Identification of a Comprehensive Spectrum of Genetic Factors for Hereditary Breast Cancer in a Chinese Population by Next-Generation Sequencing

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Abstract

The genetic etiology of hereditary breast cancer has not been fully elucidated. Although germline mutations of high-penetration genes such as BRCA1/2 are implicated in development of hereditary breast cancers, at least half of all breast cancer families are not linked to these genes. To identify a comprehensive spectrum of genetic factors for hereditary breast cancer in a Chinese population, we performed an analysis of germline mutations in 2,165 coding exons of 152 genes associated with hereditary cancer using next-generation sequencing (NGS) in 99 breast cancer patients from families of cancer patients regardless of cancer types. Forty-two deleterious germline mutations were identified in 21 genes of 34 patients, including 18 (18.2%) BRCA1 or BRCA2 mutations, 3 (3%) TP53 mutations, 5 (5.1%) DNA mismatch repair gene mutations, 1 (1%) CDH1 mutation, 6 (6.1%) Fanconi anemia pathway gene mutations, and 9 (9.1%) mutations in other genes. Of seven patients who carried mutations in more than one gene, 4 were BRCA1/2 mutation carriers, and their average onset age was much younger than patients with only BRCA1/2 mutations. Almost all identified high-penetration gene mutations in those families fulfill the typical phenotypes of hereditary cancer syndromes listed in the National Comprehensive Cancer Network (NCCN) guidelines, except two TP53 and three mismatch repair gene mutations. Furthermore, functional studies of MSH3 germline mutations confirmed the association between MSH3 mutation and tumorigenesis, and segregation analysis suggested antagonism between BRCA1 and MSH3. We also identified a lot of low-penetration gene mutations. Although the clinical significance of those newly identified low-penetrance gene mutations has not been fully appreciated yet,
these new findings do provide valuable epidemiological information for the future studies. Together, these findings highlight the importance of genetic testing based on NCCN guidelines and a multi-gene analysis using NGS may be a supplement to traditional genetic counseling.

Introduction

Germline mutations in BRCA1 (MIM# 113705) and BRCA2 (MIM# 600185) are the most common cause of hereditary breast cancer, and early diagnosis and preventive interventions have been confirmed to improve the survival rate of mutation carriers [1]. However, BRCA1 and BRCA2 germline mutations are present in only half of all families with a strong family history of breast cancer. Other high-penetrance genes that also play an important role in the genetic etiology of breast cancer include TP53, PTEN and CDH1 [2–4]. Additionally, germline mutations in many moderate-to-low-penetrance genes can also lead to hereditary breast cancer [5].

In clinical practice, the families of breast cancer probands present with multiple types of malignant tumors that are not limited to breast and ovarian cancers. Moreover, the correlation between breast cancer and several other hereditary cancer syndromes remains unclear. Breast cancer may not represent the primary phenotype of these hereditary cancer syndromes; however, it occurs frequently [6]. In traditional cancer genetic counseling, prior to genetic testing, families are divided into different hereditary cancer syndromes based on the characteristics of the probands and their family history. Subsequently, the related genes are tested to determine the etiology of the disease. This approach is useful for identifying high-risk populations and susceptibility genes and for improving detection efficiency; however, some individuals may not be successfully identified using this method [7, 8]. Currently, a mature genetic counseling system in China is not available, and there is a lack of relevant systematic studies.

In recent years, the rapid development of next-generation sequencing (NGS) technologies has enabled the simultaneous sequencing of multiple genes, and the cost is comparable to that of single-gene sequencing using traditional sequencing techniques. Studies have suggested that such cost-effective approach could be used to the genetic testing and personalized risk assessment for individuals at high risk of breast cancer [9]. Our study utilized two important approaches. First, we randomly selected family members of breast cancer probands and did not limit the cancer types in the family history. Second, we selected 152 genes associated with hereditary cancer and performed comprehensive testing using NGS. Through these efforts, we aimed to obtain comprehensive results regarding the genes for hereditary breast cancer susceptibility in the Chinese population and to lay a foundation for the development of cancer genetic counseling in China. The value of NGS applied in genetic counseling also needed to be evaluated.

Materials and Methods

Ethics statement

This study was approved by the Scientific and Ethical Committee of the Shanghai Cancer Center, Fudan University. Written informed consent was obtained from all participants.

Patient/Sample Selection

Breast cancer patients were consecutively enrolled after diagnosis at the Shanghai Cancer Center of Fudan University from June 2011 to July 2012. To be eligible for the study, the breast cancer patients were required to meet one of the following inclusion criteria:
1. Age younger than or equal to 35 years with at least one other blood relative suffering from any type of cancer
2. Age older than 35 and younger than or equal to 50 years with ≥2 blood relatives in the same lineage suffering from any type of cancer
3. Age older than 50 years with ≥3 blood relatives in the same lineage suffering from any type of cancer.

Pedigrees and medical records were collected via questionnaires at study entry. Pathologies and other medical reports were reviewed for the probands and their blood relatives when available. For each patient, blood and formalin-fixed paraffin-embedded (FFPE) specimens were collected from each patient, and samples from relatives of the probands who carried candidate mutations were obtained when available. Genomic DNA was extracted using a Chemagic STAR DNA Blood 4k Kit (PerkinElmer, Waltham, Massachusetts, USA) or a QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer’s recommended protocol.

Gene Selection
In total, 152 genes were tested, selected as cancer susceptibility genes based on a PubMed keyword search of (“Neoplasms” [Mesh] AND “germline mutation”) in 8334 studies published up to June 2012. Among these genes, 115 are known to be associated with hereditary cancer syndromes. A summary of the genes is provided in S1 Table.

Targeted Capture and Massively Parallel Sequencing
A sample of 3 μg of genomic DNA was fragmented to 150–200 bp using sonication (Covaris Inc., Woburn, MA), and adapters were subsequently ligated to both ends of the resulting fragments. After purification and amplification, target-region capture was performed using the pre-designed Human SureSelect XT Custom Kit (700 Kb-34 Mb, Agilent Technologies) according to the recommended protocols. Captured libraries were analyzed using an Agilent 2100 Bioanalyzer to estimate the magnitude of enrichment and were sequenced with 90-bp paired end reads on a HiSeq2000 platform subsequently (Illumina, San Diego, CA, USA). We independently performed high-throughput sequencing of each captured library to ensure that each sample met the desired average fold coverage. Coverage was calculated by counting the number of sequenced bases that mapped to the target regions. Bases mapping to regions within a 200-bp range of a target were considered “near target”.

Bioinformatic Analysis of DNA Variants
Raw image files were processed using Illumina base-calling software 1.7 with default parameters, and the sequences for each individual were generated as 90-bp paired-end reads. After filtering, high-quality reads were aligned to the human genome (GRCH37, UCSC hg19) using the Burrows-Wheeler Aligner program [10]. SAMtools [11] and SOAPsnp [12] were used for the identification of small insertions or deletions (InDels) and single nucleotide polymorphisms (SNPs), respectively. SNPs were called using SOAPsnp with options: -r 0.00005 -e 0.0001 -t -u -L 90-Q L. Next, filters of quality score (≥20), neighbor distance (≥5) and depth (≥4) were applied to the SNPs calling results. Finally, ANNOVAR [13] was used for the annotation of InDels and SNPs.

Filtering of Variants
Variants were selected for follow-up when all of the following criteria applied:
1. Variants were located in the exonic region or were within -2 bp away from an exon/intron boundary (splicing).

2. Variants were InDels, nonsynonymous (missense or nonsense) or splicing variants.

3. Variants did not occur within the 1000 Genomes (1000G) data with a minor allele frequency (MAF) greater than 0.01[14].

4. Variants were consistent with the known pattern of inheritance of the respective gene.

   If prioritization resulted in a single strong candidate allele (a nonsense or canonical splice site variant) in a recessively acting gene, a manual literature search was performed to confirm the pathogenicity of the heterozygous mutation.

**Determination of Pathogenicity of Variants**

Frameshift InDels, non-frameshift InDels, nonsense mutations, and splicing mutations were considered to be pathogenic. For missense mutations, we developed a classification system based on *in silico* evidence, which contained two different features: (1) missense prediction software (acting at the amino acid level) and (2) evolutionary conservation (acting at the nucleotide level).

1. In the case of missense prediction programs, SIFT [15] and PolyPhen-2 [16] were used. A variant was considered to be “damaging” when either prediction program predicted it as “damaging.”

2. For classification based on evolutionary conservation, LJB_PhyloP [17] and LJB_LRT [18] were used. A variant was considered to be “damaging” when either prediction program predicted it as “conserved.”

Variants considered to be “damaging” by both classifications (prediction tools and conservation) were predicted as “damaging” and were manually referenced to gene-specific mutation databases or published studies. Those with clear pathogenic impact, as reported by previous studies, were selected for further analysis.

**Variant Validation and Segregation Analysis**

All candidate variants were manually referenced to gene-specific mutation databases or published studies and were validated using conventional Sanger sequencing (ABI PRISM 3730XL Genetic Analyzer). Where available, DNAs from additional family members were sequenced to enable segregation analysis. The sequencing results were evaluated using Chromas software, version 2.4.1 (Technelysium Pty Ltd., South Brisbane QLD, Australia).

**Functional Studies of MSH3 Germline Mutation**

For patients with mutations in the DNA mismatch repair (*MMR*) gene *MSH3*, the expression of Msh3 protein was evaluated by immunohistochemistry (IHC) using an anti-Msh3 rabbit monoclonal antibody (clone EPPR4334(2); Epitomics, USA) at a dilution of 1:500. The expression of Msh3 was scored by two specialized pathologists (W.T.Y. and X.Y.Z.) as follows: 0, no staining; 1+, weak staining; 2+, intermediate staining; and 3+, strong staining. Microsatellite instability (MSI) was determined using fluorescent multiplex PCR of paired normal and tumor DNA samples by employing the MSI analysis system on an ABI PRISM 3500 Avant Genetic Analyzer (Applied Biosystems, Carlsbad, California, USA). Fluorescent multiplex PCR was performed using the RT-PCR Quick Master Mix Kit (Toyobo, Osaka, Japan). Five consensus
National Cancer Institute (NCI) microsatellite markers (BAT25, BAT26, D2S123, D5S346, and D17S250), two additional dinucleotide repeat markers (D18S64 and D18S69) and seven elevated microsatellite alterations at selected tetra-nucleotide repeat (EMAST) markers (MYCL1, D20S82, D20S85, L17835, D8S321, D9S242 and D19S394) were used. The results were analyzed by two specialized pathologists (W.T.Y. and X.Y.Z.). The criteria for the determination of MSI were those described by Haugen et al. [19].

Statistical Analysis

The average age of onset of patients carrying a single BRCA1/2 mutation and those carrying other gene mutations in addition to a BRCA1/2 mutation were compared using a t-test. The analysis was performed using SPSS software, version 19.0 (IBM institute). All P values were two-sided, and any P value <0.05 was considered significant.

Accession Codes

The GenBank reference sequences used for variant annotation are summarized in S2 Table. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence. The initiation codon is codon 1.

Results

Patient Characteristics

Of the 3,102 breast cancer patients who underwent surgery in our hospital, 134 met the inclusion criteria. Thirty-five patients refused enrollment, and 99 patients entered the study. These patients are all independent with no blood relationship. They are recruited from families with at least 2 relatives suffering from certain type of cancer to ensure that germline mutations, rather than somatic mutations or environmental exposures, are the determinations of cancer. The characteristics of the 99 probands and their families are presented in Fig 1. Infiltrating ductal carcinoma was the most prominent histological type and accounted for approximately 92% of all types. Thirteen patients exhibited bilateral disease, and 11 patients in addition to breast cancer also suffered from another malignant tumor. Breast cancer was the most common malignancy within the family history (59.6%). According to the National Comprehensive Cancer Network (NCCN) guidelines (version 4.2013), 88, 11 and 15 families fulfilled the criteria for hereditary breast and ovarian cancer syndrome (HBOCS), Li-Fraumeni syndrome (LFS, according to classic LFS criteria or Chompret criteria), and Lynch syndrome (LS, according to the revised Bethesda criteria), respectively.

Mutation Detection and Characterization

A total of 99 breast cancer patients were screened for germline mutations in 2,165 coding exons of 152 genes. The average sequencing depths of the targeted regions were 93 to 123. Over 98% coverage of the targeted regions was achieved for each proband. The coverage of the targeted exons for the >20× reads ranged from 87.60% to 92.60% and its standard deviations was 0.93%. Thus, the coverage should have been adequate to reliably detect DNA variants within the majority of the targeted regions. The NGS results, including the number of reads, sample coverage, and sequencing depth, are summarized in S3 Table. Overall, 4883 InDels and 57377 SNPs were obtained. Systematic filtering of variants was accomplished as described in Materials and Methods and is summarized in Fig 2. A total of 42 putative deleterious germline mutations in 21 genes (Fig 3) were identified in 34 breast cancer patients, including 22 frameshift InDels, 5 non-frameshift InDels, 8 nonsense mutations, 5 missense mutations and 2 splicing...
mutations. Features of the mutations, family characteristics, segregation analysis and age of diagnosis for each of the patients carrying a mutation are provided in Table 1.

### Mutation Analyses of BRCA1/2, TP53, and MMR Genes

Eighteen (18.2%) patients were found to carry BRCA1/2 germline mutations (7 in BRCA1 and 11 in BRCA2) (Table 1). Two recurrent mutations, BRCA1 c.5468-1_5474del8 and BRCA2 c.2442delC, were each identified in two unrelated patients. Based on their family history, all patients with BRCA1/2 mutations met the criteria for HBOCS, and these patients accounted for 20.5% (18/88) of the families who fulfilled the HBOCS criteria. The frequency of triple-negative breast cancer was 71.4% (5/7) in BRCA1 mutation carriers and 9.1% (1/11) in BRCA2 mutation carriers.

Three TP53 germline mutations were identified. One was novel and another two were detected within the germline and somatic mutations in the p53 mutation database, respectively [20] (Table 1). One family with TP53 mutation met the criteria for LFS and accounted for 9.1% of all families who fulfilled the LFS criteria. Two of the carriers were diagnosed with breast...
cancer under the age of 30, and they accounted for 10% (2/20) of all very young (<30 years) breast cancer patients in our study. One carrier of a TP53 mutation whose family history did not meet the LFS criteria was diagnosed at age 37 and reported a family history of malignant phylloides tumor of the breast (PTB). Another TP53 mutation carrier whose family met the criteria for LFS also reported a family history of PTB.

Deleterious mutations within the MMR genes MLH1, MLH3, and MSH3 were detected in five distinct families. Three mutations were novel and two were previously reported (Table 1). Two families met the criteria for LS and accounted for 13.3% (2/15) of the families fulfilling the LS criteria.

In summary, all BRCA1/2 mutations occurred in patients with a family history of HBOCS, but two TP53 mutations occurred in patients without a family history of LFS, and three MSH3 mutations occurred in patients without a family history of LS (Table 2).

Mutation Analyses of Other Genes

One deleterious mutation was found in CDH1 and this mutation has been previously reported (Table 1). The patient carrying the CDH1 mutation had infiltrating ductal carcinoma of the breast, and her two blood relatives in the same lineage were diagnosed with gastric cancer; however, the histological types of the two gastric cancers were unknown.
Six Fanconi anemia (FA) pathway genes were found to carry loss-of-function mutations (Table 1). These genes were \textit{RAD50}, \textit{PALB2}, \textit{FANCD2}, \textit{FANCI}, \textit{SLX4} and \textit{RAD51C}. They accounted for 31.6% (6/19) of all of the non-\textit{BRCA} mutation genes, which confirmed that multiple genes in the FA pathway are associated with breast cancer risk [21].

Nine deleterious mutations were identified in eight other genes (Table 1), \textit{RGSL1}, \textit{CDKN2A}, \textit{SPINK1}, \textit{TNFRSF13B}, \textit{FGFR3}, \textit{WRN}, \textit{MUTYH} and \textit{CYP17A1}. The last three genes exhibit an autosomal recessive mode of inheritance. Studies have reported that these gene mutations can also increase the risk of breast cancer or other cancers [22–29].

**Patients with Mutations in Two or More Genes**

Seven patients were found to carry mutations in two or more genes (20.6%, 7/34). Among these patients, four were carriers of \textit{BRCA1/2} mutations. Details about the patients carrying mutations in two or more genes are provided in Table 3 and Fig 1. In comparison to patients with single mutations in \textit{BRCA1/2}, the average age of onset of patients carrying mutations in other genes in addition to \textit{BRCA1/2} was 7 years earlier, regardless of whether the comparison
| ID  | Age (yrs) | Gene          | DNA change       | Protein change | Chr | Mutant site start | Mutant site end | Segregation | 1000G MAF³ | Missense prediction SIFT² | Missense prediction PolyPhen-2° | Evolutionary conservation PhyloP² | Evolutionary conservation LRT° | Reference |
|-----|----------|---------------|------------------|----------------|-----|-------------------|----------------|-------------|-------------|--------------------------------|---------------------------------|--------------------------------|---------------------------------|-----------|
| 56  | 35       | HBOCS        | BRCA1            | c.754delC      | p.R252Vfs*46 | 17  | 41246794          | 41426794       | 2/2         | 0                       | --                               | --                              | --                              | Novel     |
| 6   | 32       | HBOCS        | BRCA1            | c.1410_1411delG | p.S469*     | 17  | 41246334          | 41426334       | 2/3         | 0                       | --                               | --                              | --                              | Novel     |
| 22  | 37       | HBOCS        | BRCA1            | c.2121_2111delAA | p.N704fs*7  | 17  | 41245408          | 41424549       | 5/6         | 0                       | --                               | --                              | --                              | BIC       |
| 5   | 40       | HBOCS        | BRCA1            | c.422delG      | p.E1410fs*5  | 17  | 41245651          | 41424551       | 3/4         | 0                       | --                               | --                              | --                              | Novel     |
| 29  | 39       | HBOCS        | BRCA1            | c.5503delT     | p.R1835*    | 17  | 411957784         | 411957784      | 3/3         | 0                       | --                               | --                              | --                              | BIC       |
| 26  | 40       | HBOCS        | BRCA1            | c.5468_1_5474delII | p.A1823_303*9 | 17  | 41197813          | 41197820       | 3/3         | 0                       | --                               | --                              | --                              | [33]      |
| 52  | 34       | HBOCS        | BRCA1            | c.5468_1_5474delII | p.A1823_303*9 | 17  | 41197813          | 41197820       | 6/6         | 0                       | --                               | --                              | --                              | [33]      |
| 3   | 46       | HBOCS        | BRCA2            | c.2442delC     | p.M819fs*10 | 13  | 32910934          | 32910944       | 2/3         | 0                       | --                               | --                              | --                              | [33, 54] |
| 4   | 42       | HBOCS, LS   | BRCA2            | c.2442delC     | p.M819fs*10 | 13  | 32910934          | 32910944       | 3/3         | 0                       | --                               | --                              | --                              | [33, 54] |
| 68  | 38       | HBOCS        | BRCA2            | c.2808_2811delAAA | p.A903fs*21 | 13  | 32911300          | 32911303       | UA          | 0                       | --                               | --                              | --                              | BIC       |
| 2   | 37       | HBOCS        | BRCA2            | c.5682delG     | p.Y1894*    | 13  | 32914174          | 32914174       | 2/2         | 0                       | --                               | --                              | --                              | BIC       |
| 7   | 30       | HBOCS        | BRCA2            | c.5699delG     | p.S1900*    | 13  | 32914191          | 32914191       | 12          | 0                       | D                                | B                              | C                              | NC        |
| 54  | 70       | HBOCS        | BRCA2            | c.700delA      | p.R2336H    | 13  | 32921033          | 32921033       | UA          | 0                       | D                                | B                              | C                              | NC        |
| 24  | 26       | HBOCS, LS   | BRCA2            | c.714delC      | p.P238fs*13 | 13  | 32929132          | 32929132       | 5/6         | 0                       | --                               | --                              | --                              | Novel     |
| 45  | 43       | HBOCS        | BRCA2            | c.740_9dupT    | p.T2471_11*4 | 13  | 32929399          | 32929399       | UA          | 0                       | --                               | --                              | --                              | [31]      |
| 42  | 34       | HBOCS, LS   | BRCA2            | c.848_50C>T    | p.G2829*    | 13  | 32944692          | 32944692       | 2/2         | 0                       | --                               | --                              | --                              | BIC       |
| 13  | 32       | HBOCS        | BRCA2            | c.8517C>A      | p.Y2839*    | 13  | 32945122          | 32945122       | UA          | 0                       | D                                | B                              | C                              | C         |
| 71  | 28       | HBOCS        | BRCA2            | c.895_897delAA | p.I286_906*3 | 13  | 32953889          | 32953889       | UA          | 0                       | --                               | --                              | --                              | Novel     |
| 65  | 30       | HBOCS, LS   | TP53             | c.320dupA     | p.Y107*     | 17  | 7579367           | 7579367        | 2/2         | 0                       | D                                | PD                             | C                              | C         |
| 83  | 20       | HBOCS        | TP53             | c.523_524delG | p.R175_105 | 17  | 7578407           | 7578407        | 2/2         | 0                       | D                                | PD                             | C                              | C         |
| 38  | 37       | HBOCS        | TP53             | c.839_905delC | p.R280T    | 17  | 7577099           | 7577099        | UA          | 0                       | D                                | PD                             | C                              | C         |
| 46  | 52       | HBOCS, LS   | MLH1             | c.194delA     | p.G650      | 3   | 3703817           | 3703817        | UA          | 0                       | D                                | PD                             | C                              | C         |
| 67  | 46       | HBOCS        | MLH1             | c.1189_1191delTAT | p.I397del | 14  | 75515169          | 75515171       | UA          | 0                       | --                               | --                              | --                              | Novel     |
| 22  | 37       | HBOCS        | MSH2             | c.162_170del18 | p.A57_62del | 5   | 79650730          | 79650725       | 5/6         | 0                       | --                               | --                              | --                              | Novel     |
| 49  | 32       | HBOCS, LS   | MSH3             | c.199_207del9 | p.R67_76del9 | 5   | 79650745          | 79650753       | 5/5         | 0                       | --                               | --                              | --                              | Novel     |
| 75  | 51       | HBOCS        | MSH3             | c.230_315delG | p.V769*     | 5   | 80071564          | 80071564       | 2/3         | 0                       | --                               | --                              | --                              | Novel     |
| 94  | 47       | HBOCS        | CDH1             | c.1296delG    | p.N432K    | 16  | 68847374          | 68847374       | UA          | 0                       | D                                | PD                             | NC                             | C         |
| 86  | 52       | HBOCS        | RAD51C           | c.343dupG    | p.V115Gts*24 | 17  | 56772489          | 56772489       | 2/2         | 0                       | --                               | --                              | --                              | Novel     |
| 51  | 45       | HBOCS        | PALB2            | c.1050_1051del18TCT | p.Q350fs*11 | 16  | 23646815          | 23646815       | 3/4         | 0                       | --                               | --                              | --                              | [96]      |
| 77  | 34       | HBOCS        | RAD50            | c.1291_1295delGAGAT | p.E431Kfs*3 | 5   | 13192568          | 131925672      | 1/2         | 0                       | --                               | --                              | --                              | Novel     |
| 35  | 30       | HBOCS        | FANCJ            | c.2693_2704dupG3CAAT | p.G90_1_392fsWQ | 15  | 89843611          | 89843611       | UA          | 0                       | --                               | --                              | --                              | Novel     |

(Continued)
| ID  | Age dx (yrs) | HCS     | Gene      | DNA change   | Protein change | Chr | Mutant site start | Mutant site end | Segregationa | 1000G MAFb | Missense prediction | Evolutionary conservation | Evolutionary conservation | Reference |
|-----|--------------|---------|-----------|--------------|----------------|-----|-------------------|----------------|--------------|------------|----------------------|---------------------------|---------------------------|-----------|
| 86  | 52           | HBOCS   | SLX4      | c.3583_3585delATT | p.I1195del     | 16  | 3640054           | 3640056        | 1/2          | —          | —                    | —                         | —                         | rs199897550 |
| 7   | 30           | HBOCS   | FANCD2    | c.4234_4236delAGTGAG | p.S1412_E1413del | 3   | 10140452          | 10140457       | 1/1          | —          | —                    | —                         | —                         | Novel     |
| 77  | 34           | HBOCS   | CDKN2A    | c.480G>A      | p.W160*        | 9   | 21968748          | 21968748       | 1/2          | —          | —                    | —                         | —                         | Novel     |
| 64  | 27           | HBOCS   | CYP17A1   | c.987delC     | p.Y329*        | 10  | 104592420         | 104592420      | 1/1          | —          | —                    | —                         | —                         | Novel     |
| 88  | 45           | HBOCS   | FGFR3     | c.2073delG    | p.G691Afs*17   | 4   | 1806976           | 1806976        | 1/3          | —          | —                    | —                         | —                         | Novel     |
| 75  | 51           | HBOCS   | RGSL1     | c.1357C>T     | p.Q453*        | 1   | 182443603         | 182443603      | 2/3          | —          | —                    | —                         | —                         | Novel     |
| 22  | 37           | HBOCS   | MUTYH     | c.850-2A>G    | p.?            | 1   | 45797760          | 45797760       | 1/2          | 0.002      | —                    | —                         | —                         | [57]      |
| 96  | 35           | HBOCS   | SPINK1    | c.194-2T>C    | p.?            | 5   | 147207583         | 147207583      | 2/2          | 0.001      | —                    | —                         | —                         | [58]      |
| 60  | 47           | HBOCS, LS | TNFRSF13B | c.704_705delCT | p.P235Rfs*169  | 17  | 16855857          | 16855857       | 2/2          | —          | —                    | —                         | —                         | Novel     |
| 29  | 39           | HBOCS, LS | TNFRSF13B | c.102delC     | p.E36Kfs*48    | 17  | 16843038          | 16843039       | 3/3          | 0          | —                    | —                         | —                         | Novel     |
| 24  | 26           | HBOCS, LS | WRN       | c.424dupT     | p.D1416*       | 8   | 31030564          | 31030564       | 5/6          | 0          | —                    | —                         | —                         | Novel     |

Mutation nomenclature follows the recommended guidelines of the Human Genome Variation Society, with the nucleotide numbering based on the GenBank reference sequence indicated by its accession NCBI number. Details are listed in S2 Table.

Abbreviations: HCS, hereditary cancer syndrome; HBOCS, hereditary breast and ovarian cancer syndrome; LFS, Li-Fraumeni syndrome; LS, Lynch syndrome; U/A, unavailable; D, damaging; B, benign; C, conserved; NC, not conserved; and PD, probably damaging. BIC, breast cancer information core, URL: http://research.nhgri.nih.gov/bic/; p53 mutation database, URL: http://www-p53.iarc.fr, version R16.

a Results of segregation studies marking positive individuals out of the total number available for validation.

b All variants were queried against 1000 Genomes (1000G) data using the 1000 Genomes Browser (http://browser.1000genomes.org/index.html) which integrates SNP and InDel calls from 1,092 individuals (data released 2012 April). The minor allele frequency (MAF) is provided here.

c In silico prediction (missense prediction software and evolutionary conservation) were used for the determination of pathogenicity of missense mutations. Detailed criteria were described in Materials and Methods.

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was made among the probands alone (40.6 years vs. 33.3 years, \( P = 0.22 \)) or together with their blood relatives (48.7 years vs. 41.7 years, \( P = 0.29 \)); however, neither difference reached significance.

**Functional Analysis of MSH3 Germline Mutation and Gene-Gene Interaction**

In total, six paired (normal and tumor) breast tissue samples, one paired colon tissue sample and one paired ovarian tissue sample from three families with MSH3 mutations were available for MSI and IHC analysis. An analysis of mononucleotide and dinucleotide repeat microsatellite markers identified two MSI-low (MSI-L) cases (25%) and six microsatellite stable (MSS) cases (75%). Seven of the eight cases exhibited mutations in at least one EMAST locus (Table 4). Msh3 expression was obviously lost in tumor tissue samples of two patients with MSH3 mutation (Fig 4 and Table 4) and studies have suggested that deficient MMR protein expression in tumor tissues were implications for the present of MMR gene mutations [30].

Moreover, family No. 49 was identified as carrying a single MSH3 germline mutation, and the family history manifested as typical LS. A segregation study indicated that this mutation segregated well with the diseases (Fig 5A). However, family No. 22 was identified as carrying both MSH3 and BRCA1 germline mutations, and the family history manifested as typical HBOCS instead of LS. A segregation study indicated that the BRCA1 mutation completely segregated with the disease, whereas the MSH3 mutation incompletely segregated with the disease (Fig 5B).

**Discussion**

We performed comprehensive analysis of 152 genes associated with hereditary cancer in Chinese families who were at high risk for breast cancer, using targeted capture and NGS. We identified 34 (34/99, 34.3%) families who carried putative deleterious germline mutations. BRCA2 mutations were more common than BRCA1 mutations in our study, which is consistent with other Chinese studies [31, 32]. Two recurrent mutations were separately detected in two unrelated breast cancer patients in our study, and we have previously reported that the BRCA1 c.5468-1_5474del8 mutation, together with another recurrent mutation, 1100delAT, accounted for 34.8% of all BRCA1 mutations [33]. The founder effects of these recurrent mutations in BRCA1 and BRCA2 require further investigation.

Two topics regarding TP53 germline mutation detection are currently under debate. First, is the selection of patients for TP53 mutation analysis effective according to the LFS or the

### Table 2. Analysis of BRCA1/2, TP53, and MMR gene mutation carriers fulfilling and not fulfilling the HBOCS, LFS, and LS criteria according to the NCCN guidelines.

| gene          | BRCA1/2 | TP53 | MMR |
|---------------|---------|------|-----|
| No. of mutation carriers identified in this study using NGS | 18 | 3 | 5 |
| No. of mutation carriers fulfilling the HBOCS, LFS or LS criteria* | 18 | 1 | 2 |
| No. of mutation carriers not fulfilling the HBOCS, LFS or LS criteria* | 0 | 2 | 3 |

Abbreviations: HBOCS, hereditary breast and ovarian cancer syndrome; LFS, Li-Fraumeni syndrome; LS, Lynch syndrome; NCCN, national comprehensive cancer network; MMR, mismatch repair; and NGS, next-generation sequencing.

* HBOCS, LFS and LS criteria used were according to the NCCN guidelines (version 4.2013).

**Table 2. Analysis of BRCA1/2, TP53, and MMR gene mutation carriers fulfilling and not fulfilling the HBOCS, LFS, and LS criteria according to the NCCN guidelines.**

| gene          | BRCA1/2 | TP53 | MMR |
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| No. of mutation carriers not fulfilling the HBOCS, LFS or LS criteria* | 0 | 2 | 3 |

Abbreviations: HBOCS, hereditary breast and ovarian cancer syndrome; LFS, Li-Fraumeni syndrome; LS, Lynch syndrome; NCCN, national comprehensive cancer network; MMR, mismatch repair; and NGS, next-generation sequencing.

* HBOCS, LFS and LS criteria used were according to the NCCN guidelines (version 4.2013).
According to our results, the TP53 mutation detection rates in families fulfilling the classic LFS and the Chompret criteria were 50% and 9.1%, respectively. Previous studies have reported that the corresponding TP53 mutation detection rates were approximately 56%-73% and 21%-36%, respectively [2, 35, 36]. More importantly, two of the three families with a TP53 germline mutation met neither the classic LFS criteria nor the Chompret criteria. Other studies reported similar findings. Mouchawar et al. examined early-onset breast cancer patients and found that three of five TP53 mutation carriers did not meet the classic LFS or LFL (Li-Fraumeni-like) syndrome criteria [7]. Second, is it necessary for early-onset (<30 years) breast cancer patients to undergo TP53 gene testing [37]? In a multiethnic Asian cohort, Lee et al. reported that the frequency of the TP53 germline mutation was approximately 5% in

Chompret criteria [34]? According to our results, the TP53 mutation detection rates in families fulfilling the classic LFS and the Chompret criteria were 50% and 9.1%, respectively. Previous studies have reported that the corresponding TP53 mutation detection rates were approximately 56%-73% and 21%-36%, respectively [2, 35, 36]. More importantly, two of the three families with a TP53 germline mutation met neither the classic LFS criteria nor the Chompret criteria. Other studies reported similar findings. Mouchawar et al. examined early-onset breast cancer patients and found that three of five TP53 mutation carriers did not meet the classic LFS or LFL (Li-Fraumeni-like) syndrome criteria [7]. Second, is it necessary for early-onset (<30 years) breast cancer patients to undergo TP53 gene testing [37]? In a multiethnic Asian cohort, Lee et al. reported that the frequency of the TP53 germline mutation was approximately 5% in

| ID | Age dx (yrs) | Gene | Associated syndrome or diseases | Nucleotide change | Protein change | Exon | FH of cancer and age of onset (yrs) |
|----|-------------|------|---------------------------------|------------------|---------------|------|-----------------------------------|
| 7  | 30          | BRCA2| HBOCS                           | c.5699C>G        | p.S1900*      | 11   | Paternal aunt (Br,57)             |
|    |             |      | FANCD2                          | c.4234_4239delAGTGAG | p.S1412_E1413del | 43   | Paternal aunt (Lung,58)          |
| 22 | 37          | BRCA1| HBOCS                           | c.2110_2111delAA | p.N704Cfs*7   | 11   | Mother (Br,60; Lym,61)           |
|    |             |      | MSH3                            | p.A57_A62del     |               | 1    | Maternal aunt (Br,49)            |
|    |             |      | MUTYH                           | c.850-2A>G       | p.?           | 1    | Maternal aunt (Br,34)            |
|    |             |      |                                 |                  |               |      | Maternal aunt (Br,50)            |
|    |             |      |                                 |                  |               |      | Maternal aunt (Br,68)            |
| 24 | 26, 36      | BRCA2| HBOCS                           | c.7142delC       | p.P2381Hfs*13 | 14   | Father (rectum,64)              |
|    |             |      | WRN                             | c.4245dupT       | p.D1416*      | 35   | Paternal aunt (rectum,47)        |
| 29 | 39          | BRCA1| HBOCS                           | c.5503C>T        | p.R1835*      | 24   | Sister (Br,36)                   |
|    |             |      | TNFRSF13B                       | c.102delC        | p.E36Kfs*48   | 5    | Mother (Bladder,64)             |
|    |             |      |                                 |                  |               |      | Maternal aunt (Br,54)            |
| 75 | 51          | MSH3| LS                              | c.2305delG       | p.V769*       | 16   | Sister (Br,53)                   |
|    |             |      | RGSL1                           | c.1357C>T        | p.Q453*       | 6    | Father (Liver,51)                |
|    |             |      |                                 |                  |               |      | Paternal aunt (Br,75)            |
| 77 | 34          | RAD50| Br, ovary                       | c.1291_1295delGAGAT | p.E431Kfs*3   | 9    | Sister (Br,32)                   |
|    |             |      | CDKN2A                          | c.480G>A         | p.W160*       | 4    | Mother (Br,41)                   |
| 86 | 52          | RAD51C| FA                             | c.343dupG        | p.V115Gfs*24  | 2    | Sister (Br,57)                   |
|    |             |      | SLX4                            | c.3583_3585delATT | p.I1195del    | 12   | Mother (Ovary,63)                |
|    |             |      |                                 |                  |               |      | Maternal uncle (Liver,45)        |
|    |             |      |                                 |                  |               |      | Paternal uncle (Liver,65)        |
|    |             |      |                                 |                  |               |      | Father (Lym,79)                  |
|    |             |      |                                 |                  |               |      | Paternal aunt (Lung,65)          |
|    |             |      |                                 |                  |               |      | Grandfather (Stomach,81)         |

Mutation nomenclature follows the recommended guidelines of the Human Genome Variation Society with the nucleotide numbering based on GenBank reference sequence indicated by its accession NCBI number. Details were listed in S2 Table. Abbreviations and definitions are as follows: FH, family history; HBOCS, hereditary breast and ovarian cancer syndrome; Br, breast; FA, Fanconi anemia; Lym, lymphoma; LS, Lynch syndrome; FAP2, familial adenomatous polyposis, 2; WS, Werner syndrome; and CVID2, common variable immune deficiency,2.

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| Family No. | Patient Type | Site of disease | MSH3 IHC | MSH3 IHC Tumor | MSI BAT25 | MSI BAT26 | MSI D2S123 | MSI D17S250 | MSI D18S64 | MSI D18S69 | MSI D19S394 | MSI D20S82 | MSI D20S85 | MSI D9S242 | MSI D9S248 | MSI D17S35 | MSI D19S394 | MSI NCI | MSI NCI D status | EMAST status |
|-----------|--------------|----------------|----------|----------------|-----------|-----------|------------|-------------|------------|------------|-------------|-----------|-----------|------------|-----------|------------|-------------|----------|----------------|-------------|
| 22        | Proband     | Breast         | 3+       | 2+             | -         | -         | -          | -           | -          | -          | +           | +         | +         | -          | -         | -          | +           | S         | S             | E           |
| 22        | Mother      | Breast         | 2+       | 2+             | -         | -         | -          | -           | -          | +          | -           | -         | -         | -          | -         | -          | -           | -         | L             | non-E       |
| 49        | Proband     | Breast         | 3+       | 2+             | -         | -         | -          | -           | +          | -          | -           | -         | -         | +          | -         | -          | +           | S         | S             | E           |
| 49        | Mother      | Breast         | 1+       | 1+             | -         | -         | -          | -           | -          | +          | -           | -         | -         | -          | -         | -          | -           | S         | S             | E           |
| 49        | Mother      | Colon          | 2+       | 2+             | -         | -         | -          | -           | -          | -          | -           | -         | +         | -          | -         | -          | -           | S         | S             | E           |
| 75        | Proband     | Breast         | 3+       | 3+             | -         | -         | -          | -           | -          | +          | -           | -         | -         | -          | -         | -          | -           | S         | S             | E           |
| 75        | Sister      | Breast         | 3+       | 1+             | -         | -         | -          | -           | +          | -          | +           | -         | -         | +          | -         | -          | -           | S         | L             | E           |

For MSH3 IHC, “3+” indicates strong staining, “2+” indicates intermediate staining, “1+” indicates week staining, and “0” indicates no staining. For MSI profiles, it includes MSI data for five consensus National Cancer Institute (NCI) microsatellite markers (two mono-A repeats, BAT25 and BAT26, and three dinucleotide repeats, D2S123 through D17S250), two additional dinucleotide (D) markers (D18S69 and D18S64), and seven EMAST markers (MYCL1 through D8S321). For MSI data, “-” indicates the absence of the mutation, and “+” indicates the presence of the mutation. For MSI using the NCI panel (NCI), S indicates MSS. For MSI using the NCI panel and two additional dinucleotide markers (NCI and D status), S indicates MSS, and L indicates MSI-L. For EMAST status, E indicates EMAST positive, and non-E indicates EMAST negative.

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breast cancer patients who were diagnosed at \( \leq 35 \) years of age [38]. In an Australian population-based cohort, Mouchawar et al. found that 2/52 (4\%) of very-early-onset (\( \leq 30 \) years) breast cancer patients who were unselected for family history carried a germline TP53 mutation, and 3/42 (7\%) of early-onset (31 to 39 years) breast cancer patients with a family history of breast or ovarian cancer carried a TP53 germline mutation [7]. Our results indicated that 10\% (2/20) of very-early-onset (\( \leq 30 \) years) breast cancer patients with a family history of cancer carried a TP53 mutation, which is consistent with the results of Mouchawar et al. Since 2009, the NCCN guidelines have recommended that breast cancer patients who are diagnosed before the age of 30 and who are BRCA1/2 negative should also be offered genetic counseling and testing for TP53, in addition to those fulfilling LFS or LFL syndrome criteria. Additionally, 2 of the 99 patients reported a family history of PTB and both carried a TP53 germline mutation. Although PTB is not identified as a component of LFS according to the NCCN guidelines, Birch et al. [39] have reported that the detection rate of a TP53 germline mutation in families with PTB was significantly high, which is consistent with our findings.

In the Chinese population, nearly all of the pathogenic mutations in LS families were identified in common MMR genes (MSH2, MLH1 and MSH6) [40], which is different from that identified in our study. This difference may be attributed to the fact that, in current clinical practice, MSH3 and MLH3 are not routinely tested in patients suspected of having LS, but we conducted a comprehensive examination of multiple genes and obtained different results. The
The association between MLH3 germline mutation and LS has been confirmed [41]. Meanwhile, it has been reported that MSH3 deficiency is associated with EMAST and with low levels of instability at dinucleotide repeat loci [19], and MSH3 deficiency may elevate susceptibility to certain neoplastic diseases and contribute to tumor initiation [42, 43]. In our study, an MSH3 germline mutation was related to EMAST and MSI-L at dinucleotide repeat loci (Table 4), which is consistent with previous studies [19]. Moreover, we identified a typical LS family with an MSH3 germline mutation, and segregation analysis indicated that this mutation segregated well with the familial diseases. Therefore, our findings suggested the association between MSH3 germline mutation and LS. In our study, the mutation detection rate of common MMR genes in families fulfilling the revised Bethesda guidelines was 6.7% (MLH1: 1/15). In Western studies with large sample sizes, the corresponding common MMR gene mutation detection rate was only 2.5%-3.8% [44, 45]. Moreover, three (60%) of five MMR gene mutations were identified in patients who did not fulfill the revised Bethesda guidelines. Hampel and colleagues also found that approximately 28% of LS cases did not fulfill the revised Bethesda guidelines [8].

Fig 5. Pedigrees and results of segregation studies of family No. 49 and family No. 22. Subjects with cancers are indicated with solid symbols. Sites of cancer are breast (BC), ovary (OC), rectum (RC), colon (CC), and lymphoma (Lym). Ages under symbols indicate age at cancer diagnosis. Probands are indicated with arrows. Individuals having the gene mutation were indicated as M/w, and those not having such gene mutation were indicated as w/w. A, the pedigree and results of segregation analysis of family No. 49. B, the pedigree and results of segregation analysis of family No. 22.

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guidelines, which were introduced in 2002, are more sensitive than the Amsterdam criteria in identifying individuals who are at risk for LS [46], the revised Bethesda guidelines still miss carriers.

A missense mutation c.1296 C>G (N432K) in CDH1 was detected in 1 of these 99 patients. This germline mutation has been previously reported in gastric cancer [47]. It can generate the E-cadherin exon9-skipping and was supposed to be a disease-causing mutation. The CDH1 gene appears to be rare in Chinese with breast cancer. Zhu et al. [48] found no germline disease associated mutations in a patient with familial diffuse gastric cancer and lobular breast cancer. More research is required in order to describe the role of CDH1 mutations in Chinese at high risk of breast cancer.

Except for BRCA1/2, FA pathway-related genes accounted for 28.6% (6/21) of the remaining genes containing loss-of-function mutations in our study. However, to date, three FA genes (FANCD1, FANCN, FANCJ) have been shown to be bona fide breast cancer susceptibility genes. The sequencing of DNA from members of families with heightened breast cancer risk has not revealed mutations in other FA genes [49]. It is unclear why some FA gene mutations impose a higher risk of cancer than others. We also detected heterozygous germline mutations in three genes that follow an autosomal recessive mode of pathogenesis, which include WRN, MUTYH, and CYP17A1. It is generally believed that recessive genes are not pathogenic in heterozygotes. However, some studies have reported that heterozygous mutations of certain genes are associated with increased cancer risk. Wang et al. [27] reported the association of a WRN heterozygous mutation with breast cancer susceptibility in Chinese women. Nielsen et al. observed a significantly increased risk of breast cancer in female MUTYH associated polyposis coli patients [28]. Hopper et al. [29] detected a deleterious CYP17A1 germline mutation, p. R239X, in three sisters with early-onset breast cancer, and the breast cancer risk associated with this mutation has been reported to be more than 20 times the population incidence.

A high proportion (20.6%, 7/34) of patients carrying mutations in two or more genes was detected in our study. Walsh et al. stated that a single deleterious mutation in any one of the breast cancer susceptibility genes is sufficient to significantly increase breast cancer risk [5]. However, different findings have emerged as high-throughput sequencing technology has increased in popularity. Whole-exome sequencing studies conducted in BRCA1/2-negative familial breast cancer patients revealed that each sequenced individual harbored as many as 35 deleterious germline mutations on average [50]. Due to the difficulty inherent in functional studies, determining which mutations are pathogenic is problematic. In our study, all the tested genes are hereditary cancer susceptibility genes, therefore, their germline mutations can be considered more relevant to breast cancer risk. However, the analysis of gene-gene interactions is also complex. Currently, there are few systematic studies of gene-gene interactions among breast cancer susceptibility genes. Lavie et al. [51] reported that when compared to those carrying a single BRCA1 or BRCA2 mutation, women with double mutations had a much younger mean age at breast cancer diagnosis, which is similar to our findings. However, the effect of gene-gene interactions is not only confined to synergism. It is possible that antagonism may also exist, as indicated in this study (see Fig 5). Borg et al. [52] identified a family with both MLH1 and BRCA1 mutations, and they found that the double heterozygotes developed breast cancer but not colorectal or endometrial cancer. These authors proposed that the BRCA1 deficiency had a potential protective effect against carcinogenic events in the colon. Thus, gene-gene interactions are very complex and require further study.

Pleiotropy occurs when one gene has an effect on multiple phenotypes. Sivakumaran et al. performed a systematic review and reported pleiotropy is a common property of genes and SNPs associated with disease traits [53]. Indeed, we observed several incidents where relatives from the same family developed different cancer types (see Fig 1), suggesting a potential role of
pleiotropy. Even though it is hard to test for pleiotropy with our limited sample size, it is a crucial component of disease etiology which will need further investigation.

In summary, we identified comprehensive genetic factors for Chinese hereditary breast cancer using NGS. Our findings reconfirmed the importance of traditional genetic testing based on NCCN guidelines because almost all high-penetrance gene mutations were detected in the families that fulfill the typical phenotypes of hereditary cancer syndromes listed in the NCCN guidelines. Furthermore, NGS also identified a few gene mutations in the families not fulfill the NCCN guidelines, as indicated in our study (see Table 2), especially in families carrying mutations in two or more genes, in which a potential gene mutation does not present typical phenotypes (see Fig 5). So we think NGS may be a supplement of traditional genetic counseling. We also identified a lot of low-penetrance gene mutations which clinical significance has not been fully cleared yet, and these findings provide valuable epidemiological information for the future studies. Large sample sizes and multi-center population should be studied in the future to obtain more comprehensive data. We believe that our extensive findings will be helpful to the development of breast cancer genetic counseling in China.

Supporting Information
S1 Table. List of genes tested in this study. 
(DOCX)

S2 Table. Genomic information of genes referenced in this study. 
(DOCX)

S3 Table. Next generation sequencing metrics and coverage. 
(DOCX)

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Author Contributions
Conceived and designed the experiments: ZH X. Yang. Performed the experiments: X. Yang MHS WTY XIX YZ XYZ XYC X. Yan. Analyzed the data: ZH X. Yang DQL X. Hu ZGC XIX YZ. Contributed reagents/materials/analysis tools: ZH JW JSL GYL GHD CMC YFH X. Huang ZBL QXH Z. Shen Z. Shao. Wrote the paper: ZH X. Yang DQL.

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