Whole-exome sequencing insights into synchronous bilateral breast cancer with discordant molecular subtypes

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Received May 10, 2024; Accepted September 5, 2024

DOI: 10.3892/ol.2024.14728

Abstract. The incidence of synchronous bilateral breast cancer (SBBC) is very low, and SBBC with discordant molecular subtypes is even more uncommon. As such, little is known about the pathogenesis of SBBC with discordant molecular subtypes, and reports about this entity are scarce. In the present study, the case of a 72-year-old female patient who presented with SBBC with discordant molecular subtypes is reported, with a stage IA hormone receptor negative {human epidermal growth factor receptor-2 [HER2(+)]} tumor in the left breast and a stage IIIA hormone sensitive tumor [HER2(-)] in the right breast. Whole-exome sequencing was performed to identify the differential genetic variations in the BBC tissues. A total of 8 key mutated cancer susceptibility genes (ALK, BRCA1, FAT1, HNF1A, KDR, PTCH1, SDHA and SETBP1) were screened, and mutations were found in 10 vital cancer driver genes, including BRCA1, EBF1, MET, NF2, NUMA1 RALGAPA1, ROBO2, SMYD4, UBR5 and ZNF844. The high-frequency mutated genes mainly contained missense mutations, among which single nucleotide variants were the most common mutations, with C > T and C > A as the main forms. The pathways associated with the high frequency mutated genes were further elucidated by functional category and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses. Heterogeneity in the hormone receptor and HER2 status of SBBC poses unique therapeutic challenges. Future studies should aim to identify the optimal management strategy for this disease.

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Key words: synchronous bilateral breast cancers, discordant molecular subtypes, whole-exome sequencing, BRCA1, nuclear mitotic apparatus protein 1

Introduction

Breast cancer (BC) is one of the most prevalent malignant tumors in women, representing 30% of all female cancers (1). The disease can be divided into unilateral BC (UBC) and bilateral BC (BBC). The overall morbidity rate of BBC is low; however, with the improvement in BC diagnostics and therapeutics and the prolongation of postoperative survival times in recent years, the cumulative occurrence of this disease has increased annually. BBC can be divided into synchronous BBC (SBBC) and metachronous BBC (MBBC) according to the interval between the first and second tumor diagnosis (2). The incidence of SBBC is 1.39% among the general population (3), and the rate of remote metastasis is significantly higher than that of UBC (4). Hormone receptors are biomarkers for characterizing the prognosis of patients with BC and predicting treatment responses (5); for example, a transition from positive to negative estrogen receptor (ER) status after neoadjuvant chemotherapy is associated with a poor prognosis (6). A retrospective study found that the 5-year disease-free survival (DFS) and 5-year overall survival (OS) estimates for patients with persistent ER positivity after neoadjuvant chemotherapy were 88 and 92%, respectively, meanwhile the survival for patients with ER-positive conversion (85 and 83%) differed significantly (7). ER, progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2) have been established as strong predictors of the effects of various molecular targeted therapies, such as endocrine and anti-HER2 treatments (8). Selecting the optimal therapeutic regimen based on the molecular subtypes of SBBC is challenging, particularly given the inconsistency in molecular subtypes.

Materials and methods

Immunohistochemical (IHC) staining. The surgically resected tissues were fixed with 10% neutral buffer formalin and dehydrated with gradient alcohol, then immersed in the embedding molds filled with paraffin at 58˚C and solidified in the freezer. IHC staining was performed on 4 μm-thick paraffin sections of the tumor tissues according to the manufacturer's instructions. The ready-to-use primary antibodies for ER, PR, HER2, CK5/6, P63, Ki-67, P120, CK34βE12 and E-cadherin (cat. nos. Kit-0012, Kit-0013, Kit-0043, MAB-0744, MAB-0694, MAB-0672, MAB-0621, Kit-0020 and MAB-0738,

respectively) were purchased from MXB Biotechnologies Co., Ltd. The sections were incubated with the primary antibodies for 2 h at 37˚C. An EliVision™ plus Polyer HRP kit (cat. no. Kit-9901; MXB Biotechnologies Co., Ltd.) was used for secondary antibody incubation and chromogenic reaction. IHC staining sections were observed under an optical microscope (BX53; Olympus Corp.), and the representative images were collected.

Fluorescence in situ hybridization (FISH). FISH assay was performed on 3 μm-thick paraffin sections of the tumor tissues according to the instructions of the human HER2 gene amplification detection kit (cat. no. FP-001; Wuhan HealthCare Biotechnology Co., Ltd.), which contains a HER2 gene probe (300 kb Spectrum Orange-directly labelled DNA probe for 17q12-21.1) with a probe for centromeric enumeration of chromosome 17 (CEP17; Spectrum Green-directly labelled fluorescent DNA probe). The paraffin was removed from the sections with a 15-min wash in dewaxing agent at 68˚C. The sections were dehydrated in 100% ethanol for 5 min, immersed in permeabilization buffer for 20 min at 90˚C, and rinsed in purified water for 1 min. After incubation in a protease solution at 37˚C for 15 min, the enzymatic reaction was stopped by placing the sections in 2X saline sodium citrate (SSC) wash buffer twice for 5 min. Subsequently, the sections were dehydrated through graded alcohols, and 10 μl dual HER2/CEP17 probe was applied to the sections. The probe and target tissue were then co-denatured for 5 min at 85˚C and allowed to hybridize for 2 h at 42˚C using a hybridization instrument (ThermoBrite; Leica Biosystems). The sections were washed in 2X SSC/0.1% NP-40 for 2 min at 68˚C, rinsed in purified water for 1 min at 37˚C, and counterstained with 10 μl 4',6-diamidino-2-phenylindole (DAPI). FISH analysis (x1,000 magnification) was performed under a fluorescence microscope (BX53; Olympus Corp.).

Whole-exome sequencing (WES). The research team submitted the application for project review and the research protocol to the Ethics Committee of the First Affiliated Hospital of Dali University (Dali, China) before the initiation of the project. The Ethics Committee held a meeting to validate the reasonableness and safety of the project and approved the use of biospecimens obtained in clinical diagnosis and treatment to conduct the research (approval no. DYF20230309). After obtaining ethical approval for using patient samples, the authors commissioned BGI Technology Co., Ltd. (Shenzhen, China) to perform WES on bilateral tumor tissues. The genomic DNA in the tissue samples was extracted using a DNA extraction kit (cat. no. 940-000972-00; MGI Tech Co., Ltd.). The concentration of DNA samples was quantified by Qubit fluorimeter (Thermo Fisher Scientific, Inc.), and the integrity of DNA samples was detected by 1% agarose gel electrophoresis and visualized by ethidium bromide. The DNA samples were mechanically fragmented to 200-300 bp using an ultrasonic cell disruption system (Covaris, LLC) and purified with an Agencourt AMPure XP kit (cat. no. A63881; Beckman Coulter, Inc.). Qualified samples can be used for DNA library preparation, and the fragment size and concentration of DNA library were examined with a DNA 1000 kit (cat. no. 5067-1504; Agilent Technologies, Inc.) on a 2100 Bioanalyzer instrument (Agilent Technologies, Inc.). The loading concentration of the final library was 10 pM for DNA sequencing. The exome microarray (Agilent_V6; Agilent Technologies, Inc.) was applied to capture the DNA library, and paired end sequencing (PE150) was finally performed using a DNBSEQ instrument (MGI Tech Co., Ltd.). The MuTect2 tool [version 4.1.4.1; genomic analysis toolkit (GATK) team], the Funcotator tool (version 4.1.4.1; GATK team), the FACETS (9) software (version 0.6.2; Memorial Sloan Kettering Cancer Center), and MuSic (10) software (version 0.4; Washington University) were used to analyze the sequencing data.

Literature retrieval. A systematic literature search was conducted in the PubMed database (https://pubmed.ncbi.nlm.nih.gov/) to identify all relevant studies published up to September 2023. The following search strategy was applied to obtain relevant titles and abstracts: 'BC' AND 'bilateral' AND ('discordant' OR 'discordance' OR 'heterogeneous'). A manual search of all references cited in full-text papers was performed to identify additional studies for inclusion.

Results

Case presentation. The patient was a 72-year-old woman who found a right breast mass without any persistent pain 2 months earlier and was referred to the local hospital in July 2022. Mammography showed a right breast mass (category 4B) without redness, swelling, or rupture and no chills, fever, nausea, or vomiting. The patient was hospitalized at the First Affiliated Hospital of Dali University on July 25, 2022 (Dali, China) for further diagnostics and treatment. The physical examination revealed symmetric breasts with no redness of the surface skin, no nipple indentation, and no abnormal elevation or depression on the surface of the mammary glands. A 2.5x2 cm lump was palpated in the right breast, with medium quality, no compression pain, activity investigated, poorly defined border, and no nipple discharge on extrusion. An ~3x2 cm enlarged lymph node was detected in the right axilla, no lump was palpated in the left breast, and no enlarged lymph node was detected in the left axilla. Magnetic resonance imaging (MRI) showed two small shallowly lobulated nodules in the left breast, and multiple nodules in the right breast with flocculent abnormal signals around the lesions. Local infiltration was possible (Fig. 1).

Bilateral modified radical mastectomy for BC and anterior sentinel lymph node biopsy were performed on day 9 after hospital admission. The left mastectomy specimen was found in the inner lower quadrant 2 cm from the nipple with a 1 cm diameter scooped area. No definite mass was observed around the excavation area, and the rest of the mastectomy tissue was grayish yellow, grayish-white, solid and medium textured, with an axillary fat size of 9x8x3 cm. A total of 19 nodules, 0.4-1.3 cm in diameter, were found within it. The excised right breast specimen showed a mass measuring 5.5x3x2 cm in size in the outer upper quadrant 2 cm from the nipple, which was grayish, solid, hard, and poorly demarcated on the cut surface. The rest of the breast tissue was grayish, grayish reddish, substantial, and medium in texture. The axillary fat measured 10x9x2.5 cm, and 18 nodes measuring 0.8-3 cm in diameter were detected in its interior.

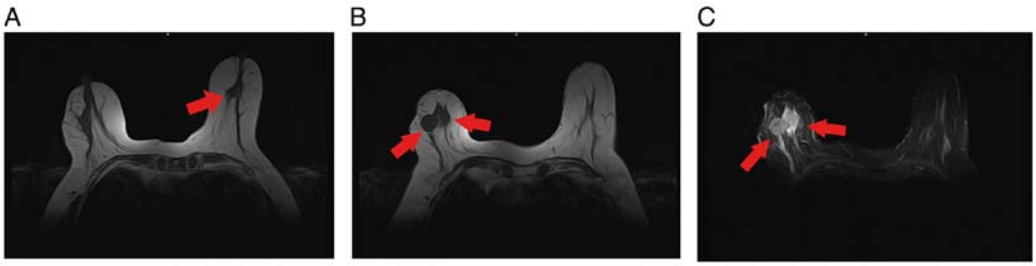

Figure 1. Magnetic resonance imaging of the patient's breasts. (A) Two small nodules in the left breast. (B and C) Multiple nodules in the right breast.

The tumor was diagnosed as invasive ductal carcinoma with high grade intraductal carcinoma. Microscopically, the tumor showed infiltrative growth; the tumor cells in invasive ductal carcinoma area of bilateral breasts were arranged in a trabecular or nested pattern with reduced interstitial stroma, abundant eosinophilic cytoplasm, vacuolated nuclei of varying sizes, extensive nuclear division, and remarkable heterogeneity; and a small number of lymphocytes were infiltrated in the interstitial stroma (Fig. 2A and B). The dilated mammary duct and the tumor cells with high proliferative activity in the high-grade intraductal carcinoma area of the left breast is shown in Fig. 2C; necrotic material with calcification could be observed in the center of the lumen, and there were large polymorphic cells around the necrotic area; the tumor cells with irregular nuclei are arranged disorderly. In the high-grade intraductal carcinoma area of the right breast, the slightly enlarged tumor cells were typically arranged in either solid or cribriform pattern, with round or oval nuclei of uniform size (Fig. 2D). The surgical pathology report revealed fibro-adenopathy in the tissue surrounding the tumor with no cancer in the peripheral margins, base and nipple of the left breast and no carcinoma metastasis in the ipsilateral axillary lymph nodes; the tissue in the right breast showed fibro-adenopathy in the peripheral tissue surrounding the tumor, and the peripheral margins, base and nipple were not cancerous. A total of 7 out of 14 axillary lymph nodes were positive for metastasis, and 4 other carcinomatous nodes were visualized.

IHC staining revealed that the left invasive ductal carcinoma was negative for ER, PR, cytokeratin5/6 (CK5/6), and P63, but positive for HER2 (2+), Ki-67 (10%+), P120, CK34βE12 and E-cadherin (Fig. 3A-I). Myoepithelial cells were positive for CK5/6 and P63 in the intraductal carcinoma area (Fig. 3J and K). FISH assays further confirmed HER2 gene amplification in the invasive ductal carcinoma area of the left breast (Fig. 3L). The right invasive ductal carcinoma was positive for ER (90%+), PR (20%+), Ki-67 (50%+), P120, CK34βE12 and E-cadherin, but negative for HER2, CK5/6 and P63 (Fig. 4A-I). In the right intraductal carcinoma area, myoepithelial cells were positive for P63 and CK5/6, while tumor cells were negative for HER2 but highly positive for ER and PR (Fig. 4J-N). The patient was diagnosed with bilateral simultaneous BC of discordant molecular types: The molecular subtype of the left BC was HER2 positive [HR(-)], and the molecular subtype of the right BC was luminal B [HER2(-)]. The tumor-node-metastasis staging was based on the 8th edition of the American Joint Committee on Cancer (AJCC) BC staging system (2), with T1bN0M0 IA in the left breast and T3N2M0 IIIA in the right breast.

Because this patient did not undergo pathological examination through ultrasound-guided percutaneous biopsy before surgery, although the tumor size and regional lymph node involvement could be determined, it was not available to determine the specific subtype of BC as well as some microscopic metastasis lesions before surgery. For example, M1 staging was defined by the distant metastasis detected through clinical and imaging examinations or metastasis lesion >0.2 mm under microscopy through histopathological examinations. The identification of BC subtypes requires detection of immunohistochemical markers, including ER, PR, HER2 and Ki-67, on the histopathological sections. Therefore, the description of case presentation in the study was based on this.

After the surgery, a multidisciplinary diagnosis and treatment team helped to determine the further adjuvant therapeutic options for the patient. The patient's scars healed well, with no erythema, tenderness, nodules, or palpable abnormal masses, and no enlarged lymph nodes were detected bilaterally in the axillae, supraclavicular region, or neck. She then underwent the first postoperative chemotherapy on September 2, 2022, with an albumin-bound paclitaxel + trastuzumab + pertuzumab (THP) regimen. On September 13, 2022, a specialist examination revealed infection with necrotic corruption and pus in the left breast incision. The patient received anti-infective therapy, and received chemotherapy with trastuzumab + pertuzumab (HP) regimen on September 22, 2022. From October 2022 to January 2023, the patient received four times of THP regimens, with 5-10 years of endocrine therapy and targeted therapy prescribed. During the follow-up period from January 2023 to April 2024, the patient continued to receive a combination therapy of trastuzumab, pertuzumab and letrozole. The patient has been in favorable physical condition without tumor recurrence up to now.

Differential variations in BBC tissues. WES was performed to explore possible different molecular alterations between the bilateral tumor tissues. By comparing the mutations in the left and right BC tissues, the differential single nucleotide variants (SNVs), insertions and deletions (InDels), and cell copy number variations (CNVs) in the bilateral tumor tissues of discordant molecular subtypes were obtained. SNVs are widely found in the human genome and are associated with many phenotypic differences and susceptibility to drugs or diseases. InDels refer to insertions and deletions of small fragments in the genome. InDels in a coding region or splice site can alter protein translation. The MuTect2 tool (version 4.1.4.1; GATK team) was employed to find SNV and InDel sites and annotated them using the Funcotator tool (version

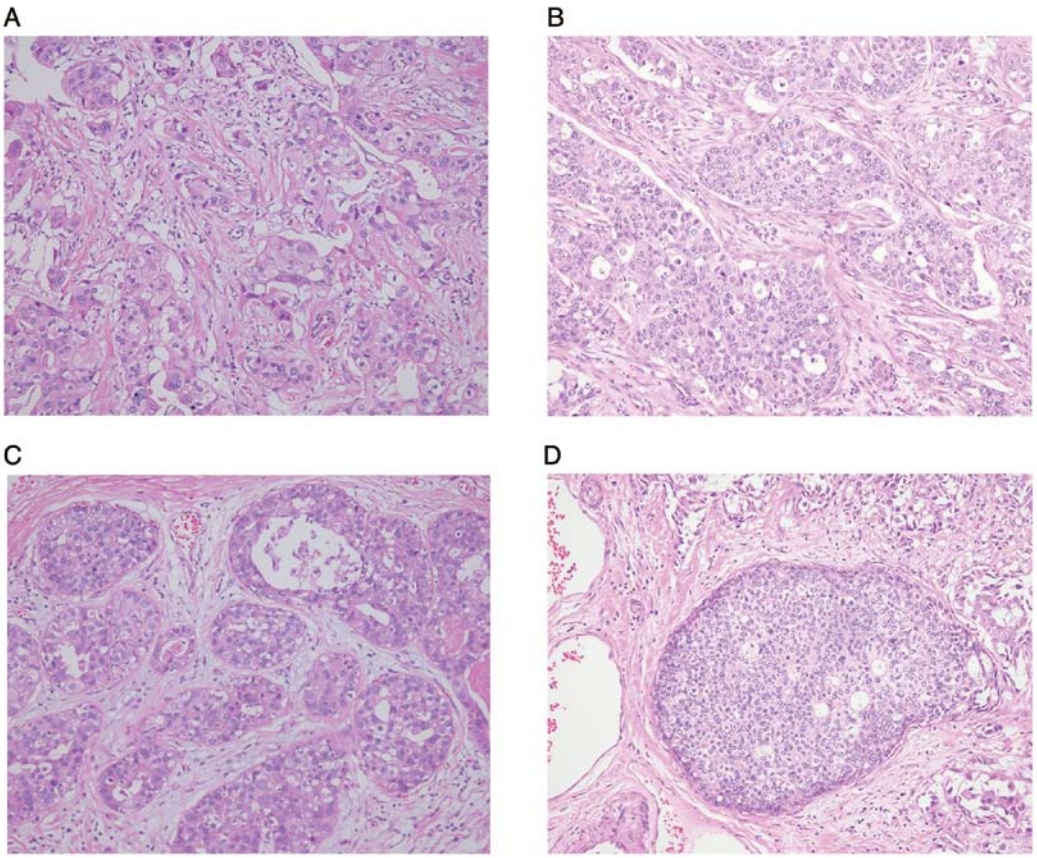

Figure 2. Morphological characteristics of the bilateral breast cancer tissues shown by hematoxylin and eosin staining (magnification, x200). (A) Invasive ductal carcinoma in the left breast. (B) Invasive ductal carcinoma in the right breast. (C) High-grade intraductal carcinoma in the left breast. (D) High-grade intraductal carcinoma in the right breast.

4.1.4.1; GATK team). A total of 324 differential SNVs, annotated on 189 genes, were found. The SNVs were primarily distributed in introns (41.36%) and intergenic regions (31.79%), and there were 19 missense mutations (5.86%) that did not influence initiation or termination codons (Fig. 5A). A total of 16 differential InDels were found, annotated on 12 genes, which were mainly distributed in gene introns (intron, 31.25%), intergenic regions (intergenic region, 18.75%), 3'-non-coding regions [3' untranslated region (UTR), 12.5%], and 5'-flanking regions (5'Flank), with one insertion causing a code-shift mutation that did not affect the initiation and termination codons (Fig. 5B). CNV manifests as an increase or decrease in the copy number of genomic fragments, and deletions and amplifications at the chromosome level have become a focus in tumor research. FACETS software (version 0.6.2) (9) was used to compare CNVs in the left and right BCs. A total of 159 differential CNVs were identified, of which 34 were deletions and 125 were duplications. The comparison of copy variation multiplicity at different chromosomal locations is shown in Fig. 5C, indicating that the overall copy number variation was higher in right BC than in left BC.

Variation identification of cancer susceptibility and driver genes in BBC. Susceptibility genes mediate inherited diseases or result in acquired susceptibility to diseases under appropriate environmental stimuli. Potential cancer susceptibility genes were screened by comparing the mutated genes using the Cancer Gene Census (CGC) database (https://cancer.sanger.ac.uk/census). The following 8 key mutated tumor susceptibility genes were selected: ALK, BRCA1, FAT1, HNF1A, KDR, PTCH1, SDHA and SETBP1 (Table I). KDR demonstrated missense mutations and structural interaction variants, while the remainder contained missense mutations (Table I). Mutations in these genes in the CGC database (11) are associated with susceptible tumor types, such as neuroblastoma, BC, ovarian cancer, pancreatic cancer, melanoma, hepatic adenoma, hepatocellular carcinoma, skin basal cell carcinoma, medulloblastoma, paraganglioma and neuroepithelial tumors (Table I).

Cancer is the consequence of an accumulation of gene mutations; however, not all gene mutations in cancer cells are involved in the occurrence and development of cancer. Some of the mutations involved in this process are referred to as driver mutations, and the genes in which they are located are named driver genes. The driver genes in this case were identified by comparing the differential gene mutations in the BBC tissues with the known driver genes reported in the IntOGen database (https://www.intogen.org/), CGC database and literature (11-15). A total of 10 key mutated tumor driver genes were screened, including BRCA1, EBF1, MET, NF2, NUMA1, RALGAPA1, ROBO2, SMYD4, UBR5 and ZNF844 (Table II). In particular, the SMYD4 mutation was missense, and the ZNF844 mutation was located in the

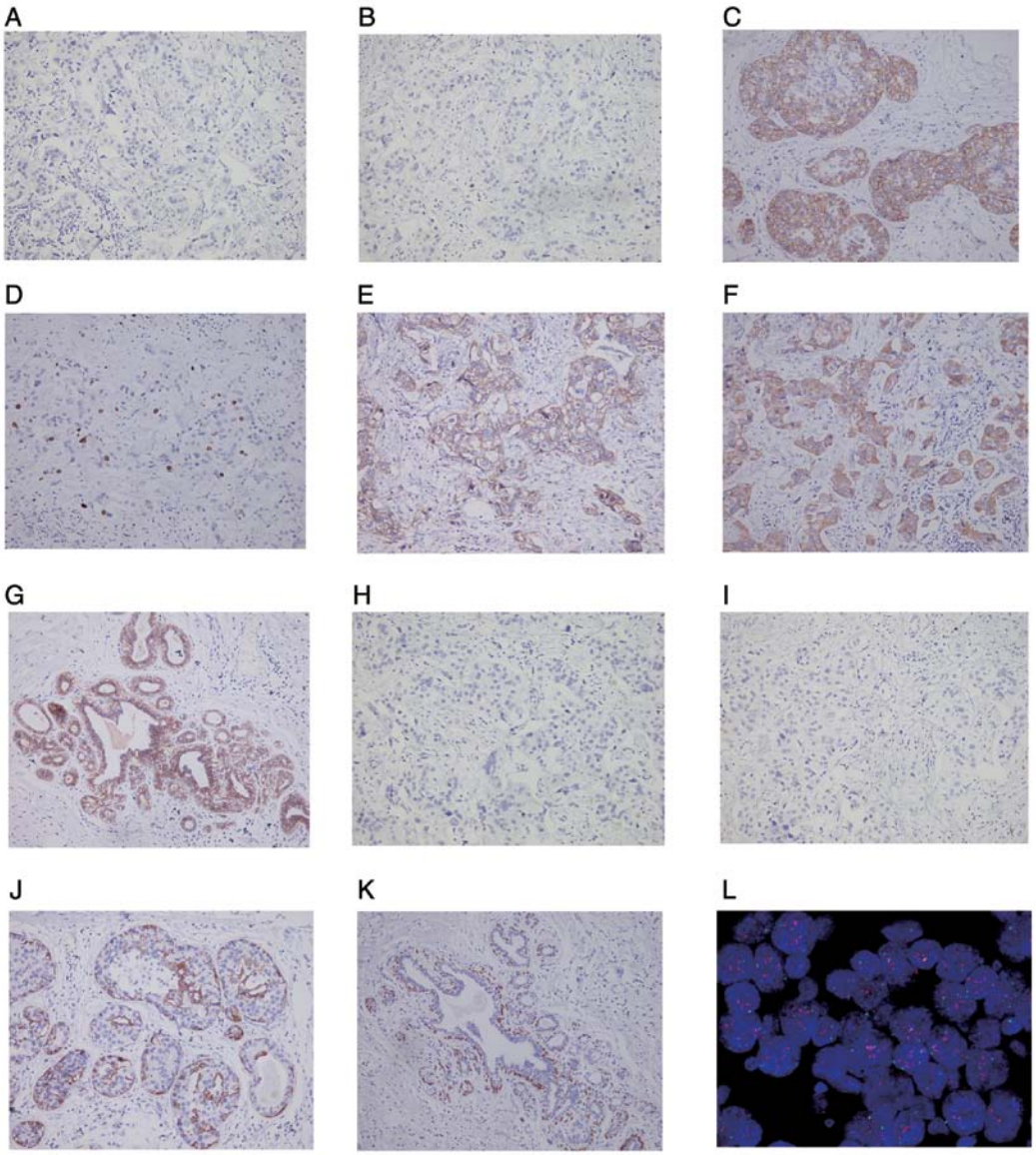

Figure 3. IHC staining (magnification, x200) and FISH assay (magnification, x1,000) in the left breast cancer tissues. IHC staining showed that the left invasive ductal carcinoma cells were negative for (A) ER, (B) PR, (H) CK5/6 and (I) P63 but positive for (C) HER2, (D) Ki-67, (E) P120, (F) CK34βE12 and (G) E-cadherin. IHC staining showed that the myoepithelial cells in the left high-grade intraductal carcinoma area were positive for (J) CK5/6 and (K) P63. (L) HER2 gene amplification in the left invasive ductal carcinoma was detected by FISH. The red signal shows focal amplification of HER2 gene, and the green signal shows the centromeric enumeration of chromosome 17. IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization; ER, estrogen receptor; PR, progesterone receptor; CK5/6, cytokeratin5/6; HER2, human epidermal growth factor receptor-2.

3'UTR; the remaining mutations were located in intronic regions (Table II). The IntOGen and CGC databases revealed that these gene mutations could drive the development of BC, ovarian cancer, cervical cancer, colorectal adenocarcinoma, lymphoma, melanoma and other tumors. Among them, BRCA1 and NUMA1 have been identified as key driver genes in BC (Table II).

Analysis of significantly mutated genes (SMGs) and mutated sample classification in BBC. SMGs are genes with a mutation frequency that is significantly higher than the background mutation rate. MuSic software (version 0.4) (10) was used to analyze the differential high frequency mutations in the BBC tissues, and the convolution test was used to conduct a statistical test for each mutation type. The high-frequency mutated genes mainly contained missense mutations, among which SNV was the most common mutation, with C > T and C > A as the main forms (Fig. 6A-C). The top 10 high frequency mutated genes were TRIM49, DGKG, FBXO40, FBXL8, EPG5, EIF2B3, DOCK11, COL6A6, ANKRD36B and ANAPC15 (Fig. 6D). The most important biochemical processes and signal transduction pathways involved in high frequency mutated genes were identified through significant functional categories and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses. These high frequency mutated genes were related to 3 functional categories, comprising signal transduction, global and overview maps, and lipid metabolism (Fig. 6E). In addition, the high frequency mutated genes were mainly involved in metabolic pathways such as glycerolipid metabolism, glycerophospholipid metabolism, the phosphatidylinositol signaling system and the phospholipase D signaling pathway (Fig. 6F).

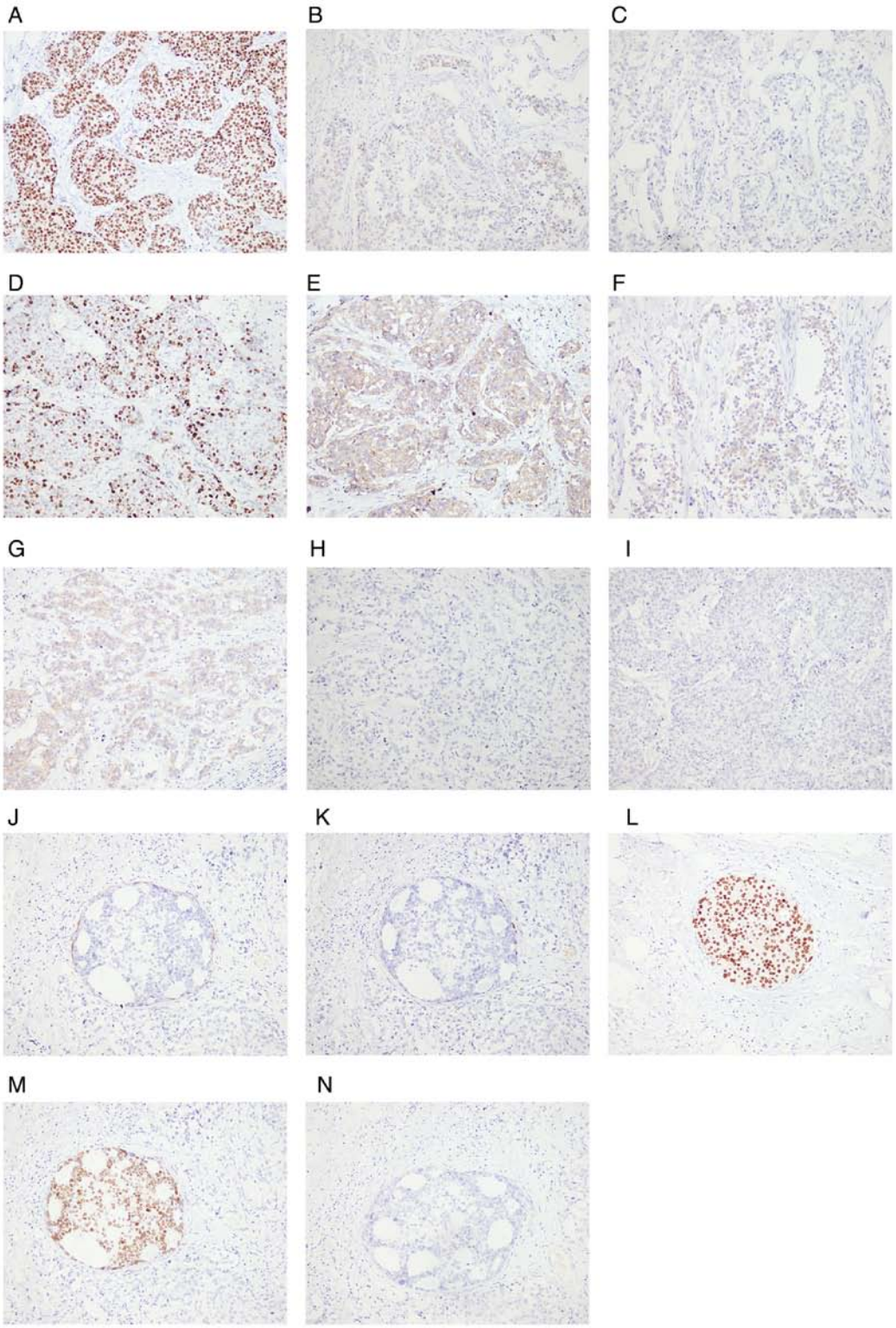

Figure 4. IHC staining (magnification, x200) of the right breast cancer tissues. IHC staining showed that the right invasive ductal carcinoma cells were positive for (A) ER, (B) PR, (D) Ki-67, (E) P120, (F) CK34βE12 and (G) E-cadherin but negative for (C) HER2, (H) CK5/6 and (I) P63. IHC staining showed that in the right intraductal carcinoma area, myoepithelial cells were positive for (J) CK5/6 and (K) P63, while tumor cells were positive for (L) ER and (M) PR but negative for (N) HER2. IHC, immunohistochemistry; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor-2; CK5/6, cytokeratin5/6.

Discussion

In 2020, BC accounted for 11.7% of all female cancer cases worldwide and previously surpassed lung cancer as the most common cancer in women (16). Breasts are paired organs in humans; BC can simultaneously or heterochronously occur in the bilateral breast because both sides can be exposed to the same internal and external carcinogenic factors and there is lymphatic transportation between the mammary glands. A retrospective analysis revealed that only 14.3% of patients

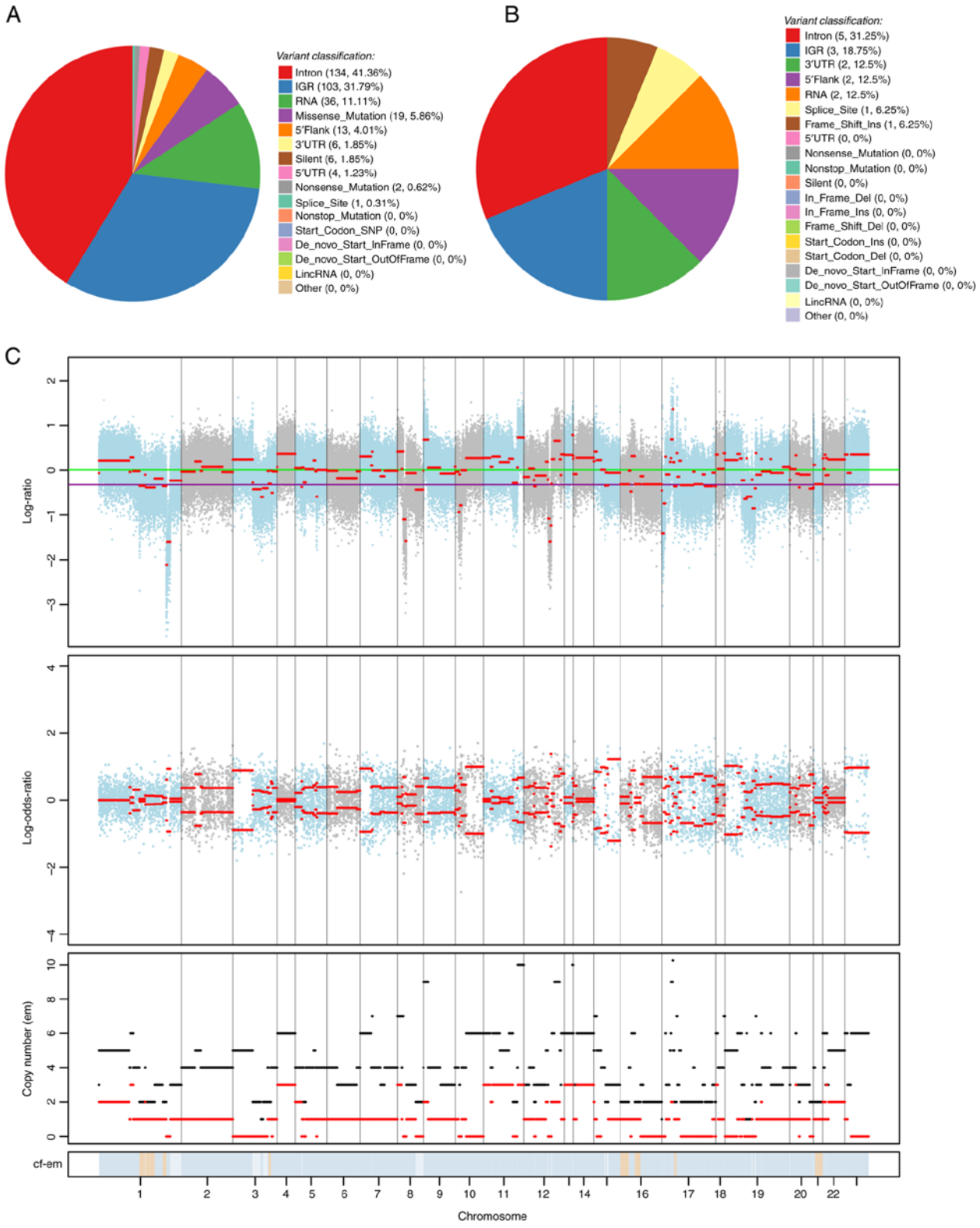

Figure 5. Differential variations in bilateral breast cancer tissues. (A) The distribution proportion of SNVs. (B) The distribution of InDels. (C) Comparison of CNVs in the bilateral breast cancers. The horizontal coordinates represent the chromosome regions, and the upper and middle panels are the log2 values based on the ratio of the coverage depths of the two sample reads at different chromosome locations, with the red line representing the corrected result. The vertical coordinate of the lower panel represents the copy number variation: The black line represents the major copy number variation, and the red line represents the minor copy number variation. A value >2 indicates an increased copy number, while a value <2 indicates a decreased copy number. SNV, single nucleotide variants; InDels, insertions and deletions; CNV, copy number variations.

with SBBC display bilateral breast symptoms at first diagnosis. By contrast, at least 28.6% were asymptomatic bilateral cases, highlighting the importance of population-based mammography in detecting asymptomatic or occult BBCs that may be overlooked (17). Furthermore, there is no uniformity in the definition of the time interval between occurrences, and

Table I. WES profile of cancer susceptibility gene variations in the bilateral cancers.

Genes	Chromosome	Position	Transcript ID	Ref.	Alteration	Variant classification	Sequencing	Coding	CGC-Cancers
ALK	Chr2	29221210	ENST00000453137	G	T	Missense variant	c.237C>A	p. Phe79Leu	Neuroblastoma
BRCA1	Chr17	43071077 43092418	ENST00000471181	T	C	Missense variant	c.4900A>G; c.3113A>G	p. Ser1634Gly; p. Glu1038Gly	Breast cancer; ovarian cancer
FAT1	Chr4	186621601 186709436	ENST00000614102	T G	C A	Missense variant	c.4991A>G c.392C>T	p. Asn1664Ser p. Ala131Val	Pancreatic cancer
HNF1A	Chr12	120999418	ENST00000541395	T	C	Missense variant	c.1652T>C	p. Leu551Ser	Hepatic adenoma; hepatocellular carcinoma
KDR	Chr4	55106807 55113391	ENST00000263923	T C	A T	Missense variant Structural interaction variant	c.1416A>T c.889G>A	p. Gln472His	Melanoma
PTCH1	Chr9	95447312	ENST00000331920	G	A	Missense variant	c.3944C>T	p. Pro1315Leu	Skin basal cell carcinoma; medulloblastoma
SDHA	Chr5	224372	ENST00000264932	T	C	Missense variant	c.163T>C	p. Tyr55His	Paraganglioma
SETBP1	Chr18	44876688	ENST00000426838	G	A	Missense variant	c.664G>A	p. Ala222Thr	Neuroepithelial tumors

WES, whole exome sequencing; CGC, Cancer Gene Census; ALK, anaplastic lymphoma kinase; BRCA1, BRCA1 DNA repair associated; FAT1, FAT atypical cadherin 1; HNF1A, HNF1 homeobox A; KDR, kinase insert domain receptor; SDHA, succinate dehydrogenase complex flavoprotein subunit A; SETBP1, SET binding protein 1.

Table II. WES profile of known driver gene variations in the bilateral breast cancers.

Genes	Chromosome	Position	Ref.	Alteration	Variant classification	Coding	IntOGen-Cancers	CGC-Cancers	Literature
BRCA1	Chr17	43063183	-	CCAGCCAATAACG GAATTATTAAAAA CTTATTTTAACAG AAGGCAGGTAAGA	Intron	-	Breast cancer; Ovarian cancer	Ovarian cancer	High confidence driver
EBF1	Chr5	158924607	G	C	Intron	-	Large B-cell lymphoma; lung squamous cell carcinoma, Non-Hodgkin lymphoma	Lipoma	-
MET	Chr7	116743773 116743776	T C	A T	Intron	-	-	Papillary renal; head and neck squamous cell	Oncogene
NF2	Chr22	29658418	C	A	Intron	-	Cervical cancer; head and neck cancer; mesothelioma; ovarian cancer; pancreatic adenocarcinoma; renal cell carcinoma; hepatocellular carcinoma; skin squamous cell carcinoma	Meningioma; acoustic neuroma; renal cell carcinoma	-
NUMA1	Chr11	72035654	A C	G T	Intron	-	Breast cancer	-	High confidence driver
RALGAPA1	Chr14	35771120	A	G	Intron	-	-	-	Candidate driver
ROBO2	Chr3	76485224 76485246	G G	T A	Intron	-	-	Colorectal adenocarcinoma; melanoma	-
SMYD4	Chr17	1801002	C	A	Missense mutation	p. R131I	-	-	Candidate driver
UBR5	Chr8	102346741	T	C	Intron	-	-	Mantle cell lymphoma; gastric cancer; colorectal cancer	-
ZNF844	Chr19	12077126	G	A	3'UTR	-	-	-	High confidence driver

WES, whole exome sequencing; CGC, Cancer Gene Census; BRCA1, BRCA1 DNA repair associated; EBF1, early B-cell factor 1; MET, MET proto-oncogene, receptor tyrosine kinase; NF2, neurofibromin 2; NUMA1, nuclear mitotic apparatus protein 1; RAL-GAPA1, Ral GTPase activating protein catalytic subunit alpha 1; ROBO2, roundabout guidance receptor 2; SMYD4, SET and MYND domain containing 4; UBR5, ubiquitin protein ligase E3 component n-recognin 5; ZNF844, zinc finger protein 844.

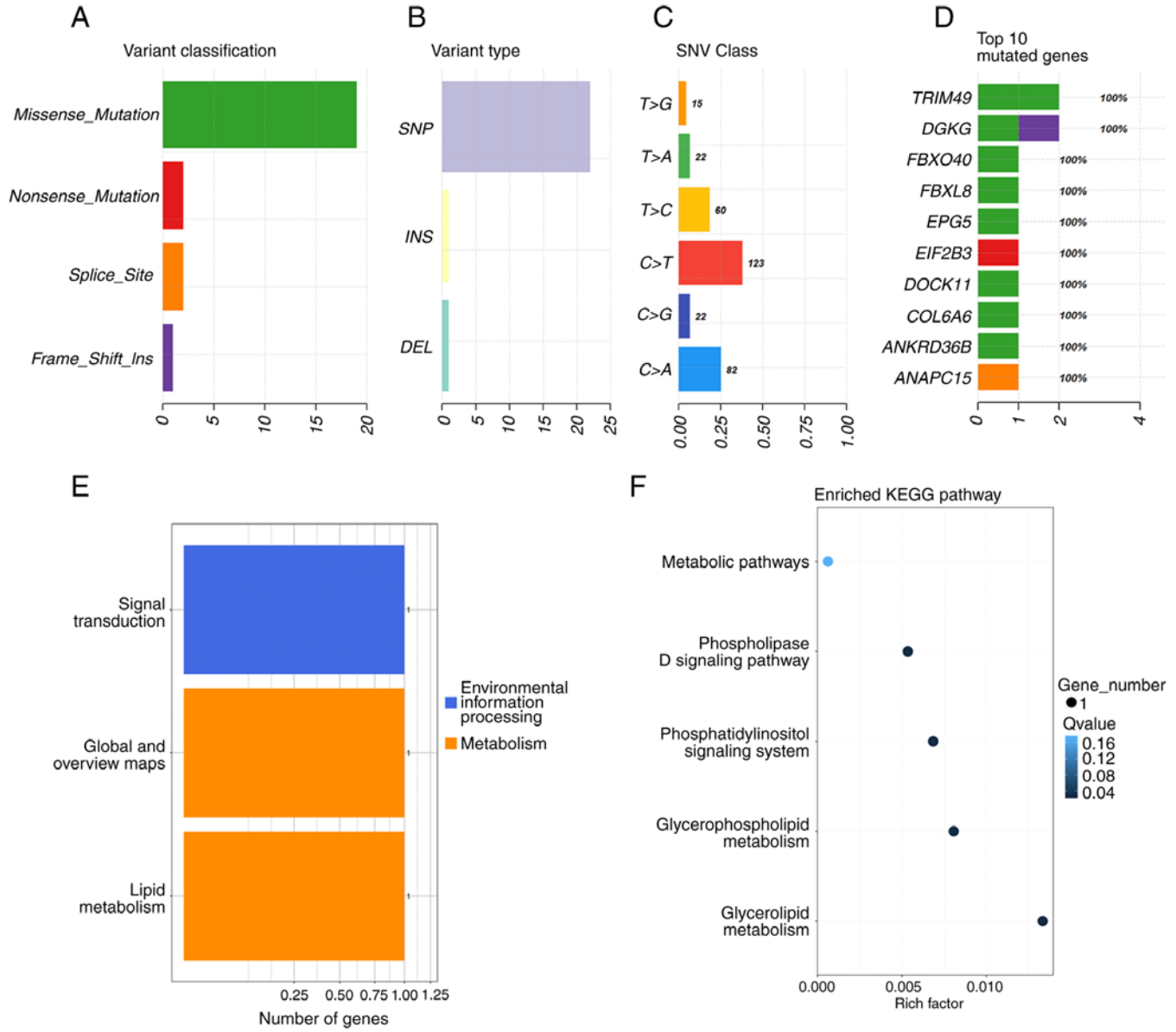

Figure 6. Significantly mutated genes in the bilateral breast cancers. (A) Variant classification, (B) variant type and (C) SNV class of the significantly mutated genes. (D) The top 10 high-frequency mutated genes. (E) Functional category enrichment analysis of the high-frequency mutated genes. Vertical coordinates represent different pathways, and horizontal coordinates represent the number of genes mutated in the corresponding functional categories. (F) KEGG pathway enrichment analysis of the high-frequency mutated genes. The vertical coordinates represent different pathways, and the horizontal coordinates represent the proportion of genes mutated in the corresponding pathway to all genes in that pathway. The circle size represents the number of genes enriched in the corresponding pathway, and the color represents the significance of enrichment. SNV, single nucleotide variants; KEGG, Kyoto Encyclopedia of Genes and Genomes.

the cutoff time for synchronization described in the literature is usually between 3 and 6 months (18). For the first time, the 8th edition of the AJCC BC Guidelines (19) defined BBCs with an interval of ≤4 months from the initial diagnosis as SBBC, while those with an interval of >4 months between diagnoses were considered MBBC. Huo *et al* (20) argued that it is more reasonable to adopt <12 months as the diagnostic criterion for concurrent SBBC, as this cutoff might better reflect the biological characteristics of the tumor.

Researchers have summarized 4 standards for the diagnosis of bilateral primary BC (21): i) Secondary tumors found as *in situ* lesions or as *in situ* carcinoma continuation into invasive carcinoma; ii) the histological types of bilateral tumors are completely different; iii) the histological differentiation of the secondary tumors is significantly higher than that of the primary side; and iv) the contralateral side reoccurs more than 5 years after the primary side's operation, with no local recurrence, lymphatic metastasis, or distant metastasis. Regarding the diagnostic criterion of Chaudary *et al* (21) of 'completely different histologic types bilaterally', a number of studies (22,23) have shown that the proportion of patients with SBBC with consistent histologic types of BBCs ranges from 44 to 73%, while that of patients with consistent histologic grading is 69%. Gong *et al* (24) also found that the histologic type consistency of SBBC (93%) was significantly higher than that of MBBC (59%) in a large-scale study of Korean patients with BC. However, this diagnostic standard has certain limitations, and some academics have supplemented it as follows (25,26):

i) The same type of histology is not necessarily non-primary; ii) primary BC is mostly located in the upper outer quadrant of the breast within the intrinsic breast tissues, whereas metastatic BC is generally located in the inner quadrant, near the midline of the chest, or in the axillary caudal adipose tissues; iii) primary lesions tend to be solitary, and metastatic lesions tend to be multiple; and iv) cases that have distant metastases at the time of diagnosis are excluded.

Only 6 studies of SBBC with discordant molecular subtypes were found by a review of the literature in the PubMed database up to September 2023 (Table III), suggesting that SBBC with discordant molecular subtypes is extremely rare. The age at diagnosis in these studies ranged from 35 to 60 years. The patient in the present study is the oldest reported to date. Hormone receptor status was discordant in 5 cases (27-31) and concordant in 1 case (32). Concordant HER2 status was observed in only 2 cases (27,28). A total of 4 patients underwent long term follow up (27,30-32), 3 were relapse free at 15, 20 and 78 months after diagnosis (27,31,32), and 1 succumbed 71 months after the initiation of treatment (30).

During treatment, SBBC can be regarded as two independent tumors occurring simultaneously. The principle of SBBC treatment is similar to that of UBC, adopting different surgical methods according to the clinical stage of each side of the BC with supplementary chemotherapy, radiation therapy, endocrine therapy and molecular targeting therapy with individualized comprehensive treatment for the more serious side after surgery if the pathological types and immunohistochemical test results of the two sides differ (4). Treatment according to the molecular subtype of SBBC involves more complex strategies but will significantly improve the prognosis of patients with BBC (33).

The Chinese Society of Clinical Oncology BC Guidelines 2023 (34) state that the neoadjuvant treatment of HER2(+) BC in stage I should be TCbHP (1A), THP*6 (2A) and THP*4 (1B) regimens, respectively (T, paclitaxel; Cb, carboplatin; H, trastuzumab; P, pertuzumab). Adjuvant therapy after neoadjuvant therapy for HER2(+) BC can involve preoperative anti-HER2 therapy using trastuzumab alone; HP (2A) is recommended for pathologic complete remission (pCR) grade I. Patients who have not reached pathologic complete remission (non-pCR) are first advised to use TDM1 (1A), followed by HP (2A); preoperative anti-HER2 therapy using trastuzumab in combination with pertuzumab can be applied. Patients with grade I showing a pCR are recommended to receive HP (2A), while those without a pCR are recommended to receive T-DM1 (2A) or HP (2A). Neoadjuvant endocrine therapy may be considered for hormone dependent patients who require preoperative neoadjuvant therapy but are not candidates for chemotherapy, are temporarily ineligible for surgery or do not require immediate operation, and are insensitive to neoadjuvant chemotherapy, with AI (1A, an aromatase inhibitor) or AI + CDK4/6 inhibitor (2B) being recommended for grade I in postmenopausal women with hormone receptor positive (HR+) BC. 1A, 1B, 2A and 2B represent recommended levels of therapeutic regimens, with level 1 prioritizing over level 2 and Class A prioritizing over Class B.

Postoperative adjuvant chemotherapy regimens for HER2 negative BC are usually based on anthracyclines, such as doxorubicin/cyclophosphamide (AC) and epirubicin/cyclophosphamide (EC); due to the cardiotoxicity of anthracyclines, the left ventricular ejection fraction must be evaluated at least once every 3 months when using anthracyclines. Another option is a sequential regimen of anthracyclines and paclitaxel, such as AC followed by paclitaxel (once a week), AC followed by docetaxel (once every 3 weeks), dose-intensive AC followed by paclitaxel (once every 2 weeks) and dose-intensive AC followed by paclitaxel (once a week). AI can be recommended as an adjuvant endocrine therapy regimen to all postmenopausal ER^- and/or PR^+ patients, with 5 years of extended AI treatment recommended for stage III patients. For patients with hormone receptor-positive/$HER2^-$ advanced BC, endocrine therapy combined with CDK4/6 inhibitor or endocrine therapy-based therapy remains the superior treatment option (34).

According to the reported molecular phenotypes of discordant SBBC, 5 patients received neoadjuvant chemotherapy, and only two did not undergo bilateral modified radical mastectomy. Regarding adjuvant therapy, all patients received chemotherapy, 2 received radiotherapy, 1 was evaluated for radiotherapy, and 3 received concurrent hormone therapy. The remaining three started trastuzumab monotherapy, and one subsequently received letrozole for 5 years. Neoadjuvant therapy has traditionally been applied to locally advanced or inoperable tumors to improve surgical outcomes (32). However, neoadjuvant therapy is now increasingly used in early-stage disease to assess the tumor response and guide future adjuvant therapy (35). In the case of the present study, after bilateral modified radical mastectomy and sentinel lymph node biopsy, chemotherapy was administered with THP and HP regimens, followed by 5-10 years of endocrine therapy and continued targeted therapy prescribed.

Certain large-sample studies (36-38) have revealed that SBBC has a poorer prognosis than UBC. The prognosis of SBBC varies with different intervals. SBBC with an interval of 3-12 months has the poorest prognosis because it responds poorly to adjuvant therapy and even develops resistance to therapy. When the interval was set at 6 months, SBBC and MBBC showed similar survival ratios (37). Mejdahl *et al* (39) used competing risk modeling to demonstrate that the combined effects of having two cancers resulted in higher mortality rates and poorer prognoses than those observed in patients with UBC. Liu *et al* (40) developed an animal model of SBBC and found that the majority of micro-metastases in the lungs comprised cells derived from the primary tumor, suggesting a high degree of metastatic cross-seeding, which could contribute to intratumor heterogeneity and treatment resistance. Different hormone receptor statuses exhibit variable responses to hormone therapy, with ER(+)/PR(+) tumors being the most responsive, and tumors with ER(-)/PR(+) or ER(+)/PR(-) mixed receptor status show a reduced response to treatment due to mixed receptor status and intrinsic resistance to hormone therapy (29); HER2 is expressed in 15-20% of primary BCs, and HER2(+) BCs have the poorest prognoses among BCs (41). In a large population based retrospective study, Ding *et al* (42) revealed that molecular subtypes were associated with a poor prognosis in patients with SBBC but not in those with MBBC.

There are known risk factors for BBC, including younger age, family history, BC susceptibility gene 1/2 (BRCA1/2)

Table III. Published case reports of SBBC with discordant molecular subtypes.

First author, year	Age, years	Sex	Side of breast	TNM stage	ER	PR	HER2	Ki-67, %	Molecular subtype	Treatment	Follow-up	(Refs.)
Ojo *et al*, 2023	45	F	Left	N/A	-	-	-	90	Triple-negative	Neoadjuvant therapy (chemotherapy and trastuzumab), bilateral MRM and SLN, and evaluation for possible radiotherapy.	15 months of follow-up, with no signs of recurrence	(32)
			Right	N/A	-	-	2+	90	HER2 positive (HR negative)			
Aranda-Gutierrez *et al*, 2020	35	F	Left	T2N1M0	+	+	-	30	Luminal A	Adjuvant therapy (chemotherapy and hormone therapy), and adjuvant radiotherapy.	78 months of follow-up, with no signs of recurrence	(27)
			Right	T2N0M0	-	-	-	20	Triple-negative			
Dhadlie *et al*, 2018	58	F	Left	N/A	+	+	3+	N/A	HER2 positive (HR positive)	Neoadjuvant therapy (chemotherapy and hormone therapy). Surgical and adjuvant management to be determined based on response to neoadjuvant therapy.	N/A	(28)
			Right	N/A	-	-	3+	N/A	HER2 positive (HR negative)			
Copur *et al*, 2017	48	F	Left	T2N0M0	+	+	1+	N/A	HER2 positive (HR positive)	Neoadjuvant therapy (chemotherapy), bilateral MRM with right ALND and left SLN, and adjuvant therapy (radiotherapy and unspecified hormonetherapy).	20 months of follow-up, with no signs of recurrence	(31)
			Right	T2N1M0	-	-	-	N/A	Triple-negative			
Esclovon *et al*, 2016	59	F	Left	N/A	+	+	-	1	Luminal A	Neoadjuvant therapy (chemotherapy and trastuzumab), bilateral MRM and right SLN, and adjuvant therapy (unspecified hormone therapy).	N/A	(29)
			Right	N/A	-	-	3+	67	HER2 positive (HR negative)			
Hayashi *et al*, 2013	60	F	Left	T4bN1M0	-	-	+	N/A	HER2 positive (HR negative)	Neoadjuvant therapy (chemotherapy and trastuzumab), bilateral MRM with bilateral ALND, and adjuvant therapy (trastuzumab and letrozole).	The patient died after 71 months of follow-up, free of recurrence	(30)
			Right	T4bN0M0	+	+	-	N/A	Luminal A			

ALND, axillary lymph node dissection; F, female; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; MRM, modified radical mastectomy; SLN, sentinel lymph node; N/A, not available; SBBC, synchronous bilateral breast cancer.

mutations, lobular histologic type and multicentricity (27). Patients with enhanced BC susceptibility usually present at an earlier stage of development with BBC or >1 cancer in an individual or a family (17). Scholars have highlighted that there is a certain association between family history and BBC, where the risk of developing the disease increases by 2-4 times when a lineal descendant experiences BC (43,44). Therefore, after the diagnosis of SBBC in this patient, it is advisable to remain alert to the risk of cancer in the patient's first-degree relatives, perform early genetic screening, and increase the family members' awareness of the importance of self-screening.

As SBBC with discordant molecular subtypes is rare, and its molecular pathogenesis has not yet been fully elucidated, no prior case studies involving analyses of the genetic mutations in SBBC were found. In the present study, analysis of genetic mutations and identification of cancer driver genes were attempted by WES. Among the 10 key mutated tumor driver genes obtained, BRCA1 and NUMA1 have been identified as the key drivers of BC tumorigenesis.

The BRCA1 gene on human chromosome 17q21 encodes a tumor suppressing protein comprising 1,863 amino acids (45). Early studies have revealed that BRCA1 is critical for the maintenance of genomic stability (46-48), which is sustained by its participation in multiple aspects of the cellular response to DNA double strand breaks, including cell cycle control, chromatin remodeling, homologous recombination repair and nonhomologous end joining (49). The prevalence of germline and somatic BRCA1 mutations in BC is 7.8 and 3.4%, respectively (50). Moreover, 2~50% of hereditary BC cases result from germline mutations in BRCA1/BRCA2 genes, which are associated with early onset BC. The cumulative risk of BC by the age of 80 years has been estimated to be 72% among BRCA1 mutation carriers (51). The NCCN recommends an annual mammographic examination of BRCA1 mutation carriers with breast MRI screening up to the age of 75 years (52). In a high-risk Chinese cohort study (53), BRCA mutation carriers were found to be more likely to have lymph node involvement after a BC diagnosis. Despite adjusting for clinical prognostic factors, these patients had significantly worse BC specific outcomes, suggesting that BRCA mutations represent an independent factor contributing to poor prognosis. In a multifactorial analysis of the 6 risk factors for hereditary BC in a cohort of all high-risk individuals in the aforementioned study, the predominance ratio of germline mutations in BBCs was 3.27, which was significant (53); thus, it may be necessary to pay special attention to those with BBC even in the absence of family history or very young age of onset. The current patient had BRCA1 somatic mutations, suggesting that she and her family should be further tested for BRCA1 germline mutations to guide PARP inhibitor targeted therapy and assess genetic risk.

Nuclear mitotic apparatus protein (also referred to as NUMA1 and NMP-22) is a hyper-molecular mass nuclear matrix protein first discovered and named by Lydersen and Pettijohn (54). The NUMA1 gene is located on chromosome 11q13.4 and encodes a 236 kDa protein essential for normal mitotic spindle organization (55). NUMA1 organizes the spindle pole in mitosis and controls spindle orientation; it is also essential for the establishment of higher order chromatin organization during epithelial cell differentiation and DNA repair by homologous recombination (56). The interaction of NUMA1 with p53 is enhanced following DNA damage, and NUMA1 upregulates p53-mediated transcription of target genes (57). NUMA1 prevents 53BP1 accumulation at DNA breaks in breast carcinoma (58). NUMA1 has been reported to be associated with acute promyelocytic leukemia (APL), and NUMA1-RARα (retinoic acid receptor α) t(11;17)(q13;q21) translocation has been observed in very rare cases of APL translocation (59). NUMA1 plays an oncogenic role in esophageal squamous cell carcinoma by regulating the ASK1-JNK signaling pathway (60). NUMA1 alternative splicing induces enhanced cell proliferation and centrosome amplification in nontumorigenic mammary epithelial cells (61). Salvador *et al* (58) found that NUMA1 levels are highly heterogeneous within and between tumors, and NUMA1 expression was significantly correlated with distal metastasis free survival in patients by Kaplan Meier analysis of microarray datasets; however, high NUMA1 expression predicted longer OS times in patients in a cohort of The Cancer Genome Atlas. In a large-scale association study, Kammerer *et al* (62) identified a non-synonymous single nucleotide polymorphism (SNP; A794G) in NUMA1 that was correlated more strongly with BC risk than the initial marker SNP, and they concluded that mutations in the NUMA1 gene might be responsible for the observed increased BC risk.

Other important genes that were obtained in the present study function as oncogene or tumor suppressor, and are also closely involved in the development of BC by regulating cell division, apoptosis, angiogenesis, tumor stem cell self-renewal and immune cell infiltration. EBF1 (early B-cell factor 1) is a transcription factor with multiple effects on cell differentiation and metabolic processes (63). A number of studies have suggested that EBF1 is an important regulator of specific methylation and gene expression programs in BC subtypes (64). Qiu *et al* (65) demonstrated that EBF1 is highly expressed in triple negative BC (TNBC) cells and that the knockdown of EBF1 blocks the growth and invasiveness of TNBC cells. Importantly, the absence of EBF1 also triggers extensive mitosis and the remodeling of cellular metabolism.

The tyrosine kinase c-Met, also called MET, is a plasma membrane protein that transduces signals from the extracellular matrix to the cytoplasm. Dysregulation of MET signaling has been identified in various malignant and premalignant lesions and is involved in the uncontrolled survival, growth, angiogenesis and metastasis of cancer cells (66). A broad range of mechanisms may lead to aberrant MET signaling in BC, including activating gene mutations, gene amplification, protein overexpression, increased ligand dependent paracrine stimulation and autocrine signaling acquisition (67). MET overexpression has been reported in 14-53.6% of patients with BC and is a significant adverse predictor of relapse-free survival and OS times in patients with BC. In addition, MET may influence the prognosis of HR(+) patients by mediating resistance to endocrine therapy, especially in the HR(+)/HER2(-) subgroup, in a HER2-independent manner (66).

The neurofibromin 2 (NF2) gene encodes two transcripts, NF2-1 and NF2-2, containing 595 amino acid residues and NF2-2 contains 590 amino acid residues, respectively (68). NF2 expression is decreased in BC tissues compared with that

in adjacent normal tissues, and low expression of NF2 associates with tumor stage, while overexpression of NF2 inhibits the formation of cellular clones and stemness (69).

ROBO is considered tumor suppressor because it is frequently inactivated in various tumors, and the SLIT/ROBO signaling pathway is reportedly involved in BC development and metastasis. Overexpression of SLIT/ROBO induces its tumor suppressive effects possibly by inactivating the β-catenin/LEF/TCF and PI3K/Akt signaling pathways or by altering β-catenin/E-cadherin-mediated cell-cell adhesion in BC cells (70). SLIT2 negatively regulates WNT signaling through ROBO2 signaling in a subpopulation of basal cells, restricting mammary stem cell renewal (71).

SMYD4 is located on human chromosome 17p13.3 and serves as a potential tumor suppressor in BC. SMYD4 has been found to significantly inhibit breast tumorigenesis by suppressing the expression of platelet derived growth factor receptor α (72). Han *et al* (73) found that miR-1307-3p significantly inhibits breast stem cell renewal by targeting SET and SMYD4 expression in BC, exerting oncogenic effects.

UBR5, a HECT structural domain E3 ubiquitin ligase, is an attractive therapeutic target for invasive BC, in which CDC73, a critical substrate of UBR5, is involved in regulating the expression of β-catenin and E-cadherin, apoptosis of tumor cells, and CD8(+) T-cell infiltration mechanisms that impede the profound tumorigenic and metastatic activity of UBR5 in TNBC (74).

In conclusion, the diagnostics and treatment optimization strategies for SBBC with discordant molecular subtypes are complex. In the present study, a 72-year-old woman patient with a heterogeneous molecular subtype of SBBC was reported, who presented with a HER2(+) [HR(-)] tumor in the left breast and a hormone sensitive [HER2(-)] tumor in the right breast was reported. The patient underwent systemic chemotherapy, followed by 5-10 years of endocrine therapy and continued targeted therapy prescribed. To the best of our knowledge, this patient is the oldest patient among the reported SBBC cases with discordant molecular subtypes. In the present study, the patient did not undergo pathological examination through ultrasound-guided percutaneous biopsy before surgery, so the molecular subtype and TNM stage of the tumors were determined after surgery. It would be more rational if a multidisciplinary therapeutic regimen was determined before surgery. WES revealed differential gene variations in the BBC tissues and identified 8 cancer susceptibility genes and 10 important cancer driver genes, including BRCA1 and NUMA1, which may be associated with the occurrence of SBBC and targeted therapy options. These findings may offer prognosis assessment and therapeutic guidance for patients with SBBC and provide a basis for the necessity of self-examination of the patients' immediate family members. Since SBBC with discordant molecular subtypes is extremely rare, WES was conducted in only one case in the present study, which may lead to certain limitations for genetic analysis, and validation is needed in more cases for future study.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 82160582), the Yunnan Fundamental Research Project (grant no. 202201AT070003) and the Scientific Research Foundation of The First Affiliated Hospital of Dali University (grant no. DYFGG2022-01).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author. The data generated in the present study may be found in the NCBI Sequence Read Archive under accession nos. SRR28840132 and SRR28840133 or under the following URLs: https://www.ncbi.nlm.nih.gov/sra/?term=SRR28840132 and https://www.ncbi.nlm.nih.gov/sra/?term=SRR28840133.

Authors' contributions

SHH and BG contributed to manuscript writing, data collection and data analysis. ZJL contributed to the pathological diagnosis. YCY contributed to data collection. BG contributed to the study design, project supervision, administrative support and manuscript revision. SHH and BG confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the First Affiliated Hospital of Dali University (Dali, China; approval no. DYF20230309). Written informed consent to participate in the present study was obtained from the patient.

Patient consent for publication

Written informed consent was obtained from the patient for the publication of the images.

Competing interests

The authors declare that they have no competing interests.

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