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Research Article

Application of Next Generation Sequencing Approach to Molecular Diagnosis Of Hereditary Colorectal Cancer: Identification Of A Novel Heterozygous Single Nucleotide Germline Deletion In Msh2 Gene Cause Lynch Syndrome

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Abstract

Germline mutations in DNA mismatch repair (MMR) genes including MSH2, MLH1, MSH6 and PMS2 causes Lynch syndrome (LS). Lynch syndrome (LS) is the autosomal dominantly inherited familial form of colorectal cancer (CRC) predisposing syndrome. In our present study, we identified and screen a five generation Chinese family with LS. According to the Amsterdam II criteria, clinical diagnosis has been done for the affected members of this family. In this study, we performed genetic molecular study for 79 members (11 patients and 68 unaffected members) of this five generation Chinese family and 100 normal healthy control individuals of same ethnic background. Targeted next generation sequencing and Sanger sequencing identified a novel heterozygous single nucleotide germline deletion (c.1427delC) in MSH2 gene in the proband, which is co-segregated well with the disease phenotype among all the affected family members. This novel heterozygous deletion (c.1427delC) in MSH2 gene leads to the formation of a premature stop codon (p.Pro476Leufs*6) which finally results into the formation of a truncated MSH2 protein. Our present finding expands the mutation spectrum for the MSH2 gene as well as establishes the significance of targeted next generation sequencing for identifying novel candidate mutations for patients with Lynch syndrome.

Keywords: Lynch Syndrome; MSH2 Gene; Targeted Next Generation Sequencing; Novel Mutation; DNA Mismatch Repair Gene.

Introduction

LS is a colorectal cancer (CRC) predisposing syndrome with an autosomal dominant mode of inheritance Lynch et al. [1] approximately, 1-13% among all the colorectal cancer patients has been suffering from LS primarily Vasen et al. [2]. The Clinical symptoms or diagnostic features of LS are early onset CRC, along with extracolonic manifestations; i.e. endometrial, pancreatic or gastrointestinal cancers Canard et al. [3]. Clinical diagnosis for the patients with LS has been done according to the Amsterdam II criteria Canard et al. [3]. Identification of germline mutations of DNA mismatch repair (MMR) genes and positive family history are also significant factor for clinical diagnosis Chung et al. [4]; Goecke et al. [5].

However, according to the previous reports, MSH2 was the first gene to be identified as associated with LS, together with MLH1 Knudsen et al. [6]. Germline mutations of MSH2 and MLH1 are together accounting for 90% cases of LS Peltomäki et al. [7]. During DNA replication, to repair the mismatched bases, the MSH2 bind with MSH6 or MSH3 to form the MutSα/β complexes and translocate into nucleus to bind to the DNA and start the mismatched base repair Boland et al. [8]. Germline mutations in the

DNA MMR genes cause formation of non-functional MMR protein which in turn increases the rate of spontaneous somatic mutation. Non-functional MMR protein associated somatic mutations are majorly occurring in microsatellite sequences Woods et al. [9]. In addition, till now, there were 500 germline mutations of DNA MMR genes have been reported and 39% of these are MSH2 mutations Vasen et al. [10].

Takeda et al [11] reported that germline loss-of-function mutation in MSH2 gene also causes synchronous endometrial and ovarian cancer (SEOC) in a 41-year-old Japanese patient with a positive family history of colorectal and gastric cancers Takeda et al. [11]. This patient was diagnosed with Lynch syndrome and it is a rare example of a patient with genetically diagnosed Lynch syndrome together with SEOC. SEOC is extremely rare and caused by Lynch syndrome. In addition, LS patients with MSH2 and MLH1 germline mutations are identified with high-degree of microsatellite instability Sanchez et al. [12]. Moreover, according to the data deposited in International Society for Gastrointestinal Hereditary Tumours (InSiGHT) database, among 3000 unique germline mutations of MMR genes, MLH1, MSH2, MSH6 and PMS2 account for 40, 34, 18, and 8 %, respectively Thompson et al. [13].

Here, in order to understand the genetic and molecular basis of the LS in this five generation Chinese pedigree, targeted next-generation sequencing has been done with a panel of 14 genes (APC, AXIN2, BMPR1A, EPCAM, MLH1, MLH3, MSH2, MSH6, MUTYH, PMS1, PMS2, PTEN, SMAD4, STK11) associated with Lynch syndrome. Targeted Next generation sequencing identified a novel heterozygous single nucleotide germline deletion (c.1427delC, p.Pro476Leufs*6) in MSH2 gene. Identified candidate variants by next generation sequencing has been validated and confirmed by Sanger sequencing. This germline deletion is co-segregated with LS phenotype among all the affected family members, with autosomal dominant mode of inheritance, in this five generation Chinese family.

Results

Clinical Description

Family recruitment and clinical examination. We identified a five generation Chinese pedigree of 89 members (Figure 1) with LS. In (Table 1), we described the detailed clinical information for all the affected and unaffected members in this family.

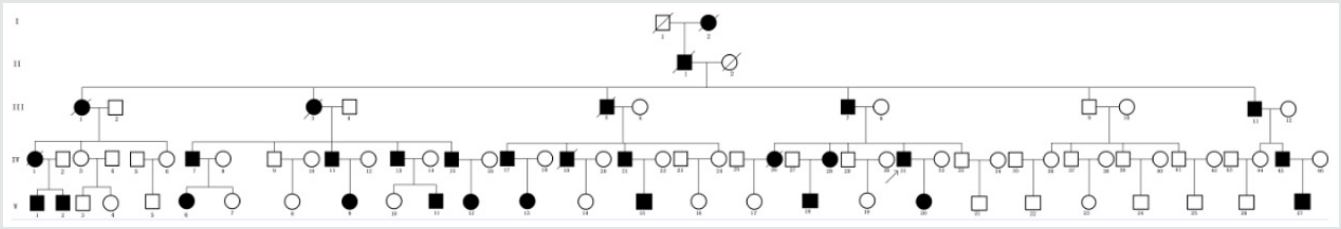

Figure 1.

Table 1: Clinical characteristics of all the affected and unaffected family members found in our study. [WT: Wild type; MT: Mutant type].

Family ID	Sex	WT/MT	Present Age (Years)	Cancer Clinical symptoms	Extra-Clinical symptoms (Diagnosed Years)
I-1	M	-	Died	-	-
I-2	F	-	Died	Intestinal cancer	-
II-1	M	-	Died	Intestinal Cancer	-
II-2	F	-	Died	Undiagnosed	-
III-1	F	-	Died	Colon cancer	-
III-2	M	WT	86	Healthy	-
III-3	F	-	Died	Endometrial cancer	-
III-4	M	WT	85	Healthy	-
III-5	M	-	Died	Intestinal cancer, Liver metastases	-
III-6	F	WT	-	Healthy	-
III-7	M	MT	84	Intestinal cancer (52), Adrenal cancer	-
III-8	F	WT	83	Healthy	-
III-9	M	WT	77	Healthy	-
III-10	F	WT	76	Healthy	-

III-11	M	MT	74	Intestinal cancer, Liver metastases	-
III-12	F	WT	71	Healthy	-
IV-1	F	-	Died	Intestinal cancer, Gastric metastases	-
IV-2	M	WT	56	Healthy	-
IV-3	F	WT	66	Healthy	-
IV-4	M	WT	62	Healthy	-
IV-5	M	WT	58	Healthy	-
IV-6	F	WT	59	Healthy	-
IV-7	M	MT	62	Colon cancer	-
IV-8	F	WT	61	Healthy	-
IV-9	M	WT	61	Healthy	-
IV-10	F	WT	59	Healthy	-
IV-11	M	MT	59	Intestinal cancer,	
Adrenal cancer	-				
IV-12	F	WT	58	Healthy	-
IV-13	M	MT	56	Intestinal cancer	-
IV-14	F	WT	56	Healthy	-
IV-15	M	MT	54	Colon cancer	-
IV-16	F	WT	53	Healthy	-
IV-17	M	MT	52	Liver cancer	-
IV-18	F	WT	52	Healthy	-
IV-19	M	-	Died (50)	Stomach cancer, Liver metastases	-
IV-20	F	WT	49	Healthy	-
IV-21	M	MT	57	Colon cancer	-
IV-22	F	WT	55	Healthy	-
IV-23	M	WT	55	Healthy	-
IV-24	F	WT	53	Healthy	-
IV-25	M	WT	55	Healthy	-
IV-26	F		Died (53)	Brain cancer	-
IV-27	M	WT	56	Healthy	-
IV-28	F	MT	57	Endometrial cancer	-
IV-29	M	WT	56	Healthy	Enteritis
IV-30	F	WT	55	Healthy	-
IV-31	M	MT	54	Adrenal cancer	-
IV-32	F	WT	53	Healthy	-
IV-33	M	MT	51	Healthy	Intestinal erosion
IV-34	F	WT	49	Healthy	-
IV-35	M	WT	53	Healthy	-
IV-36	F	WT	52	Healthy	-
IV-37	M	WT	52	Healthy	-
IV-38	F	WT	52	Healthy	-
IV-39	M	WT	46	Healthy	-
IV-40	F	WT	44	Healthy	-
IV-41	M	WT	44	Healthy	Enteritis
IV-42	F	WT	43	Healthy	-
IV-43	M	WT	44	Healthy	-

IV-44	F	WT	43	Healthy	-
IV-45	M	MT	44	Colon cancer	-
IV-46	F	WT	43	Healthy	-
V-1	M	MT	35	Intestinal cancer	-
V-2	M	MT	32	Intestinal cancer	-
V-3	M	WT	32	Healthy	-
V-4	F	WT	31	Healthy	-
V-5	M	WT	31	Healthy	-
V-6	F	MT	37	Intestinal cancer	-
V-7	F	WT	27	Healthy	-
V-8	F	WT	34	Healthy	-
V-9	F	MT	34	Intestinal Cancer	-
V-10	F	WT	35	Healthy	-
V-11	M	MT	34	Intestinal Cancer	-
V-12	F	MT	35	Intestinal Cancer	-
V-13	F	MT	38	Intestinal Cancer	-
V-14	F	WT	37	Healthy	-
V-15	M	MT	38	Intestinal Cancer	-
V-16	F	WT	37	Healthy	-
V-17	F	WT	34	Healthy	-
V-18	M	MT	32	Intestinal Cancer	-
V-19	F	WT	34	Healthy	-
V-20	F	MT	32	Intestinal Cancer	-
V-21	M	WT	25	Healthy	-
V-22	M	WT	19	Healthy	-
V-23	F	WT	22	Healthy	-
V-24	M	WT	19	Healthy	-
V-25	M	WT	26	Healthy	-
V-26	M	WT	28	Healthy	-
V-27	M	MT	31	Intestinal Cancer	-

Identification and characterization of candidate mutation

Targeted next generation sequencing identified a heterozygous novel single nucleotide deletion; c.1427delC, p.Pro476Leufs*6 in MSH2 gene [NCBI Reference sequence NM_000251] in the proband (IV-31). Segregation analysis showed that this novel heterozygous deletion is co-segregated well with the LS phenotypes among the affected members, but not present in the unaffected members of this family. In addition, this novel deletion is also not present in the 100-normal control individual of same ethnic background.

Sanger Sequencing And Confirmation

This novel heterozygous deletion; c.1427delC, p.Pro476Leufs*6 in MSH2 gene was confirmed by Sanger sequencing in proband and among all the affected family members in this family (Figure 2).

Discussion

Here, we identified a five generation Chinese family with LS. After proper clinical diagnosis, genetics screening has been done by targeted next generation sequencing and confirmatory Sanger sequencing. Targeted next generation sequencing and Sanger sequencing identified a heterozygous novel single nucleotide deletion (c.1427delC, p.Pro476Leufs*6) [NCBI Reference sequence NM_000251] in the human MSH2 gene in the proband (IV-31) and in all the affected family members in this Chinese family. This heterozygous novel deletion of MSH2 gene has not presented in the ExAC database. This deletion finally results in the formation of truncated MSH2 protein (p.Pro476Leufs*6) by the formation of a premature termination codon (Figure 2). The structural effect of this mutation has been schematically showed in (Figure 3).

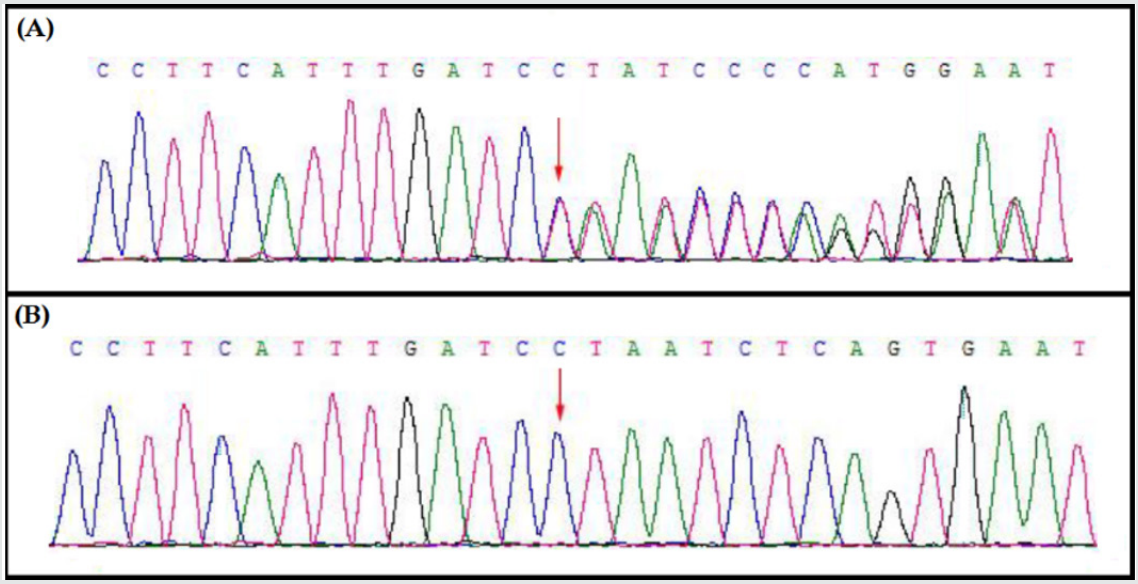

Figure 2.

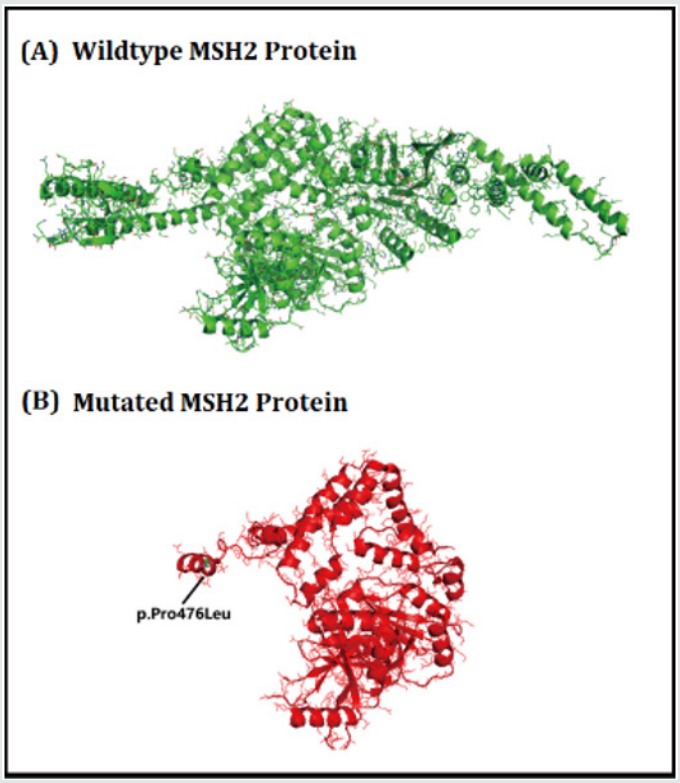

Figure 3.

The function of DNA mismatch repair genes and its correlations with the human disease is very significant. Germline mutations in the human homologue of the bacteria mutS gene (MSH2) predispose individuals to Lynch syndrome Wang et al. [14]; Pérez-Cabornero et al. [15]. DNA mismatch repair (MMR) genes are following a highly conserved biological pathway playing a significant role in maintaining genomic stability. However, primary function of MMR gene is to repairing base-base mismatches and insertion/deletion mispairs generated during DNA replication and recombination. Furthermore, recently it has been reported that MMR gene also plays a key role in homologous recombination and in DNA damage signaling in eukaryotic cells. Germline truncated mutation of MMR leads to the formation of a non-functional MMR protein which finally results into genome-wide instability, increase the spontaneous mutation rate, predisposition to certain types of cancer including lynch syndrome, resistance to certain chemotherapeutic agents, and abnormalities in meiosis and sterility in mammalian systems Baglietto et al. [16].

According to recent reports it has been showed that LS patients with germline MSH2 mutations used to develop extracolonic cancers while LS patients with MLH1 mutations never develop extracolonic manifestations Lucía Pérez et al. [15]. In addition, LS patients with MSH6 mutations have the highest risk of developing endometrial cancer Lucía Pérez et al. [15]. In South-East Asia, LS patients are usually developed endometrial and stomach cancer Li [17]. In LS patients with MSH2 germline mutations, the lifetime risk of developing colorectal cancer is ranging between 57 % - 80 % Bonadona et al. [18]; Vasen et al. [10]. Lynch syndrome is caused by the germline mutations of MSH2 gene. Most of the reported mutations of MSH2 gene were leading to the formation of truncated MSH2 protein Li [17] ; Bakry et al. [19]. Presently, in world population, 5327 sequence variants of the MSH2 gene have been reported. Among all reported pathogenic mutations of the MSH2 gene, missense, nonsense and deletion mutations are the major types. In Chinese population, till now, 119 pathogenic mutations in MSH2 gene has been reported In conclusion, our present study identified a five generation Chinese family with LS. Proband and all the affected family members are harboring a heterozygous novel single nucleotide deletion in MSH2 gene. Our study not only expands the spectrum of the germline mutations of MSH2 gene in the Chinese population but also establish again the significance of targeted next generation sequencing for identifying candidate mutations in patients with LS. Moreover, our present finding also contributes to a database of germline mutations in MSH2 gene which is very significant in future for proper clinical diagnosis, easy molecular genetic screening patients with LS.

Materials and Methods

Ethical statement

Family members of this five generation Chinese family have given written informed consent as they are participating in this study. The Ethical Committee of the Maternal and Child Health Hospital of Hainan Province, Haikou, China, reviewed and approved our study protocol in compliance with the Helsinki declaration. Diagnosis of the patients for Lynch syndrome was made by oncologists, on the basis of Amsterdam II criteria.

Patients and pedigree

A five generation Chinese family with Lynch syndrome (Figure 1), diagnosed and treated in Maternal and Child Health Hospital of Hainan Province, Haikou, China, were enrolled in our study. The diagnostic standard or criteria for patients with Lynch syndrome was as follows: Diagnosis of Lynch syndrome is based on the Amsterdam criteria Canard et al. [20]. In this study, we performed genetic molecular study for 79 members (11 patients and 68 unaffected members) of this five generation Chinese family and 100 normal healthy control individuals of same ethnic background.

Targeted exome-based next-generation sequencing and variant identification

DNA samples obtained from the proband (IV-31) were sequenced using targeted next-generation sequencing. Roche NimbleGen's (Madison,USA) custom Sequence Capture Human Array was used to designed to capture 98480 kb of targeted sequence, covering 181 exons and flanking sequence (including the 100 bp of introns) of 14 genes (APC, MLH1, MSH2, MSH6, PMS2, AXIN2, BMPR1A, EPCAM, MLH3, MUTYH, PMS1, PTEN, SMAD4, STK11) which is associated with colorectal cancer (CRC) and yielded an average of 6366534 reads per sample, with approximately 68.78% mapping to the targeted regions. The average sequencing depth of the target area is 464.68 with 99.46% coverage. The procedure for preparation of libraries was consistent with standard operating protocols. In each pooling batch, 10 to 33 samples were sequenced simultaneously on Illumina HiSeq 4000 Analyzers (Illumina, San Diego, USA) for 90 cycles (specially designed by us for this study). Image analysis, error estimation, and base calling were performed using Illumina Pipeline software (version 1.3.4) to generate raw data. The raw reads were screened to generate – clean reads‖ followed by established filtering criteria. Clean reads with a length of 90 bp were aligned to the reference human genome from the NCBI database (Build 37) using the Burrows Wheeler Aligner (BWA) Multi-Vision software package with output files in - bam‖ format. The bamdata were used for reads coverage in the target region and sequencing depth computation, SNP and INDEL calling, and CNV detection. First, a novel three-step computational frame work for CNV was applied. Then, SNPs and INDELs were called using SOAPsnp software and Sam tools pileup software, respectively. A SNP or INDEL would be filtered out if it could not follow the criterion: supported by at least 10 reads and >20% of the total reads. The frequency filter was set at 0.05. If a SNP frequency was more than 0.05 in any of the four databases (dbSNP, Hapmap, 1000 Genomes Project, the 124 healthy reference samples sequenced in this study), it would be regarded as a polymorphism, but not a causative mutation. Last, SNVs were retrieved in The Human Gene Mutation Database and the Leiden Open Variation Database and then labeled as reported or novel.

Sanger Sequencing

To validate the true positive of the mutation among all the family members (11 patients including the proband and 68 unaffected members), Sanger sequencing was performed. Primers flanking the candidate loci were designed based on the reference genomic sequences of Human Genome from GenBank in NCBI and synthesized by Invitrogen, Shanghai, China. PCR amplification was carried out in ABI 9700 Thermal Cycler. PCR products were directly sequenced on ABI PRISM 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA). Sequence data comparisons and analysis were performed by DNASTAR SeqMan (DNASTAR, Madison, Wisconsin, USA).

The novel heterozygous deletion identified in MSH2 gene through targeted next generation sequencing were verified through Sanger sequencing using the primers: F-5'- AAGGAGTTGTTCGTTTTCCACTT -3', R-5'- TTACCAAAAGCCAGGTGACATTC -3'. The reference sequence NM_000251 of MSH2 was used.

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Availability Of Data And Materials

The data used to support the findings of this study are included within the article.

Authors'contributions

MX, YZ and SB designed the study. ZL performed the molecular diagnosis by Sanger sequencing. LY carried out the clinical analysis. YZ and MX contributed to the molecular diagnosis analysis based on NGS. ZL and LY wrote the paper. This project has been done under the guidance of ZL, MX, YZ and SB. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The present study was approved by the Ethics Committee of Maternal and Child Health Hospital of Hainan Province, Haikou, China, and written informed consent was obtained from all participants.

Consent for publication

Informed consent was obtained from all individual participants included in the study.

Competing interests

The authors declare that they have no competing interests.

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