INTRODUCTION

Cancer is a major health problem worldwide; in particular, breast cancer is the most frequently diagnosed cancer and the leading cause of cancer-related death in women based on the GLOBOCAN 2018 estimates in the United States. In 2019, 1,762,450 new cancer diagnoses and 606,880 cancer deaths are predicted to occur, including approximately 62,930 new cases of female breast cancer in the United States, which accounts for 30% of all new cancer diagnoses in women. The incidence of breast cancer has been rising for most...
developing countries in over the last few decades. In China, breast cancer is also the most common cancer in women; more than 1.6 million women have been diagnosed with breast cancer, and 1.2 million women are dying of breast cancer each year. Patients in China currently account for 12.2% of all newly diagnosed breast cancers and 9.6% of all deaths from breast cancer worldwide.

Breast cancer is a multi-factorial disease that occurs because of various risk factors, such as sex, age, ethnicity, environmental factors, diet and lifestyle. Although non-hereditary factors are the significant drivers of the observed international and interethnic differences in the disease incidence, genetics, including a personal or family history of breast or ovarian cancer and inherited mutations in BRCA1 (Breast Cancer 1 gene, OMIM 113705), BRCA2 (Breast Cancer 2 gene, OMIM 600185) and other breast cancer susceptibility genes such as PALB2 (Partner And Localizer Of BRCA2, OMIM 610355) account for 6% to 10% of breast cancer cases. A woman’s life-time risk of developing breast and/or ovarian cancer is greatly increased if she inherits a harmful mutation in the genes BRCA1/2 or PALB2. Breast cancer is also influenced by somatic gene mutations and chromosome instability.

In this study, we have successfully identified a germline mutation in the BRCA2 gene in a large Chinese family with breast cancer, expanding the knowledge and information about genetic mutations related to breast cancer.

## Materials and Methods

### 2.1 Ethical statement, patient information and DNA preparation

This study was approved by the Ethical Committees in the Southwest Medical University of China with written informed consent from the participants and was conducted in line with the tenets of the Declaration of Helsinki (2013 version). A Chinese pedigree of breast cancer including a proband (Figure 1, pedigree III:11, arrow) was recruited, and the family history of breast cancer was collected by questionnaire. Clinicopathological assessments and neoadjuvant therapy information were obtained for the proband, such as haematoxylin and eosin (HE) staining, immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH). Fresh peripheral blood samples (2 ml each) were taken from each individual, and gDNA was extracted using our previously described phenol/chloroform method. Blood samples from one hundred healthy individuals were taken for DNA extraction as controls.

### 2.2 Targeted NGS, Sanger sequencing and co-segregation analysis

All of the exons and exon-intron boundaries of the BRCA1/2 and PALB2 genes were screened with next-generation sequencing (NGS) by a targeted gene panel using an Illumina HiSeq × 10 NGS platform (Illumina, CA, US). Gene capture was up to 500 × depth and had greater than 99% coverage. For mutation identification, the sequenced results were aligned to reference sequences of the genes BRCA1 (NM_0007294.3), BRCA2 (NM_000059.3) and PALB2 (NM_024675.3) using the Burrows-Wheeler Alignment tool. Local realignment and variant recalibration were conducted using the GATK (Genome Analysis Toolkit) Best Practices Pipeline. Variants were annotated by annovar software tools (http://annovar.openbioinformatics.org/).

PCR amplification and Sanger sequencing of variants were applied to human gDNA of the available individuals for variant verification and co-segregation analysis by designing primer pairs (BRCA2-7007L and BRCA2-7007R) through the online Primer 3 programme (http://primer3.ut.ee/) in the corresponding BRCA2 genome (Table 1). A PCR product with 553 bp for BRCA2 was amplified using gDNA as a template. The PCR products were directly subjected to sequencing with the Sanger method on an ABI-3500DX sequencer (Applied Biosystems Inc, Foster City, CA, USA) using the specific primer BRCA2-7007L (Table 1). All unrelated, ethnically matched controls were also sequenced using the aforementioned primer, and then, co-segregation analysis was performed based on the sequencing results and clinical phenotypes in this pedigree.

### 2.3 Protein structure and bioinformatic analysis

The conserved domains were identified by the online NCBI system for the BRCA2 protein (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

## Results

We recruited a Chinese breast cancer family, including 3 female patients and 25 healthy individuals (Figure 1, II:3, III:3, III:11, Table 2), from Hunan province in the central south of China. The patient II:3 was diagnosed with breast cancer at the age of 60 and died 4 years later. The proband (Figure 1, proband, III:11, molecular no.: M411) was 41 years old and diagnosed with breast cancer three years ago (Figure 1B-D). The proband and her sister with breast cancer (Figure 1, III:3) were diagnosed with advanced invasive ductal breast carcinomas. The clinical information of the two patients, including current age, the year diagnosed, unilateral status, treatment strategy, tumour grade and size, lymph node status, recurrence, IHC results for biomarkers and FISH results, is listed in Table 2. By IHC analysis based on biomarker testing of the cancer tissues, the proband’s tumour was positive for Ki67, ER, PR and HER-2, whereas other markers were negative (Table 2). Both patients were initially diagnosed with stage II disease (Table 2). Treatments were used immediately when diagnosed, and the recurrences were not found over three years for the proband (III:11) and five years for her sister (III:3) (Table 2).

Blood samples were collected from the family members, and DNA was extracted from it. After sequencing all of the exons and exon-intron boundaries of the BRCA1/2 and PALB2 genes by
NGS-based genetic diagnosis in the proband, we identified a heterozygous missense mutation of the gene *BRCA2*: NM_000059.3: c.7007G>T (p.R2336L) in exon 13, which substituted base T for base G at the base position 7007, leading CGC to be mutated into CTC (c.7007G>T), causing arginine (Arg, R) to be substituted by leucine (Leu, L) (p.R2336L). This variant was then verified by Sanger
sequencing in the proband (Figure 2A). PolyPhen-2 analysis suggested this change was benign (score 0.0011), but MutationTaster revealed the change to be disease causing (score 1). SIFT indicated it was tolerated (score 0.46), and I-Mutant2.0 for the free energy change value indicated an increase in protein stability (DDG = 0.61 kcal/mol, >0). This variant of \textit{BRCA2}: NM_000059.3: exon 13: c.7007G>T, p.R2336L, is likely pathogenic. By searching the ExAC and HGMD databases, this variant was found to be novel. The pathogenic aspects of this mutation are presented in Table 3.

Sequencing of DNA materials from nine other female members in this family including the proband’s sister with breast cancer was also performed. The representative results by Sanger sequencing are shown in Figure 2A-J. In addition to proband III:11 and her sister III:3 who were affected with breast cancer, another five women in this family, including the proband’s mother, three sisters and one niece, who all appeared to be normal, also carried the same heterozygous, missense mutation of the gene \textit{BRCA2}: c.7007G>T. The other 3 women in this family with normal phenotypes carried two wild-type alleles (Figure 2). DNA from III:13 was not available. Sequencing of DNA materials from male members of this family was also performed and revealed that IV:3 had wild-type alleles of \textit{BRCA2}, whereas IV:10 had the same heterozygous mutation of \textit{BRCA2}: c.7007G>T (Figure 2K,L, respectively). One hundred unrelated, healthy individual controls did not show this variant by Sanger sequencing (data not shown).

Searches of the Conserved Domain Database (CDD) in NCBI were executed. Comparing the human BRCA2 protein to nine other species indicated that it is highly conserved in the chimpanzee, rhesus monkey, dog, cow, mouse, rat and chicken (Figure 3A). Comprehensively, this study shows that the heterozygous variant of \textit{BRCA2}: c.7007G>T (p.R2336L) might cause breast cancer in this Chinese pedigree (Figure 3B, arrow).

4 | DISCUSSION

The \textit{BRCA2} gene, also known as \textit{BRCC2}, \textit{BROVCA2}, \textit{FAD}, \textit{FACD}, \textit{FAD1}, \textit{GLM3}, \textit{FANCD}, \textit{PNCA2}, \textit{FANCD1} and \textit{XRCC11}, functions as a tumour suppressor gene and is involved in repairing damaged DNA, and mutations of it are associated with diseases including fanconi anaemia, complementation group D1, fallopian tube cancer, primary peritoneal cancer, ovarian cancer and breast cancer. Numerous reports have indicated that inherited germline mutations in the \textit{BRCA1} and \textit{BRCA2} genes result in an increased risk of developing breast or ovarian cancer sometime during their life-times. The prevalence and clinical outcome of germline mutations in the \textit{BRCA1/2}
and/or PALB2 genes in breast cancer patients in different ethnic
groups, including populations from Turkey,26 Lebanon,27 Japan,28
Mexico,29 China,30-32 etc, have been widely reported recently. By
analysing large numbers of Chinese hereditary breast and ovarian
cancer patients and Caucasian patients, Bhaskaran et al33 recently
revealed that germline variations in the BRCA1/2 genes are highly
ethnicity-specific. Thus, identification of novel pathogenic germline
BRCA2 variants in familial Chinese breast cancers is necessary.

In the current study, NGS-based genetic diagnosis14,34-36 was
used to identify a novel heterozygous missense mutation of the gene
BRCA2: c.7007G>T, p.R2336L, in a Chinese breast cancer family. This
gene is likely pathogenic, enriching its known mutation spectrum.

Two breast cancer patients including the proband, her elder sister
III:3 and another five women (the proband’s mother, three sisters
and one niece) in this family whom appeared to be normal through
August 2019 also carried this heterozygous, missense mutation of
BRCA2: c.7007G>T. Because of financial and medical constraints,
the carriers declined to go to hospitals for breast cancer screening.

Nevertheless, it may be used as a biomarker for the BRCA2 variant
(c.7007G>T) in this family.

Women carrying BRCA1/2 mutations have an increased life-time
risk of developing breast cancer, and multiple risk factors, such as
sex, age, ethnicity, environmental factors, diet and lifestyle, affect
the penetrance, progression and development of breast cancer.7,37-39
Antoniou et al40 for example, reported that the breast cancer risk for
female carriers of PALB2 mutations was increased by eight to nine
times among those younger than 40 years of age, six to eight times
among those 40 to 60 years of age and five times among those older
than 60 years of age, when compared to the general populations.

Environmental factor, being overweight/dietary choices, having a first
child at an older age, few or no childbirths and the lack of or only a
short period of breastfeeding should increase the risk or penetrance
for developing breast cancer in this family.41-42 Therefore, it is not

41 surprising that the proband’s mother, as a carrier, is still unaffected at
76 years of age, as she gave birth to seven children with her first baby
at the age of 22, and they were all breastfed. But the other carriers
in this family should be warned that they have a higher risk of breast
cancer, especially the proband’s 9-year-old niece. In this regard,
this knowledge may provide viable strategies for breast cancer prevention
through avoiding some of the other risk factors such as those afore-
mentioned. Thus, genetic counselling and long-term follow-up should
be provided for this family, especially the patients and carriers of this
variant of BRCA2: c.7007G>T (p.R2336L).

Offspring of individuals with a germline BRCA1/2 or PALB2
pathogenic variant have a 50% chance of inheriting the variant. The
male individuals IV:1, IV:2, IV:3, IV:4 and IV:10 have a 50% chance
of inheriting the c.7007G>T variant, and if they have a daughter,
she may be at high risk of breast cancer. In addition, men who carry
BRCA2 variants also have an increased risk of developing breast can-
cer.20 Thus, we collected samples from men, and Sanger sequencing
revealed that IV:3 had wild-type alleles of BRCA2, whereas IV:10
carried the heterozygous missense mutation of the gene BRCA2:
c.7007G>T (Figure 2L). Unfortunately, the male individuals IV:1, IV:2

| Table 3 | Characteristics of BRCA2 variant and analysis of predicted protein structure and disease-causing effects |

| Gene  | Exon | Variation | Nucleotide* | Amino acid* | Type   | Status  | Polyphen-2 | Mutation Taster | ExAC |
|-------|------|-----------|-------------|-------------|--------|---------|------------|----------------|------|
| BRCA2 | 13   | c.7007G>T | p.R2336L    |             | Missense| Heter   | B (0.01)   | T (0.46)       | Novel |

Note: B: benign; BRCA2: breast and ovarian cancer susceptibility protein 2 (NM_000059.3); c, variation at cDNA level; DC, disease causing; DDG > 0, increase stability; ExAC, the Exome Aggregation Consortium.; Heter, heterozygote; p, variation at protein level; R2336L, leucine substitution; T, tolerated.

*All nucleotide and amino acid are abbreviated according to the International Union of Pure and Applied Chemistry (IUPAC).
and IV:4 could not be tested. Prenatal testing is possible for pregnancies if a pathogenic variant is known; however, requests for prenatal diagnoses of mutations linked to breast cancer are not common and require careful genetic counselling.

In conclusion, we have successfully identified a novel germline heterozygous, likely pathogenic variant of $BRCA2$: c.7007G>T (p.R2336L) in a Chinese family with breast cancer by NGS-based genetic diagnosis, enriching its known mutation spectrum. Genetic counselling and long-term follow-up should be provided for this family and in other patients and carriers carrying this $BRCA2$ variant: c.7007G>T (p.R2336L).

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AUTHORS’ CONTRIBUTIONS
JC, JF, PT, XD, CW, JP and HC recruited patients, collected samples and conducted experiments. JF and MAK wrote the manuscript; and JF revised the manuscript. JF supervised the project. All authors reviewed and approved the manuscript.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE
The study was approved by Southwestern Medical University, and written informed consent was obtained from all patients, in compliance with the recommendations of the Helsinki Declaration.

CONSENT FOR PUBLICATION
Written informed consent was obtained from the participants for publication of their medical data and images.
CONFLICTS OF INTEREST
The authors declare that they have no conflicts of interest.

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DATA AVAILABILITY STATEMENT
All data used to support the findings of this study are available from the corresponding authors upon request.

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