ABSTRACT

Purpose: Triple-negative breast cancer (TNBC) does not have defined therapeutic targets and is currently treated with chemotherapy only. Kinase dysregulation triggers cancer cell proliferation and metastasis and is a crucial therapeutic target for cancer. In this study, targeted kinome sequencing of TNBC tumors was performed to assess the association between kinome gene alterations and disease outcomes in TNBC.

Methods: A kinome gene panel consisting of 612 genes was used for the targeted sequencing of 166 TNBC samples and matched normal tissues. Analyses of the significantly mutated genes were performed. Genomic differences between Asian and non-Asian patients with TNBC were evaluated using two Asian TNBC datasets (from Seoul National University Hospital [SNUH] and Fudan University Shanghai Cancer Center [FUSCC]) and three non-Asian TNBC datasets (The Cancer Genome Atlas [TCGA], METABRIC, and Gustave Roussy). The prognostic value of kinome gene mutations was evaluated using tumor mutational burden (TMB) and oncogenic pathway analyses. Mutational profiles from the TCGA were used for validation.

Results: The significantly mutated genes included TP53 (60% of patients), PIK3CA (21%), BRCA2 (8%), and ATM (8%). Compared with data from non-Asian public databases, the mutation rates of PIK3CA p.H1047R/Q were significantly higher in the SNUH cohort ($p = 0.003, 0.048$, and $0.032$, respectively). This was verified using the FUSCC dataset ($p = 0.003, 0.078$, and $0.05$, respectively). The TMB-high group showed a trend toward longer progression-free survival in our cohort and the TCGA TNBC cohort ($p = 0.041$ and $0.195$, respectively). Kinome gene alterations in the Wnt pathway in patients with TNBC were associated with poor survival in both datasets ($p = 0.002$ and $0.003$, respectively).

Conclusion: Comprehensive analyses of kinome gene alterations in TNBC revealed genomic alterations that offer therapeutic targets and should help identify high-risk patients more precisely in future studies.

Keywords: Mutation; Protein Kinases; Survival; Triple Negative Breast Neoplasms
INTRODUCTION

Triple-negative breast cancer (TNBC) is a breast cancer subtype characterized by a lack of estrogen and progesterone receptors and the absence of human epidermal growth factor receptor 2 (HER2) gene overexpression. TNBC accounts for 15%–20% of all breast cancers and is characterized by its aggressiveness, earlier age of onset, and poor clinical outcomes compared to other subtypes [1,2]. Many clinical trials investigating therapeutic targets in TNBC have shown disappointing results, so chemotherapy remains the only standard treatment option [3]. Only recently have immunotherapies achieved a modest increase in the pathological complete response rate when added to neoadjuvant chemotherapy for the treatment of early TNBC [4,5]. However, immunotherapies for TNBC still lack predictive biomarkers.

Kinome refers to a single superfamily of 518 protein kinases encoded in the human genome, constituting approximately 1.7% of all human genes [6]. Kinases play critical regulatory roles in cell growth, differentiation, migration, and survival. Dysregulation of kinase activity is a major mechanism underlying cancer progression and is an attractive therapeutic target [7]. Currently, approximately one-third of all protein targets being studied in the context of cancer treatment are kinase-based [8]. The development of HER2-targeted therapies has significantly improved the survival of patients with HER2-overexpressing breast cancer. Recently, a phosphoinositide 3-kinase (PI3K) inhibitor was also shown to be effective in PIK3CA-mutated hormone receptor-positive advanced breast cancer [9]. However, no targeted therapy using kinase inhibitors has been successful in patients with TNBC. Thus, there is an urgent need to identify kinase targets and predictors of kinase inhibitor sensitivity in these patients.

In this study, a comprehensive somatic genetic profiling of patients with TNBC was conducted using a target kinome sequencing panel. The genetic profile of the TNBC cohort was compared to that of The Cancer Genome Atlas (TCGA) TNBC cohort, and the prognostic value of kinome gene alterations was analyzed to identify potential therapeutic targets.

METHODS

Patients and samples

A total of 166 TNBC tissues, each with matched normal breast tissue or peripheral blood samples, were collected at Seoul National University Hospital (SNUH). Fresh frozen tissues and peripheral blood samples were obtained prospectively at the time of surgery between 1995 and 2010 and were retrieved from the SNUH Laboratory of Breast Cancer Biology Biorepository. Formalin-fixed paraffin-embedded (FFPE) tumor samples were collected from surgical specimens obtained between 2003 and 2013 and stored at the SNUH Tumor Bank. There were 41 fresh frozen tumor samples and 129 FFPE tumor samples collected. Clinicopathological data were acquired from the prospectively maintained online database of SNUH Breast Care Center. This study was approved by the Institutional Review Board of SNUH (No. 1210-072-434), and the requirement for informed consent was waived by the committee.

Kinome sequencing

Genomic DNA was extracted from the samples, and 1 μg of the genomic DNA extract was fragmented via nebulization. The fragmented DNA was repaired by ligating an ‘A’ to their 3’ ends, then Illumina adapters were ligated to the fragments. Each sample was size-selected, where products 350–400 base pairs long were preferred. The size-selected products were
polymerase chain reaction-amplified, and the final products were validated using an Agilent Bioanalyzer (2100 Bioanalyzer Instrument; Agilent Technologies, Santa Clara, USA). Target enrichment was performed using an Agilent SureSelect Human Kinome panel, which targets a large set of kinases and kinase-related genes for enrichment (612 genes, including more than 500 kinases). Paired-end libraries were sequenced using an Illumina HiSeq 2000 instrument.

**Sequence data processing and discovery of somatic variants**

The paired-end reads of the tumor and normal matched FASTA files obtained from sequencing were mapped to the human genome reference using BW A-MEM [10]. The aligned reads were sorted using SAM tools [11]. After duplicate reads of the aligned BAM files were marked and removed using Picard, the base quality of reads in the BAM files was recalibrated using the Genome Analysis Tool Kit (version 4.1.0.0) [12]. The Mutect2 best practice pipeline for somatic variants was used to identify candidate somatic mutations. Sequencing artifacts were removed by filtering out exome variants labeled as “bad_haplootype,” “chimeric_original_alignment,” “base_quality,” “duplicate_evidence,” “fragment_length,” “low_avg_alt_quality,” “mapping_quality,” “multiallelic,” “n_ratio,” “read_orientation_artifact,” “read_position,” “str_contraction,” “strand_artifact,” or “strict_strand_bias” in the variant call format files.

Only loss-of-function variants (missense, nonsense, splice site variants, in-frame, frame insertion, and deletions) in the target regions of the Agilent SureSelect Human Kinome panel were chosen. Normal population database-based filtering was used to remove germline variants. If the population allele frequency of the variants was more than 1% in any subpopulation among the 1000 Genomes Project, Exome Aggregation Consortium, Korean Variant Archive, Genome Aggregation Database, and Korean Genome Project data, the variants were excluded as germline mutations [13-17]. The Korean 1,000 depression exome data was also used as a normal population panel, and the variants were filtered [18]. All quality-passed variants were annotated with Sorting Intolerant from Tolerant, Polylphen2, and Combined Annotation Dependent Depletion algorithms using ANNOVAR software to evaluate the pathogenicity of each variant [19-22].

**Validation cohorts**

The TCGAmc3 data of patients with TNBC were classified by TNBCtype and used to compare Asian and non-Asian TNBC patients [23,24]. These results were validated using whole-exome sequencing (WES) data of Chinese TNBC patients from the Fudan University Shanghai Cancer Center (FUSCC), French TNBC patients from Gustave Roussy, and METABRIC data [25-27]. The same bioinformatics pipeline for SNUH was applied to the Gustave Roussy dataset. However, Mutect2 without matched normal sample pipelines was used to generate somatic variant candidates for the FUSCC TNBC cohort because of the unavailability of matched normal samples. The sample germline variant filtering step used in this study cohort was also applied to the FUSCC somatic variant data. Somatic mutations in patients with TNBC from the METABRIC database were downloaded from cBioPortal [28]. These data also intersected with the same regions of the kinome panel.

Recurrent somatic mutations were selected from the COSMIC (v88) coding mutation database and were defined as somatic mutations that occur in breast cancer more than 100 times compared to the normal population (Supplementary Table 1). These recurrent somatic mutations were generated using the original tumor-normal matched pipeline and Mutect2 without normal sample pipelines in the TCGA and SNUH datasets to assess the confidence...
of Mutect2 without normal sample pipelines. Both datasets showed high agreement (Cohen's kappa value 0.91 in TCGA and 0.96 in the SNUH dataset) for recurrent somatic mutations in breast cancer (Supplementary Figure 1A and B).

**Analyses of significantly mutated genes**

Significantly mutated genes were identified using two algorithms, MutSigCV and OncodriveCLUST [29,30]. MutSigCV identifies significantly mutated genes in cancer genomes using a model with mutational covariates. It identifies significantly mutated cancer genes by considering the sample-specific mutation frequencies, gene-specific mutation rates, expression levels, and replication times. In MutSigCV, a gene is considered a statistically significant mutated gene if its $p$-value is $< 0.05$ using GenePattern [31]. OncodriveCLUST was used to identify genes with a significant mutation bias within the protein sequence. If the Q-value obtained from OncodriveCLUST using maftools was $< 0.05$, it was considered statistically significant [32].

**Tumor mutational burden**

The tumor mutational burden (TMB) was calculated as the ratio of the number of somatic mutations to the total coding region within the kinome panel target region. UCSC RefSeq genes were used as the source of gene-coding region information. All coding sequences comprised multiple 3-mer sequences that began with the start codon (ATG) and ended with stop codons (TAA, TAG, or TGA) with unique mRNA (NM) IDs. The total number of coding regions in the target region was 1,528,857 bases. The mutation counts of each sample were divided by the total coding region, multiplied by megabases, and rounded up to the nearest integer for downstream analysis. The TMB-low and TMB-high groups were divided according to the median TMB of each cohort.

**Oncogenic cell pathway analyses**

Exploratory analyses of oncogenic cell pathways were performed for mutations in each kinome gene previously reported as gain-of-function or loss-of-function mutations [33]. The following ten canonical signaling pathways with frequent genetic alterations were analyzed: cell cycle, Hippo, myc, Notch, oxidative stress response/nuclear factor erythroid 2-related factor 2, PI3K, receptor tyrosine kinase/RAS/mitogen-activated protein kinase, transforming growth factor-beta, p53, and β-catenin (encoded by CTNNB1)/Wnt signaling. Detailed gene lists are provided in Supplementary Table 2.

**Statistical analyses**

Variant- and gene-wise comparisons were performed using Fisher's exact test to compare mutation frequencies between the Asian and non-Asian TNBC cohorts. Progression-free survival was defined as the time from the date of diagnosis to the date of local recurrence, distant metastasis, diagnosis of a new primary tumor, death from any cause, or the last outpatient follow-up. Survival curves were drawn using the Kaplan–Meier method, and the log-rank test was used to assess survival differences. Cox proportional hazards regression models were used for multivariate survival analyses, adjusted for age at diagnosis and tumor stage. All statistical tests were performed using R software (R Foundation for Statistical Computing, Vienna, Austria) [32].
RESULTS

Patient characteristics
The baseline characteristics of the TNBC SNUH cohort are shown in Table 1. The median patient age was 50 years (range, 28–83 years), and most patients had stage I or II disease (88%). All patients were Asian, compared to only 10 (5.9%) Asian patients included in the TCGA cohort. The median follow-up duration was 73.9 months (range, 3–257 months) in the SNUH cohort.

Somatic genetic alteration profiling
The median target region sequencing depth and median on-target rate of the preprocessed BAM files were 388.21x and 98.72%, respectively. Sequencing coverage and quality statistics are provided in the supplementary data. A total of 5,378 somatic single-nucleotide variants and 54 insertions or deletions were identified in the kinome sequencing data of the SNUH TNBC cohort (n = 166). At least one genetic alteration was identified in 163 (98.2%) cases, with a median of five (range: 0–63) alterations detected per case. The most frequently altered genes were TP53 (60% of patients), followed by TTN (36%), PIK3CA (21%), OBSCN (15%), BRCA2 (8%), PRKDC (8%), and ATM (8%) (Figure 1). Among these genes, TP53, PIK3CA, BRCA2, and ATM were significantly mutated in the MutSigCV analysis (Supplementary Figure 2). PIK3CA was the only significant gene in the OncodriveCLUST analysis (q = 0.006). The differences in genomic features between the SNUH and TCGA non-Asian TNBC cohorts were also analyzed. The SNUH cohort had a significantly higher PIK3CA mutation rate than the TCGA cohort (21.7% vs. 11.2%; p = 0.01). Notably, the PIK3CA p.H1047R and p.H1047Q mutation rates were significantly higher in the SNUH cohort than in the TCGA cohort, according to Fisher’s exact test (13.3% vs. 3.3%; p = 0.003). To verify these results, the same analyses were performed on other Asian (FUSCC) and non-Asian (Gustave Roussy, METABRIC) TNBC datasets. The trends were consistent among all analyses (Supplementary Table 3 and Supplementary Figure 1C).

Table 1. Baseline characteristics of the SNUH and TCGA TNBC cohorts

| Variable                        | SNUH TNBC (n = 166) | TCGA TNBC (n = 169) |
|---------------------------------|---------------------|---------------------|
| Age at diagnosis (yr)           |                     |                     |
| Mean                            | 49.4                | 55.13               |
| Median (range)                  | 50.0 (28–83)        | 54 (29–90)          |
| Race, No. (%)                   |                     |                     |
| Asian                           | 166 (100.0)         | 10 (5.9)            |
| Black                           | -                   | 47 (27.8)           |
| White                           | -                   | 106 (62.7)          |
| NA                              | -                   | 6 (3.6)             |
| Stage, No. (%)                  |                     |                     |
| 1-2                             | 147 (88.6)          | 140 (82.8)          |
| 3-4                             | 19 (11.4)           | 25 (14.8)           |
| NA                              | -                   | 4 (2.4)             |
| Progression free interval, No. (%) |                 |                     |
| Event                           | 34 (20.4)           | 28 (16.6)           |
| Censored                        | 132 (79.5)          | 141 (83.4)          |
| Follow-up (days)                |                     |                     |
| Mean                            | 2,401               | 1,221               |
| Median (range)                  | 2,218 (92–7,707)    | 858 (0–7,777)       |

SNUH = Seoul National University Hospital; TNBC = triple-negative breast cancer; TCGA = The Cancer Genome Atlas; NA = not applicable.
The TCGA TNBC dataset was analyzed to compare TMB values from the kinome target panel and whole-exome regions to determine whether the kinome TMB value could be used to accurately assess the whole-exome TMB. The TMB values calculated using these two methods were highly correlated ($R = 0.91$; Supplementary Figure 3). The median TMB was four mutations/Mb in both cohorts (Supplementary Figure 4). The SNUH and TCGA TNBC cohorts were then divided into two groups based on the median split of TMB values. In the TCGA cohort, the TMB-high group had a significantly better survival rate than the TMB-low group ($p = 0.041$; Figure 2). Similar results were found in the SNUH cohort, in which the TMB-high group showed a trend toward improved survival ($p = 0.195$). The median TMB was three mutations/Mb in the TCGA WES data, and a non-significant trend favoring improved survival in the TMB-high group was also demonstrated ($p = 0.182$, Supplementary Figure 5).

The on-target rate and average depth on-target were both higher in the kinome sequencing dataset compared to the WES dataset resulting in lower median TMB in the TCGA WES data (Supplementary Figure 6).

Survival analyses of kinome genes in oncogenic pathways
Survival analysis of kinome genes in oncogenic pathways was performed to determine whether specific genetic alterations in TNBC confer a survival advantage. Gene mutations were grouped according to curated oncogenic pathways, as previously described [33]. Among the ten oncogenic pathways, patients with TNBC in the SNUH and TCGA cohorts
with alterations in the β-catenin/Wnt signaling pathways had poor survival (Figure 3). This association remained after correcting for age and stage according to the Cox proportional hazards model (Supplementary Figure 7). The kinome genes included in the Wnt pathway were CTNNB1, APC, and GSK3β; less than 10% of the TNBC cohorts had alterations in these genes (Supplementary Figure 8).

**DISCUSSION**

This study aimed to identify prognostic factors for TNBC that may serve as potential therapeutic targets. Here, we analyzed target kinome sequencing data from 166 TNBC cases and the TCGA TNBC dataset. Kinome sequencing was used to this end, considering the important regulatory role of kinomes in cancer initiation and progression and their potential therapeutic role.

The SNUH TNBC cohort had a higher PIK3CA mutation rate than several non-Asian TNBC cohorts in this study. This difference was also observed when comparing the FUSCC Chinese TNBC cohort with publicly available data from non-Asian TNBC cohorts. This observation was consistent with a recent report by Xiao et al. [34] on another Chinese breast cancer cohort, indicating that Asian patients with TNBC have a higher PIK3CA mutation rate than non-Asian patients with TNBC. A distinct characteristic of our study was the high rate of PIK3CA p.H1047R and p.H1047Q mutations in Asian patients with TNBC, which has not been reported in previous TNBC studies. Recently, alpelisib, a PI3K inhibitor, was approved by the United States Food and Drug Administration to treat patients with PIK3CA-mutated, hormone receptor-positive advanced breast cancers. Although the role of PI3K inhibitors in TNBC with
PIK3CA mutations is unknown, many clinical trials are ongoing to unravel this association [35]. The relatively high rate of PIK3CA mutations in the Asian population suggests that PI3K inhibitors may play an essential role in treating Asian patients with TNBC.

The current gold standard for TMB measurements is the application of WES data. In this study, a kinome panel consisting of 612 genes was used for TMB evaluation. The TCGA cohort was used to compare TMB values between the subset of genes in the kinome panel and the original whole-exome region showing strong correlation. Although WES data are the gold standard for TMB measurements, they are not widely utilized in routine clinical practice because of their high cost, time consumption, and labor-intensive processing. Currently, precision oncology platforms are primarily based on targeted gene panels. Similar to this study, analyses of several commercialized gene panels have revealed strong correlations between WES- and panel-based TMB quantification using TCGA datasets [36,37]. Additionally, the relationship between recurrence and TMB value was clearer for kinome-based TMB values which could be related to a significantly higher on-target rate and average depth on-target in the kinome sequencing dataset. The predictive value of panel-based TMB value for immunotherapy response is demonstrated in other studies also [38].

This study suggests that TNBC patients with high TMB have a better likelihood of survival compared to patients with low TMB. TMB-low and TMB-high were defined in relation to the median TMB value (four mutations per Mb). Although the commonly used definition of high TMB is ≥ 10 mutations/Mb, this is a predictive value for immune checkpoint inhibitors originating from the KEYNOTE-158 trial [39]. In this study, we investigated the prognostic value of TMB unrelated to immunotherapy. The number of mutations that define TMB-high
varies across cancer types, and previous studies have demonstrated that 10 mutations/ Mb cannot be a universal definition for a prognostic/predictive factor in all cancer types [40,41].

TMB is closely related to neoantigen burden and T-cell infiltration, and is a marker of tumor antigenicity [38]. A high TMB is associated with high response rates to immunotherapy and is recognized as a predictive factor for immune checkpoint inhibitor efficacy in various cancers [38]. However, the prognostic role of TMB in TNBC has not been established. Garrido-Castro et al. [42] reported that a high TMB is significantly associated with improved overall survival in patients with de novo metastatic TNBC. In the GeparNuevo trial, patients who underwent neoadjuvant chemotherapy for early TNBC tumors had a significantly higher TMB value when pathologic complete response was achieved [43]. In contrast, the survival rate did not differ according to TMB values among patients with early breast cancer in the US001062 study [44]. In general, cancers with a high TMB also have a higher tumor-infiltrating lymphocyte (TIL) count, and because of the prognostic role of TIL in TNBC, we can also assume that TMB has a prognostic role in breast cancer. However, this warrants further investigation.

Survival analyses of cancer signaling pathways revealed that Wnt pathway alterations were related to poor prognosis in TNBC. The kinome genes altered in the Wnt pathway include CTNNB1, APC, and GSK3β. Inactivating mutations in APC and GSK3β and activating mutations in β-catenin lead to the mutational inactivation of the β-catenin destruction complex, the archetypal mode of Wnt pathway activation in cancer. Several reports have demonstrated that Wnt pathway activation is associated with extensive metastasis and poor prognosis for TNBC [45-47]. Geyer and colleagues [47] suggested that β-catenin/Wnt pathway activation is not related to CTNNB1 mutations, as no exon 3 CTNNB1 mutations were observed in 19 invasive breast carcinoma samples with β-catenin nuclear expression. Alternatively, the β-catenin/ Wnt pathway may be activated by other exons of the CTNNB1 gene or other genes in the Wnt pathway, which could be the underlying mechanism of the Wnt pathway alteration observed in this study. Further investigation is needed using RNA or immunohistochemistry expression data to determine whether Wnt pathway alterations correlate with Wnt pathway activation. The poor outcomes of TNBC patients with Wnt pathway alterations in this study suggest that the Wnt pathway might be a valuable therapeutic target for TNBC. However, despite identifying numerous Wnt pathway inhibitors, no drugs have been approved to target this pathway. A major challenge when targeting the Wnt pathway is avoiding toxicity in healthy tissues, considering its role in maintaining stem cells and the regeneration of tissues and organs [48].

Targeted sequencing is more practical for clinical applications than whole-exome and genome sequencing. Screening a limited but clinically important gene set reduces the turnaround time and provides high-depth sequencing. It also helps reduce costs and minimize the complexity of data interpretation and reporting. However, targeted panels may not be suitable for research purposes because of their narrow coverage since only a small part of the human genome is covered.

Here, we only focused on small mutations using targeted sequencing. A limitation of this study was the absence of any analysis of copy number alterations, the tumor microenvironment, and the presence of fusion genes. We could not consider TNBC subtype classifications based on gene expression data because of a lack of RNA expression data in the SNUH cohort. In addition, the definition of high TMB is still not optimized and varies widely by tumor type and the number or type of selected genes [49]. Lastly, the low number of patients with altered Wnt pathway in the two cohorts limit the clinical significance of our
results and additional studies are needed to determine the prognostic value of Wnt pathway alterations in patients with TNBC.

In conclusion, we characterized the somatic mutation landscape in patients with early TNBC using a targeted kinome sequencing approach. We found a higher \( \text{PIK3CA} \) mutation rate in Asian patients than in non-Asian patients with TNBC, especially mutations in \( \text{PIK3CA} \ p.H1047R \) and \( p.H1047Q \). In this study, TNBC patients with high TMB showed a trend toward better clinical outcomes, whereas Wnt pathway alterations were related to a poor survival rate. These genomic traits are associated with disease recurrence and can be considered therapeutic targets. Furthermore, they may also help identify high-risk patients in future studies.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1
Recurrent somatic mutations in breast cancer from the COSMIC (v88) coding mutation database

Click here to view

Supplementary Table 2
Log-rank test results of the SNUH cohort according to kinome gene alterations in the oncogenic pathways

Click here to view

Supplementary Table 3
Fisher’s exact test results for the \( \text{PIK3CA} \ p.H1047Q/R \) mutation between Asian and non-Asian datasets

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Supplementary Figure 1
Contingency table of the number of mutations and normal DNA sequences generated for recurrent somatic mutations using a tumor-normal matched and Mutect2 without normal sample pipelines in the TCGA (A) and SNUH (B) datasets. Fisher’s exact test results of \( \text{PIK3CA} \ p.H1047Q/R \) mutations between Asian and non-Asian populations without a normal sample pipeline are shown in (C).

Click here to view
Supplementary Figure 2
MutsigCV result of the SNUH cohort. The genes that have a q-value < 0.05 are labeled.

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Supplementary Figure 3
Scatter plot of the TMB and mutation count in the kinome sequencing and WES data.

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Supplementary Figure 4
Distribution of TMB with the kinome data in the SNUH and TCGA cohorts. Outlier samples with a TMB value > 50 (60 and 189) in the SNUH cohort were excluded from the plot.

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Supplementary Figure 5
Kaplan-Meier curves of the TMB-low and TMB-high groups in the TCGA WES data.

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Supplementary Figure 6
A comparison of the on-target rate (A) and average depth on-target (B) between WES and kinome sequencing in the TCGA dataset.

Click here to view

Supplementary Figure 7
Forest plot of the Cox proportional hazard models.

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Supplementary Figure 8
Kinome genes (green highlighted) in the Wnt β-catenin pathway.

Click here to view

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