



Genomic Profiling Comparison of Germline *BRCA* and Non-*BRCA* Carriers Reveals *CCNE1* Amplification as a Risk Factor for Non-*BRCA* Carriers in Patients With Triple-Negative Breast Cancer

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Background: Differences in genomic profiling and immunity-associated parameters between germline *BRCA* and non-*BRCA* carriers in TNBC with high tumor burden remain unexplored. This study aimed to compare the differences and explore potential prognostic predictors and therapeutic targets.

Methods: The study cohort included 21 consecutive TNBC cases with germline *BRCA1/2* mutations and 54 non-*BRCA* carriers with a tumor size ≥ 2 cm and/or ≥ 1 affected lymph nodes. Differences in clinicopathological characteristics and genomic profiles were analyzed through next-generation sequencing. Univariate Kaplan–Meier analysis and Cox regression model were applied to survival analysis. Immunohistochemistry was used to confirm the consistency between *CCNE1* amplification and cyclin E1 protein overexpression.

Results: The cohort included 16 and five patients with germline *BRCA1* and *BRCA2* mutations, respectively. Patients with germline *BRCA1/2* mutations were diagnosed at a significantly younger age and were more likely to have a family history of breast and/or ovarian cancer. Six non-*BRCA* carriers (11.11%) carried germline mutations in other cancer susceptibility genes, including five mutations in five homologous recombination repair (HRR) pathway genes (9.26%) and one mutation in *MSH3* (1.85%). Somatic mutations in HRR pathway genes were found in 22.22 and 14.29% of the non-*BRCA* and *BRCA* carriers, respectively. *PIK3CA* missense mutation ($p = 0.046$) and *CCNE1* amplification ($p = 0.2$) were found only in the non-*BRCA* carriers. The median tumor mutation burden (TMB) was 4.1 Muts/Mb, whereas none of the cases had high microsatellite instability (MSI). *BRCA* status did not affect disease-free survival (DFS, $p = 0.15$) or overall survival (OS, $p = 0.52$). *CCNE1* amplification was an independent risk factor for DFS in non-*BRCA* carriers with TNBC (HR 13.07, 95% CI 2.47–69.24,

$p = 0.003$). Consistency between *CCNE1* amplification and cyclin E1 protein overexpression was confirmed with an AUC of 0.967 for cyclin E1 signal intensity.

Conclusions: We found differences in genetic alterations between germline *BRCA* and non-*BRCA* carriers with TNBC and a high tumor burden. TMB and MSI may not be suitable predictors of TNBC for immune checkpoint inhibitors. Notably, *CCNE1* amplification is a novel potential prognostic marker and therapeutic target for non-*BRCA* carriers with TNBC. Cyclin E1 may be used instead of *CCNE1* to improve clinical applicability.

Keywords: triple negative breast cancer, *BRCA1/2*, *CCNE1*, genomic profiles, tumor mutation burden, microsatellite instability

INTRODUCTION

Triple-negative breast cancer (TNBC) has been defined as a subtype of breast cancer negatively expressing estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (1), accounting for ~15–20% of newly diagnosed breast cancer (2). Women with TNBC have a higher rate of early distant recurrence and worse 5-years prognosis than those with other subtypes of breast cancer (3, 4).

Hereditary breast cancer differs from sporadic breast cancer because of the impact of germline genetic variation. Testing for germline mutations in breast cancer predisposition genes has become a standard practice (5). *BRCA*-associated breast cancer is the most common type of hereditary breast cancer (6, 7). In addition to *BRCA1/BRCA2*, some other germline mutations in high- and moderate-penetrance genes play significant roles in increasing breast cancer risk for mutation carriers (8). However, testing for other breast cancer predisposition genes such as *TP53*, *PTEN*, and *PLAB2* is still required to determine family history or specific clinical features (9, 10) compared with other cancer predisposition genes. Not all patients with germline mutations have a known family cancer history or specific clinical features, which results in about 50–80% of at-risk individuals not being successfully identified as such (11). Thus, these criteria alone may not provide sufficient information for testing and assessment of other genetic cancer predisposition genes (12) in non-*BRCA* carriers with TNBC.

Recently, next-generation sequencing (NGS) has been increasingly used in cancer risk assessment in clinical practice (13). Sequencing with multigene panels may identify significant differences to further understand the relationship between genetic profiling and tumor biology (14). TNBC is highly intertumorally and intratumorally heterogeneous and

incorporates various molecular, and clinical pathological features and distinct clinical outcomes (15). Hence, sequencing with multigene panels can be used to recognize the heterogeneity of TNBC through genomic profiling.

The POSH study (16) showed the early survival advantage of patients with *BRCA* mutation carriers with TNBC, who have a lower likelihood of dying from early breast cancer than non-*BRCA* carriers. This advantage might reflect the higher sensitivity of DNA repair deficiency associated with *BRCA*-mutant breast cancers to chemotherapy, particularly that of a higher response to platinum-based drugs (17, 18) or the greater visibility of *BRCA*-mutant breast cancers to host immune attack (19). Although poly-ADP-ribose polymerase (PARP) inhibitors displayed clinical efficiency for approved *BRCA*-mutant patients with TNBC (20), the potential targeted therapeutic treatments for non-*BRCA* carriers with TNBC need to be explored considering the growing evidence associating germline mutations with cancer predisposition as well as the availability of targeted therapies (21–23).

Tumor mutation burden (TMB) (24) and microsatellite instability (MSI) (25) have been extensively investigated in breast cancer. Given the lack of effective targeted therapies for TNBC, immunotherapeutic approaches and associated predictive markers, such as TMB, MSI, and programmed cell death ligand 1 (PD-L1) (26), remain a focus of great interest. Romualdo et al. (27) found that a high TMB was associated with clinical benefit in patients with metastatic TNBC receiving anti-programmed death 1(PD-1)/PD-L1 therapy. Yoshiya et al. (28) evaluated MSI in 63 patients with TNBC exhibiting a high number of tumor-infiltrating lymphocytes and found that MSI-high (MSI-H) tumors were absent among those with enriched PD-L1 responding to immunotherapy. There are currently no specific guidelines for assessing TMB and MSI in TNBC. In addition, a high TMB and MSI-H tumors are not uncommon in patients with TNBC, who are potential candidates for treatment with immune checkpoint inhibitors (ICIs) (29). It remains necessary to study the role of TMB and MSI (30) in relation to immunotherapy in Chinese patients with TNBC.

Previous studies have reported the overall genomic landscape of unselected (without any specific tumor characteristics) TNBC cases in Chinese (14, 31, 32), American, and European populations (32–34). For example, Jiang (31) classified TNBCs

Abbreviations: NGS, Next-generation sequencing; TNBC, Triple-negative breast cancer; LVI, Lymphovascular invasion; PUMCH, Peking Union Medical College Hospital; ER, Estrogen receptor; PR, Progesterone receptor; DFS, Disease-free survival; OS, Overall survival; HRR, Homologous recombination repair; PARP, Poly-ADP-ribose polymerase; CNV, Copy number variations; TMB, Tumor mutation burden; MSI, Microsatellite instability; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; TCGA, The Cancer Genome Atlas; FFPE, Formalin-fixed and paraffin-embedded; IHC, Immunohistochemistry; AUC, Area under the curve.

into four transcriptome-based subtypes by comprehensively analyzing the clinical, genomic, and transcriptomic data of a cohort of 465 unselected primary triple-negative breast cancer patients. However, the genomic profiles of selected TNBC cases with specific tumor characteristics such as large tumor size or involved lymph nodes remain to be explored. In addition, these studies lack data about differences in genomic profiles via the comparison between germline *BRCA* and non-*BRCA* carriers. Even Chen et al. (35) mainly identified the differences in somatic mutation profiles between *BRCA*, non-*BRCA* germline mutation, and non-carriers with unselected breast cancer not focused on TNBC. Immunity-associated parameters, potential predictors, and therapeutic targets for non-*BRCA* carriers with TNBC were not included in these studies, either. Furthermore, TNBCs with a high tumor burden were associated with worse prognosis (36) and required the identification of distinct prognostic predictors and potential therapeutic targets.

In the present study, we performed a capture-based, targeted NGS utilizing a panel comprising 508 cancer-associated genes in Chinese patients with TNBC with high tumor burden. We aimed to identify potential prognostic and therapeutic markers in non-*BRCA* carriers with TNBCs exhibiting a high tumor burden by comparing differences in genomic profiling together with immunity-associated parameters in patients with different germline *BRCA* status.

METHODS

Ethics

All the procedures performed in this study involving human participants were conducted following the ethical standards of the institutional and national research committees and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. The study was approved by the Ethics Committee of Peking Union Medical College Hospital (No. HS-1623), and written informed consent was obtained from all participants.

Patients and Specimens

Triple negative breast cancer patients that met the eligibility criteria including $T \geq 2$ cm and/or number of affected lymph nodes ≥ 1 were acquired from Peking Union Medical College Hospital between October 2013 and April 2019 and were selected to form a consecutive cohort including 87 primary TNBC patients. Triple negative breast cancer was defined as negative ER, negative PR and negative human epidermal growth factor receptor-2 (HER2). ER, PR, HER2, and other receptors in each specimen were routinely evaluated by immunohistochemistry (IHC) staining at the Department of Pathology in Peking Union Medical College Hospital. HER2 status was confirmed by IHC and/or fluorescence *in situ* hybridization according to the 2018 American Society of Clinical Oncology/College of American Pathologists Clinical Practice guidelines (37). We excluded patients with a diagnosis of other malignant tumors such as gastric, lung, and ovarian cancer.

Primary tumor formalin-fixed and paraffin-embedded (FFPE) tissue and paired peripheral blood samples were obtained along

with their clinical and therapeutic information in the same batch. NGS with a panel of 508 cancer-associated genes was applied to both specimens and blood samples within 1 year after the initial treatment. Clinicopathological characteristics and treatment regimen, including age; T stage; histological grade; TNM stage; lymph node status; lymphovascular invasion (LVI); ER/PR/HER2 status; and treatment variables, including surgery, chemotherapy, and radiation therapy, were collected.

Study Design and Endpoints

Patients with TNBC were allocated to groups according to the germline *BRCA1/2* mutation status. Disease-free survival (DFS) was defined as the time from the date of surgery until first disease recurrence at a local, regional, or distant site or the diagnosis of contralateral breast cancer. Overall survival (OS) was defined as the time from the date of surgery until death from any cause. Patients without any events were censored at the date of last follow-up.

Targeted Exome Sequencing of TNBCs

Genomic DNA (gDNA) was extracted from FFPE and peripheral blood samples using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) as per the manufacturer's protocol. DNA concentration and quality were assessed by Qubit (Life Technologies, Gaithersburg, MD, USA) and agarose gel electrophoresis. gDNA (250 ng) was used for sequence library construction using a previously described method (38). The hybridization product was subsequently purified, amplified, and quantified. Finally, sequencing of 508 key cancer-related genes was performed with a paired-end 100 and 8 bp barcodes on a MGISEQ-2000 sequencer following the manufacturer's protocol.

Raw data were first filtered by SOAPnuke to exclude low-quality reads. Clean reads were then aligned to the reference human genome (UCSC hg19) using the BWA MEM algorithm. Single-nucleotide variants were detected by the Genome Analysis Toolkit (GATK) Unified Genotyper. Small insertions and deletions were identified using GATK Haplotype. Copy number variations (CNVs) were identified using read-depth analysis. All above variants were further filtered by quality depth, strand bias, mapping quality, and read position. Each variant was finally annotated with respect to gene location.

All deleterious germline mutations were confirmed by Sanger sequencing in duplicate samples. Pathogenic mutations were defined as those leading to a truncated protein or those that have been previously reported to be associated with disease.

Analysis of MSI

MSI analysis was determined by next generation sequencing of 16 microsatellite loci, including BAT25, BAT26, NR24, D2S123, D5S346, NR21, MONO27, BAT40, BAT34c4, D18S55, D1S2883, D17S261, D17S799, D18S35, D18S58, and D17S250 (39–41). The sequences were compared with matched peripheral blood samples. The mSINGS, MSISensor, and MANTIS algorithms were used to determine if extent of the detected instability was significant, and the samples were categorized as MSI-H, MSI-low, or microsatellite stable.

Immunohistochemistry (IHC)

Cyclin E antibody (clone HE12) was obtained from Milipore (Temecula, CA, USA). FFPE tissue sections were deparaffinized and hydrated. Antigen retrieval was performed using pH9 Antigen Retrieval Solution (DAKO). Peroxide blocking was performed using 3% H₂O₂ in methanol at room temperature for 5 min. The slides were incubated with primary antibody for 30 min for all antibodies at a concentration of Cyclin E 1:2,000. EnVision Flex+ (DAKO) was used as the detection system following the manufacturer's instructions and was developed using freshly prepared 0.05% 3',3'-diaminobenzidine tetrahydrochloride.

Finally, the slides were counterstained with hematoxylin, dehydrated, and mounted. Positive and negative controls of placenta tissues were performed in each run. Cyclin E immunohistochemical expression was quantified by two independent pathologists who were blinded to the identity of the samples using a four-value intensity score (0, 1+, 2+, and 3+), expression score (H score), and the percentage extent of reactivity. A consensus value on both intensity and extension was reached by the two independent observers. A final consensual score was obtained by multiplying both intensity and extension values (range, 0–300) (42).

Comparison of CCNE1 Status Using Public Databases

Difference of *CCNE1* amplification between patients with/without TNBC was compared via the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) (43) and the Cancer Genome Atlas (TCGA) (44) datasets. All *CCNE1* profiling data from METABRIC or TCGA was obtained from analyzed NGS data provided by cBioPortal (www.cbioportal.org) (45). *CCNE1* expression of 150 samples from METABRIC, including 131 TNBC cases with normal *CCNE1* expression and 19 TNBC cases with *CCNE1* amplification, were used for comparison of OS.

Statistical Analysis

Categorical variables were analyzed by Pearson's chi-square test or Fisher's exact test. Kaplan–Meier (K–M) curves were used to display DFS and OS. Log-rank test was applied to test survival difference among groups with various genomic and clinical characters. Cox uni- and multivariate analyses were used to construct the risk model that predicted disease recurrence as well as cancer-related death. To determine whether IHC staining for cyclin E1 could be used instead of genetic testing for *CCNE1*, the consistence was assessed by calculating area under the ROC curve (AUC) with bootstrap correction.

RESULTS

Study Population

A total of 87 Chinese female patients with TNBC who met the eligibility criteria including a tumor size of ≥ 2 cm and/or ≥ 1 affected lymph nodes were included in the study. By NGS, the prevalence of germline *BRCA1/2* mutation was 24.1% (21/87) in the study cohort. Five patients with a diagnosis of other

malignant tumors including gastric carcinoma ($n = 2$), lung cancer ($n = 2$), and ovarian cancer ($n = 1$) were excluded from the study. Furthermore, three patients with incomplete medical records and four patients who were lost to follow-up were excluded. Finally, 21 germline *BRCA* carriers and 54 non-*BRCA* carriers were enrolled in the study (Supplementary Figure 1). The median follow-up was 30 months (range, 6–66 months), with a 4.6% of the patients lost to follow-up. The DFS and OS rates in *BRCA1/2* and non-*BRCA* carriers are shown in Supplementary Table 2.

Patient Characteristics and Treatment History

We first analyzed the clinicopathological characteristics to understand predisposing factors associated with germline *BRCA1/2* mutations in TNBC. Breast cancer was diagnosed at a significantly younger age in germline *BRCA* carriers than in non-*BRCA* carriers ($p = 0.007$, Table 1). Moreover, germline *BRCA1/2* carriers were more likely to have a family history of breast and/or ovarian cancer ($p = 0.008$, Table 1). However, other clinical characteristics showed no further significant correlations (Supplementary Table 1) between germline *BRCA1/2* mutation status and other clinicopathological factors, including histology ($p = 0.83$), T stage ($p = 0.65$), lymph node status ($p = 0.93$), histological grade ($p = 0.09$), LVI ($p = 0.44$), and Ki-67 index ($p = 0.48$), the same to surgical management of breast and axilla ($p = 0.88$ and 0.53 , respectively) and systemic treatment including chemotherapy and radiotherapy ($p = 0.48$ and 0.46 , respectively).

Overall Landscape of Germline BRCA Mutation and Somatic Mutation in This Chinese Cohort

Twenty-one patients were found carrying *BRCA1/2* mutations in individuals (Supplementary Figure 2B). Details for germline *BRCA1/2* mutations were shown in Table 2, in which no

TABLE 1 | Clinicopathological characteristics of Chinese female patients with triple-negative breast cancer according to *BRCA* germline mutation status in this study cohort ($p < 0.05$).

Characteristics (N = 75)	Parameter	No. (%)		p-value
		BRCA germline mutation	BRCA non-carrier	
Age, years	≤ 35	8 (38.1)	14 (25.9)	0.007
	36–45	11 (52.4)	12 (22.2)	
	46–55	1 (4.8)	18 (33.4)	
	>55	1 (4.7)	10 (18.5)	
Family history	None	11 (52.4)	45 (83.3)	0.008
	BRCA-related	9 (42.9)	6 (11.1)	
	Non-BRCA-related	1 (4.7)	3 (5.6)	

P-values were derived from the Pearson's Chi-square test, Fisher's exact test and Continuity Correction chi-square test.

synonymous variation was found in all single nucleotide variants (SNV).

The average target coverage depths were $\geq 500 \times$ for all specimens and blood samples. Among the TNBCs with genomic profiling data, 480 somatic mutations were identified (**Supplementary Figure 2A**), comprising 13 Delins (2.71%), 48 Frameshifts (10.00%), 253 Missenses (52.71%), 38 Nonsenses (7.92%), 17 Splices (3.54%), one Start-Alt (0.21%), and 110 CNVs (22.9%), including 102 copy number gains (21.25%) and eight copy number losses (1.67%). Copy number variations (CNVs) were identified by comparing sequence coverage of targeted regions in a tumor sample relative to the normal sample using CONTRA (46). First, read-depth statistics (log-ratio) were calculated from baits originating in the same exon. Then, Adjacent exons were merged into larger segments if the read depths of their component baits were not significantly different by *t*-test, and log-ratio were recalculated for the larger segments. We call segment a CNV event, if the segment has a log-ratio >0.3 (gain) or <-0.3 (loss). CNV analysis identified an average of 0.1 (range, 0–3) CNV loss and 1.4 (range, 0–10) amplified genes per patient (**Supplementary Figure 2B**).

Comparison of the Genomic Profiles of Germline BRCA and Non-BRCA Carriers With TNBC in a Chinese Cohort

Comparison of Germline Mutations

In the BRCA carrier group, 16 and five patients harbored BRCA1 and BRCA2 mutations, respectively. Conversely, in the

non-BRCA carrier group, six patients (11.11%, 6/54) harbored a total of six mutations in other cancer susceptibility genes beyond BRCA1/2. Of these, five mutations (9.26%, 5/54) were in homologous recombination repair (HRR) pathway genes, including PALB2 (1.85%, 1/54), BLM (1.85%, 1/54), NBN (1.85%, 1/54), RAD51C (1.85%, 1/54), and RAD51D (1.85%, 1/54), and one mutation was in MSH3 (1.85%, 1/54) related to Lynch syndrome. None of the patients harbored simultaneous BRCA mutations and other germline mutations ($p = 0.17$). Interestingly, among these eight genes, three belonged to the Fanconi anemia gene family, including BRCA2, PALB2, and RAD51C, with 25.92% (7/27) of patients carrying germline mutations in any of these genes.

Comparison of Somatic Mutations

Genomic alterations with variation allele frequency $\geq 4\%$ were listed (**Figure 1A**) to understand the intertumoral heterogeneity between the two TNBC subgroups. No somatic PIK3CA missense was detected in any of the patients with germline BRCA1/BRCA2 mutations (**Table 3**, $p = 0.046$). GRM3 mutation was found more in BRCA carriers (**Table 3**, $p = 0.03$). In addition, several somatic mutations also showed non-significantly enriching tendencies in non-BRCA carriers, including NOTCH2, B4GALT3, BCOR, WHSC1L1, NCOR1, and EPHA5 (**Supplementary Table 3**). Interestingly, somatic CCNE1 and IKBKB amplification in patients with TNBC were mutually exclusive with germline BRCA1/BRCA2 mutation ($p = 0.20$ and 0.20 , respectively, **Supplementary Table 3**) as well as other germline-mutated

TABLE 2 | BRCA mutation in details of 21 patients with TNBC in the cohort.

Patient no.	Gene_symbol	c.HGVS	p.HGVS	Variation type	Variation type	Synonymous or Non-synonymous
3	BRCA2	c.7975A>G	p.R2659G	SNV	Missense	Non-synonymous
8	BRCA1	c.2572C>T	p.Q858*	SNV	Nonsense	Non-synonymous
12	BRCA1	c.4698_4704del TGGAAATC	p.G1567Afs*32	Indel	Frameshift	/
32	BRCA2	c.3860delA	p.N1287Ifs*6	Indel	Frameshift	/
45	BRCA1	c.4801A>T	p.K1601*	SNV	nonsense	Non-synonymous
46	BRCA1	c.5521delA	p.S1841Vfs*2	Indel	Frameshift	/
47	BRCA2	c.3085_3087delATGinsTA	p.M1029Yfs*14	Indel	Frameshift	/
48	BRCA1	c.17_18delTT	p.L6Pfs*3	Indel	Frameshift	/
49	BRCA1	c.441+2T>A	/	splice	Splice	/
50	BRCA1	c.2751delC	p.K918Sfs*82	Indel	Frameshift	/
55	BRCA1	c.5470_5477del	p.I1824Dfs*3	Indel	Frameshift	/
58	BRCA1	c.4222C>T	p.Q1408*	SNV	Nonsense	Non-synonymous
62	BRCA1	c.4756G>T	p.E1586*	SNV	nonsense	Non-synonymous
63	BRCA1	c.3756_3759delGTCT	p.S1253Rfs*10	Indel	Frameshift	/
65	BRCA2	c.2059_2063del	p.D687*	Indel	Frameshift	/
67	BRCA1	c.5470_5477delATTGGGCA	p.I1824Dfs*3	Indel	Frameshift	/
69	BRCA1	c.5470_5477delATTGGGCA	p.I1824Dfs*3	Indel	Frameshift	/
70	BRCA2	c.9122C>G	p.Ser3041*	SNV	Nonsense	Non-synonymous
71	BRCA1	c.3G>T	p.0	SNV	Start loss	/
72	BRCA1	c.2572C>T	p.Q858*	SNV	Nonsense	Non-synonymous
73	BRCA1	c.4801A>T	p.K1601*	SNV	Nonsense	Non-synonymous

SNV, single nucleotide variant; Indel, insertion-deletion.



FIGURE 1 | Somatic mutation spectra among different groups. **(A)** Somatic mutation spectrum between germline *BRCA* and non-*BRCA* carriers (mutation frequency equal to or more than 4% in the whole cohort). **(B)** Somatic mutation spectrum among *gBRCA1*, *gBRCA2*, other *gHRR*, and non-*gHRR* carriers. Each column represents a patient and each row represents a gene. In **(A)**, the number on the right represents the percentage of patients with mutations in a specific gene in (Continued)

FIGURE 1 | the whole cohort. The top plot represents the overall number of mutations detected in a patient. Different colors denote different types of mutation. The annotation at the top depicts the germline mutations carried by the patients. HRR, homologous recombination repair.

TABLE 3 | Comparison of somatic mutations between *BRCA* germline mutation carriers and non-carriers of triple-negative breast cancer (mutation frequency equal to or more than 4% in the whole cohort, p -value < 0.05).

	mut (N = 21)	wild (N = 54)	Total (N = 75)	p -value
<i>PIK3CA</i>				0.046
mut	0 (0.0%)	9 (16.7%)	9 (12.0%)	
Wild	21 (100.0%)	45 (83.3%)	66 (88.0%)	
<i>EPHA3</i>				0.08
mut	0 (0.0%)	7 (13.0%)	7 (9.3%)	
Wild	21 (100.0%)	47 (87.0%)	68 (90.7%)	
<i>GFM3</i>				0.03
mut	3 (14.3%)	1 (1.9%)	4 (5.3%)	
Wild	18 (85.7%)	53 (98.1%)	71 (94.7%)	

mut, mutation.

P -values were derived from the Pearson's Chi-square test, Fisher's exact test and Continuity Correction chi-square test.

genes involved in the homologous recombination repair (HRR) pathway (*PALB2*, *BLM*, *NBN*, *RAD 51C*, and *RAD 51D*) (**Figure 1B**), although there was no statistical significance.

Because other germline mutations in HRR pathway genes were very common in non-*BRCA* carriers, somatic mutations were also compared due to different germline *BRCA* status. In this study cohort, 19 somatic mutations in 12 genes were involved in the HRR pathway from 15 patients (20.0%, 15/75) including *ATRX*, *ATM*, *ATR*, *BARD1*, *BRCA1*, *BRCA2*, *CHEK1*, *MRE11A*, *PALB2*, *RAD52*, and *FANCL*. In particular, *PALB2* dominated in four patients (26.67%, 4/15) (**Figure 2**). There were three *BRCA* carriers (3/21, 14.29%) accompanying somatic HRR-mutant genes compared to 12 non-*BRCA* carriers (22.22%, 12/54) ($p = 0.53$, **Supplementary Table 4**). *ATR* ($p = 0.02$) was only detected in *BRCA* carriers (**Table 4**). In contrast, *ATRX*, *ATM*, *CHEK1*, *MRE11A*, and *FANCL* were only found in non-*BRCA* carriers even without statistical significance (all $p = 0.53$, **Supplementary Table 4**). One patient with germline *BRCA1* mutation had co-occurring somatic *ATR* and *PALB2* mutations, while another patient with germline *BRCA2* mutation had co-occurring somatic *ATR* and *BARD1* mutations and a third patient with germline *BRCA2* mutation had co-occurring somatic *RAD52* mutation. No concomitant germline and somatic *BRCA* or other HRR gene mutations occurred in any other patients. Moreover, missense mutation dominated in somatic mutation types involved in HRR pathway genes (81.48%, 22/27, **Figure 2**).

Comparison of TMB and MSI Between Germline *BRCA* and Non-*BRCA* Carriers in Chinese Patients With TNBC

Median TMB (4.1 Muts/Mb) remained the same in both germline *BRCA* and non-*BRCA* carriers with TNBC in this study cohort ($p = 0.38$), with a range of 1.79–24.62 Muts/Mb for the former

TABLE 4 | Comparison of somatic mutant genes involved in the homologous recombination repair pathway between *BRCA* germline mutation carriers and non-carriers of triple-negative breast cancer ($p < 0.05$).

	mut (N = 21)	wild (N = 54)	Total (N = 75)	p -value
<i>ATR</i>				0.02
mut	2 (9.5%)	0 (0.0%)	2 (2.7%)	
Wild	19 (90.5%)	54 (100.0%)	73 (97.3%)	

mut, mutation.

P values were derived from the Pearson's Chi-square test, Fisher's exact test and Continuity Correction chi-square test.

and 0.51–17.95 Muts/Mb for the latter. Interestingly, TMB of the 4 patients with *CCNE1* amplification were all lower than 4.1 Muts/Mb.

Only one case of low MSI was detected in germline *BRCA* carriers compared with two cases in non-*BRCA* carriers ($p = 0.63$). The rest belonged to microsatellite stability, and no MSI-H cases were found in this study cohort.

Survival Analysis

Survival Analysis Between Germline *BRCA* and Non-*BRCA* Carriers With TNBC

There were no significant differences in DFS and OS between the germline *BRCA* and non-*BRCA* carriers with TNBC ($p = 0.15$ and $p = 0.52$, respectively) (**Figures 3A,B**). In addition, germline mutations involving HRR pathway genes did not affect either DFS or OS too ($p = 0.06$ and $p = 0.39$, respectively, **Figures 3C,D**).

Survival Analysis and Risk Factors for DFS and OS in Non-*BRCA* Carriers With TNBC

Survival analysis showed that both TMB < 4.1 Muts/Mb and abnormal CNV were associated with worse DFS ($p = 0.01$ and $p = 0.02$, respectively, **Figures 4A,B**). Approximately 80% (102/120) of abnormal CNVs belonged to CNV gain (**Supplementary Figure 2A**). As mentioned above, the TMB value of those four patients with *CCNE1* amplification were all found lower than 4.1 Muts/Mb. K–M curves showed that DFS in patients with TNBC was affected by *CCNE1* amplification ($p = 0.0002$, **Figure 4C**). Univariate analysis for correlation of DFS and OS was applied to clinicopathological characteristics, TMB, CNV, HRR pathway genes, high-frequency somatic mutation genes, and genes with somatic mutation detected only in non-*BRCA* carriers (**Supplementary Tables 5, 6**). The results revealed that advanced T stage ($p = 0.007$) and TNM stage ($p = 0.02$), low TMB ($p = 0.01$), abnormal CNV ($p = 0.03$), *GATA3* mutation ($p = 0.009$), and *CCNE1* amplification ($p = 0.001$) were associated with worse DFS (**Supplementary Table 5**), whereas advanced T

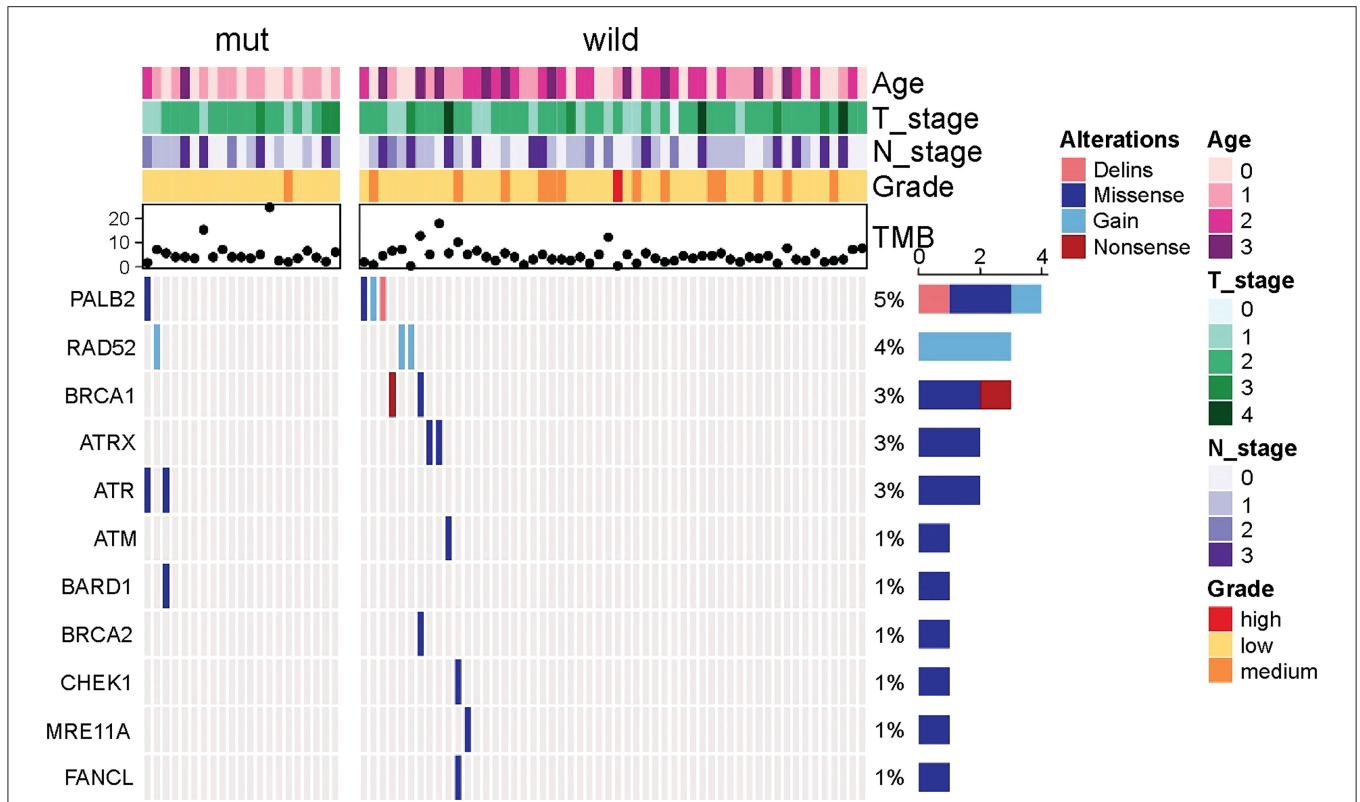


FIGURE 2 | Comparison of somatic mutant genes involved in the homologous recombination repair pathway including somatic *BRCA* mutations between germline *BRCA* and non-*BRCA* carriers.

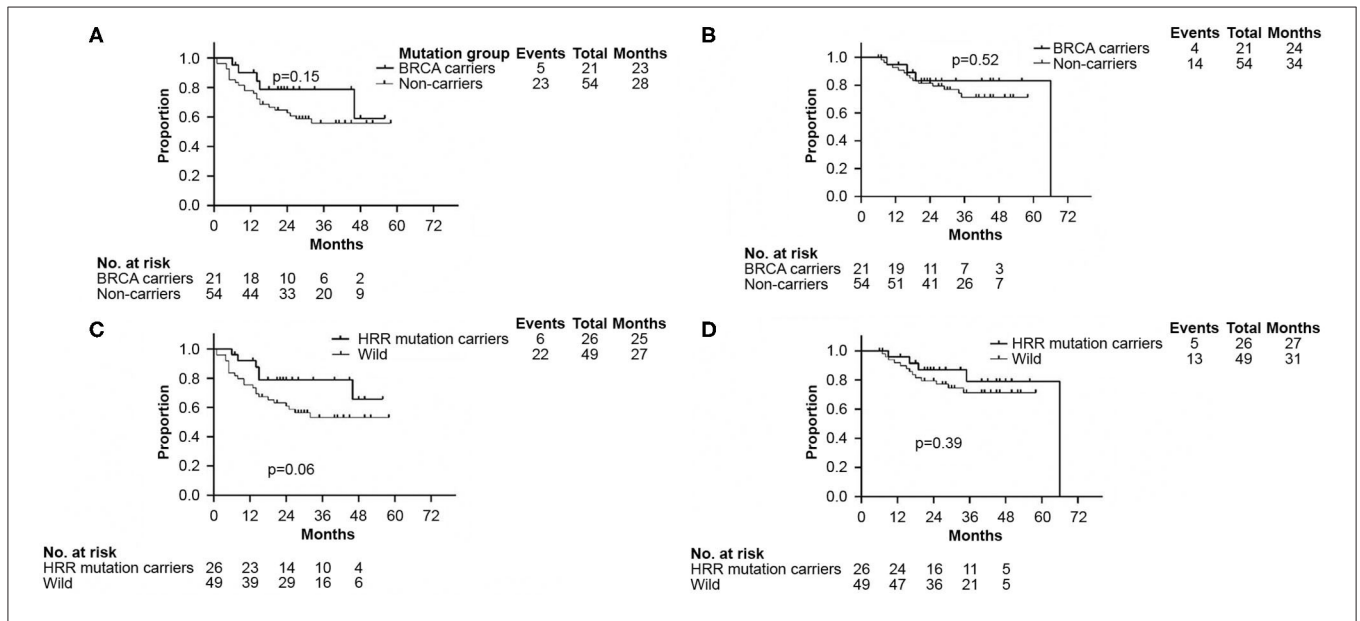


FIGURE 3 | Kaplan-Meier analyses performed to confirm neither *BRCA* germline mutation status nor HRR germline mutation status affected DFS and OS. **(A)** Kaplan-Meier curve of DFS according to *BRCA* germline mutation status. **(B)** Kaplan-Meier curve of OS according to *BRCA* germline mutation status. **(C)** Kaplan-Meier curve of DFS according to genes involved in HRR pathway mutation status. **(D)** Kaplan-Meier curve of OS according to genes involved in HRR pathway mutation status. DFS, disease free survival; OS, overall survival. HRR, homologous recombination repair.

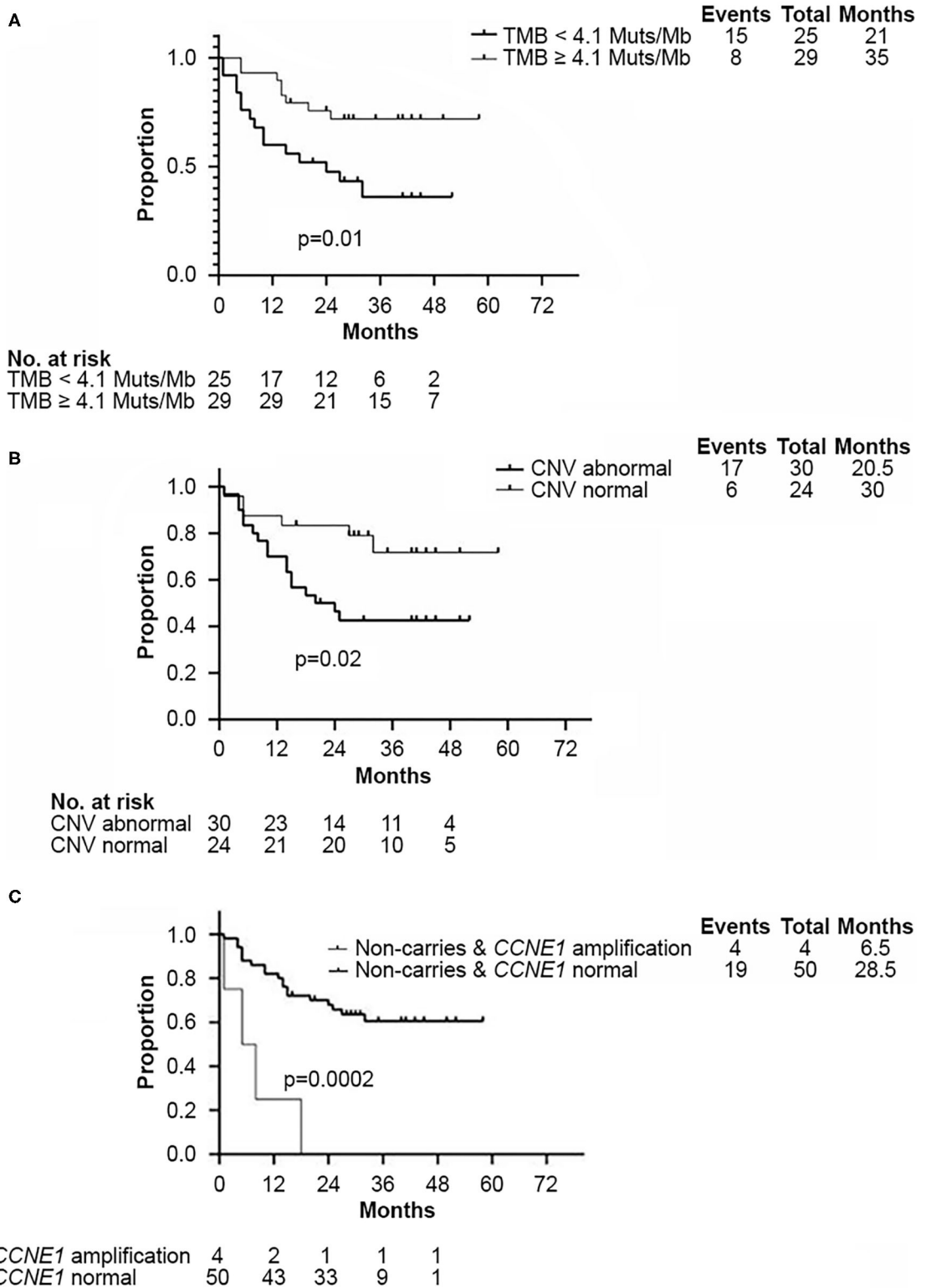


FIGURE 4 | Kaplan–Meier analysis showed disease-free survival in non-BRCA carriers with TNBC in this cohort. **(A)** Kaplan–Meier curve of DFS according to median TMB (4.1 Muts/Mb) in this cohort. **(B)** Kaplan–Meier curve of DFS according to CNV status. **(C)** Kaplan–Meier curve of DFS according to CCNE1 CNV status. DFS, disease free survival; CNV, copy number variation; TMB, tumor mutation burden.

stage ($p < 0.001$), N stage ($p = 0.01$), and TNM stage ($p = 0.01$) were associated with worse OS (**Supplementary Table 6**).

Based on the results of univariate analysis, Cox regression modeling was performed to evaluate the risk factors associated with DFS and OS in non-*BRCA* carriers. We found that T stage, TNM stage, and *CCNE1* amplification were the independent risk factors for DFS [T stage, HR = 2.34 (95% CI, 1.26–4.38), $p = 0.007$; TNM stage, HR = 3.20 (95% CI, 1.16–8.81), $p = 0.024$; and *CCNE1*, HR = 13.07 (95% CI, 2.47–69.24), $p = 0.003$] (**Figure 5A**). T stage was the only independent risk factor for OS [HR = 3.58 (95% CI, 1.61–7.98), $p = 0.002$]. Even *CCNE1* amplification was the independent risk factor for DFS in any patient with TNBC [HR = 13.48 (95% CI, 2.62–69.23), $p = 0.002$] (**Figure 5B**).

CCNE1 Amplification in Public Databases

CCNE1 amplification was more frequently detected in TNBCs (10%, 21/209 in METABRIC and 13%, 16/119 in TCGA) compared to non-TNBCs (1.9%, 34/1,771 in METABRIC, $p < 0.0001$, **Supplementary Figure 3A**; and 2.9%, 28/961 in TCGA, $p < 0.0001$, **Supplementary Figure 3B**). In addition, the distinction translated into significant OS differences that TNBC with amplified *CCNE1* was associated with worse overall survival ($p = 0.016$, **Supplementary Figure 3C**) in METABRIC.

IHC Confirmed Strong Intensity of Cyclin E1 in TNBC With CCNE1 Amplification

IHC staining for cyclin E1 was performed in 42 non-*BRCA* carriers with TNBC, including four patients with *CCNE1* amplification. A final consensus score for cyclin E1 staining by IHC was obtained by multiplying the intensity and extension values to achieve a score ranging from 0 to 300. As shown by the ROC curve, there was a good consistency between the IHC staining for cyclin E1 and the somatic mutation status of *CCNE1*, with an AUC of 0.967 (95% CI, 0.9174–1, **Figure 6A**). Based on a cutoff cyclin E1 consensus score of 235, 42 non-*BRCA* carriers were divided into two groups, those with strong and weak cyclin E1 signals. All four patients with *CCNE1* amplification were in the strong cyclin E1 signal group (**Figure 6B**), and the strong signal intensity of cyclin E1 was confirmed for all four patients with *CCNE1* amplification (**Supplementary Figure 4**). Based on the IHC staining for cyclin E1, strong cyclin E1 signal intensity by IHC tended to be associated with worse DFS (**Supplementary Figure 5**).

DISCUSSION

In contrast to the previous comprehensive mutational spectrum in TNBC (31, 34, 47), we performed an NGS-based analysis by comparing 21 germline *BRCA* carriers and 54 non-*BRCA* carriers in Chinese patients with TNBC. To the best of our knowledge, our study is the first to elucidate a more comprehensive comparison that included clinicopathological characteristics, genomic profiles, immunity-associated parameters and survival analysis. In addition, based on the eligibility criteria including a tumor size of ≥ 2 cm and/or ≥ 1 affected lymph nodes, we described the genomic profiles of TNBC cases with high

tumor burden and worse prognosis compared to comprehensive unselected TNBC (48). The genomic profiles in this study was rare (49). In the current cohort, 14 of the 54 non-*BRCA* carriers (25.9%) and four of the 21 *BRCA* carriers (19.0%) died even though the follow-up was not long.

The prevalence of *BRCA1/2* mutations in the current cohort of TNBC cases reached 24.1%, which was close to the reported rate of 21.4% in unselected Chinese populations (50), indicating that *BRCA1/2* mutations were not related to high tumor burden or worse prognosis, in agreement with the findings of the POSH study (16). Our analysis revealed a mutation rate of 11.11% (6/54) in other cancer predisposition genes beyond *BRCA1/2* in non-*BRCA* carriers, which was only slightly lower than that in the germline *BRCA1/2* mutation carriers [14.19% (21/148)] in TNBC. Therefore, in addition to *BRCA1/2*, we should not overlook the clinical value of other germline mutation tests in TNBC patients in China. Among other germline mutations, one (1.85%, 1/54) mismatch repair gene mutation (*MSH3*) was found, which was parallel to that reported in a previous study (51), although the correlation between the germline *MSH3* mutation and TNBC remains to be explored (52). Except for *MSH3*, the rest of the germline mutant genes in non-*BRCA* carriers with TNBC were involved in the HRR pathway, including *BLM*, *PALB2*, *NBN*, *RAD51C*, and *RAD51D*, with the mutation rate of 9.26% (5/54), which increased the risk of other cancers, such as *PLAB2* for pancreatic cancer (53). The germline mutation rate of the members of the Fanconi anemia gene family (including *BRCA2*, *PALB2*, and *RAD51C*) was 25.93% (7/27) among patients with germline mutations in TNBC, which was lower than those in unselected breast cancer (35). These data strongly support the inclusion of not only *BRCA1/2* but also other germline mutations, such as HRR pathway genes, Lynch Syndrome, and Fanconi anemia (54), in the assessment of germline mutations in patients with TNBC.

In addition to *BRCA1/2*, growing evidence indicates that other germline mutations involved in HRR pathway genes may improve sensitivity to therapeutic agents such as platinum-based chemotherapy (55) and PARP inhibitors (56), implying that a few non-*BRCA* carriers with TNBC with other accompanying germline mutations involved in HRR pathway genes may benefit from platinum-based chemotherapy or PARP inhibitors.

Furthermore, our study has identified distinct somatic mutations among germline *BRCA* and non-*BRCA* carriers. *GRM3* mutation was detected more in *BRCA* carriers, while somatic *PIK3CA* missense was only detected in non-*BRCA* carriers. *GRM3* was reported to be the only genetic marker associated with bipolar disorder (57). However, the relationship between germline *BRCA* mutation and somatic *GRM3* alteration remains to be explored. Alpelisib has been approved for the treatment of advanced breast cancer with *PIK3CA* mutation in positive hormone receptor (58), and clinical trials are investigating the possible response of TNBC with *PIK3CA* mutation to alpelisib (59).

We specifically investigated the association between somatic mutations involved in HRR pathway genes and germline *BRCA1/2* status and found coexistence between germline *BRCA1* mutation and somatic *PLAB2* pathogenic variation in one

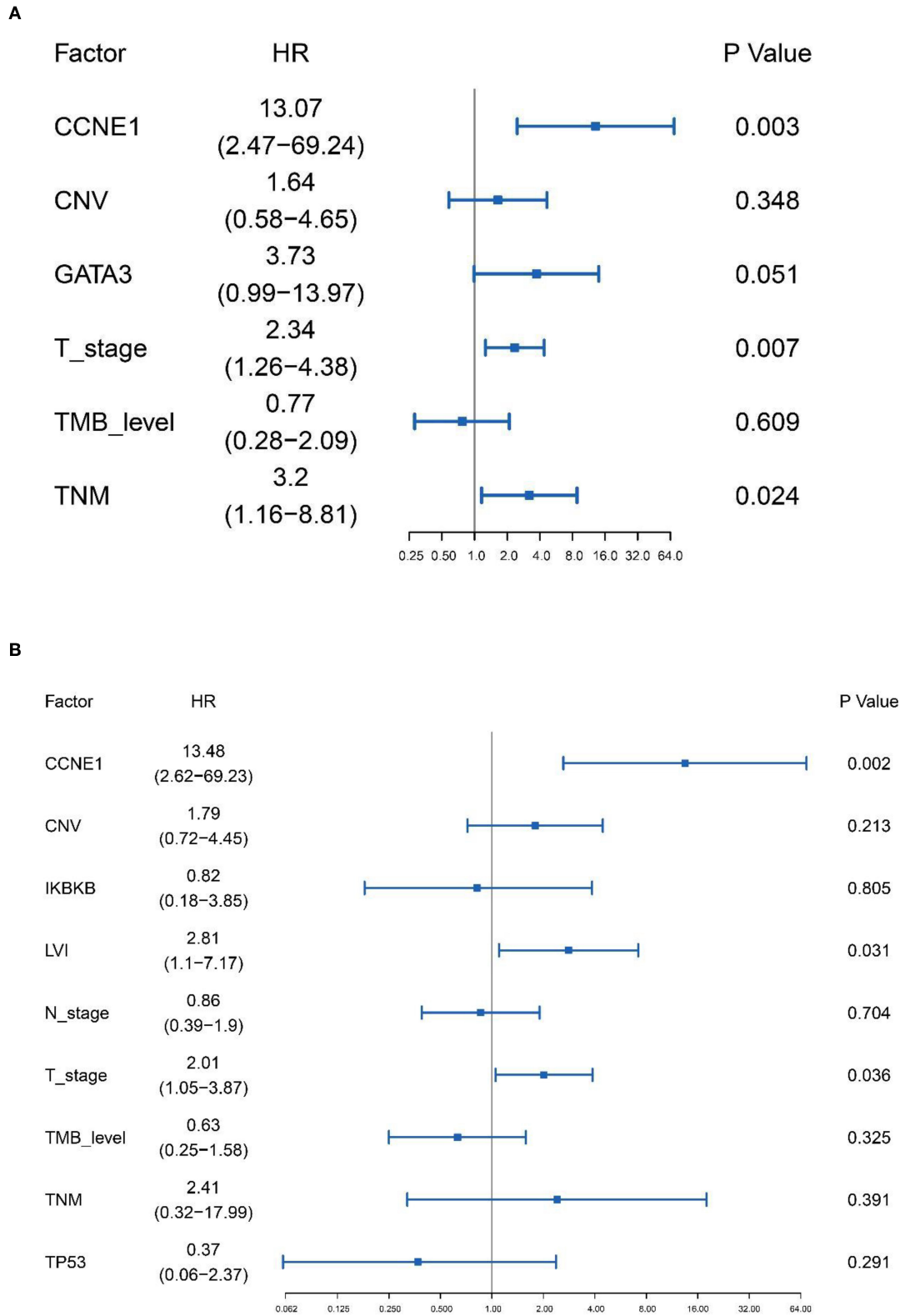
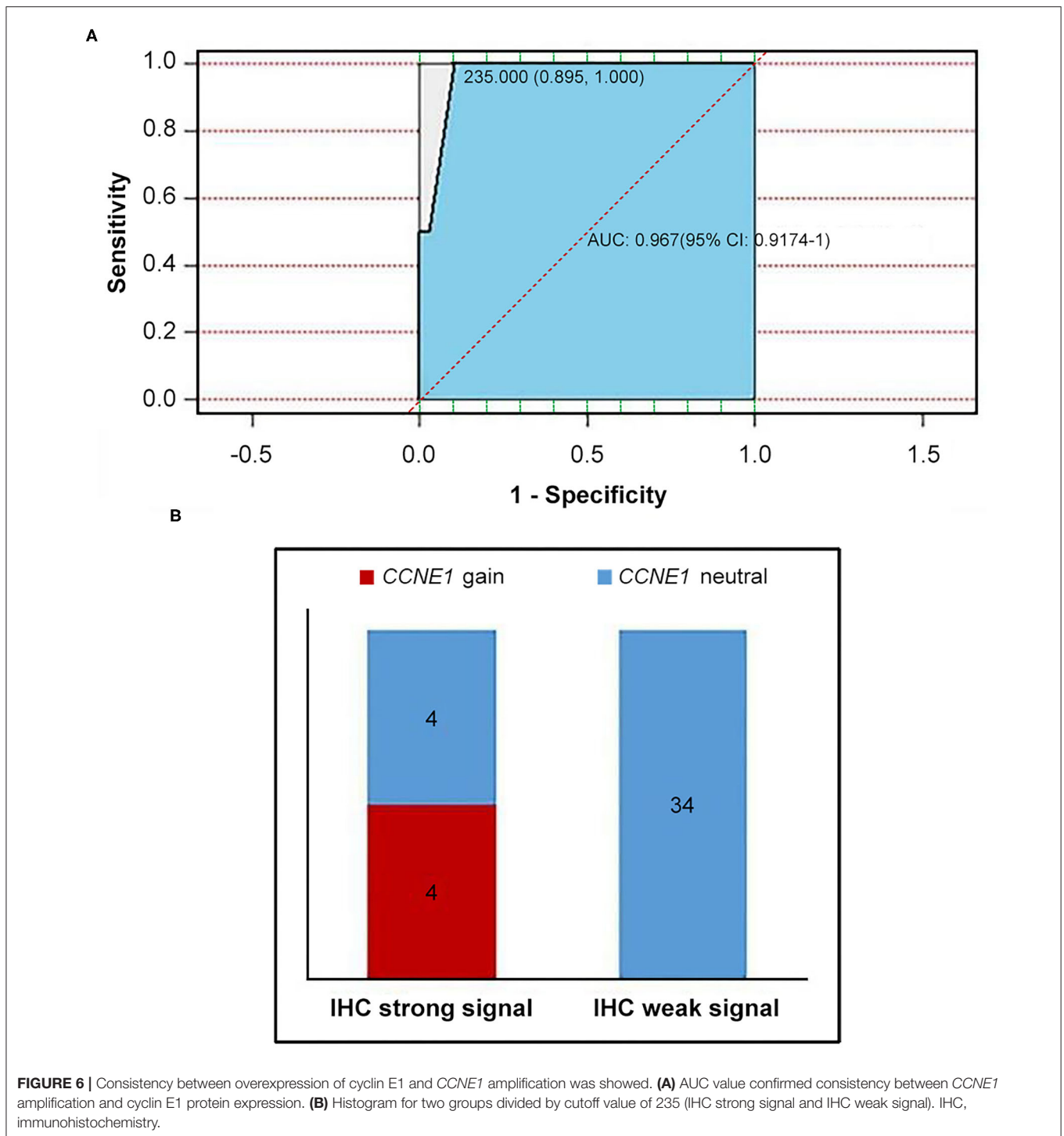


FIGURE 5 | Cox proportional hazards regression model performed to determine the risk factors associated with disease-free survival in non-BRCA carriers with TNBC and all TNBC patients in this cohort. **(A)** Cox proportional hazards regression model for non-BRCA carriers with TNBC. **(B)** Cox proportional hazards regression model for all TNBC patients. HR, hazard ratio; CNV, copy number variation; TMB, tumor mutation burden.



patient. Although it did not directly conform to Knudson’s “two-hit” paradigm (60), *PALB2* may be an example of a cancer predisposition gene (61).

Although a significant portion (22.22%, 12/54) of non-*BRCA* carriers also carry other somatic mutations involving HRR pathway genes, platinum-based chemotherapy, or PARP

inhibitors may have no response in these cases as missense dominates the mutation type and this type might not result in homologous recombination defect (33, 62).

PD-1/PD-L1 blockade has been shown to have an acceptable safety profile and antitumor activity for TNBC in the phase Ib KEYNOTE-012 study (63), suggesting that ICIs are a promising

therapeutic approach for TNBC cases with accumulated mutations. We aimed to investigate whether a high TMB and MSI-H, which are considered as predictive markers for survival after immunotherapy in other tumors (64–66), were also suitable as predictors in patients with TNBC. The median TMB, which was 4.1 Muts/Mb in both the *BRCA* and non-*BRCA* carriers in the present study, was very similar to the median TMB of 3.8 Muts/Mb in patients with breast cancer reported by Chalmers et al. (67); even lower than Japanese population reported by Nagahashi et al. (68) which was 11.5 (3.9–56.2)/Mb; far lower than TMB in melanoma (65) and non-small cell lung cancer (66) which was at least higher than 10 Muts/Mb. Thus, a high TMB was rare in TNBC. Furthermore, MSI-H was absent in this study. MSI-H was reported uncommon (0.9%) in TNBCs for Japanese population (69). Thus, we presume that neither a high TMB alone nor MSI-H alone is a suitable predictive marker for immunomodulation in TNBC because they do not represent the comprehensive immune environment in TNBC. These findings suggest that a combination of ICIs and other therapies should be considered as treatment approaches for TNBC. Several clinical trials are testing specific combinations of ICIs and PARP inhibitors such as olaparib, niraparib, and talazoparib, with preliminary data indicating their efficacy (70, 71).

There is an urgent need to discover new potential predictors and therapeutic targets for patients with TNBC, particularly for those whose prognosis is poor and who have poor response to chemotherapy (17) and no response to PARP inhibitors (20). Patients with high tumor burden, such as those with large tumor size (≥ 2 cm) and/or a higher proportion of affected lymph nodes (at least one affected lymph node) may have worse prognosis. Meanwhile, those without germline *BRCA* mutation may not respond to PARP inhibitors and may have poor response to platinum-based chemotherapy.

Our comparison of genomic profiling in *BRCA* and non-*BRCA* carriers led us to the unexpected discovery that *CCNE1* amplification was only detected in non-*BRCA* carriers. By comparing the survival of non-*BRCA* carriers with TNBC based on *CCNE1* status, those with *CCNE1* amplification showed worse DFS and a tendency of worse OS. Therefore, we focused our attention on *CCNE1* and were surprised to find that *CCNE1* amplification was an independent risk factor in non-*BRCA* carriers with TNBC even as an independent risk factor in TNBC.

Available large-scale public genomic databases from The Cancer Genome Atlas (TCGA) project (44) and the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) (43) have significantly improved our understanding of breast cancer tumor biology. To strength the importance of *CCNE1* amplification for TNBC, we also found the significant difference of amplified *CCNE1* between TNBCs and non-TNBCs both in METABRIC and TCGA databases. Surprisingly, *CCNE1* amplification was also confirmed to be associated with worse OS in METABRIC which was first reported.

There are few studies on the association between *CCNE1* amplification and TNBC prognosis. Only Zhao et al. (72) reported that *CCNE1* amplification may confer resistance to chemotherapy and is associated with poor overall survival in

patients with TNBC. Although we drew the same conclusion, we went a step further that we confirmed amplified *CCNE1* was the independent risk factor for non-*BRCA* carriers with TNBC who really need attention due to being lack of distinct prognostic marker and therapeutic target. *CCNE1* amplification was mutually exclusive to germline *BRCA1/2* mutation which has been previously reported (35, 72). We determined its predictor role in the sub-group of TNBC, i.e., non-*BRCA* carriers with fewer options for treatment, such as PARP inhibitors. To translate and facilitate the application from DNA to protein level, IHC was used to confirm the consistency of *CCNE1* amplification and cyclin E1 overexpression in TNBC samples. With a good sensitivity of 100% and specificity of 89.5%, cyclin E1 IHC staining may have great potential for use instead of *CCNE1* amplification testing to facilitate routine clinical application on the basis that worse tendency of overall survival was still associated with strong intensity of Cyclin E1 according to IHC staining of Cyclin E1. This is the first to translate *CCNE1* at DNA level to Cyclin E1 at protein level.

Cyclin E1, as encoded by *CCNE1*, is the key kinase complex for cell cycle regulation from G1 to S phase. *CCNE1* amplification has also been observed in some other tumors (73, 74) and may lead to continuously activated DNA and centrosome replications, inducing chromosomal instability and tumor growth. Although cyclin E1-specific pharmacological inhibitors are not yet available, preclinical investigations as well as trials indirectly targeting *CCNE1* are both underway. Dariush Etamadmoghadam (75) showed the specific sensitivity of proteasome inhibitor bortezomib to *CCNE1*-amplified tumor cells. Furthermore, results of a study that applied proteasome inhibitor bortezomib as first-line therapy against multiple myeloma (76) indicate that it could also be considered as a potential therapeutic approach for TNBC with *CCNE1* amplification.

There are three limitations of our study. First, all 75 patients were from a single hospital. Second, the sample size for germline *BRCA1/2* mutation subgroup was <30 and certain mutation subtypes were very limited. Third, not all 75 patients had been followed up for more than 5 years. Therefore, future studies using larger sample sizes and long-term follow-up procedures should be conducted to investigate the correlation between specific mutations and survival outcomes.

CONCLUSION

We explored intertumoral heterogeneity by comparing the differences in genomic profiles and immunity-associated parameters between germline *BRCA* and non-*BRCA* carriers in TNBC with high tumor burden. We revealed that both a high TMB and MSI-H were rare in patients with TNBC, indicating that these would not act as suitable predictors for TNBC for immune checkpoint inhibitors. Most notably, we discovered that amplified *CCNE1* may be a novel potential prognostic marker and therapeutic target for TNBC without germline *BRCA1/2* mutations. Overexpression of cyclin E1 may become a replacement for *CCNE1* amplification, which will facilitate its clinical application.

DATA AVAILABILITY STATEMENT

The dataset have been deposited to: <http://db.cngb.org/> and the accession number is CNP0001304.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The study was approved by the Ethics Committee of Peking Union Medical College Hospital (No. HS-1623). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

QS contributed to conceptualization and design of the study. XH, DS, CZ, and CC organized the database. XH acquired funding. XH, DS, YZ, YL, and CW contributed the patients' resources. QS, XH, DS, DG, CZ, and BZ performed statistical analysis, immunochemistry, and NGS data analysis. XH, DS, YZ, YL, and TL investigated the patients. XH and DS wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fonc.2020.583314/full#supplementary-material>

Supplementary Figure 1 | Flow diagram of the patients in the study and analyses.

Supplementary Figure 2, related to Figure 1 and Table 2 | Somatic alterations in the genomes of 75 Chinese patients with TNBC and germline *BRCA1/2* mutations in 21 Chinese patients with TNBC. **(A)** Percentages of types of somatic mutations. **(B)** Numbers of somatic mutations and germline *BRCA* mutations in individual patients. The numbers of genes with CNV loss per patient ranged from 0 to 3, whereas the numbers of amplified genes per patient ranged from 0 to 10. CNV, copy number variation; TNBC, triple-negative breast cancer.

Supplementary Figure 3 | TNBCs exhibited more frequency with amplified *CCNE1* compared with non-TNBCs (Fisher's exact test) in **(A)** METABRIC: TNBC ($n = 209$) and non-TNBC ($n = 1,771$) ($p < 0.0001$) and in **(B)** TCGA: TNBC ($n = 119$) and non-TNBC ($n = 961$) ($p < 0.0001$). While TNBC with amplified *CCNE1* showed worse overall survival in METABRIC database: **(C)** Kaplan–Meier curve of OS with TNBC according to *CCNE1* CNV status in METABRIC. AMP, amplification; OS, overall survival; TNBC, triple-negative breast cancer.

Supplementary Figure 4, related to Figure 6 | High-intensity levels of Cyclin E1 correlated positively with TNBC and *CCNE1* amplification under both low (40×) and high (100×) magnification in 4 patients in this study cohort. **(A)** Patient No. 56 **(B)** Patient No. 26 **(C)** Patient No. 28 **(D)** Patient No. 64.

Supplementary Figure 5 | Kaplan–Meier analysis showed disease-free survival in non-*BRCA* carriers with TNBC according to IHC staining of Cyclin E1. IHC, immunohistochemistry.

Supplementary Table 1, related to Table 1 | Clinicopathological characteristics of Chinese female patients with triple-negative breast cancer according to *BRCA* germline mutation status in this study cohort ($p \geq 0.05$).

Supplementary Table 2 | Events in *BRCA1/2* mutation carriers and non-*BRCA* carriers.

Supplementary Table 3, related to Table 3 | Comparison of somatic mutations between *BRCA* germline mutation carriers and non-carriers of triple-negative breast cancer (mutation frequency equal to or more than 4% in the whole cohort, $p \geq 0.05$).

Supplementary Table 4, related to Table 4 | Comparison of somatic mutant genes involved in the homologous recombination repair pathway between *BRCA* germline mutation carriers and non-carriers of triple-negative breast cancer ($p \geq 0.05$).

Supplementary Table 5 | Univariate analysis of correlations between clinicopathological factors and genomic alterations and disease-free survival in non-*BRCA* carriers of triple-negative breast cancer.

Supplementary Table 6 | Univariate analysis of correlations between clinicopathological factors and genomic alterations and overall survival in non-*BRCA* carriers of triple-negative breast cancer.

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Conflict of Interest: DS and CZ were employed by the company BGI Genomics, Shenzhen, China.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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