Original Article

Multiple cancer susceptible genes sequencing in BRCA-negative breast cancer with high hereditary risk

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Background: Hereditary factors contributed to breast cancer susceptibility. Low *BRCA* mutation prevalence was demonstrated in previous *BRCA* mutation screening in Chinese breast cancer patients. Multiple-gene sequencing may assist in discovering detrimental germline mutation in *BRCA*-negative breast cancers.

Methods: A total of 384 Chinese subjects with any two of high-risk factors were recruited and screened by next-generation sequencing (NGS) for 30 cancer susceptible genes. Variants with a truncating, initiation codon or splice donor/acceptor effect, or with pathogenicity demonstrated in published literature were classified into pathogenic/likely-pathogenic mutations.

Results: In total, we acquired 39 (10.2%) patients with pathogenic/likely-pathogenic germline mutations, including one carrying two distinct mutations. Major mutant non-*BRCA* genes were *MUTYH* (n=11, 2.9%), *PTCH1* (n=7, 1.8%), *RET* (n=6, 1.6%) and *PALB2* (n=5, 1.3%). Other mutant genes included *TP53* (n=3, 0.8%), *RAD51D* (n=2, 0.5%), *CHEK2* (n=1, 0.3%), *BRIP1* (n=1, 0.3%), *CDH1* (n=1, 0.3%), *MRE11* (n=1, 0.3%), *RAD50* (n=1, 0.3%) and *PALLD* (n=1, 0.3%). A splicing germline mutation, *MUTYH* c.934-2A>G, was a hotspot (9/384, 2.3%) in Chinese breast cancer.

Conclusions: Among *BRCA*-negative breast cancer patients with high hereditary risk in China, 10.2% carried mutations in cancer associated susceptibility genes. *MUTYH* and *PTCH1* had relatively high mutation rates (2.9% and 1.8%). Multigene testing contributes to understand genetic background of *BRCA*-negative breast cancer patients with high hereditary risk.

Keywords: Germline mutation; *BRCA*-negative; hereditary breast cancer; multigene sequencing

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Introduction

Breast cancer susceptibility is demonstrated to be associated with hereditary background, and it is estimated that hereditary and genetic factors contributed to 27% of breast cancer incidences (1,2). *BRCA1* and *BRCA2* germline mutations are the most common cause of hereditary breast cancer. In our previous study, comprehensive screening in Chinese breast cancer patients with high hereditary risk in our cancer centre showed a low *BRCA* mutation prevalence (3), which suggesting the majority of Chinese hereditary breast cancer is associated with other susceptible genes. Apart from the first discovery of *BRCA1* and *BRCA2*, other breast cancer associated susceptibility genes have been identified constantly, including high-penetrance susceptible genes (*TP53* and *PTEN*), moderate-penetrance susceptible genes (*CDH1*, *STK11*, *NF1*, *PALB2*, *CHEK2*, *ATM* and *NBN*), and low-penetrance susceptible genes (*BARD1*, *FANCC*, *MRE11A*, *MUTYH* heterozygotes, *RECQL*, *RAD50*, *RET1*, *SLX4*, *SMARCA4*, *XRCC2* and so on) (4-6). Despite the fact that breast cancer susceptible genes have been extensively studied and multiple genes testing have been widely performed in Caucasians, Ashkenazi Jewish and African Americans, insufficient data supports the knowledge of hereditary background in Chinese breast cancer patients.

Many retrospective studies proved that clinicopathologic features and outcomes of breast cancer varied between Chinese and Caucasian population. Chinese patients had a younger age at diagnosis of breast cancer, whose peak age onset was between 45 and 55 years old, compared to an average of between 60 and 70 years old in Caucasian breast cancer patients (7). Besides, Chinese patients had a lower rate of incidence of invasive lobular breast cancer. Genomic profiling studies also demonstrated disparities between breast cancers of different ethics. One study compared gene expression and microRNA profiles between Chinese and Italian breast cancers and found lower prevalence of Luminal A subtype among Chinese breast cancers (8). A more recent study revealed a higher mutational prevalence for *TP53* and *AKT1* in Chinese patients (9).

The National Comprehensive Cancer Network (NCCN) has set criteria of hereditary risk evaluations for breast cancer patients since 2014 (6,10-12). Main concerns in NCCN guidelines include early-age onset breast cancer, triple negative breast cancer under 60 years old, primary bilateral breast cancer, male breast cancer and breast cancer with certain family history. The NCCN guidelines recommend multigene testing should ideally be offered in the context of professional genetic expertise for pre- and post-test counselling, and warranted' in those who have tested negative for a single inherited syndrome (6,10,11). However, no consensus or guidelines regarding the identification of hereditary mutation (beyond *BRCA1* and *BRCA2*) carriers and clinical management options has been integrated for Chinese breast cancer patients.

Next-generation sequencing (NGS) is driving growth and possibilities in genomic researching, providing reading lengths as long as the entire genomes, reducing the cost of sequencing, and enabling the application of genetic testing as a clinical tool (13,14). Moreover, NGS allows for the sequencing of multiple genes simultaneously at an unprecedented speed. Multiple gene panel testing could not only include high-penetrance susceptible genes associated with a specific cancer, but also include moderate- and low-penetrance susceptible genes as well (15). Meanwhile, multiple gene panels for inherited cancer risk have proved to be a more time- and cost-efficient approach in hereditary risk management.

In our present study, we are aiming to provide more information about and get better knowledge of mutational spectrum in Chinese population, to identify novel mutations in high hereditary risk breast cancer patients with *BRCA1* and *BRCA2* testing negative, and to aid in updating the clinical recommendations for genetic testing.

Methods

Pathologic data

A triple-negative breast cancer (TNBC) case was defined as a patient whose tumour sample was negative for oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expression upon immunohistochemical (IHC) staining. ER or PR immunostaining was considered positive when >1% of the tumour cells showed positive nuclear staining. Patients showing HER2 expression (IHC, score equal to 2+) were subjected to florescence *in situ* hybridization (FISH) to determine *HER2* gene amplification. The HER2 over-expression subgroup was defined as those patients who were FISH-positive or presented an IHC staining score equal to 3+.

Cases and samples

We selected the breast cancer patients with high-risk hereditary background who was previously tested negative

in *BRCA1* and *BRCA2* genes. Breast cancer patients with any two of the five following risk criteria were defined to harbour high-risk hereditary background in the present study: (I) pathological diagnosis of TNBC, (II) male breast cancer, (III) primary bilateral breast cancer, (IV) early-age onset breast cancer (less than or equal to 40 years of age at diagnosis), or (V) positive family history of breast and/or ovarian cancer. All the cases were collected from three independent hospitals in China, which were Fudan University Shanghai Cancer Center, the Affiliated Union Hospital of Fujian Medical University, and Shanghai First Maternity and Infant Hospital. Finally, a total of 384 patients were enrolled and peripheral blood samples were collected. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Fudan University Shanghai Cancer Center (No. 050432-4-1212B) and informed consent was taken from all the patients.

Multigene testing

The Multigene panel includes 30 breast cancer associated susceptibility genes (*Table 1*). All coding regions and exon-intron boundaries of the genes were screened. The average intronic sequence length was 70 bp (ranging from 5 to 204 bp).

Multiplex PCR

Genomic DNA was isolated from peripheral lymphocytes using a TGuide M16 automatic extraction machine (Tiangen Biotechnology, Beijing, China). The DNA concentration was quantified using a NanoDrop ND2000 (NanoDrop Technologies, Wilmington, DE, USA) spectrophotometer, and the samples were diluted to 20–50 ng/μL if the DNA concentration was higher than 50 ng/μL. Thirty-microliter aliquots of the DNA samples were transferred to the wells of a 96-well-plate. A total of 384 extracted genomic DNA samples were used for target capture and sequencing.

All DNA samples were amplified in two separate multiplex PCR assays. Each amplification reaction was prepared by mixing 3 μL of the genomic DNA, 8 μL of each primer panel, 12.5 μL of the KAPA2G Robust hot start ready mix (Kapa Biosystems, Wilmington, MA, USA) and 1.5 μL of H_2O. The PCR program was 95 ℃ for 4 min followed by 18 cycles of 98 ℃ for 15 s and 60 ℃ for 4 min. The PCR products were cleaned up using AMPure XP Beads (Beckman Coulter, Pasadena, CA, USA). The procedure was performed according to the manufacturer's protocol and described in the supplementary materials.

Barcoding and Illumina sequencing

Barcoding was performed in a 20-μL reaction mixture that contained 8 μL of the cleaned PCR products, 10 μL of KAPA2G Robust hot start ready mix (Kapa Biosystems, Pasadena, CA, USA), 1 μmol/L barcode F primers and 1 μmol/L barcode R primer. The reaction was performed in a conventional PCR thermal cycler using the following conditions: 95 ℃ for 30 seconds; 5 cycles of 95 ℃ for 15 seconds, 55 ℃ for 15 seconds, and 72 ℃ for 1 minute; and a completion step at 72 ℃ for 5 minutes.

The barcoded PCR products from the various samples were cleaned up using AMPure XP Beads (Beckman Coulter, Pasadena, CA, USA). The procedure was performed according to the manufacturer's protocol and described in the supplementary materials. The purified PCR product library was quantified using a Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Based on library quantitation, the PCR products were pooled together in equal molar ratios. The purified libraries were routinely sequenced on a NextSeq 500 sequencer (Illumina, San Diego, CA, USA) using the 2×150 bp end sequencing protocol.

Analysis of sequencing data

Demultiplexed, compressed FASTQ files were generated from BCL using bcl2fastq Conversion Software v1.8.4 (Illumina, San Diego, CA, USA). For all successful sequencing runs, the read depth was 30× at any given position, with 100× mean coverage across the entire targeted sequence and Q30 at greater than 75% of reads. The variant calling and coverage of each captured region were analysed using an in-house-developed bioinformatics pipeline based on the general analysis algorithm pipeline. Briefly, the reads were mapped to the hg19 version of the human reference genome (GRCh37) and then filtered to remove off-target and poor-quality reads. Variants were identified and annotated. The variants and annotation results were transferred into Excel spreadsheets.

Interpretation of the mutation testing results

The mutations were classified as benign, likely-benign,

Table 1 The multigene panel of 30 breast cancer susceptibility genes

Breast cancer susceptibility genes	Reference sequence	Breast cancer relative risk or selection criterion	Genetic and biological background
APC	NM_001127511	Familial adenomatous polyposis	*APC* encodes a multi-domain protein that has been implicated in many cellular functions including cellular proliferation, differentiation, cytoskeleton regulation, migration and apoptosis. Inactivating *APC* mutations cause familial adenomatous polyposis, classically characterized by hundreds to thousands of adenomatous colorectal polyps and cancer (16,17)
ATM	NM_000051	2.2–3.7	*ATM* encodes a PI3K-related serine/threonine protein kinase that helps maintain genomic integrity and plays a central role in the repair of DNA double-strand breaks. Germline mutations of *ATM* result in the well-characterized ataxia telangiectasia syndrome (18)
BARD1	NM_000465	Breast cancer association reported	*BARD1* encodes a BRCA1-interacting protein, and heterodimerization of BARD1-BRCA1 via the RING domain is crucial in the homologous recombination repair and transcriptional regulation functions of BRCA1 (19)
BMPR1A	NM_004329	Breast cancer association reported	*BMPR1A* encodes a receptor involved in the bone morphogenetic protein signaling pathway, and is found in the germline of patients with Cowden Syndrome (20)
BRIP1	NM_032043	1.2–3.2	*BRIP1* encodes a helicase-like protein that was identified via its direct binding to the BRCA1 BRCT domains, and is known to contribute to DNA repair via homologous recombination (21)
CDH1	NM_004360	2.2–19.9	*CDH1* encodes E-cadherin, a cell-cell adhesion glycoprotein that acts as a critical invasion suppressor. Loss-of-function germline mutations in the CDH1 tumour-suppressor gene is the cause of hereditary diffuse gastric cancer syndrome (22)
CDK4	NM_000075	Breast cancer association reported	*CDK4* is a potential oncogene, which acts early in the cell cycle and is involved in the transition from G to S phase. All *CDK4* reported mutations are located in exon 2, which codes for the $p16^{INK4A}$ binding site (23)
CDKN2A	NM_000077	1.1–1.7	*CDKNA* encodes the cyclin-dependent kinase inhibitor $p16^{INK4a}$ and the p53 activator $p14^{ARF}$ which are both involved in the negative control of cell proliferation (24)
CHEK2	NM_001005735	2.6–3.5	*CHEK2* encodes a kinase that, when activated, blocks cell-cycle progression in response to DNA damage, and prevents cell transformation and carcinogenesis. The mostly prevalent recurrent mutation in CHEK2 is 1100delC (25)
EPCAM	NM_002354	Breast cancer association reported	*EPCAM* encodes a membrane-bound protein that is localized to the basolateral membrane of epithelial cells and is overexpressed in some tumors. Monoallelic deletions of the 3' end of EPCAM that silence the downstream gene, MSH2, cause a form of Lynch syndrome (26)
MEN1	NM_000244	Breast cancer association reported	*MEN1* encodes a610-amino acid protein referred to as menin. Menin is predominantly a nuclear protein that has roles in transcriptional regulation, genome stability, cell division, and proliferation (27)

Table 1 (*continued*)

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Breast cancer susceptibility genes	Reference sequence	Breast cancer relative risk or selection criterion	Genetic and biological background
MLH1	NM_000249	0.2–2.0	*MLH1* is a tumor suppressor gene involved in DNA mismatch repair. Germline mutations in this gene are known to cause Lynch syndrome. The most common malignancies in Lynch syndrome are colorectal and endometrial carcinomas (28)
MRE11A	NM_005590	Breast cancer association reported	*MRE11A* encodes the part of the tri-molecular MRE11A/RAND50/NBS1 complex, functions as an exonuclease and endonuclease, contributes to single- and double-strand break repair, processes damaged DNA ends and activates the ATM protein, cell cycle checkpoints and apoptotic responses (29)
MSH2	NM_000251	1.2–3.7	*MSH2* encodes the component of post-replicative DNA mismatch repair system which forms two different heterodimers: MutS alpha (MSH2-MSH6 heterodimer) and MutS beta (MSH2-MSH3 heterodimer) which binds to DNA mismatches thereby initiating DNA repair (30)
MSH6	NM_000179	0–13.0	*MSH6* encodes the component of post-replicative DNA mismatch repair system which heterodimerizes with MSH2 to form MutS alpha, which binds to DNA mismatches thereby initiating DNA repair (31)
MUTYH	NM_001048171	1.0–3.4	*MUTYH* encodes for a base excision repair DNA glycosylase. Mutations in this gene cause the *MUTYH*-associated polyposis syndrome, an autosomal recessive inherited condition commonly characterized by the presence of few to hundreds of colonic adenomatous polyps and an increased colorectal cancer risk at young age (32)
NBN	NM_002485	1.9–3.7	*NBN* encodes the part of the genome surveillance complex responsible for DNA damage repair. Homozygous carriers of *NBN* mutations are diagnosed with the Nijmegen Breakage Syndrome, which features immunodeficiency, chromosomal instability, microcephaly as well as a predisposition to various cancers (33)
NF1	NM_000267	2.1–3.2	*NF1* encodes a cytoplasmic protein, termed neurofibromin, which is a large protein containing three alternatively spliced exons (9a, 23a and 48a). The Neurofibromin protein interacts with a number of upstream regulators of Ras signaling, and has the potential to play multiple roles within neurons as part of various intracellular pathways (34)
PALB2	NM_024675	3.0–9.4	*PALB2* encodes for the partner and localizer of BRCA2, which is identified as a BRCA2-interacting protein that is crucial for key BRCA2 genome caretaker functions; it is also shown to interact with BRCA1. Biallelic germline loss-of-function mutations in *PALB2* cause Fanconi's anemia (35)
PALLD	NM_001166108	Breast cancer association reported	*PALLD* encodes a cytoskeletal protein that is required for organizing the actin cytoskeleton. The protein is a component of actin-containing microfilaments, and it is involved in the control of cell shape, adhesion, and contraction (36)
PMS2	NM_000535	Lynch syndrome	*PMS2* encodes for a key component of the mismatch repair system that functions to correct DNA mismatches and small insertions and deletions that can occur during DNA replication and homologous recombination (37,38)
PTCH1	NM_000264	Breast cancer association reported	*PTCH1* encodes a 1447-amino acid transmembrane glycoprotein, which is part of the hedgehog (Hh) pathway. The Hh pathway is a key regulator in embryonic development and tumorigenesis controlling cell differentiation, tissue polarity, and cell proliferation (39)

Table 1 (*continued*)

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Breast cancer susceptibility genes	Reference sequence	Breast cancer relative risk or selection criterion	Genetic and biological background
PTEN	NM_000314	2.0–5.0	*PTEN* encodes a dual-specificity phosphatase that can dephosphorylate both protein and phospholipid substrates. Germline *PTEN* mutations underpin the PTEN Hamartoma-Tumor Syndrome, an umbrella term that includes a range of autosomal-dominant clinical syndromes mainly including Cowden syndrome, presenting in adulthood, and Bannayan-Riley-Ruvalcaba syndrome in children (40)
RAD50	NM_005732	Breast cancer association reported	*RAD50* encodes the RAD50 protein. It plays key roles in DNA double strand breaks repairs, which are crucial to safeguarding genome integrity and sustaining tumor suppression (41)
RAD51C	NM_002876	1.5–7.8	*RAD51C* encodes a crucial protein in homologous recombination, which is involved in loading Rad51 at sites of DNA double-stranded breaks, mediating strand exchange and homologous pairing of DNA sequences. A bi-allelic missense mutation in *RAD51C* causes a Fanconi Anemia-like phenotype (42)
RAD51D	NM_001142571	Breast cancer association reported	*RAD51D* encodes a member of the RAD51 protein family and a constituent of DNA repair mechanism by homologous recombination through the BCDX2 complex formation, which binds to single-stranded DNA after damage and provides homology detection between the damaged and wild-type strand in the repair process (43)
RET	NM_020630	Breast cancer association reported	*RET* encodes a transmembrane receptor and member of the tyrosine protein kinase family of proteins. Binding of ligands such as glial cell-line derived neurotrophic factor and other related proteins to the encoded receptor stimulates receptor dimerization and activation of downstream signaling pathways that play a role in cell differentiation, growth, migration and survival (44)
STK11	NM_000455	2.0–4.0	*STK11* encodes a serine/threonine kinase involved in the regulation of cell growth, polarity and motility. Its inactivation has been initially described in human tumors associated with Peutz-Jeghers hereditary syndrome (45)
TP53	NM_001126115	62.0–165.0	*TP53*, which encodes p53, is a tumor suppressor gene that is frequently mutated in sporadic cancers. The tumor suppressor p53 is a key player in stress responses that preserve genomic stability, responding to a variety of insults including DNA damage, hypoxia, metabolic stress and oncogene activation (46)
VHL	NM_000551	Breast cancer association reported	*VHL* encodes a multifunctional protein that shuttles between the nucleus and cytoplasm whose function links to the pathogenesis of von Hippel-Lindau disease (47)

variants of uncertain significance, likely-pathogenic, and pathogenic. If applicable, detailed information was obtained using the gene-specific databases dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP), ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/). Subsequently, a manual literature search was performed using a Google search in PubMed, Science-Direct, and BioMed Central to confirm that there had been no previous reports on each specific mutation. Novel mutations were defined when there was no match to the reference single-nucleotide polymorphism (RS) numbers in the dbSNP database. Mutations were classified according to American College of Medical Genetics and Genomics recommendations (48) and interpreted as positive for a oncogenic mutation when (I) frameshift insertions or deletions resulted in the expression of an abnormal or truncated protein product; (II) mutations in noncoding intervening sequence at splicing sites caused abnormal processing of the mRNA transcript; or (III) missense mutations and non-frameshift insertions or deletions were defined as pathogenic in a database and/or published study. The mutations with clear oncogenic impacts reported in previous studies were selected for further analysis.

Variant confirmation

A subset of variants, including known variants that were pathogenic or likely pathogenic and newly identified variants with functional damage, was confirmed by conventional Sanger sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA). Variants that could not be confirmed were excluded from further analysis.

Statistical analysis

The Chi-square test, *t*-test and Fisher's exact test were applied in statistical analysis. The statistical analyses were performed using SPSS software version 20.0 (IBM institute, Chicago, IL, USA). All P values in the study were two-sided, and $P<0.05$ was considered statistically significant.

Results

Description of the NGS dataset

Our NGS analysis revealed 18,435 candidate variants in the 30 genes' coding regions and the adjacent splice sites, with a range of 34–78 genetic variants in individual samples. These candidate variants included 27 splicing variants, and 18,408 exonic variants. The exonic variants represented 7,266 missense variants, 11,102 silent variants, 11 stop-gain variants, 3 stop-loss variants, and 26 insertion variants.

Associations between clinical characteristics and mutation status

As it was described above, a total of 384 Chinese breast cancer patients with high hereditary risks were recruited. All the participants were tested to be *BRCA*-negative who came from our previous study (3). The baseline characteristics of breast cancer patients and its relationship with oncogenic mutations were showed in *Table 2*.

A total of 39 (39/384, 10.2%) mutation carriers were identified in our multigene screening. Most kinds of clinical characteristics didn't have statistically significant associations with multigene mutation status, except that breast cancer patients with HER2 positive tended to have a higher mutation prevalence than those with HER2 negative (20% versus 9%, P=0.049).

In our study, the average age at diagnosis of breast cancer was similar between patients with and without germline mutations in these *BRCA*-negative cases (42 versus 39, P=0.431; *Table 3*). However, we found the average age at diagnosis of breast cancer was significantly older for patients with deleterious *RET* mutations than the patients without germline mutations (49 versus 39, P=0.028; *Table 3*). We further evaluated whether patients with mutations in the 30 predisposition genes were associated with a stronger family history of breast or ovarian cancers than non-mutated patients. In particular, all patients with *RET* mutations were enriched for a family history of breast cancer (100% versus 49%, P=0.014; *Table 3*). However, no carriers had a family history of ovarian cancer.

We also evaluated associations between mutation status of single predisposition gene and clinical stages (*Table 4*) as well as tumor pathology (*Table 5*). Overall, carriers and non-carriers had similar tumor stages (*Table 4*). When each receptor was examined alone, we observed *PALB2* mutation carriers were more likely to be ER-positive than non-carriers (80% versus 28%, P=0.027; *Table 4*). Notably, *TP53*-mutated breast cancers were significantly more likely to be ER–, PR– and HER2-positive (100% versus 28%, P=0.024 for ER; 100% versus 27%, P=0.020 for PR; 100% versus 9%, P=0.001 for HER2; *Table 5*).

Table 2 Characteristics of breast cancer patients and mutation carriers

Characteristics	No. of patients	Non-carriers (N=345)		Mutation carriers (N=39)		P
		No.	%	No.	%	
Family history of breast cancer						
Negative	193	175	91	18	9	0.588
Positive	191	170	89	21	11	
Family history of other neoplasms						
Negative	267	236	88	31	12	0.141
Positive	117	109	93	8	7	
Histologic classification						
Carcinoma *in situ*	44	38	86	6	14	0.435
Invasive carcinoma	340	307	90	33	10	
ER status						
Negative	270	246	91	24	9	0.205
Positive	113	98	87	15	13	
Unknown	1	1		0		
PR status						
Negative	275	252	92	23	8	0.069
Positive	108	92	85	16	15	
Unknown	1	1		0		
HER2 status						
Negative	341	310	91	31	9	0.049#
Positive	40	32	80	8	20	
Unknown	3	3		0		
Ki67 status						
<15%	59	50	85	9	15	0.103
≥15%	262	241	92	21	8	
Unknown	63	54		9		
Tumor size						
≤2 cm	185	169	91	16	9	0.395
>2 cm	186	165	89	21	11	
Unknown	13	11		2		
Tumor grade						
I–II	99	92	93	7	7	0.548
III	175	159	91	16	9	
Unknown	110	94		16		

Table 2 (*continued*)

Table 2 (*continued*)

Characteristics	No. of patients	Non-carriers (N=345)		Mutation carriers (N=39)		P
		No.	%	No.	%	
Cancer emboli						
Negative	287	258	90	29	10	0.907
Positive	95	85	89	10	11	
Unknown	2	2		0		
Lymph nodes status						
Negative	262	237	90	25	10	0.546
Positive	121	107	88	14	12	
Unknown	1	1		0		
Stage						
0–II	325	293	90	32	10	0.613
III–IV	49	43	88	6	12	
Unknown	10	9		1		

[#], denote two-sided P<0.05.

Table 3 Gene-based age at diagnosis and family history of cancer

Gene	No. of Mutations	Age at diagnosis (years)*			Family history of cancer[†]							
					Breast				Ovarian			
		Mean	Range	P	Yes	No	Positive %	P	Yes	No	Positive %	P
Mutated genes	39	42	20–92	0.431	21	18	54	0.616	0	39	0	1.000
BRIP1	1	30	30–30	–	0	1	0	1.000	0	1	0	1.000
CDH1	1	32	32–32	–	0	1	0	1.000	0	1	0	1.000
CHEK2	1	34	34–34	–	0	1	0	1.000	0	1	0	1.000
MRE11	1	34	34–34	–	0	1	0	1.000	0	1	0	1.000
MUTYH	11	51	23–92	0.145	7	4	64	0.378	0	11	0	1.000
PALB2	5	38	27–54	0.725	4	1	80	0.172	0	5	0	1.000
PALLD	1	38	38–38	–	0	1	0	1.000	0	1	0	1.000
PTCH1	7	40	34–62	0.901	3	4	43	1.000	0	7	0	1.000
RAD50	1	30	30–30	–	0	1	0	1.000	0	1	0	1.000
RAD51D	2	48	36–59	0.293	0	2	0	1.000	0	2	0	1.000
RET	6	49	34–81	0.028[#]	6	0	100	0.014[#]	0	6	0	1.000
TP53	3	28	20–38	0.073	2	1	67	0.549	0	3	0	1.000
Wildtype	345	39	21–77	Referent	170	175	49	Referent	9	336	3	Referent

[#], denote two-sided P<0.05. *, associations with age at diagnosis were evaluated by *t*-test. [†], associations with family history of breast or ovarian cancer were evaluated by Fisher's exact test.

Table 4 Association between mutation status and clinical stages

Gene	No. of Mutations	Clinical stages																				
		T*							N*							TNM stage*						
		≤2 cm	%	>2 cm	%	Unknown	%	P	Positive	%	Negative	%	Unknown	%	P	I–II	%	III–IV	%	Unknown	%	P
BRIP1	1	0	0	1	100	0	0	0.493	1	100	0	0	0	0	0.313	1	100	0	0	0	0	1.000
CDH1	1	0	0	0	0	1	100	–	0	0	1	100	0	0	1.000	0	0	0	0	1	100	0.131
CHEK2	1	0	0	0	0	1	100	–	0	0	1	100	0	0	1.000	0	0	0	0	1	100	0.131
MRE11	1	1	100	0	0	0	0	1.000	0	0	1	100	0	0	1.000	1	100	0	0	0	0	1.000
MUTYH	11	5	45	6	55	0	0	0.768	5	45	6	55	0	0	0.333	9	82	2	18	0	0	0.641
PALB2	5	4	80	1	20	0	0	0.372	1	20	4	80	0	0	1.000	4	80	1	20	0	0	0.501
PALLD	1	0	0	1	100	0	0	0.493	1	100	0	0	0	0	0.313	1	100	0	0	0	0	1.000
PTCH1	7	3	43	4	57	0	0	0.720	2	29	5	71	0	0	1.000	6	86	1	14	0	0	1.000
RAD50	1	0	0	1	100	0	0	0.493	0	0	1	100	0	0	1.000	1	100	0	0	0	0	1.000
RAD51D	2	0	0	2	100	0	0	0.243	1	50	1	50	0	0	0.527	2	100	0	0	0	0	1.000
RET	6	2	33	4	67	0	0	0.444	3	50	3	50	0	0	0.383	4	67	2	33	0	0	0.180
TP53	3	1	33	2	67	0	0	0.618	0	0	3	100	0	0	0.555	3	100	0	0	0	0	1.000
Wildtype	345	170	49	164	48	11	3	Referent	107	31	237	69	1	0	Referent	293	85	43	12	38	11	Referent

*, associations were evaluated by Fisher's exact test.

Table 5 Association between mutation status and tumor pathology

Gene	No. of Mutations	tumor pathology																					
		ER*							PR*							HER2*							
		Positive	%	Negative	%	Unknown	%	P	Positive	%	Negative	%	Unknown	%	P	Positive	%	Negative	%	Unknown	%	P
BRIP1	1	0	0	1	100	0	0	1.000	0	0	1	100	0	0	1.000	0	0	1	100	0	0	1.000
CDH1	1	0	0	1	100	0	0	1.000	0	0	1	100	0	0	1.000	0	0	1	100	0	0	1.000
CHEK2	1	0	0	1	100	0	0	1.000	0	0	1	100	0	0	1.000	0	0	1	100	0	0	1.000
MRE11	1	0	0	1	100	0	0	1.000	0	0	1	100	0	0	1.000	0	0	1	100	0	0	1.000
MUTYH	11	4	36	7	64	0	0	0.520	5	45	6	55	0	0	0.180	2	18	9	82	0	0	0.287
PALB2	5	4	80	1	20	0	0	0.027#	0	0	5	100	0	0	0.331	0	0	5	100	0	0	1.000
PALLD	1	0	0	1	100	0	0	1.000	0	0	1	100	0	0	1.000	0	0	1	100	0	0	1.000
PTCH1	7	1	14	6	86	0	0	0.678	1	14	6	86	0	0	0.680	0	0	7	100	0	0	1.000
RAD50	1	1	100	0	0	0	0	0.287	1	100	0	0	0	0	0.270	1	100	0	0	0	0	0.096
RAD51D	2	0	0	2	100	0	0	1.000	0	0	2	100	0	0	1.000	0	0	2	100	0	0	1.000
RET	6	2	33	4	67	0	0	1.000	2	33	4	67	0	0	0.661	1	17	5	83	0	0	0.452
TP53	3	3	100	0	0	0	0	0.024#	3	100	0	0	0	0	0.020#	3	100	0	0	0	0	0.001#
Wildtype	345	98	28	246	71	1	0	Referent	92	27	252	73	1	0	Referent	32	9	310	90	3	1	Referent

*, associations were evaluated by Fisher's exact test. #, denote two-sided P<0.05. ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

Table 6 Distribution of patients according to selection criteria

Selection criteria	Enrolled patients, No.	Non-carriers (N=345)		Mutation carriers (N=39)	
		No.	%	No.	%
Harboring two hereditary risks					
Triple-negative BC: male BC	2	2	100	0	0
Triple-negative BC: primary bilateral BC	15	13	87	2	13
Triple-negative BC: early-age onset BC	147	137	93	10	7
Triple-negative BC: family history of BC or OC	57	51	89	6	11
Male BC: early-age onset BC	3	2	67	1	33
Primary bilateral BC: early-age onset BC	18	14	78	4	22
Primary bilateral BC: family history of BC or OC	13	11	85	2	15
Early-age onset BC: family history of BC or OC	99	88	89	11	11
Total	354	318	90	36	10
Harboring three hereditary risks	30	27	90	3	10

BC, breast cancer; OC, ovarian cancer.

Associations between hereditary risk factors and mutation status

According to the study design, all patients were specifically chosen to harbour at least two known risk factors of hereditary background. Breast cancer patients with two risk factors took the main part of our cohort (354/384, 92%), while breast cancer patients with three risk factors took the rest (30/384, 8%). We didn't observe any person who harboured four or five risk factors as described in the selection criteria. In the meanwhile, no male patients with primary bilateral breast cancer or a positive family history of breast/ovarian cancer could be enrolled in our cohort. In our study, most of the participants included were early-age onset patients with triple negative (147/384, 38%), followed by early-age onset patients with a positive family history of breast cancer or ovarian cancer (99/384, 26%) (*Table 6*).

Though the number of patients is rare, male breast cancer patients under 40 years old were very likely to be tested positive in multigene screening (1/3, 33%). The early-age onset patients with primary bilateral breast cancer showed a high prevalence of germline mutation (4/18, 22%), followed by primary bilateral breast cancer with a positive family history of breast/ovarian cancer (2/13, 15%). Interestingly, multigene mutation frequency was similar between breast cancer patients with two risk factors (36/354, 10%) and those with three factors (3/30, 10%).

Multigene germline mutations

Among the 39 patients (39/384, 10.2%) with pathogenic/likely-pathogenic germline mutations, one participant (patient code, 295860) carried two distinct mutations, which were *RET* c.341G>A and *MUTYH* c.C55T (*Table 7*). The major mutant non-*BRCA* genes were *MUTYH* (n=11), *PTCH1* (n=7), *RET* (n=6) and *PALB2* (n=5). Other mutant genes included *TP53* (n=3), *RAD51D* (n=2), *CHEK2* (n=1), *BRIP1* (n=1), *CDH1* (n=1), *MRE11* (n=1), *RAD50* (n=1) and *PALLD* (n=1). We identified 4 novel mutations which were never reported before, including *PALB2* c.2964_2965insAA, *PALB2* c.T1352G, *RAD50* c.C1966T and *RAD51D* c.331_332insTA. A splicing germline mutation, *MUTYH* c.934-2A>G, was demonstrated to be a hotspot (9/384, 2.3%) in Chinese breast cancer. Besides, we observed two recurrent mutations in our cohort, including *RET* c.341G>A (4/384, 1.0%) and *PTCH1* c.2479A>G (6/384, 1.6%) mutations.

The association between distribution of multigene germline mutations and hereditary risks was not statistically apparent. We could merely tell *PALB2* and *RET* mutations possibly tend to occur in breast cancer patients with family history of breast or ovarian cancer, for all those mutations were only observed in groups carrying risk factor of a positive family history of breast or ovarian cancer (*Table 8*). Similarly, *TP53* mutations might associate with breast cancer taking place at a young age for they were all falling

Table 7 The mutations identified as pathogenic/likely pathogenic in our multigene panel screening

Patients code	Gene symbol	Chromosome position (on assembly GRCh37)	RS number	Reference nucleotide base	Alternation nucleotide base	Mutation type	Systematic nomenclature	HGVS protein change
380038	*TP53*	chr17:7577538	rs11540652	C	T	Nonsynonymous SNV	NM_000546.5:c.743G>C	R116Q
297311	*TP53*	chr17:7574034	rs587782272	C	G	Splicing	NM_000546.4:c.994-1G>A	–
303498	*TP53*	chr17:7578407	rs138729528	G	C	Nonsynonymous SNV	NM_000546.5:c.C523G	R175G
253180	*PALB2*	chr16:23619236	rs1567206813	G	GT	Frameshift insertion	NM_024675.3:c.3298dupA	T1100fs
281943	*PALB2*	chr16:23634321	–	C	CTT	Frameshift insertion	NM_024675.3:c.2964_2965insAA	V989fs
305158	*PALB2*	chr16:23646515	–	A	C	Stopgain	NM_024675.3:c.T1352G	L451X
388870	*PALB2*	chr16:23646815	rs886039738	GTT	G	Frameshift insertion	NM_024675.3:c.1050_1051del	Q350fs
341870	*PALB2*	chr16:23647116	rs180177091	G	A	Stopgain	NM_024675.3:c.751C>T	Q251X
382275	*CHEK2*	chr22:29091846	rs531398630	G	A	Nonsynonymous SNV	NM_007194.3:c.1111C>T	H342Y
371054	*RET*	chr10:43597793	rs76397662	G	A	Nonsynonymous SNV	NM_020975.4:c.341G>A	R114H
345675	*RET*	chr10:43597793	rs76397662	G	A	Nonsynonymous SNV	NM_020975.4:c.341G>A	R114H
295860	*RET*	chr10:43597793	rs76397662	G	A	Nonsynonymous SNV	NM_020975.4:c.341G>A	R114H
291491	*RET*	chr10:43597793	rs76397662	G	A	Nonsynonymous SNV	NM_020975.4:c.341G>A	R114H
374885	*RET*	chr10:43601830	rs34682185	G	A	Nonsynonymous SNV	NM_020975.4:c.874G>A	V292M
252737	*RET*	chr10:43601830	rs34682185	G	A	Nonsynonymous SNV	NM_020975.4:c.874G>A	V292M
398850	*MUTYH*	chr1:45797760	rs77542170	T	C	Splicing	NM_001128425.1:c.934-2A>G	–
367026	*MUTYH*	chr1:45797760	rs77542170	T	C	Splicing	NM_001128425.1:c.934-2A>G	–
360832	*MUTYH*	chr1:45797760	rs77542170	T	C	Splicing	NM_001128425.1:c.934-2A>G	–
334744	*MUTYH*	chr1:45797760	rs77542170	T	C	Splicing	NM_001128425.1:c.934-2A>G	–
316506	*MUTYH*	chr1:45797760	rs77542170	T	C	Splicing	NM_001128425.1:c.934-2A>G	–
311452	*MUTYH*	chr1:45797760	rs77542170	T	C	Splicing	NM_001128425.1:c.934-2A>G	–
304731	*MUTYH*	chr1:45797760	rs77542170	T	C	Splicing	NM_001128425.1:c.934-2A>G	–
304587	*MUTYH*	chr1:45797760	rs77542170	T	C	Splicing	NM_001128425.1:c.934-2A>G	–
291710	*MUTYH*	chr1:45797760	rs77542170	T	C	Splicing	NM_001128425.1:c.934-2A>G	–
345039	*MUTYH*	chr1:45798130	rs34126013	G	A	Nonsynonymous SNV	NM_001128425.1:c.721C>T	R241W
295860	*MUTYH*	chr1:45800165	rs587780088	G	A	Stopgain	NM_001128425.1:c.C55T	R19X
389336	*BRIP1*	chr17:59876486	rs587780226	G	A	Stopgain	NM_032043.2:c.1315C>T	R439X
335773	*CDH1*	chr16:68846047	rs116093741	A	G	Nonsynonymous SNV	NM_004360.4:c.1018A>G	T340A

Table 7 (*continued*)

Table 7 (*continued*)

Patients code	Gene symbol	Chromosome position (on assembly GRCh37)	RS number	Reference nucleotide base	Alternation nucleotide base	Mutation type	Systematic nomenclature	HGVS protein change
371693	*MRE11*	chr11:94211948	rs587782308	G	A	Nonsynonymous SNV	NM_005591.3:c.497C>T	P166L
314705	*PTCH1*	chr9:98211548	–	TG	T	Frameshift deletion	NM_000264.3:c.3606del	S1203fs
400359	*PTCH1*	chr9:98229479	rs199476092	T	C	Nonsynonymous SNV	NM_000264.3:c.2479A>G	S827G
394246	*PTCH1*	chr9:98229479	rs199476092	T	C	Nonsynonymous SNV	NM_000264.3:c.2479A>G	S827G
383458	*PTCH1*	chr9:98229479	rs199476092	T	C	Nonsynonymous SNV	NM_000264.3:c.2479A>G	S827G
371518	*PTCH1*	chr9:98229479	rs199476092	T	C	Nonsynonymous SNV	NM_000264.3:c.2479A>G	S827G
337089	*PTCH1*	chr9:98229479	rs199476092	T	C	Nonsynonymous SNV	NM_000264.3:c.2479A>G	S827G
164622	*PTCH1*	chr9:98229479	rs199476092	T	C	Nonsynonymous SNV	NM_000264.3:c.2479A>G	S827G
274548	*RAD50*	chr5:131930733	–	C	T	Stopgain	NM_005732.3:c.C1966T	R656X
289068	*PALLD*	chr4:169589381	rs769584673	A	ATTCAAATCCACT GTGAGGGAGGG	Frameshift insertion	NM_001166108.1:c.949_950ins TTCAAATCCACTGTGAGGGAGGG	I317fs
392489	*RAD51D*	chr17:33434458	–	T	TTA	Frameshift insertion	NM_001142571:c.331_332insTA	K111fs
316014	*RAD51D*	chr17:33434458	–	T	TTA	Frameshift insertion	NM_001142571:c.331_332insTA	K111fs

SNV, single nucleotide variant.

Table 8 Distribution of germline mutations in breast cancer patients according to selection criteria

Selection criteria	*BRIP1*	*CDH1*	*CHEK2*	*MRE11*	*MUTYH*	*PALB2*	*PALLD*	*PTCH1*	*RAD50*	*RAD51D*	*RET*	*TP53*
Harboring two hereditary risks												
Triple-negative BC: male BC												
Triple-negative BC: primary bilateral BC					1					1		
Triple-negative BC: early-age onset BC	1	1	1	1	1		1	3		1		
Triple-negative BC: family history of BC or OC					3	1		1			2	
Male BC: early-age onset BC					1							
Primary bilateral BC: early-age onset BC					1	1			1			1
Primary bilateral BC: family history of BC or OC					1						1	
Early-age onset BC: family history of BC or OC					3	3		1			2	2
Total	1	1	1	1	11	5	1	5	1	2	5	3
Harboring three hereditary risks								2			1	

BC, breast cancer; OC, ovarian cancer.

into groups carrying risk factor of early-age onset.

Discussion

The present study demonstrated about 10% of Chinese breast cancer patients with high hereditary risk who were previously tested *BRCA*-negative could benefit from multigene testing. Our study contributed to the knowledge of germline variations in multiple cancer susceptible genes in Chinese population. In previous studies, beyond *BRCA1* and *BRCA2*, the prevalence of germline mutations varied from 4.3% to 34.3% according to different recruiting criteria, gene panels or sequencing methods (49-55). Li *et al.* conducted a multi-centre study to investigate mutational frequency in Chinese patients with high hereditary risk breast cancer patients, and the study showed 23.8% of participants contained germline mutations, including 6.8% in 38 other non-*BRCA* genes (52). Similarly, the study also defined multiple hereditary risks as selection criteria. A more recent study carried out by Wang *et al.* found 8.5% of patients harboured non-*BRCA* oncogenic mutations through a 22-gene panel screening, which were mainly found in *ATM*, *CHEK2*, *PALB2*, and *BRIP1* genes (55). In a much larger study with more susceptibility genes testing, the data from 8,085 cases demonstrated a mutation frequency of 2.9% in non-*BRCA* susceptibility genes (54). In spite of the fact that a more general gene panel was applied, the mutation frequency didn't go up as the rising number of sequenced genes. However, it seemed quite different when genes number crossing over one hundred. There is another study using a panel of 152 genes associated with hereditary cancer, and the study identified 16.1% of hereditary breast cancer patients as non-*BRCA* germline mutation carriers. Taken together, these collective evidences suggested criteria should be carefully chosen when using a small gene panel to detect genetic variations in hereditary breast cancer patients.

In our previous study, we observed *BRCA* mutation frequency raised up with hereditary risk factors added up (3). However, the theory didn't work well in non-*BRCA* mutations. It was noted that multigene mutation frequency was similar between breast cancer patients with two risk factors (36/354, 10%) and those with three factors (3/30, 10%) in our present cohort. Due the limited sample size and the lack of comparable study, it is hard to tell a difference for now, so more data and larger studies await to demonstrate such phenomenon.

PALB2 germline mutation frequency was demonstrated to be 1.3% in our study, and the results varied from 0.7–1.2% in other Chinese studies (52,56,57). We further observed a potential association between *PALB2* mutation carriers and breast cancer with a positive history of breast/ovarian cancer, and other studies also proved the conclusion (56,57). Wu *et al.* performed *PALB2* mutation screening a large Chinese breast cancer cohort, and demonstrated that compared with non-carriers, *PALB2* mutation carriers were significantly more likely to have a familial aggregation of breast cancer and/or ovarian cancer (27.8% *vs.* 8.4%, P<0.001) (57). In the meanwhile, we also *RET* mutations tended to occur in breast cancer patients with family history of breast or ovarian cancer, but no further studies support the conclusion for *RET* mutations were less studied in breast cancer. A previous study only found one *RET* mutation carriers out of 8,085 consecutive unselected Chinese breast cancer patients (54). It seemed *RET* mutations could be more prevalent in breast cancer with high hereditary risk which needed to be confirmed by further investigation.

As mentioned before, we identified a hotspot germline mutation, *MUTYH* c.934-2A>G, in Chinese breast cancer. *MUTYH* is a human base excision repair gene involved in preventing 8-oxo-dG-induced mutagenesis (58). Bi-allelic germline mutations of the *MUTYH* gene lead to autosomal recessive colorectal adenomatous polyposis and very high colorectal cancer risk in Caucasian population (59,60). *MUTYH* c.934-2A>G was first found in Japanese familial gastric cancer patients and also demonstrated to cause a splicing abnormality that led to the production of an aberrant mRNA transcript encoding a truncated MYH protein and lead to an impaired ability of excision repair (61). Interestingly, experts hold converse opinion about the *MUTYH* mutation, saying that some support its pathogenicity (62-65), while some do not (52,66,67). Notably, a Chinese study reported a relatively high variant rate (4.2%, 5/120) of *MUTYH* c.892-2A>G in their high-risk group, but lower rate (0.8%, 1/120) in their breast cancer group (66). According to the 5-tier rating system in American College of Medical Genetics and Genomics recommendations, *MUTYH* c.934-2A>G is likely pathogenic (48). Besides, another Chinese study also noticed 8 *MUTYH* mutation carriers out of 937 patients with high hereditary risk breast cancer (52). Moreover, a more recent study identified a *MUTYH* germline pathogenic variant and somatic loss of the wild-type allele which contributed to tumorigenesis (65). Considering all above, with currently available evidence suggesting that the variant is pathogenic, but the available data is insufficient

to prove that conclusively. Therefore, this variant was classified as likely pathogenic in our study.

We also explored whether the mutation status could impact the survival in these *BRCA*-negative breast cancer (data not showed), but no significant results were observed in comparing disease-free survival (DFS) or overall survival between the germline mutation carriers and non-carriers. Previous studies came to inconsistent conclusions about *BRCA* mutation status as a prognostic factor in breast cancer (68-73). Among other predisposition genes, *CHEK2* 1100delC was demonstrated to be associated with increased risk of second breast cancer and a worse long-term recurrence-free survival rate (74). Another study indicated *CHEK2* H371Y mutation carriers were more likely to respond to neoadjuvant chemotherapy than non-carriers (75). However, we failed to identify these two mutations in our cohort. Moreover, breast cancer patients with *PALB2* mutations were considered to be at a higher risk of death from breast cancer compared with non-carriers (76). A more recent study involved 16 DNA-repair genes including *ATM*, *BLM*, *CHEK2*, *FANCC*, *MER11A*, *MLH1*, *MSH2*, *MSH6*, *MUTHY*, *NBN*, *PALB2*, *PMS2*, *RAD50*, *RAD51C*, *RAD51D* and *TP53* (77), where most genes were also comprised in our study. The study concluded that 3.4% of *BRCA*-negative breast cancer patients carried germline mutations in the 16 DNA-repair genes, and the DNA-repair gene mutation carriers exhibited an aggressive phenotype and had poor survival compared with non-carriers. By virtue of the germline mutations, breast cancers harboring these mutations had unique mechanisms that could be rationally targeted for therapeutic opportunities. Increasing evidences demonstrated mutations in *non-BRCA1/2* DNA-repair genes contributed to sensitivity to PARP inhibitors, which suggested carriers of mutated DNA-repair genes could undergo treatment with PARP inhibitors (78). Besides PARP, there were other key components, like *PTEN* (79-81), *ATM* (82), *MSH2* (83,84) and *APC* (85), showing potentials for targeted therapy.

In conclusion, appropriately selected patients may gain benefit from multigene sequencing, and comprehensive gene panels could help understand hereditary mutations in genetic counselling, for hereditary breast cancer could be associated with more than breast cancer specific susceptibility genes especially when it was tested *BRCA*-negative. As the costs of genomic testing decline and the benefits of sequencing appearing, it is inevitable that the use of gene-panel testing, even whole-exome and whole-genome sequencing, will become widespread and come into daily clinical practice in China.

Conclusions

Our study demonstrated 10% of Chinese breast cancer patients with high hereditary risk who were previously tested *BRCA*-negative could benefit from multigene testing. Comprehensive gene panels could help understand hereditary mutations in genetic counselling, for hereditary breast cancer could be associated with more than breast cancer specific susceptibility genes when it was tested *BRCA*-negative.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was

conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Fudan University Shanghai Cancer Center (No. 050432-4-1212B) and informed consent was taken from all the patients.

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