Gene Panel Sequencing in a Chinese High-risk Breast Cancer Cohort

Xianyu Zhang[1$], Xiaohong Wang[2,6$], Bingbing Song[3$], Kang Shao[2,6$], Guibo Li[2,6$], Wenjing Jian[4$], Cong Lin[2], Min Wang[5], Xiaofei Ye[2,6], Jingjing Xie[1], Bingshu Xia[1], Shouping Xu[1], Boyang Cao[2,6], Liyun Xiao[2,6], Zhao Zhang[2,6], Meng Liu[2,6], Enhao Fang[2,6], Haoxuan Jin[2,6], Xiaofeng Wei[2,6], Michael Dean[7], Jian Wang[2,8], Huanming Yang[2,8], Xianming Wang[4,5*], Shida Zhu[2,6*], Yong Hou[2,6*], Da Pang[1,3*]

1) Department of Breast Surgery, Harbin Medical University Cancer Hospital, Harbin 150081, China;

2) BGI-Shenzhen, Shenzhen 518083, China;

3) Heilongjiang Academy of Medical Sciences, Harbin 150081, China;

4) Department of Breast and Thyroid Surgery, Shenzhen Second People's Hospital, Shenzhen 518035, China.

5) Clinical College of Shenzhen Second Hospital, Anhui Medical University, Shenzhen 518035, China.

6) China National GeneBank, BGI-Shenzhen, Shenzhen, China

7) Laboratory of Translational Genomics, Division of Cancer Epidemiology & Genetics, National Cancer Institute, Gaithersburg, Maryland, USA;

8) James D. Watson Institute of Genome Sciences, Hangzhou 310058, China.

[$]These authors contributed equally to this work.

[*] Corresponding authors:

Xianming Wang, Department of Breast Surgery, Shenzhen Second People's Hospital, Sungang West Road 3002, Shenzhen, China. Tel: +86 0755 83366388; E-mail: wxm60@aliyun.com.

Shida Zhu, BGI-Research, BGI-Shenzhen. Beishan Industrial Zone, Yantian District, Shenzhen, China.

Tel: +86 0755 25273620; Fax: +86 0755 25274207; E-mail: zhushida@genomics.cn.

Yong Hou, BGI-Research, BGI-Shenzhen. Beishan Industrial Zone, Yantian District, Shenzhen, China.

Tel: +86 0755 25273620; Fax: +86 0755 25274207; E-mail: houyong@genomics.cn.

Da Pang, Department of Breast Surgery, Harbin Medical University Cancer Hospital, Haping Road 150, Harbin 150081, China. Tel.:+86 451 86298410; Fax: +86 451 86663760; E-mail: pangda@ems.hrbmu.edu.cn.

Abstract

Background: Currently, over 20 genes have been defined that can confer susceptibility for high-risk breast cancer. Although research has proved the utility of multiple-gene sequencing in the assessment of breast cancer risk, there is little data from China patients. Here, we use a multiple-gene sequencing panel to identify the variant spectrum in Chinese high-risk breast cancer subjects.

Findings: A total of 829 Chinese high-risk breast cancer patients participated in the research. The coding regions of 115 hereditary cancer susceptibility genes were captured and sequenced using a next generation sequencing platform from Complete Genomics and Illumina. Single Nucleotide Polymorphism and small Insertion/deletion variants were defined and analyzed. We developed a semi-automatic interpretation pipeline which enables accurate variants classification. In total, 193 pathogenic variants were identified in 45 genes from 177 patients. The pathogenic variant carrier rate is 21.4%: with 10.5% patients carrying *a BRCA1* or *BRCA2* mutation only, 10.0% of patients carried non-BRCA gene mutations only, while 1.0% of patients carried both a *BRCA1/2* and a non-BRCA gene mutation. Variants of uncertain significance (VUS) totaling 2632 were identified in 115 genes from 787 of 829 patients: 82.5% patients carried more than one VUS, and only 5.1% patients did not carry any VUS. Families carrying pathogenic

variants were tracked and adenoma was founded in three of them.

Conclusions: Our data provide a comprehensive analysis of potential susceptibility variations of high-risk for breast cancer in a Chinese population. This data will be useful for the comparison of the susceptibility variation spectrum between different populations and to discover potential pathogenic variants to improve the prevention and treatment of high-risk breast cancer.

Keywords

Breast cancer, high-risk breast cancer, susceptibility gene, multiple-gene testing, next generation sequencing, *BRCA1, BRCA2*.

Introduction

Breast cancer is the most common cancer in women worldwide with over 1.6 million new cases diagnosed and over 520,000 deaths annually (Globocan 2012, http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx). Improved treatment has contributed to steady declines in breast cancer mortality in developed countries (1). However, breast cancer incidence is continuously rising in nearly all countries and mortality rate stays high in most Asian countries (Globocan 2012). In China, there is an estimated 272,000 total cases annually (269,000 in women) and 70,000 total deaths (69,000 in women) (2).

Up to 10% of breast cancer is caused by the inheritance of germline mutations in susceptibility genes (3). BRCA1 and BRCA2 are the predominate genes, while other genes such as PALB2, TP53, PTEN may also contribute to genetic risk and screening susceptible population for such mutations can lead to reduced mortality from breast and ovarian cancer (4-7). There is a considerable diversity in mutations in BRCA1, BRCA2 and other genes in different populations, requiring studies worldwide (3, 8-11). Studies of germline mutation spectrum have been carried out in Asian populations, but

comparatively few in Chinese populations (12-18). This study aims to identify the spectrum of germline variants in a large panel of cancer genes in selected Chinese breast cancer families.

Method

Participants and Selection Criteria

A total of 829 breast cancer patients who met genetic risk evaluation criteria according to National Comprehensive Cancer Network (NCCN) guidelines for breast and/or ovarian cancer genetic assessment (version1.2014) were recruited from 2010 to 2016. All patients met the high risk criteria: 1.early age-of-onset, people suffer breast cancer under age 45; 2.patients who have at least two primary breast cancer; 3.with a family history: whose first degree relatives have breast cancer or ovarian cancer. Of the 829 breast cancer patients, 593 came from Harbin Medical University Cancer Hospital, and 236 came from Shenzhen Second People's Hospital of China. All of them did not have previous breast cancer susceptibility gene testing.

Ethics, consent and permissions

Every participant signed an institutional review board-approved informed consent document offered by Harbin Medical University Cancer Hospital, Shenzhen Second People's Hospital or BGI Shenzhen. The consent informed the participants that their test data would be used for research. Clinical data and family disease history information were including patient gender, age at diagnosis of breast cancer, breast cancer molecular subtype, site, personal history of other cancers, and family history of cancer in close blood relatives: diagnosis age, tumor type, and health status. The clinical information is shown in Supplementary Table S1.

Gene Panel

All breast cancer samples were subjected to the target sequencing using a multiple-gene panel and subsequent variant analysis. A total of 115 target genes were selected through a review of databases (HGMD: Human Gene Mutation Database, NCBI ClinVar database) or published articles on the role of the genes in hereditary cancer. The main types of hereditary cancer include Breast cancer, Colorectal cancer, Gastric cancer, Prostate cancer, Thyroid cancer, Renal cancer and other cancers which have a genetic risk. The Breast cancer susceptibility genes (27 genes) are shown in Table 1; the other 88 cancer susceptibility genes involved in 35 cancer types are shown in Supplementary Table S2. The panel has been used for previous research (19), but is not currently commercially available. Interested individuals can contact us for more details.

Table1. Breast cancer susceptibility gene list and inclusion criteria.

No.	Gene name	Breast Cancer Relative Risk	References
		High-penetrant Genes	
1	*BRCA1*	4.0-7.0	Chen, S.(20)
2	*BRCA2*	4.0-7.0	Chen, S.(20)
3	*CDH1*	5.9-7.3	Pharoah, P. D. (21)
4	*PTEN*	2.0-5.0	Tan, M. H. (22)
5	*STK11*	4.0-6.7	Apostolou, P. (23)
6	*TP53*	4.3-9.3	Gonzalez, K. D.(24)
		Moderate-penetrant Genes	
7	*ATM*	1.5-3.8	Renwick, A.(25)
8	*BARD1*	Unknown	Apostolou, P. (23)
9	*BRIP1*	1.2-3.2	Seal, S.(26)
10	*CHEK2*	2 fold, 25-37% lifetime risk	Apostolou, P.(23)
11	*HMMR*	Unknown	Pujana, M. A.(27)
12	*MLH1*	0.2-2.0	Win, A. K.(28)
13	*MLH3*	Unknown	Conde, J.(29)
14	*MSH2*	1.2-3.7	Win, A. K.(28)
15	*MSH6*	0-13	Win, A. K.(28)
16	*MUTYH*	1.0-3.4	Rennert, G.(30)
17	*NBN*	1.4-6.6	Bogdanova, N.(31)
18	*NQO2*	Unknown	YU, K. D.(32)
19	*PALB2*	1.4-3.9	Rahman,N.(33)
20	*PHB*	Unknown	Jupe, E. R.(34)
21	*PMS1*	Unknown	Kobayashi, H.(35)

22	*PMS2*	Unknown	Kobayashi, H.(35)
23	*PPM1D*	Unknown	Ruark, E.(36)
24	*RAD50*	Unknown	Apostolou, P.(23)
25	*RAD51*	Unknown	Li, W.(37)
26	*RAD51C*	1.5-7.8	Meindl, A.(38)
27	*XRCC3*	Unknown	Kuschel, B.(39)

Sample Treatment, Next-Generation Sequencing and Variants Calling

Sample preparation and DNA sequencing were performed at BGI Shenzhen. The samples were separated into two groups and analyzed on different sequencing platforms. A total of 634 samples from Harbin Medical University Cancer Hospital or Shenzhen were sequenced on the Blackbird platform (Complete Genomics, a BGI Company). An additional 195 samples from Shenzhen were sequenced on the Hiseq 2500 platform (Illumina) with the Paired-end 91 bp strategy.

DNA was extracted from participant's peripheral blood sample by Qiagen DNA Blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer instructions. Qubit Fluorometer (Life Technologies) and agarose gel electrophoresis were used to detect DNA concentration and integrity.

For the samples on Blackbird platform, the DNA amount used for library construction was 1ug. DNA was randomly fragmented to 200-400bp and the A-adaptor was added. The coding region and coding region ±30 boundaries of 115 genes were captured by a BGI capture array (produced by BGI). Double strand DNA was cyclized after the PCR amplification. Then the B-adaptor was added, and the sample converted to a circular single-stranded DNA. After rolling circle replication, the DNB (DNA nanoball) was loaded onto the chip and subsequent DNA sequencing was performed according to published protocols to at least an average depth of 390X and 99% coverage on target regions [38]. Over 0.6G base of data was generated for every sample. Variants were detected using Small Variant Assembler Methods which was available on the CG website (http://www.completegenomics.com/documents/Small_Variant_Assembler_Methods.pdf).

Next, variants were filtered by allele depth, allele frequency, mapping quality and region of variation (major parameters set as follows: alteration allele > 1, Allele depth > 8, BAF > 30%, not in highly repeat region, no Indels in 10bp and other parameters set to default).

For the samples on the Hiseq platform, genomic DNA with initial amount above 1ug was randomly fragmented to 200-300bp by Covaris E210 (Massachusetts, USA). Then the library was constructed as follows: end-repair, A-tailing, adapter ligation, and PCR amplification. PCR products were captured by the same BGI chip as in Blackbird platform. Then quantified by quantitative PCR and pooled for sequencing on the Hiseq 2500 (Illumina) according to the manufacturer's protocols (https://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/hiseq2500. Over 0.6G base data was generated for every sample with an average depth of about 200X and over 99% coverage on target regions. Reads were filtered by SOAPnuke1.5.0 with parameters -l 5 -q 0.5 -n 0.1and assembled by BWA 0.7.12. Samtools 1.2 and picard MarkDuplicates 1.138 with standard parameters used in Bam file processing and duplication marking. The base quality recalibration and local realignment were performed by GATK 3.4. Variants were called by GATK 3.4 and further filtered by quality depth, strand bias, mapping quality and reads position (major parameters were -filter "QD < 2.0 || FS > 60.0 || MQ < 40.0 || SOR > 4.0 || MQRankSum < -12.5 || ReadPosRankSum < -8.0" for SNP_filter, and -filter "QD < 2.0 || FS > 200.0 || ReadPosRankSum < -20.0 || SOR > 10.0" for INDEL_filter).

Variant Classification

Variants were annotated by ANNOVAR and then classified as pathogenic, variants of uncertain significance (VUS) and benign according to American College of Medical Genetics (ACMG) recommendations(40, 41) by a semi-automatic pipeline called VCE for interpretation of sequence variants.

The detailed evidence can be found in Supplementary Table S4. Most pathogenic variants detected by next-generation sequencing were confirmed by conventional PCR-Sanger sequencing, except variants located in repeat regions.

Gastroscopy of mutation carrier

Mutation carrier families accepted the clinical screening such as gastroscopy, fibercolonoscopy, breast ultrasound and serum tumor marker detection (CEA, CA199, CA742) based on the hereditary cancer susceptibility gene research progress. Up to now, gastroenteric precancerous lesions have been observed in 3 families with ATM, PMS2, and PALB2 pathogenic variants respectively.

Functional analysis of Variant of uncertain significance

Functional analysis of variants from BRCA genes was conducted by Ranomics Inc. To validate variants occurred in RING domain, they measured if mutated BRCA1 showed decreased ligase activity and weaker protein-protein interactions in its heterodimer with the BARD1 protein(42) They also validated variants in BRCT domain by comparing growth speed between dysfunctional variants and normally functioning variants in the BRCA1 BRCT domain(43).

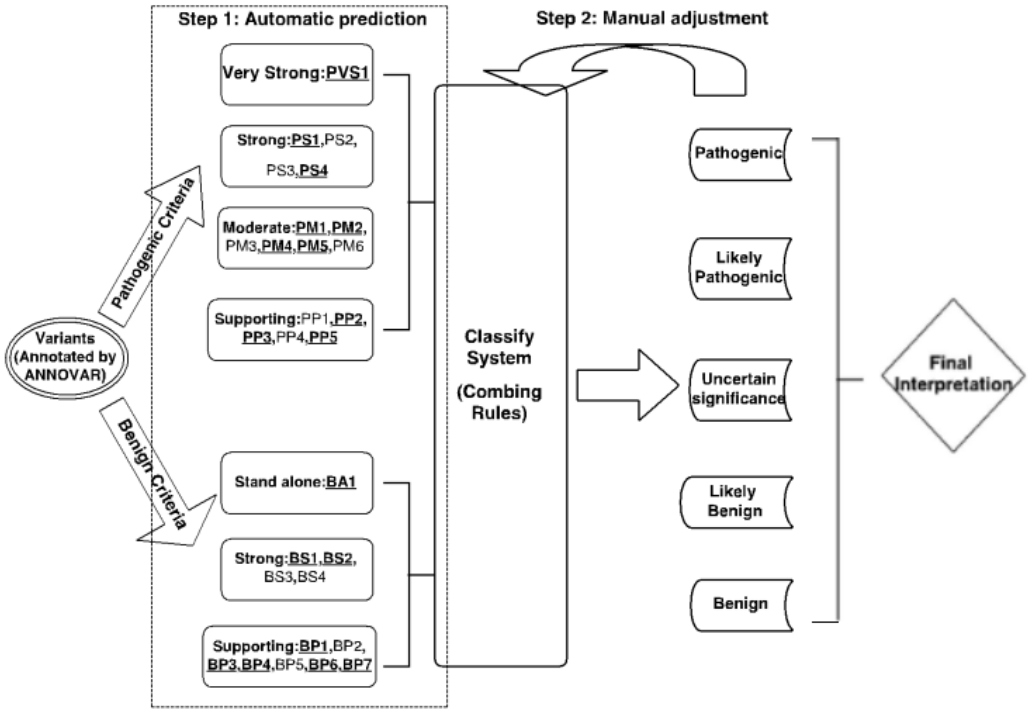
Statistical analysis

The statistics in this research is performed by R 3.5.1. The significance between mutation prevalence and characteristics is compared by Chi square test or Fisher exact test depending on case number.

Results

Variants classification pipeline.

The panel we used in this research contains a high number of genes, leading a low efficiency in classification by manual analysis. We consequently developed a semi-automatic pipeline called Variant Clinical Explanation (VCE) for clinical classification of germline variants related with cancer by ACMG/AMP 2015 guideline. VCE will judge each variant with all the evidence and assign it to a rough class. Some variants that need manual check would be indicated as shown in the figure 1A. Compared with another variants pathogenicity interpreting and predicting software CharGer (Characterization of Germline variants)(44), VCE 's difference lies in more gene number, more support database and silicon prediction software and hints for variants that need manual review. So it is suitable for border gene panel and could provide more assistant information for manual review (Figure1B). In total, the variants need to be manual checked is about 10%.



Module	Description	CharGer	VCE

PVS1	Truncation in loss-of-function intolerant genes	152 cancer predisposition genes	1449 genes which have pathogenic truncation variants reported at least 10 times in ClinVar
PS1	Same amino acid change as known pathogenic missense variants	ClinVar/compiled gene-specific databases	Clinvar/HGMD validated pathogenic missense variants excluding those affecting splicing
PSC1	PS1 when in recessive mode of inheritance	152 cancer predisposition genes	None,
PM1	located in a somatic mutation hotspot	TCGA/HotSpot3D	Same ad CharGer's database
PMC1	Truncations when not in 152 susceptibility gene list	None	None
PM2	Absent or extremely low frequency in the general population (MAF<0.0005)	ExAC	ExAC, 1000genomes, NIFTY (A BGI database including normal cohort allele frequency)
PM4	Protein length changes due to inframe indels, dominant mode	152 cancer predisposition genes	all 1449 gens
PM5	Different amino acid change of a pathogenic variant at the same amino acid residue	ClinVar/compiled gene-specific database	Clinvar/HGMD validated pathogenic missense variants excluding those affecting splcing
PP2	Missense variant in a gene that has a low rate of benign missense variation	152 cancer predisposition genes (points: 1)	760 genes where most pathogenic variants (>80%) are missense and a small proportion (<10%) of missense variants are benign
PP3	Multiple lines of in silico evidence of deleterious effect	> 1 (SIFT/PolyPhen/Blosum62/Compara/VEPImpact/MaxEntScan/GeneSplicer)	all (SIFT, PolyPhen 2 HDIV, PolyPhen 2 HVar, MutationTaster, MutationAssessor)
BA1	High allele frequency in the general population	>0.05 in ExAC	> 0.01 in ExAC, 1000genomes, NIFTY (BGI database)
BSC1	Peptide change is known to be benign	ClinVar/compiled gene-specific databases	ClinVar (BP6 in ACMG)
BMC1	Peptide change at the same location of a known benign change	ClinVar/compiled gene-specific databases	None (Not mentioned in ACMG)
BP4	Multiple lines of in silico evidence of none deleterious effect	> 1 (SIFT/PolyPhen/Blosum62/Compara/VEPImpact/MaxEntScan/GeneSplicer)	all (SIFT, PolyPhen 2 HDIV, PolyPhen 2 HVar, MutationTaster, MutationAssessor)

BP1	missense variant while most pathogenic variants are truncating variants	None	784 genes that truncation variants are majority of pathogenic variants
BP7	a synonymous variant with no impact on splicing	None	dbscSNV_ADA, dbscSNV_RF

Figure1. (A)The Figure above is work flowchart of VCE. The variants were first annotated by annotation software ANNOVAR, and then submitted to automatic prediction. Variants were classified to different clinical pathogenicity, and the variants which may be reclassified by supplementary evidence would be highlighted for manual check. (B) The table below is the different interpretations on evidence between VCE and CharGer. VCE could apply to more genes and different database.

Total Distribution of Pathogenic Variants

Mutation analysis of 115 susceptibility gene for hereditary cancer was performed in 829 breast cancer patients from Northeast of China or Shenzhen of China. In total, 193 pathogenic variants were identified in 45 genes from 174 different patients (Fig2 A), and the pathogenic variant carrier rate is 21.4%: with 87 (10.4%) patients carrying a *BRCA1 or BRCA2* mutation, 38 (4.6%) patients carried other breast cancer susceptibility gene mutations, 38 (4.6%) patients carried other cancer susceptibility gene mutations, and 12 (1.5%) patients carried both a *BRCA1/2* and Other BC susceptibility gene mutation (Fig2 B). Among those variants in breast cancer susceptibility genes, 11 recurrent (detected in more than one patient) pathogenic variants were identified in 5 genes: 4 in *BRCA1*, 5 in *BRCA2*, 1 in *HMMR*, *PALB2* and *PTEN* respectively. 8 recurrent pathogenic variants were identified in 7 genes: 2 in *ZFHX3*, 1 in *FANCG*, *MPL*, *PRF1*, *NF1*, *NTRK1,* and *SBDS* respectively. Detailed information can be found in Supplementary Table S4.

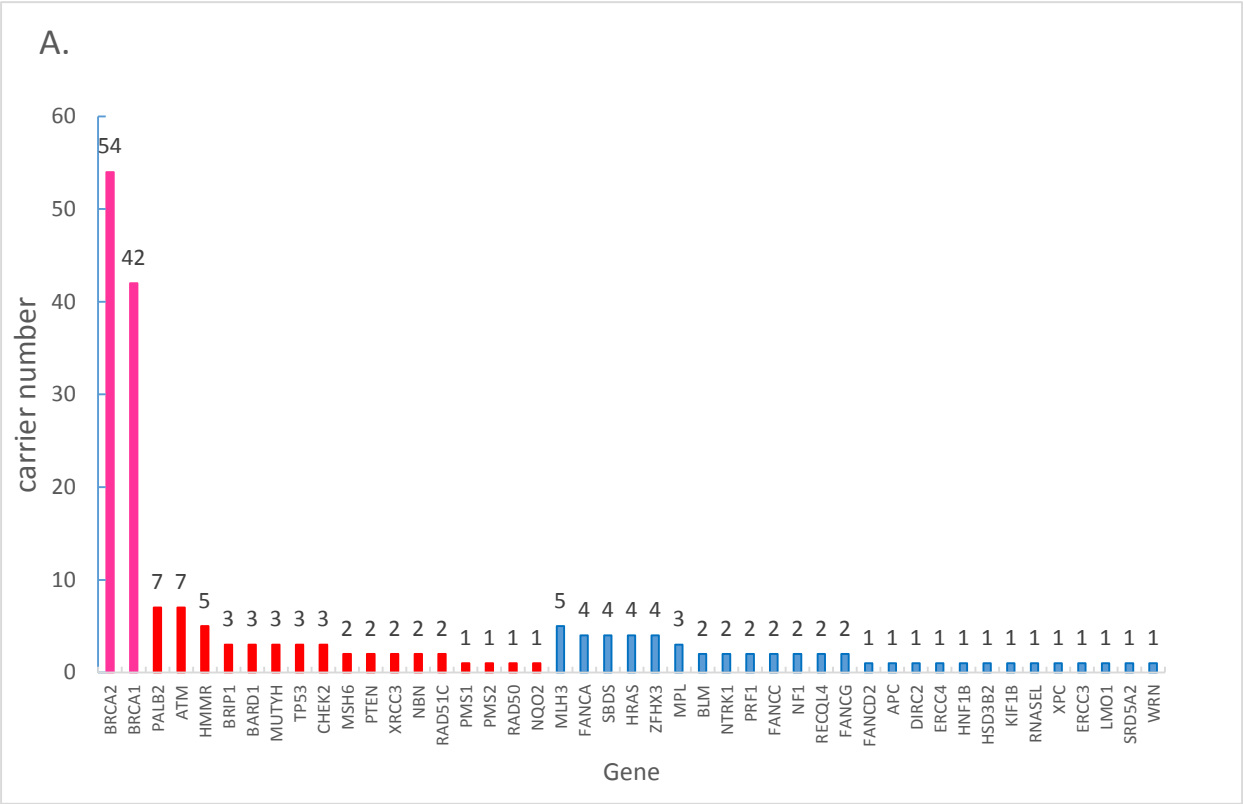


Figure 2A The pathogenic distribution of BRCA1/2 (pink), breast cancer risk related other BC susceptibility genes (red) and other cancer susceptibility genes (blue).

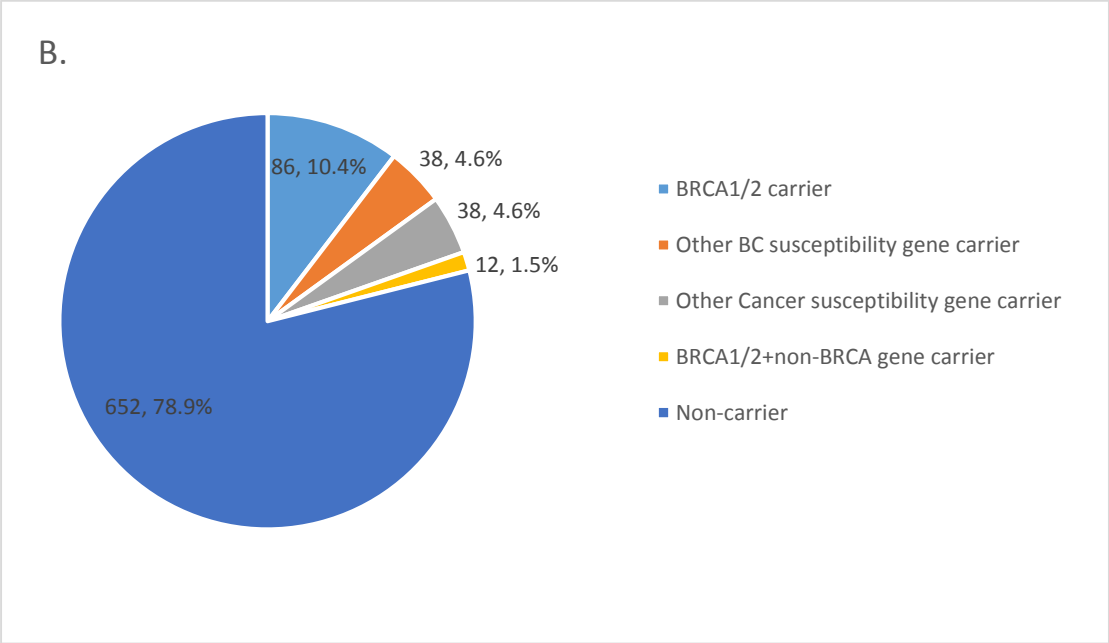


Figure 2B The pathogenic variants distribution in the cohort. Variants were classified by 4 main groups. BRCA1/2 group, other breast cancer susceptibility gene group, other cancer susceptibility gene group,

and non carrier. There a also a small proportion of people carried more than one variants that belongs to different groups.

***BRCA1* and *BRCA2* Pathogenic Variants and Clinical characteristic**

The prevalence of *BRCA1* and *BRCA2* pathogenic variant in 829 breast cancer patients is 5.0% and 6.5% respectively. The *BRCA1/2* mutation frequency found in our research is similar with the results from other studies with Chinese population but distinct from reports based on other populations (Table1).

Table1. *BRCA1* and *BRCA2* mutation frequency in high-risk breast cancer patients of different nationality/race.

Nationality/race	Case number	BRCA1/2 mutation frequency(%) [a]			Reference
		BRCA1/2	BRCA1	BRCA2	
Chinese	133	15.1	6.8	8.3	Po-Han Lin(45)
	99	18.1	7	11.1	Xiaochen Yang(46)
	651	10.6	4.5	6.1	Ava kwong(47)
	409	10.5	3.9	6.6	Juan Zhang(48)
Indian	61.	27.88	24.6	3.28	Vaidyanathan K(49)
Japanese	135	26.7	12.6	14.1	Kokichi Sugano(50)
	113	31.9	13.3	18.6	Noriko Ikeda(51)
Singaporean	90.	15.6	6.7	8.9	Peter Ang(52)
Pakistani	176	17.1	13.1	4.0	Muhammad U. Rashid(53)
Spanish	35	37	17	20	Gemma Llort(54)
	53	24.5	15.1	9.4	Torres Diana(55)
Poly-ethnic [b]	1805	22.5	11.8	10.7	Allison W. Kurian(56)
	1781	9.3	--	--	Nadine Tung, MD(57)
	40355	12.5	--	--	Michael J. Hall, MD, MS(3)

[a] The criteria of high-risk breast cancer are slightly different in the studies and this may affect the detection rate of *BRCA1/2* mutations. [b] The main race of poly-ethnic is White/Caucasian.

We found 9 recurrent pathogenic variants in *BRCA1* and *BRCA2*: 4 in *BRCA1* (NM_007294:c.1465G>T, NM_007294:c.3294delT, NM_007294:c.5156delT and NM_007294:c.5470_5477delATTGGGCA) and 5 in *BRCA2* (NM_000059:c.5864C>G, NM_000059:c.6698_6699insTTTT, NM_000059:c.7617+1G>A, NM_000059:c.9070_9073delAACA and NM_000059:c.9382C>T). *BRCA1* NM_007294:c.1465G>T and *BRCA2* NM_000059:c.5864C>G were previously reported in Chinese high-risk breast cancer[ref], and

the other variant *BRCA1* NM_007294:c.5470_5477delATTGGGCA was reported several times in Asian populations.[ref] However, these variants have not been reported in other ethnic groups. *BRCA2* NM_000059:c.7617+1G>A and BRCA2 NM_007294:c.5470_5477delATTGGGCA were previously reported in American or European subjects[ref]. The remaining four (*BRCA1* NM_007294:c.3294delT, *BRCA1* NM_007294:c.5156delT, *BRCA2* NM_000059:c.6698_6699insTTTT and *BRCA2* NM_000059:c.9070_9073delAACA) are novel variants, these variants need to be further validated in large-scale.

Among enrolled 829 patients,815 (98.3%) of them are female. 386 (46.5%) of the females are younger than age 40, 422 (50.9%) are older than age 40. The age information of the rest 21 patients (2.6%) is missing. The number of BRCA1/2 carrier is not significantly high in younger. But BRCA1/2 carrier number is significantly higher in patient with BC family history and BRCA1 carrier is significantly high in triple negative breast cancer patients.

	Basic info	Case num(ratio)	BRCA1	BRCA2	PALB2	ATM	P1	P2	P3
Gender	Male	14 (1.7%)	1	4	0	1	0.5	0.01	0.39
	Female	815 (98.3%)	39	50	7	6			
Diagnostic age	20-29	61 (7.3%)	4	3	1	0	0.31	0.093	0.73
	30-39	325 (39.2%)	12	16	1	2			
	40-49	276 (33.3%)	16	18	3	4			
	⩾ **50**	146 (17.6%)	8	15	2	1			
	Unknown	21 (2.6%)	0	2	0	0			
Breast cancer Pos	Unilateral	747 (90.1%)	33	46	7	6	0.12	0.3	0.76
	Bilateral	66 (8.0 %)	6	6	0	0			
	Unknown	16 (1.9%)	1	2	0	1			
Molecular subtype	Luminal A	209 (25.2%)	6	11	2	3			
	Luminal B	245 (29.6%)	7	20	2	1			
	HER2+	66 (8.0%)	2	1	0	0			
	TNBC	146 (17.6%)	19	9	1	1	<0.001	0.96	0.002
	Unknow	163 (19.6%)	6	13	2	2			

Family member	with BC*	267 (32.2%)	17	32	5	4	0.027	<0.001	0.562
	with other C	174 (21.0%)	13	8	0	1	0.013	0.64	0.175
	with no C	376 (45.4%)	10	14	2	2			
	Unknown	12 (1.4%)	0	0	0	0			
Total		829 (100%)	40	54	7	7			

Table2. Characteristics of patient information and comparison of BRCA1/2 mutation prevalence between different characteristic. P1 means p-value of BRCA1 carriers VS. non-carriers, P2 means p-value of BRCA2 carriers VS. non-carriers, P3 means p-value of BRCA1 carriers VS. BRCA2 carriers. *: families with BC compared with families with no Cancer

Non-BRCA Gene Pathogenic Variants

In all, 98 pathogenic variants were identified in 43 non-BRCA genes from 90 patients, the prevalence of non-BRCA gene variants in 829 patients is 10.9%. The number of variant loci is 82, and 18 variant loci were reported in the NCBI ClinVar database or published literature, the rest of the 64 (78%) variants is novel. Detailed information can be found in Supplementary Table S4. In addition, 11 variant loci appeared two or more times, including *FANCG* NM_004629:c.572T>G, *HMMR* NM_012484:c.1989_1990insA, *MPL* NM_005373:c.1908A>G, *NF1* NM_001128147:c.1736_1737insT, *NTRK1* NM_001007792:c.627+2T>C, *PALB2* NM_024675:c.2167_2168delAT, *PRF1* NM_005041:c.65delC, *PTEN* NM_000314:c.697C>T, *SBDS* NM_016038:c.258+2T>C, *ZFHX3* NM_001164766:c.6842_6843insT and *ZFHX3* NM_001164766:c.6847_6848insAG. There were 44 variants in the 18 genes (40.9%, including *ATM, BARD1, BRIP1, CHEK2, HMMR, MLH3, MSH6, MUTY, NBN, NQO2, PALB2, PMS1, PMS2, PTEN, RAD50, RAD51C, TP53* and *XRCC3*) that have been reported in high-risk breast cancer studies, the remaining 26 (59.1%) genes need further investigation and validation in high-risk breast cancer disease.

Table3. Pathogenic variation of other cancer genes

Gene	Carrier Num.	Cancer type
MLH3	5	Colorectal Cancer(58)
FANCA*	4	AML, ALL(59-61)
SBDS*	4	MDS, AML(62, 63)
MPL*	3	MDS, AML(64, 65)
BLM*	2	ALL(66, 67)
NTRK1*	2	Thyroid Cancer(68)
PRF1	2	Lymphoma, Leukemia(69-71)
FANCC*	2	AML, ALL(60)
NF1	2	neurofibroma, glioma(72, 73)
RECQL4*	2	Osteosarcoma, AML(74, 75)
FANCG*	2	AML, leukemia(60)
HRAS	2	neuroblastoma, bladder cancer(76)
ZFHX3	2	Prostate(77)

*** Means genetic defects in this gene will result in autosomal recessive disorder.**

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome

Clinical screening of mutation carrier

Some mutation carrier families (other genes pathogenic mutation carriers, excluding BRCA1, BRCA2 or PALB2) received further clinical screening as described in method section. Pre-cancer lesions were found in three families. In the first family, one PMS2 pathogenic variant carrier (proband, study ID: 30033, diagnosed with breast cancer at 55 years old) was found to have colon polyp (diagnosed at 58 years old). Her sister (study ID: 30033FM1), who is also a breast cancer patient (diagnosed at 44 years old), was found to have colon villioustublar adenoma with low-grad intraepithelial neoplasia (diagnosed at 52 years old) and the lesion was removed by endoscopic resection [Figure 3A]. They were also both found to have stomach fundus polyp and gastritis. Their father and father's sister already died because of stomach cancer. They were recommended to do examination again one year later. In the second family, one ATM

gene mutation carrier who was diagnosed with breast cancer at age 81, (proband study ID30491) refused the clinical screening because of advanced age. But he has two daughters with ATM gene mutation. One of them (study ID: 30491FM2) had been diagnosed with breast cancer (diagnosed at 39 years old). The other one (study ID: 30491FM3) was found to have colon tubular adenoma, atrophic gastritis and stomach fundus erosion in this screening (at 58 years old) [Figure 3B]. In the third family, the proband (study ID: 30306) is both ATM and PALB2 mutation carrier (diagnosed with breast cancer at 47 years old). She was found to have colon villioustublar adenoma and superficial gastritis with erosion (diagnosed at 48 years old) [Figure 3C]. Her niece (study ID: 30306FM2) was found to have rectal tubular adenoma (diagnosed at 39 years old), however, she is not a mutation carrier. All adenoma were removed during colonoscopy.

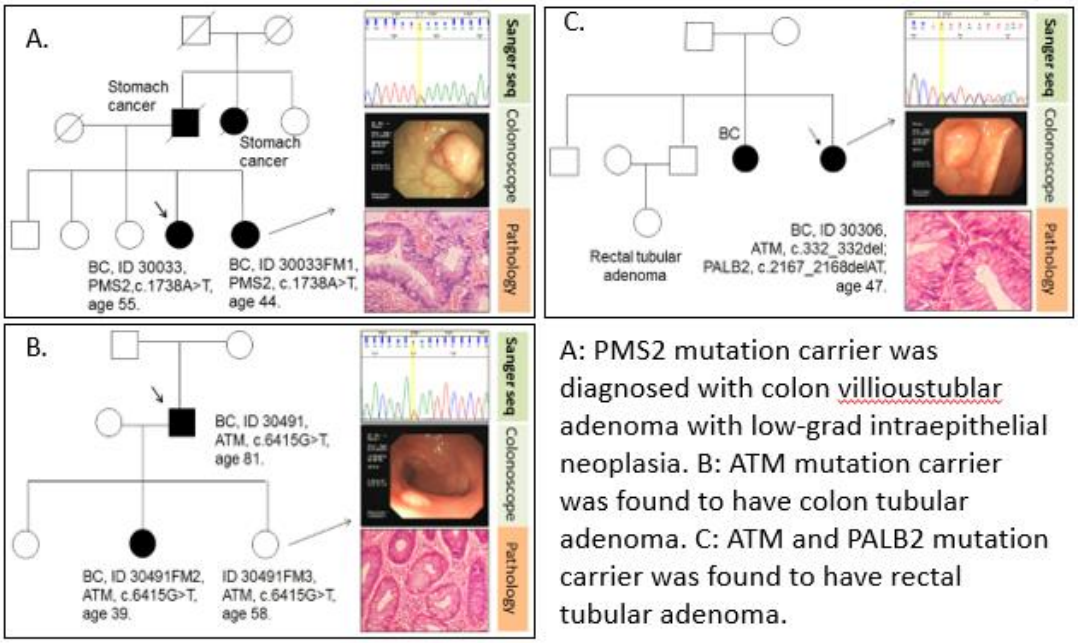


Figure3. The Clinical screening of three pathogenic variants carrier families

VUS distribution

All VUS in 115 hereditary cancer susceptibility genes were detected and analyzed. In total, 2632 VUS were identified in 115 genes from 787 of 829 patients. The total VUS number in 27 breast cancer susceptibility genes was 708 (Fig4 A), of which *BRCA1* and *BRCA2* had 5.1% and 12.1% VUS respectively.

Meanwhile, we analyzed the VUS frequency distribution in all patients and found that 684 (82.5%) patients carried more than one VUS, and 42 patients did not carry any VUS (Fig4 B). Detailed information can be found in Supplementary Table S5.

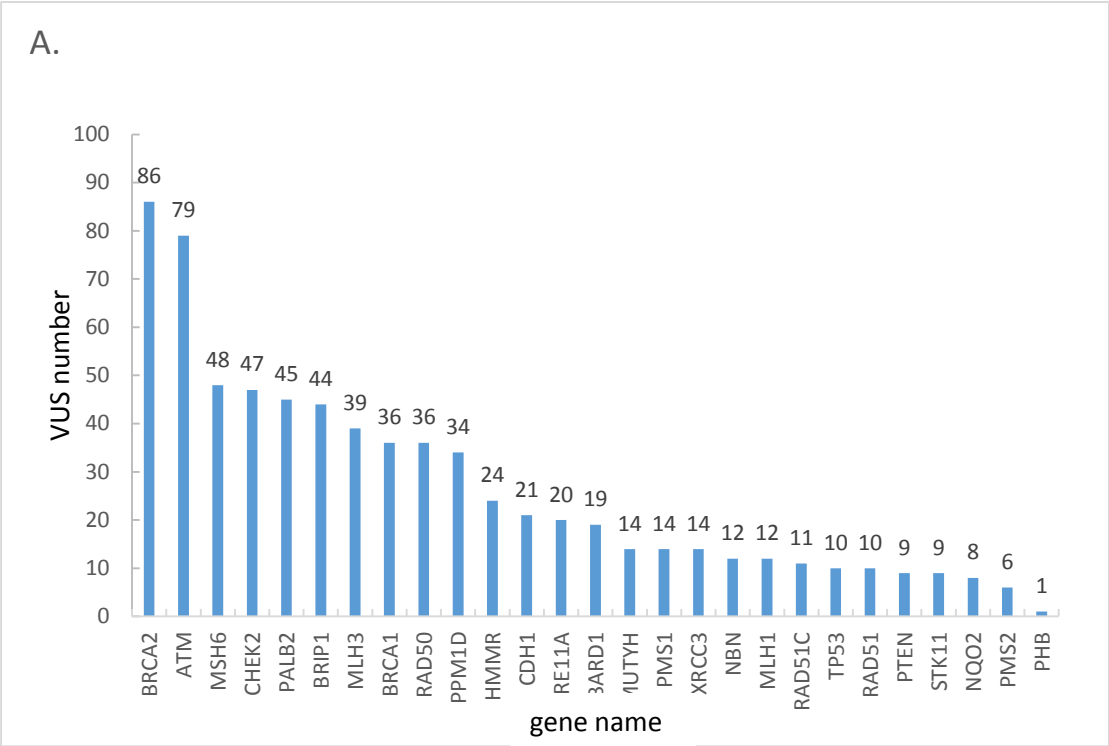


Figure 4A Total VUS distribution in genes.

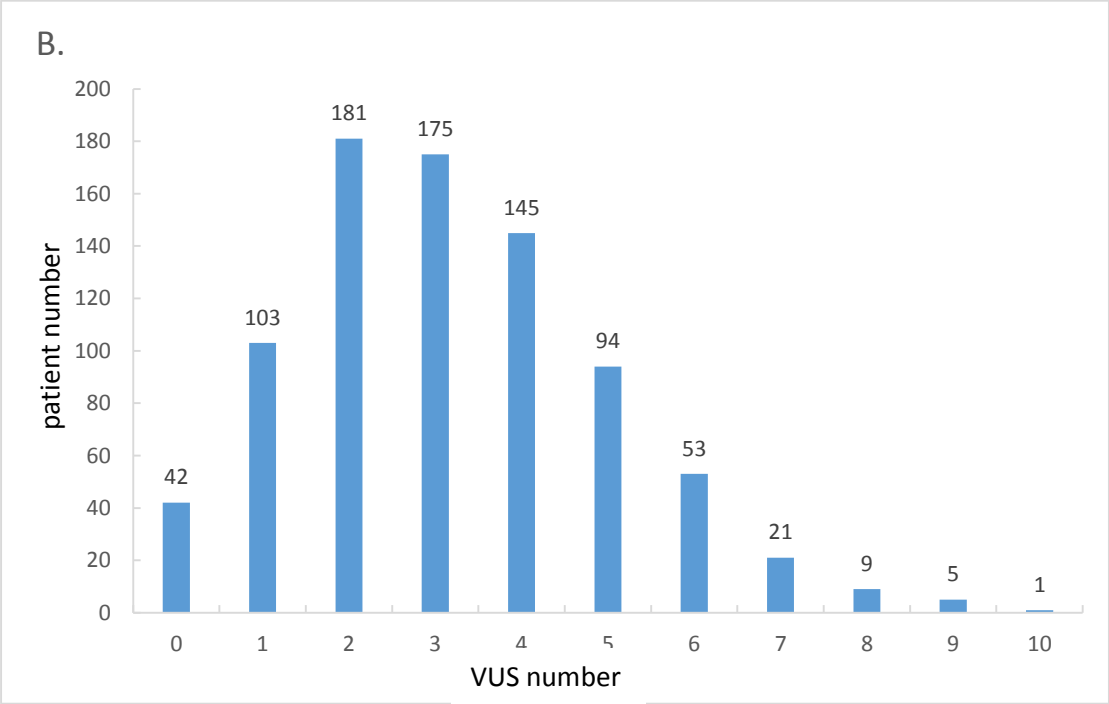


Figure 4B The average VUS number distribution in VUS carriers.

Functional validation of VUS

All 5 VUS in BRCA1 RING domain and BRCT domain were (seeing in table 4) submitted to a company for functional validation, which was conducted with biochemical function assay (RING domain) and phenotype recapitulation assay (BRCT domain).

Table 4. VUS functional validation results

Gene variant	RING/BRCT domain in BRCA1	Classification
p.Cys24Arg	RING	Pathogenic
p.Leu1679Val	BRCT	Benign
p.Val1809Phe	BRCT	Benign
p.Ile1807Val	BRCT	Benign
p.Leu1679Val	BRCT	Benign

One variants (p.Cys24Arg) was defined as pathogenic, and Other 4 variants were defined as benign. The result is consistent with recently reported massive BRCA1 functional analysis[ref].

Discussion

The major hurdle in utilizing NGS data lies in how to interpret the genotype-phenotype relationships, especially in clinical settings. First key element is automatic: A larger number of germline variants are called after WES/WGS sequencing, it will be time-consuming if we completely depend on manual judgement. Secondly, the standardization: A high proportion of variant classifications are discrepant between intra- and inter-laboratory settings resulting from different understanding on ACMG guideline and a lack of unified standard(78). So the experts review based on comprehensive evidence is also necessary. And the classification evidence should be easily exhibited for peer review. Taken above concerns into

consideration, we developed VCE to classify variants identified in our study. It could do the classification automatic based on objective evidence and marked variants that need manual check. It is also open to the further extend of literature database, it will become more applicable and less manual intervention will be needed.

There is a breast cancer cumulative risk of people carrying a *BRCA1*, *BRCA2* or *PALB2* pathogenic variation (79, 80). which demonstrated that the carriers of mutations in these susceptibility genes have a significantly higher risk of breast cancer. Additional hereditary breast cancer-related susceptibility genes have also been studied by multiple gene panel sequencing previously(56). However, only a limited number of such studies have been conducted in China, due to the high cost of massive sequencing. Our data demonstrates a pathogenic mutation frequency in BRCA1 and BRCA2 of 11%, similar to other studies. Another large Chinese study was published from Shanghai, reporting a 9.1% frequency in women with at least one breast cancer risk factor(14). They identified the BRCA1 c.5470_5477del mutation as common in their population, a mutation we also report as a recurrent mutation. Besides BRCA1 and BRCA2, PALB2 and ATM also showed high prevalence as Xie et al ‘s research(11) based on unselected breast cancer patient. But HMMR and BRAD1 shows higher positive rate which remind those 2 genes may be important for high risk patients. The total positive rate of breast cancer related genes is 15.4% in our cohort, a median rate between familial breast cancer and early onset breast cancer. In our cohort, patients with age less than 40 is not associate with BRCA1/2 mutation carrier.

Shao et al ‘s research(14) indicates the cut off value may be set to 45. Our results confirm that conclusion. The susceptibility genes of other cancer are mainly about gastric cancer and AML. Those patients are enrolled in our research mainly by their family history. Our clinical follow up also confirm that. So, a

comprehensive clinical genetic consulting including multiple department is needed. Otherwise the patient's susceptibility genes of other cancer may be not well-treated.

Another problem in variants interpretation is VUS. In our cohort, a total of 2632 VUS spots on 115 genes were identified in 787 out of 829 subjects, among which 684(82.5%) patients carried more than one VUS. According to other data from 1112 Shenzhen HBC high risk people, VUS rate of 21 breast cancer related gene is 55.04%. BRCA1 and BRCA2 in our data had (5.1%) and (12.1%) VUS rate respectively. Therefore, Chinese cohorts generally present a higher VUS rate than data from Myraid(81). It would be important to lower the frequencies of VUSs in Chinese cohorts. Recently, the large scale of variants validation on BRCA1 is conducted(82). This research provided new evidence for RING and BRCT domain in BRCA1. But there are still some short comes when applying in clinical. For instance, all the editing was conducted in same cell line. However, the real patient will have a more complicate genetic background. It's critical to confirm if those validation is consistent with clinical observation.

Our research is a multiple-gene test based hereditary breast cancer research with a big data set in China, which provides a comprehensive perspective of the breast cancer pathogenic variation in susceptibility gene as well as VUS spectrum in Chinese. With our data, we present the variant spectrum of 115 hereditary cancer-related susceptibility genes in 829 high-risk breast cancer patients.

In this research, we develop a semi-automatic variation classification tools and identified the function of five BRCA1 VUS in Chinese cohort. By analyzing our data, we provide a comprehensive overview of the pathogenic variant detection rate and the difference between Chinese and other populations. We have established screening pipeline and explorer the usage of multiple-gene hereditary cancer test in clinical practice of China. This work will aid the prevention and treatment of breast cancer in the Chinese people.

Abbreviations

NCCN: National Comprehensive Cancer Network, **HGMD:** Human Gene Mutation Database**, VUS:** variants of uncertain significance, **ACMG:** American College of Medical Genetics,

Declarations

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Competing Interests

The authors declare that they have no competing interests.

Authors' contributions

Conception and direction: Da Pang, Guibo Li, Shida Zhu, Yong Hou, Xianming Wang, Michael Dean, Xiaofei Ye, Huanming Yang, Jian Wang.

Acquisition of pedigree information and blood: Xianyu Zhang, Bingbing Song, Min Wang, Wenjing Jian,

Jingjing Xie, Bingshu Xia, Shouping Xu.

Sample treatment, sequencing and data analysis: Xiaohong Wang, Boyang Cao, Kang Shao, Meng Liu,

Liyun Xiao, Zhao Zhang, Enhao Fang, Haoxuan Jin, Xiaofeng Wei.

Manuscript writing: Xiaohong Wang, Kang Shao, Michael Dean, Cong Lin

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Supplementary

Comparison of the two sequencing platforms

DNA was extracted from YH cells – a normal cell line, used to construct a library, captured by the same BGI Chip, and sequenced on the two different platforms, Blackbird and Hiseq 2500, respectively. To compare the divergence in variant calls on these sequencing platforms, 0.6G base data were generated with at least 400X and 200X depth by Blackbird and Hiseq 2500 platform respectively, and 99% coverage on target regions. Variants were then called by the corresponding pipelines. In total, 409 SNPs and 38 Indels were identified on both the Blackbird and Hiseq 2500 platforms; 8 SNPs and 7 Indels were specifically detected on the Blackbird platform. 36 SNPs and 51 indels were called on Hiseq 2500 platform specifically. Then all the SNPs, all 7 indels detected by Blackbird and 28 of 51 indels detected by Hiseq were selected for sanger sequencing.

Sanger sequencing was used to validate selected variants from these sequencing platforms. Pairs of primers for amplification and Sanger sequencing were designed to target regions flanking 29 out of 44 SNPs and 32 out of 35 Indels. The rest of the variants were unable to be confirmed due to their location in repeat regions. PCR amplification and Sanger sequencing were then performed. On the Blackbird platform, 7 SNPs and 2 Indels were validated, 1 Indel was not, and 4 Indels were unable to be confirmed due to the duplication of A or T. However on the Hiseq 2500 platform, 12 SNPs and 3 Indels were both validated, 2 SNPs and 2 Indels not, and 8 SNPs and 20 Indels unable to call.(Supplementary Figure S1 and Table S3).