed from the proband's paternal side where his grandmother (Fig. 2b; I-1) father (II-9), three uncles (II-1, II-7, II-8), and three cousins (III-1, III-5, III-11) were affected with Ca colon. One aunt (II-5) was affected with Ca breast and

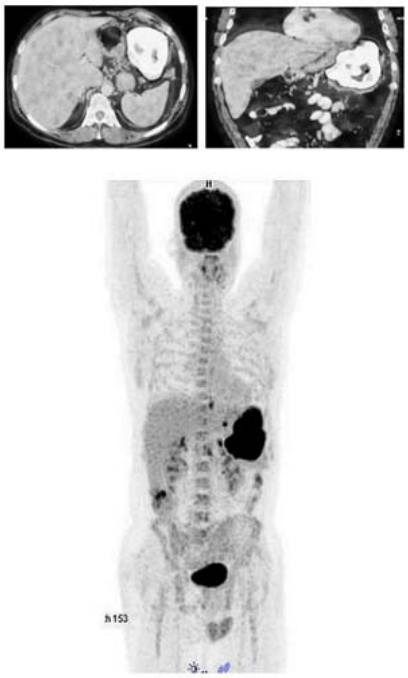

Fig. 2a. PET CT Scan showed the involvement of the transverse colon and the spleen

one aunt (II-4) with Ca uterus. The grandmother, three uncles, aunt affected with Ca breast and two cousins had succumbed to the disease. The proband underwent FDG PET scan (Fig. 2a) that showed a 12 x 5.5 cm long circumferential mass lesion involving transverse colon and splenic flexure with a SUV value of 26.8. The report suggested metabolically active pericolonic, gastrocolic and retroperitoneal lymphnodes suggesting metastasis. Surgery was planned and robotic assisted transverse hemicoloctomy was done. The postoperative course was eventful. The HPE of the operated specimen was reported as moderately differentiated adenocarcinoma and was staged as pT3N1M0-IIIB. Molecular testing revealed mutations in KRAS, PIK3CA and FBXW7. Based on the investigations, the patient received adjuvant chemotherapy with Oxaliplatin with Xeloda for 6 cycles and is doing well at present with recent PETCT scan showing no evidence of the disease. The IHC finding for MSI was non-contributory because of tissue depletion on the FFPE block. The proband's presentation and the family history fulfilled the criteria that enabled the selection of the families that are at risk for any familial syndrome. After obtaining the informed consent, genetic testing was performed to check if any genes responsible for CRC was mutated which also included the MMR genes.

RESULTS AND DISCUSSION

It has been reported that inherited CRCs accounts for five to ten percent of total CRCs which includes LS, FAP, MAP and hamartomatous polyposis syndromes (Rohlin et al. 2016). LS accounts for about three percent of all CRC cases and its prevalence is higher compared to oth-

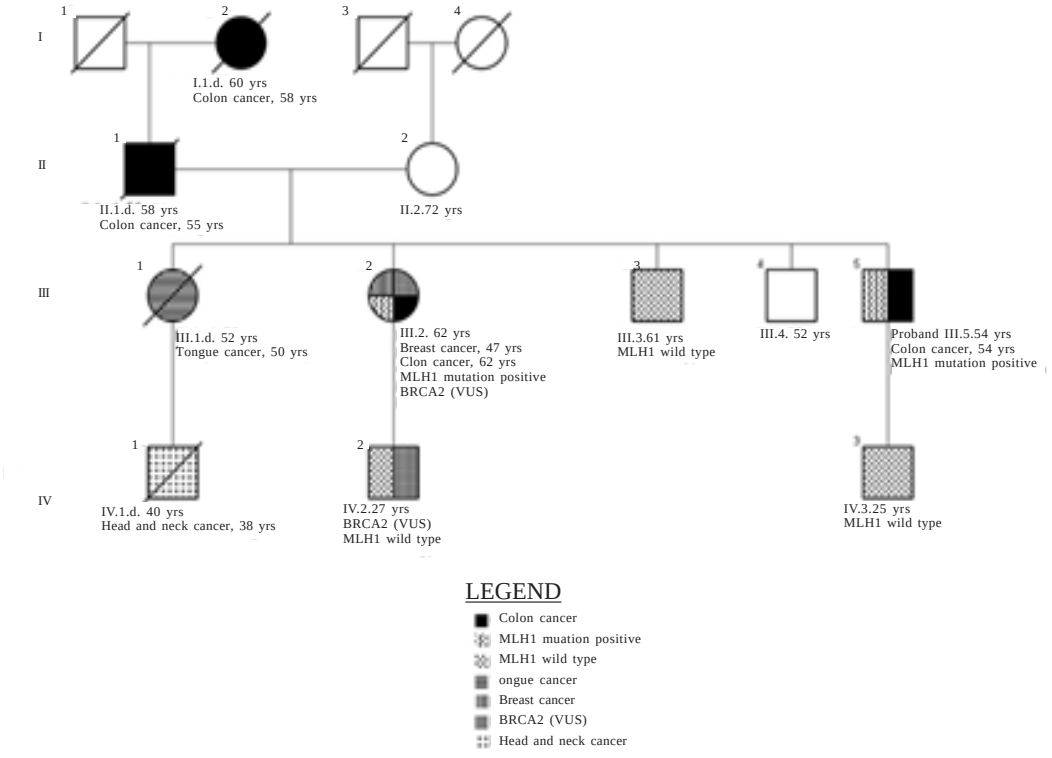

Fig. 3. Post-test pedigree chart of case 1. The proband (III-5) and his sister (III-2) were positive for MLH1 mutation

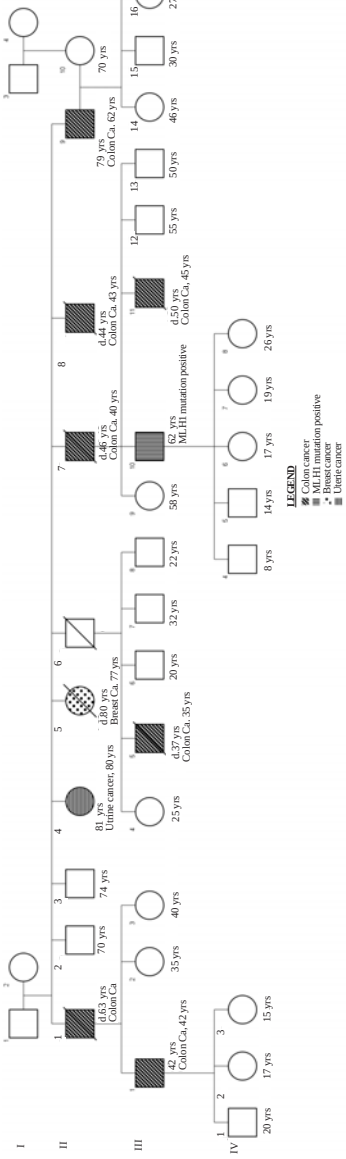

Fig. 2b. Pre-genetic test pedigree chart of case 2 (III-19). Eight family members among three generations were affected with Ca Colon (I-1, II-1, II-7, II-8, II-9, III-1, III-5, III-11)

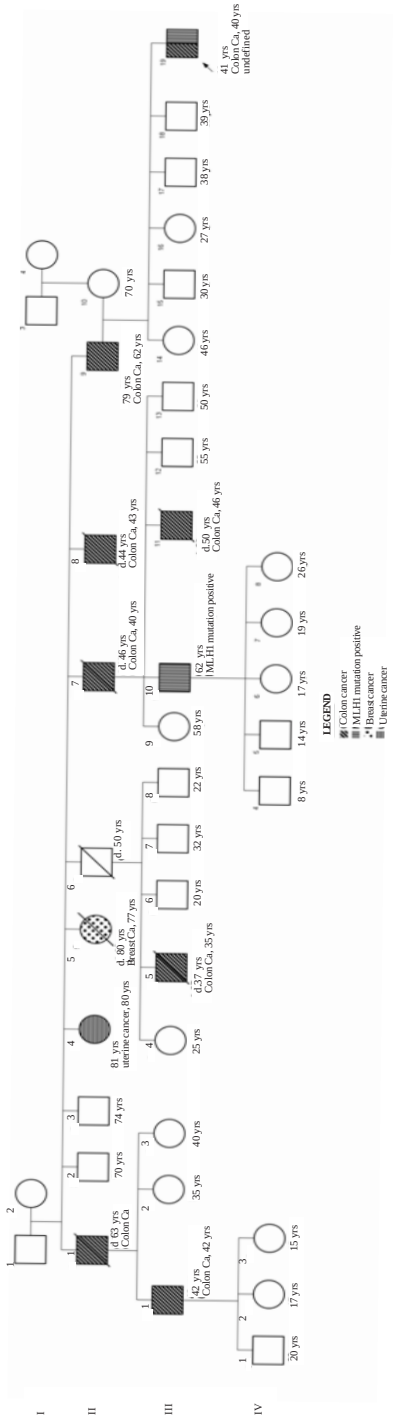

Fig. 4. Post-test pedigree chart of case 2. The proband (III-19) and his cousin (III-10) were positive for MLH1 mutation

er inherited CRC syndromes (Vasen and Boland 2005). Defect in the DNA MMR genes like MLH1, MSH2, MSH6 and PMS2 is the hallmark of LS. Majority of CRCs are related to LS, whereas, less than ten percent of sporadic CRCs show MSI (Kawakami et al. 2015). It is also reported that individuals carrying pathogenic variations in MLH1 or MSH2 genes are at highest risk of developing CRC at an early age (Jia et al. 2018).

Here, the researchers report two families with LS confirmed through genetic counselling and genetic testing. Although the Amsterdam Criteria II and Revised Bethesda Criteria were fulfilled for both the cases which confirmed LS, the researchers wanted to make a definite diagnosis with genetic testing. In the first case, the proband, his father and grandmother suffered from primary colon cancer and one of his sisters was diagnosed with two primary cancers, Ca breast in 2003 and Ca colon in 2017. His eldest sister diagnosed with Ca tongue had succumbed to the disease. Her son diagnosed with head and neck cancer had also succumbed to the disease two years after diagnosis. For this case, MSI analysis was performed by IHC on the tissue block. The results of IHC showed intact nuclear expression of the four markers MLH1, MSH2, MSH6 and PMS2 that were tested. The expression of MLH1 and PMS2 was weak but still detectable. Among all the technologies known, IHC staining in general is considered as the 'gold standard' and more sensitive than MSI analysis in detecting MMR gene mutations (Pinol et al. 2005; Southey et al. 2005; Hofstra et al. 2008). However, it should be noted that the missense mutations in the MMR genes resulting in loss of function, can have subtle effects on the protein products and still maintain an intact protein expression (Raevaara et al. 2005). The caveat of IHC is that it cannot detect these missense mutations and show faint staining of the proteins in such cases thus providing an ambiguous result. Therefore, genetic testing by NGS was recommended. The test results of the proband revealed the presence of a likely pathogenic heterozygous missense substitution (p.Glu102Asp) that lies in the exon 3 of the MLH1 gene which has been associated with LS predisposition (Fig. 3; III 5). The identified missense variant alters a highly conserved residue and has been shown to impair the protein function. It lies in the ATPase domain (residue 25-207) of the protein that is crucial for the MMR activity of the MLH1 protein (Wu et al. 2015). Out of the 7 in silico damaging tools, the variant is predicted to be damaging by 6 (SIFT, LRT, Mutation Taster, PolyPhen-2, Mutation Assessor and FATHMM). It has been previously reported in a patient affected with CRC and thus has been labelled as "likely pathogenic". A large collaborative study has shown that about eighty three percent germline mutations in hMSH2 were either nonsense or frameshift mutations, whereas about thirty one percent of hMLH1 germline mutations were due to missense changes as seen in this case (Peltomaki and Vasen 1997). A recent study has reported the association of MLH1 gene mutation with LS in a family followed for more than 45 years (Momma et al. 2019). As a confirmatory test, MSI by PCR was also performed. The sample was positive by PCR where MSI was detected at two or more of the five loci tested which can be a result of either germline or somatic mutations that cause a functional defect in mismatch repair genes. Immunotherapy drugs like Pembrolizumab and Nivolumab have been approved for MSI-H metastatic CRC cases that have progressed following prior treatment and who do not have satisfactory alternate options or have progressed following treatment with fluoropyrimidine, oxaliplatin and irinotecan, (Keytruda FDA label; Opvida FDA label). The genetic test results of the proband's sister (Fig. 3; III-2) revealed that she also carried the same variant of MLH1 along with a mutation in BRCA2 (p.Tyr2839His). As the carriers of MMR gene mutations have a high risk of Ca endometrium, she has been advised regular follow up. The proband's son (Fig. 3; IV-3) and one of his brother (Fig. 3; III-3) were tested but both did not carry the variant. Combined with the medical history of the patient and the results of germline testing for the proband and his family member, LS was confirmed in this family. A recent study of Japanese cancer patients has analysed the MMR gene variants by NGS where ClinVar database has been used as reference to evaluate the pathogenicity and showed a close concordance with MSI by PCR and IHC (Kiyozumi et al. 2019).

In the second case, the proband had a strong family history of cancer where his father, three

uncles, grandmother and three cousins were affected with Ca colon, one aunt with Ca breast and the other with Ca uterus (Fig. 4). The germline mutation test results of the proband identified a pathogenic mutation in MLH1 gene (Fig.4; III-19). The heterozygous missense substitution (p.Ala681Thr) identified results into alteration of a conserved residue (492-743 residues) that lies in the PMS2/MLH3/PMS1 interaction domain in the protein. This domain plays an important role in maintaining the MMR activity (Schmutte et al. 2001). The identified variant has been reported as pathogenic with respect to LS and has been previously reported in patients with Scottish and English ethnicity affected with LS related cancer (Barnetson et al. 2008; Froggatt et al. 1996). This variant has also been reported in patients from 8 Polish families affected with HNPCC which was suggested as a founder variant in Polish population (Kurzawski et al. 2006). There was no mutation detected in the other genes screened. Based on the result, the cousin of the proband who was present with him during the treatment was offered genetic counselling and recommended for screening which showed the presence of same variant confirming LS (Fig. 4; III-10). The presence of germline pathogenic variations in one of the MMR genes in both the family members confirmed the association of LS. In a study of Asian patients suspected with LS, NGS has revealed that MLH1 was most commonly involved followed by MSH2, MSH6, and PMS2 (Ow et al. 2019).

The surveillance program for an individual affected by LS includes colonoscopy every 1 to 2 years, esophagogastroduodenoscopy with extended duodenoscopy every 3 to 5 years. Annual urine analysis could be recommended for surveillance of colon, gastric, small bowel and urothelial cancer (Vasen et al. 2013). It has been shown that the application of appropriate programmes of management in families with LS leads to increased detection of early asymptomatic CRC. Prospective studies have shown sixty three percent reduction in the risk of CRC as well as a reduction in mortality (Jarvinen et al. 2000). The recommendations currently are based on colonoscopy every two years from the age of 20 (Vasen et al. 1995).

In both the cases discussed above, the remaining family members have been recommended for genetic testing to check for the inheritance of the mutation based on which surveillance measures can be recommended. Genetic counsellors played an important role in providing complete information to the family members regarding the importance of genetic testing based on clinical condition, risk of family members to carry the mutation and for early management and disease prevention. The researchers have reported earlier on the implications of genetic counselling and role in identifying two different familial cancer syndromes Cowden Syndrome (Chatterjee et al. 2016 a) and Li Fraumeni syndrome in two different families (Chatterjee et al. 2016b). The first study identified Cowden syndrome in a male breast cancer patient, a rare cancer type that accounts only one percent of the total breast cancer cases thus posing diagnosis challenges and social burdens where the individual needs more personalized therapies and genetic counselling to cope with the condition. In the second case, based on genetic background of the family and risk predisposition to disease, the genetic counsellors identified a Li-Fraumeni syndrome (LFS) which is associated with the high risk of a diverse spectrum of childhood and adult-onset malignancies with a predominance of the soft-tissue sarcomas, osteosarcoma, breast cancer, brain tumor, adrenocortical carcinomas, Wilms tumor, leukaemia and several other LFS-associated cancer types. In both the cases, Genetic counselling supported the family with timely screening for molecular evaluation and provided the family members with flawless knowledge about the genetic test and the outcome.

CONCLUSION

The inherited cancer syndromes of each type of cancer involve a similar gamut of clinical presentations and alterations in the genetic profile, due to which, the clinical diagnosis of hereditary CRC cases becomes challenging. Therefore, understanding the clinicopathogical features and molecular profile of each syndrome becomes very important. This enables the genetic counsellors to recommend the appropriate genetic testing of the affected and to screen at risk kindred in several generations in the family. Here, the researchers report two families with LS con-

firmed through genetic counselling and genetic testing. In the first case, the proband, his father and grandmother suffered from primary colon cancer and one of his sisters was diagnosed with two primary cancers, Ca breast in 2003 and Ca colon in 2017. His eldest sister diagnosed with Ca tongue had succumbed to the disease. Her son diagnosed with head and neck cancer had also succumbed to the disease two years after diagnosis. The test results of the proband revealed the presence of a likely pathogenic heterozygous missense substitution p.Glu102Asp that lies in the exon 3 of the MLH1 gene which has been associated with LS. However, the results of IHC showed intact nuclear expression of the four markers MLH1, MSH2, MSH6 and PMS2 with weak and still detectable expression of MLH1 and PMS2. An important finding from this study is that though IHC staining is considered as gold standard in detecting MSI, MMR gene mutation through inherited (germline) mutation testing should be recommended to CRC cases having a strong family history of cancers. Stand-alone IHC results are not sufficient to detect LS particularly in those cases which have missense mutations in MMR genes. The genetic test results of the proband's sister revealed that she also carried the same variant of MLH1 along with a mutation in BRCA2 (p.Tyr2839His). As the carriers of MMR gene mutations have a high risk of Ca endometrium, she has been advised regular follow up. The proband's son and one of his brother were tested but both did not carry the variant. Combined with the medical history of the patient and the results of germline testing for the proband and his family member, LS was confirmed in this family.

In the second case, the proband had a strong family history of cancer where his grandmother, father, three uncles, and three cousins were affected with Ca colon, one aunt with Ca breast and the other with Ca uterus. The germline mutation test results of the proband identified a pathogenic mutation in MLH1 gene. There was no mutation detected in the other genes screened. Based on the result, the cousin of the proband who was present with him during the treatment was offered genetic counselling and recommended for screening which showed the presence of same variant confirming LS. The results of the researchers' study clearly establishes evidence that in both the cases, the missense mutations identified are indeed likely pathogenic or pathogenic alterations that cause LS. Based on detectable germline variations, it was concluded that the families were 'at risk'. The individuals were counselled by the genetic counsellors who recommended predictive tests and surveillance program as per NCCN guidelines. Colonoscopy at the age of 20-25 years (or 2-5 years prior earliest colon cancer in the family) every 1 to 2 years in individuals detected with LS was suggested. Esophagogastroduodenoscopy with extended duodenoscopy every 3 to 5 years beginning at the age of 40 years in cases of family history of gastric cancer and/or Asian ancestry and annual urine analysis beginning age 30-35 years especially for people from families with a family history of urothelial cancer and individuals with a MSH2 mutation were recommended for surveillance of colon, gastric, small bowel and urothelial cancer. The genetic counsellor also recommended physical and neurological examination on an annual basis starting at age 25-30 years. Prophylactic risk-reducing surgeries to remove the ovaries and uterus upon completion of childbearing was suggested and were educated on possible symptoms of ovarian or uterine cancer. They were asked to report any unusual vaginal bleeding, pelvic or abdominal pain, bloating, increased abdominal girth, difficulty in eating, or increased urinary frequency or urgency to health care providers immediately. Breast and Endometrial cancer screening was also considered every 1-2 years based on family history.

RECOMMENDATIONS

Based on the findings of the paper, it was strongly recommended that genetic counselling and testing should be considered in patients with CRC, particularly if it is an early onset disease and there is a history of cancer in the close relatives. This might unfold to a greater extent the genetic background of the patient as well the family members and identify 'at risk' of predisposition of disease prevalence. Genetic counselling enables the family members to take a decision on timely screening, molecular and genetic evaluation and offers appropriate knowledge on the need of genetic testing and its outcome. Recognition of LS and implementation of

appropriate cancer monitoring /surveillance strategies can help in early detection of cancer in 'at 'risk' individuals. This in turn can greatly help in the identification of unique genotype-phenotype profiles thus improving prevention, management and survival outcome in CRC.

ABBREVIATIONS

- LS: Lynch syndrome
- HPE: Histopathological examination
- IHC: Immunohistochemistry
- CRC: Colorectal cancer
- MSI: Microsatellite instability
- MMR: Mismatch repair
- HNPCC: Hereditary nonpolyposis colorectal cancer
- FFPE: Formalin fixed paraffin embedded
- NGS: Next Generation Sequencing
- PCR: Polymerase chain reaction

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Annexure 1: Amsterdam I and II Criteria for Diagnosis of Hereditary Nonpolyposis Colorectal Cancer

Amsterdam I Criteria

1. Three or more relatives with histologically verified colorectal cancer, 1 of which is a first-degree relative of the other two. Familial adenomatous polyposis should be excluded.
2. Two or more generations with colorectal cancer.
3. One or more colorectal cancer cases diagnosed before the age of 50 years.

Amsterdam II Criteria

1. Three or more relatives with histologically verified HNPCC-associated cancer (colorectal cancer, cancer of the endometrium, small bowel, ureter, or renal pelvis), 1 of which is a first-degree relative of the other 2. Familial adenomatous polyposis should be excluded.
2. Cancer involving at least 2 generations.
3. One or more cancer cases diagnosed before the age of 50 years.

Annexure 2: Revised Bethesda Guidelines

1. CRC diagnosed at younger than 50 years.
2. Presence of synchronous or metachronous CRC or other LS-associated tumors.[a]
3. CRC with MSI-high pathologic-associated features (Crohn-like lymphocytic reaction, mucinous/signet cell differentiation, or medullary growth pattern) diagnosed in an individual younger than 60 years old.
4. Patient with CRC and CRC or LS-associated tumor[a] diagnosed in at least 1 first-degree relative younger than 50 years old.
5. Patient with CRC and CRC or LS-associated tumor[a] at any age in 2 first-degree or second-degree relatives.

[a]LS - associated tumors include tumor of the colorectum, endometrium, stomach, ovary, pancreas, ureter, renal pelvis, biliary tract, brain, small bowel, sebaceous glands, and kerotoacanthomas.