Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Three cases of colon cancer in four generations of the Saudi family, caused by endogamous germline mutations

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ARTICLE INFO

Original paper

Article history:
Received: April 25, 2023
Accepted: July 07, 2023
Published: August 31, 2023

Keywords:

Whole-exome sequencing, colon cancer, four generations, germline mutations, endogamy

ABSTRACT

Various research pieces of evidence have been published in recent years, establishing the increasing prevalence of early colon cancer among young people. In this background, the current study aimed to analyze the reasons behind colon cancer recurrence among endogamous consanguineous cases in four generations of a single Saud family. For this study, the authors conducted the whole-exome sequencing analysis to screen for germline mutations in DNA samples from consanguineous cases within the family. After collecting the colon samples, it was analyzed histologically and immunohistochemically with the help of Breast Cancer antibodies (BRCA2 and 1 correspondingly) and H&M staining (hematoxylin and eosin). For this study, 26 at-risk consanguineous cases were considered. Three cases were diagnosed with malignant colon cancer, two with breast cancer, and 17 with germline mutations, yet remain unaffected by cancerous tumors. The rest, four consanguineous cases, are healthy and non-carriers of the mutations. However, as per the exome analysis outcomes, 15 cases inherited germline mutations in nine genes. Nine substitution mutations were present in six of the nine inherited genes in these inherited germline mutations. Furthermore, it also presented six insertion and deletion frameshift mutations in five of nine inherited genes. The immunohistochemical staining process achieved positive staining outcomes for BRCA1 and 2. Therefore, germline mutations inherited from the nine genes of endogamous consanguineous cases of mutation carriers remain the primary reason behind colon cancer recurrence in the same family.

Doi: http://dx.doi.org/10.14715/cmb/2023.69.8.25

Introduction

Various factors contribute to the recurrent and historical colorectal cancer cases, such as the history of other cancer types, type of tumor, age-of-onset, inherited mutation of the oncogenes and tumor suppressor genes, endogamy, causes, and date of death. As per the literature (1–3), some of the cofactors that contribute to colorectal cancer include a history of chronic colitis, obesity (body mass index (BMI) ≥ 25 kg), gender, and smoking history. Colon cancer is a heterogeneous disease with three unique subtypes: 1) colitis-associated, 2) hereditary, and 3) sporadic colon cancer. Among these, 10-15% of all colon cancers fall under the hereditary type (3). This type of cancer is induced by cancer genes that are passed from one generation to the other. Despite this known genetic association, it is still unclear what is the specific gene that causes hereditary colorectal cancer. If the members of a single family are repeatedly affected by colorectal cancer, it might be attributed to the genetic association that passes from the parents to their offspring (3, 6).

Hereditary colon cancer patients tend to express germline mutations through adenomas colonic mucosa. In this biological process, germline mutations occur in the patients within oncogenes and tumor suppressor genes (1, 2, 4).

In general, the whole exome sequencing (WES) analysis is conducted to analyze the heritable nature of the malignant tumors and the associated genetic diseases. This analysis is executed by screening the nucleotide sequences of the related cases. Furthermore, some other cancer types associated with this mutation can also be identified through traditional histopathology, immunohistochemistry, and molecular analysis (5–10,11). The most

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preferred and suitable method for sequence identification is exome analysis, which is highly sensitive and detects germinal mutations with high accuracy (10).

The current study using WES analysis aims to determine the factors that exhibit the most influential causes resulting in colon cancer recurrence among all the consanguineous cases of four generations in a single Saudi family. Furthermore, the authors also evaluated the rest of the contributing cofactors, such as the clinical characteristics, traditional histopathology, immunohistochemistry tests, and pedigree analysis to have a comprehensive diagnosis of the affected patients and carriers of the consanguineous cases in the specific family.

Materials and Methods

Ethical statement and participants

The present study received ethical approval from the Deanship of Scientific Research at Princess Norah Bint Abdulrahman University and the National Committee of Bioethics at King Abdulaziz City for Science and Technology, Kingdom of Saudi Arabia (Study number H-01-R059, IRB LOG number 20-0287). Informed consent was obtained from all participants prior to sample collection.

Case selection and sample collection

Data for this study were collected from a single Saudi family residing in Riyadh, Saudi Arabia. The authors selected 26 consanguineous cases spanning four generations, including affected carriers and patients. In 2020, at King Fahad Medical City in Riyadh, Saudi Arabia, three biopsy samples were collected from 24 relatives diagnosed with malignant colon cancer.

Table 1 summarizes the demographic profiles of all the patients (consanguineous cases) across the four generations and their respective mutated parents. Table 2 provides detailed information on the affected patients with malignant colon cancer (consanguineous cases), including tumor node, metastasis (TNM) classification, and more.

Whole exome sequencing

Among the study population considered for the study, i.e., relatives cases belonging to four generations of a single Saudi family, blood samples were collected, and DNA was extracted from it using the Qiagen DNA Isolation Kit (Cat No./ID: 69506, Qiagen, Hilden, Germany), according to the instructions given by the manufacturer. The concentration and quality of the DNA ratio in the samples were analyzed using a NanoDrop Spectrophotometer system (Thermo Scientific, USA).

The authors prepared ~2 µg of the DNA sample for library design and sequencing using the Agilent SureSelect Human All Exon V6 kit (Agilent Technologies, CA, USA). This sample was then enriched, and the library was prepared as per the Agilent SureSelect protocols. The samples were enriched with paired-end sequencing (2 x 150 bp) on the Illumina NovaSeq 6000 platform (Illumina, San Diego, USA). The authors conducted dual 20-h biotinylated bait-based hybridizations, and each process was followed by three steps: the streptavidin magnetic bead binding, a washing step, and an elution step. Once the second elution got completion, a 10-cycle Polymerase chain reaction (PCR) enrichment was conducted. Then, the quality control analysis was conducted on the enriched libraries using the DNA-1000 kit (Agilent, USA). Finally, the sample's quantity was measured using the Quant-it PicoGreen dsDNA Assay Kit following the manufacturer's protocol (Invitrogen, Life Technologies, USA).

The sequencing of the paired-end libraries (2 × 100 base pair) was performed using the Illumina HiScan SQ (Illumina, San Diego, USA), according to the manufacturer's instructions. This process was conducted to generate an average of 60 million 100 bp paired-end raw reads per sample, with its coverage determined on hg19 RefSeq non-redundant exome length, ranging from 35× to 108×. The quality of raw reads was checked with the help of FastQC V0.10.0 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

The authors aligned and identified the mutations using Illumina DRAGEN version 3.5.7 upon the BaseSpace Sequence Hub cloud platform (12) with a median of 80 X coverage per base. Several mapped reads' pairs that possessed similar external coordinates were collapsed to eliminate the potential PCR duplicates with the help of the SAM tools command (13). With the help of the Genome Analysis Toolkit (GATK), the authors conducted the recalibration of the mapping quality score and local realignment in the regions where insertions and deletions (indels) occurred. Based on the GATK Unified-Genotyper, small indels, and single nucleotide variants were rendered separately (14). The mutations were verified using the snap gene viewer through manual checking, and the exome analysis was referred against the National Center for Biotechnology Information (NCBI) Reference Sequences (RefSeq) Database.

Pedigree analysis

Colon cancer occurs due to various factors, i.e., inherited mutations that occur in *MLH1, MSH2, ATM, PTEN, TP53, BRCA1, BRCA2, STK11*, and *CARD9* genes, and a few other factors such as the age-of-onset, type of tumors, endogamy and history of other cancers. In this study, pedigree analysis was conducted to determine the presence of colon cancer among four generations of the patients under consideration. The study registered the cancer incidence and pedigree data using a computerized database. The traceable consanguineous cases were those patients alive between 2019 and 2020.

Tissue and histological preparation

Through small biopsies, the tissue specimens measuring 7-10 mm in diameter were collected from the consanguineous cases of the patients diagnosed with colon cancer. Then, the tissue specimens were fixed in 100% formalin and processed. Later the tissue was kept in an automated processor for about 24 hours, and the paraffin blocks were sectioned. Three to five µm sectioned specimens were obtained with the help of traditional histopathology. The hematoxylin and eosin (H&E) technique was used as per the traditional histopathology (11).

Immunohistochemical stating for BRCA1&2 antibodies

By screening the immunohistochemical expression of the genes BRCA1 and BRCA2 followed by the confirmation of the germline mutations inherited by these genes, it can be inferred that the deficiency of these genes can

Table 1. Benchmark demographic profiles showing clinical characteristics of the participating endogamous consanguineous cases in four generations with their mutated parents.

Cases #	Age at diagnosis (years)	Gender	Family history with other types of cancer	Diagnosed with colon cancer	Smoking history	Chronic colitis history	Obesity (BMI ≥ 25 kg)
1	79	M	Unknown	YES	-	YES	-
2	76	F	-	-	-	-	YES
3	59	M	YES – pancreatic	-	YES	YES	-
4	57	F	YES – breast	-	-	-	-
5	57	F	YES – pancreatic	-	-	YES	YES
6	55	M	YES – pancreatic & breast	-	-	-	-
7	53	F	YES – pancreatic & breast	-	-	YES	YES
8	51	M	YES – pancreatic & breast	-	YES	YES	-
9	48	F	YES – pancreatic & breast	-	-	-	YES
10	46	F	YES – pancreatic & breast	-	-	-	-
11	39	M	YES – pancreatic, colon & breast	-	-	YES	-
12	39	F	YES – pancreatic and & breast	-	-	YES	-
13	36	F	YES – pancreatic, colon & breast	-	-	YES	-
14	37	M	-	-	YES	-	-
15	34	F	YES – pancreatic, colon & breast	YES	-	YES	-
16	31	M	YES – pancreatic & breast	-	-	-	YES
17	29	F	YES – pancreatic, colon & breast	-	-	-	YES
18	32	M	YES – pancreatic, colon & breast	-	YES	-	YES
19	25	F	YES – pancreatic, colon & breast	-	-	-	-
20	17	F	YES – pancreatic, colon & breast	YES	-	YES	YES
21	14	M	YES – pancreatic, colon & breast	-	-	-	-
22	12	F	YES – pancreatic, colon & breast	-	-	-	-
23	10	F	YES – pancreatic, colon & breast	-	-	-	-
24	7	M	YES – pancreatic, colon & breast	-	-	-	-
25	6	F	YES – pancreatic, colon & breast	-	-	-	-
26	6	F	YES – pancreatic, colon & breast	-	-	-	-
	Total		**22/26**	**3/26**	**4/26**	**10/26**	**8/26**

Table 2. Pathological characteristics (tumor, node, metastasis (TNM) classifications) for the participating consanguineous cases with malignant colon cancer.

Stage (TNM Classification)	Number of Patients (%)
T1 - Tumor affects the muscularis propria (1–2 cm)	2
T2 - Tumor affects the submucosa (3 cm)	1
Total	3

be linked to biological mechanisms. In this study, all the samples (collected from the colon cancer-confirmed consanguineous cases subjects and embedded in paraffin wax) underwent immunohistochemical research. The authors obtained sections measuring 5 μm thickness from the paraffin-embedded wax blocks. These sections were then placed in saline-covered glass slides and air-dried for a night at 37°C. Then, they were deparaffinized in xylene af-

ter rehydrating the same in graded alcohol (70 and 100%). After, the slides were kept under incubation at 3% methanol/ H_2O_2 for 10 minutes and washed using PBS. For this study, the authors used the following primary antibodies, anti-BRCA1 (MS110, mouse monoclonal, 1:200, Abcam, Cambridge, UK), anti-BRCA2 (MAB2476, mouse monoclonal, 1:500; R&D Systems, Inc. Minneapolis, MN, USA) at a diluted ratio of 1:10. Afterwards, the solution was designated as a negative (absent or greatly reduced) one for the brown-like nuclear stain, indicating < 20%. But, on the other hand, the nuclear staining of > 20% was cited as a positive one (15). Then, the incubation of the sections was performed for half a day in the *BRCA2* and *BRCA1* primary antibodies at 4°C. After washing the sections using PBS, a light microscope was utilized to visualize the immunostained sections.

Statistical analysis

For this study, the analyses were conducted using the SigmaStat programming adaptation 3.5 (Systat Software, San Jose, CA, USA). The quantitative analysis results are portrayed as means and SD and while $p < 0.05$ was regarded as statistically significant.

Results

Whole-exome sequencing analysis

WES was conducted to diagnose 26 participants with consanguineous cases. In this study, the authors considered the study population in which the ratio of relatives between male and female is as follows; 7 out of 10 are male, and 10 out of 16 are female carriers (who remain unaffected) for the inherited germline mutations (17/26, 65.38%). Additionally, colon and breast cancers were diagnosed among 1 out of 10 males and 4 out of 16 females (5/26, 19.23%). Moreover, one male and two females were diagnosed with colon and breast cancer, respectively (out of the cases, one was alive, and one died after the end of the study). On the contrary, 1 out of 10 males and 3 out of 16 were non-carriers of the mutations (4/26, 15.38%).

The current study observed a total of 15 inherited germline mutations in nine genes such as mutl homologous 1, mutl homologous 2, ataxia-telangiesctasia, phosphates and tension homolog, tumor protein 53, Breast Cancer 1 and 2, serine/threonine kinase 1, and caspase recruitment domain-containing protein (*MLH1, MSH2, ATM, PTEN, TP53, BRCA1* and *2, STK11, and CARD9,* respectively). This finding was among 22 out of the 26 endogamous consanguineous cases belonging to four generations of a single Saudi family, as listed in Table 3. The researchers detected the distributions of the mutated genes for every individual case in all four generations against their mutated parents, as tabulated in Table 4. The table also shows the number of inherited germ-like mutations for all the cases. Figure 1 portrays the mutated gene frequencies

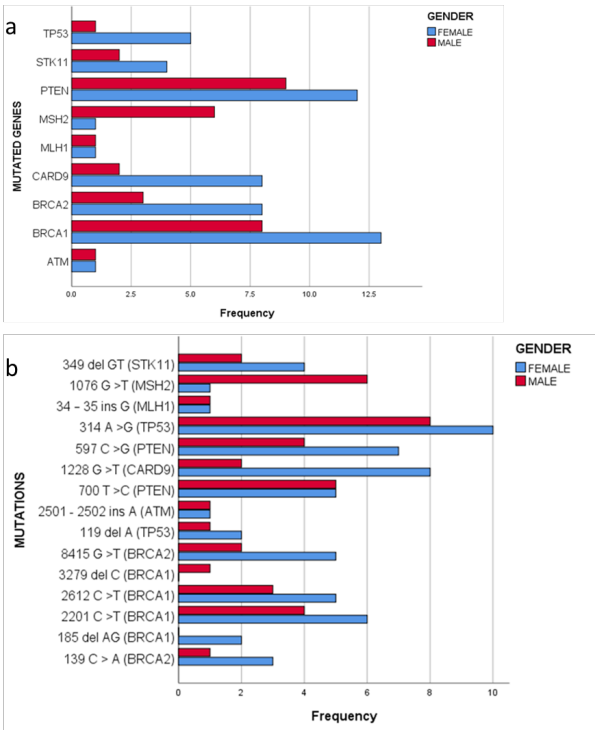

Figure 1. Inherited mutations in nine genes were recorded among 26 male and female consanguineous cases of four generations from a single endogamous Saudi family. Where (a) shows the mutated genes' frequencies (*MLH1, MSH2, ATM, PTEN, TP53, BRCA1, BRCA2, STK11*, and *CARD9*), and (b) shows the germline gene mutations' frequencies among consanguineous cases.

Table 3. Inherited mutations of genes were recorded among consanguineous cases of four generations from a single endogamous Saudi family.

Gene	Mutations	Mutation's types	As reported earlier in other populations
MLH1	34 – 35 ins G	Insertion	Yes
MSH2	1076 G >T	Substitution	Yes
ATM	2501 - 2502 ins A	Insertion	Yes
PTEN	597 C >G	Substitution	Yes
	700 T >C	Substitution	Yes
TP53	119 del A	Deletion	Yes
	314 A >G	Substitution	Yes
BRCA1	185 del AG	Deletion	Yes
	2201 C >T	Substitution	Yes
	2612 C >T	Substitution	Yes
	3279 del C	Deletion	Yes
BRCA2	139 C > A	Substitution	Yes (reported in our previous study) (15)
	8415 G >T	Substitution	Yes
STK11	349 del GT	Deletion	Yes
CARD9	1228 G >T	Substitution	Yes

Table 4. Distributions of the mutated genes were detected for each case in four generations with their mutated parents from an endogamous Saudi family.

Case #	Age at diagnosis (years)	Case gender, generation # & (nonrelative/ relative husband or wife for the married cases) in Figure 2	Number of mutations	Name of genes
1	79	M, I	9	MLH1, MSH2, ATM, PTEN, TP53, BRCA1&2, STK11
2	76	F, I	4	PTEN, BRCA1
3	59	M, II	6	PTEN, MSH2, BRCA1&2, STK11, CARD9
4	57	F, II (relative wife for case# 3)	5	PTEN, TP53, BRCA1&2
5	57	F, II	3	PTEN, BRCA1, CARD9
6	55	M, II	4	PTEN, MSH2, BRCA1
7	53	F, II	7	PTEN, TP53, BRCA1&2, STK11, CARD9
8	51	F, II	6	PTEN, BRCA1&2, CARD9
9	48	F, II	6	ATM, PTEN, BRCA1&2, STK11
10	46	M, II	3	PTEN, BRCA1
11	39	M, III	5	PTEN, MSH2, BRCA1, CARD9
12	39	F, III (relative wife for case# 11)	4	BRCA1&2, CARD9
13	36	F, III	5	PTEN, BRCA1&2, CARD9
14	37	M, III (relative husband for case# 13)	-	-
15	34	F, III	7	PTEN, TP53, BRCA1&2, STK11, CARD9
16	31	M, III	3	PTEN, BRCA1
17	29	F, III	4	PTEN, BRCA1, CARD9
18	32	M, III (relative husband for case# 17)	2	PTEN, BRCA1
19	25	F, III	3	PTEN, BRCA1
20	17	F, IIII	8	MLH1, PTEN, TP53, BRCA1&2, STK11, CARD9
21	14	M, IIII	3	MSH2, PTEN, BRCA1&2
22	12	F, IIII	-	-
23	10	F, IIII	4	MSH2, PTEN, TP53, BRCA1
24	7	M, IIII	3	MSH2, PTEN
25	6	F, IIII	-	-
26	6	F, IIII	-	-

between male and female consanguineous cases in (Figure 1a) and the frequencies of the inherited germline mutations between the male and female consanguineous cases in Figure 1b.

However, a significant number of the genetic mutations identified in the study, i.e., nine mutations, belong to substitution type. This occurred in six of the nine inherited genes (*MSH2, PTEN, TP53, BRCA1, BRCA2, and CARD9*). On the other hand, six frameshift mutations (indels) were found in five of the nine inherited genes (*MLH1, ATM, TP53, BRCA1,* and *STK11*). These mutations include two insertion mutations such as (34–35 ins G) and (2501–2502 ins A) for *MLH1* and *ATM* genes, respectively, that are found in two of the 26 consanguineous cases (8%). In addition to these, two deletion-type mutations were detected in the BRCA1 gene (185 del AG and 3279 del C) in 2/26 (8%) and 1/26 (4%) of the consanguineous cases, respectively. Moreover, two deletion type mutations (119 del A and 349 del GT) were also detected in the *TP53* and *STK11* genes in 3/26 (12%) and 6/26 (23%) of the consanguineous cases, correspondingly.

Pedigree analysis and cancer risk estimations for all the consanguineous cases in four generations of the Saudi family

Of the total sample size considered for the study, only one Saudi family fulfilled the criteria for hereditary non-polyposis colorectal cancer/ Lynch syndrome phenotype. The researchers observed the pedigree of the germline gene mutations in nine genes of *MLH1, MSH2, ATM, PTEN, TP53, BRCA1, BRCA2, STK11,* and *CARD9* among the consanguineous cases in four generations of a single Saudi family ancestry index case.

There were no mutations in four of the 26 consanguineous cases. These cases were as follows: twin girls in the fourth generation and their father in the third generation unrelated to their mother; one daughter in the fourth generation from related endogamous parents. The remaining 17 of 26 consanguineous cases exhibited the incidence risk of colon, breast, or any other possible cancers that are associated with germline mutations found in the nine genes such as *MLH1, MSH2, ATM, PTEN, TP53, BRCA1, BRCA2, STK11, CARD9*, when dealing with extended pedigrees.

Figure 2 summarizes the cancer incidence rate of the family members. In agreement with the outcomes of the pedigree analysis, the metachronous tumor cases had three colon cancer cases while the first, third, and fourth generations had a family history of breast and pancreatic cancers. Furthermore, two breast cancer cases were also found in the family. One of the females, diagnosed with breast cancer, died.

In Figure 2, the female patient diagnosed with breast cancer at 53 years is shown. This was done so based on case #6 in the second generation. The pro-band established the inheritance of cancer from their father (diagnosed with colon cancer at 79 years old in the first generation). A female cousin (diagnosed with colon cancer at 17 years, case #1 in the first generation) and a paternal aunt (diagnosed with colon cancer at 34 years, case #3 in the third generation) are also shown in the figure.

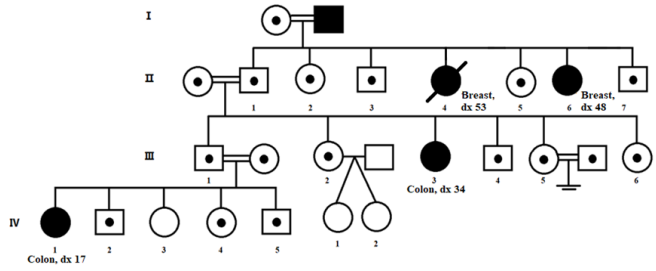

Figure 2. Pedigree indicates inherited characteristics of a family having germline mutations through four generations, entailing transmission of breast and colon cancers due to paternal and maternal lineages (by endogamy).

Histopathology

Histopathological specimens were obtained from carrier patients with BRCA2 and BRCA1 germline mutations and colon cancer. Both sections contained a remarkable number of mitosis and a few instances of pleomorphism with less formation of the glands. Additionally, the authors also observed histological formations with high-level tumor grades. Figure 3a shows the proliferation of the disorganizations than the better histological pattern of the colon section, seen in Figure 3b.

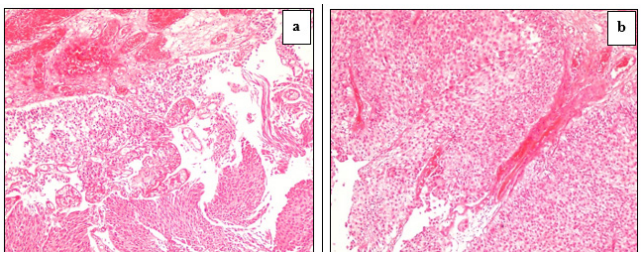

Figure 3. Photomicrographs of hematoxylin and eosin (H&E) stained sections showing the colon cancer of the relative patients using a scale bar (100 μm). Section (a) stained from a colon cancer patient diagnosed with a frameshift mutation (insertion) in the *BRCA1* gene, and section (b) from a colon cancer patient diagnosed with substitution mutations in the *BRCA2* gene. The views of both sections of colon cancer show amplified cell activity (mitotic), variable pleomorphism grades, proliferation dysregulation, no formation of the gland, and red blood cells. High-level tumor grades in section (a) include necrosis and cell pleomorphism (nuclear) with more asymmetry patterns for colon cells and mucin production.

Immunohistochemical staining for BRCA1 and 2

Figure 4 shows the immunohistochemistry outcomes of the BRCA1 and BRCA2 expressions, and the outcomes establish the moderate level of staining of these genes. However, the BRCA2 antibodies from the colon cancer patients' samples showed strong staining intensity. The BRCA1 over-expression was visible in the section of the colon tumor sample. In the section, the nuclear staining was found to be above 50% reaction of positivity with the BRCA1 antibody. This can be clearly segregated as a strong positive reaction, as shown in figure 4a. Moreover, the colon tumor sample was assessed for the expression of BRCA2 in which the nuclear staining exhibited a 20% reaction of positivity with the BRCA2 antibody. This can also be categorized as a moderate positive reaction, as shown in Figure 4b. In addition to the above, the researchers followed the antigen retrieval procedure in line with the last recorded numerical H-scores for every case. Based on this, the scores were obtained by multiplying the value against the classification, i.e., (1) over 50% (Strong), (2) 20%–40% (moderate), (3) 1%–20% (weak), and (4) 0% (none).

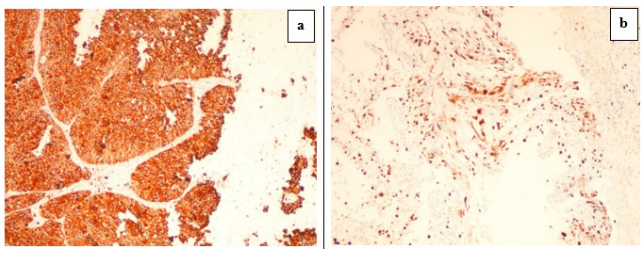

Figure 3. displays photomicrographs of immunostained sections from colon cancer patients using BRCA1 and BRCA2 antibodies. The images were captured using a light microscope, and a scale bar of 100 μm is provided. The immunohistochemical slide stained with BRCA1 mutants exhibited an over-expression of BRCA1 protein in the colon cancer tissue of a patient diagnosed with T2, indicating tumor involvement in the submucosa and a frameshift (insertion) mutation in the BRCA1 gene (a). Similarly, the slide stained with the BRCA2 antibody revealed moderate positive staining in the colon cancer tissue of a patient diagnosed with T1, indicating tumor involvement in the muscularis propria and substitution mutations in the BRCA2 gene (b).

Discussion

The current study analyzed the most important factors that contribute to the inheritance of colon cancer and found germline mutations that occur within nine genes such as *MLH1, MSH2, ATM, PTEN, TP53, BRCA1, BRCA2, STK11*, and *CARD9* through four single-family generations.

In normal conditions, the cell proliferation rate is balanced by genes such as *PTEN, ATM,* and *TP53,* while the upregulation of the tumor suppressor activities prevents tumor formation. An association exists between early-onset colon and breast cancers with the mutations in *PTEN, ATM,* and *TP53* (15). In addition, it has been previously established that mutations that occur in *MLH1, MSH2,* and *STK11* genes cause an individual to suffer from hereditary colon cancer (5). Furthermore, the CARD9 gene performs to keep the apoptosis process of the cells intact. The CARD9 gene mutations have an association

with the dysregulation of the apoptosis process, necrosis, and the initiation of cell pleomorphism and cell cycle (5).

Both BRCA1 and 2 mutations are considered important events that enhanced the distribution of colon and breast cancer (16,17) (15,18) due to heavy cell division and mitotic numbers. This functions as a reversal process of the distribution of the proliferating cells (18-20). The integrity of the genome and tumor suppressor's genes is ensured by caretaker genes such as the BRCA1/2 genes (16,18,20). Though various studies have been conducted earlier on the BRCA1/2 genes, a research gap still exists, especially in the case of Saudi Arabia. The gene mutations observed in these genes differ according to the country and the region (20,21) studied. Such gene mutations account for up to 5-10% of colon and breast cancer cases (22). The coding regions have so far reported 700 BRCA1 mutations and 600 BRCA2 mutations (20-22). As per the estimations, when the patients have inherited the mutations of the genes BRCA1/2, they are up to three-fold more susceptible to developing colon, breast, and pancreatic cancers (20-24). As a result, the cellular mechanisms associated with these inherited genes tend to change the processes involved in breast and colon tumors (18,24-26).

In this study, of the total study population, i.e., 22 consanguineous cases including colon cancer-affected patients as well as carriers, the researchers found 15 inherited germline mutations in nine genes such as *MLH1, MSH2, ATM, PTEN, TP53, BRCA1, BRCA2, STK11*, and *CARD9* among (22/26, 84.61%) the four generations of a single Saudi family, as shown in Tables 3 and 4. Figures 1a and 1b show the above findings, while Figure 2 shows the pedigree output from which it can be inferred that the germline mutations passed on from one generation to another through repeated endogamy. This passes the breast and pancreatic cancer history, as shown in Table 1, and also increases the risk of colon, pancreatic, and breast cancers. However, non-carriers of the mutations were only four out of the 26 cases (15.38%), i.e., healthy individuals without being a carrier of the mutations. These four people are twin daughters from a carrier mother, a non-related healthy father, and a healthy non-carrying female cousin of these twins.

Further, from the data regarding the immunohistochemical analysis of the colon tissue, the authors found that the BRCA1 got over-expressed, whereas the BRCA2 got moderately positively stained. These outcomes are consistent with those reported in the literature (27-29) and establish a significant relationship between the advanced stages of the tumor and the expressions of BRCA1 and 2 genes, as shown in Figures 3a and 3b.

In the current research work, the authors found a few variables to increase the incidence of colon cancer, such as age, gender, history of chronic colitis, obesity (BMI≥ 25 kg), and smoking history. Wang et al (2020) established that gender is a crucial factor to consider during diagnosis and treatment of colon cancer (3). The gender-specific diagnostic tool, developed for colon cancer in women, provides a novel method to improve the chemotherapy success rate among women (28,30)

On the contrary, the age factor was also found to have an essential role in the incidence of colon cancer. Various research investigations emphasized the contribution of a healthy lifestyle to reducing the incidence of colon cancer among teenagers (28). The current study found a similar impact of a healthy lifestyle on two associated factors, too, i.e., obesity (8/26; 30.76%) and a history of chronic colitis (10/26; 38.46%) (31). In the literature, it has been reported that obesity and chronic colitis occur due to the ingestion of hot spicy food, fast food with unhealthy nutritional values, and meals excluding vegetables and fresh fruits, etc. This is applicable, especially in the case of persons with a family history of cancer (31-34). Further, such issues can also result in a high incidence of breast and colon cancers at young ages (30).

Table 1 shows a smoking history in four of 26 study participants (16.7%). Despite this, no smokers were diagnosed with colon cancer. However, as reported earlier, the current study used smoking history to determine all the potential cofactors that may increase breast and colon cancer incidence in a single-family (32,34).

As per the current study outcomes, it can be inferred that the germline mutations that occurred due to the inheritance of the *MLH1, MSH2, ATM, PTEN, TP53, BRCA1, BRCA2, STK11*, and *CARD9* genes and the increased incidence of breast and colon cancer, across the four generations, can be correlated with repeated endogamy. This happened between the consanguineous cases which happened to be the carriers of germline mutations of these nine genes. However, such data was validated through the positive outcomes of BRCA1 and 2 expressions in the immunohistochemical data, consistent with the advanced tumors of the histopathological data. In addition to these, a few other elements, like the history of chronic colitis and obesity, may also induce the early onset of colon cancer in young people, as indicated by the consanguineous cases in the family's fourth generation.

Declarations

Ethics approval and consent to participate

All participants provided written informed consent to participate in the study. For participants under the age of 18, consent was obtained from both the participant and their parents. The study received approval from King Fahad Medical City following ICH GCP H-01-R059 guidelines, with IRB LOG number 20-0287, dated August 27, 2020.

Consent for publication

Written informed consent for publication was obtained from all participants. This included consent from participants under the age of 18 and their parents, allowing for the publication of clinical details, identifying images, genetic analyses, and other personal information.

Availability of data and material

All relevant data are provided within the paper.

Competing interests

The authors declare no conflicts of interest.

Funding

This research was funded by Princess Nourah bint Abdulrahman University Researchers Supporting Project number (PNURSP2023R227), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia.

Authors' contributions

'DA': Contributed to formal analysis, experimental work,

investigation, methodology, and resources and reviewed and edited the manuscript. 'TA': Contributed to conceptualization, formal analysis, investigation, and methodology and reviewed and edited the manuscript. 'MA': Contributed to experimental work, data curation, methodology, and resources and reviewed and edited the manuscript. 'DD': Contributed to methodology, resources, and funding acquisition and reviewed and edited the manuscript. 'AAA': Contributed to formal analysis, methodology, and software. 'FA': Contributed to experimental work, data curation, methodology, and resources and reviewed and edited the manuscript. 'GA': Contributed to experimental work, investigation, methodology, and resources and reviewed and edited the manuscript. 'WA': Oversaw project administration, formal analysis, and investigation, wrote the original draft and submitted the paper as the corresponding author. All authors have read and approved the final manuscript.

Acknowledgements
Princess Nourah bint Abdulrahman University Researchers Supporting Project number (PNURSP2023R227), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia.

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