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Inhibitory effort of MLN2238 on basal-like breast cancer: An investigation based on the gene set enrichment analysis

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ABSTRACT

Basal cell-like breast cancer (BLBC), one subtype of breast cancer, has the characteristics of a high recurrence rate and strong invasiveness. Therefore, it is necessary to exploit new drugs for the therapy of BLBC. The data on small molecular drugs were downloaded from the cancer drug sensitivity genomics (GDSC) database, and the target gene information of small molecular drugs was obtained from the SWISS website. Based on the TCGA database, a genome-wide t-value sequencing for screening differentially expressed genes (DEGs) was constructed. The bioinformatics analysis was further performed. The cell cycle was determined using flow cytometry. Western blot was performed to calculate the expression of P21 and P27. siPLK1 transfection was performed to interfere with PLK1 expression. And further cell experimental techniques were performed. The specific effect and mechanisms of the screened small molecular drugs were confirmed through clinical sample studies and in vitro experiments. MLN2238 could significantly inhibit the proliferation of HCC38, a BLBC cell line. The PPI network based on the target gene significantly up-regulated by MLN2238 shows that PLK1 is the key gene, and KEGG analysis shows that the up-regulated target gene is in the cell cycle. Flow cytometry showed that MLN2238 blocked HCC38 cells in the G2/M phase. The results of the Western blot revealed that MLN2238 inhibited the expression of P21 and P27 in HCC38 cells. The survival heat map based on the TCGA database shows that PLK1 has the greatest impact on the survival of breast cancer. Patients with high levels of PLK1 expression had a poorer overall survival rate than those with low levels of PLK1. Cell experiments in vitro revealed that the PLK1 expression decreased significantly after siPLK1 transfection. The ability of cell proliferation was significantly inhibited after SiPLK1 transfection. MLN2238 is a potential target drug for the therapy of BLBC, and PLK1 is the target gene for MLN2238 to inhibit BLBC.

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Introduction

Breast cancer (BC) is one of the most common causes of cancer-related morbidity and mortality around the world (1). Molecular phenotyping analysis based on gene expression profiling reveals that breast cancers in different patients exhibited great heterogeneity in each other. Through the array and RNA-seq analysis, several molecular subtypes have been identified which include luminal A, luminal B, HER2-enriched (HER2E) and basal-like (2, 3), etc. Basal-like breast cancers (BLBCs) are defined as breast cancer tissues lacking the expression of ER, PR and HER2(4). The clinical research showed that BLBC, which represents up to 15% of breast cancers, owned the characteristics of an aggressive and highly recurrent rate (5).

Both radiotherapy (RT) and chemotherapy showed preferable therapy effects for BLBC patients. Chemotherapy was set as the standard treatment for BLBC. However, drug resistance stayed as the major challenge for BLBC patients (6). Meanwhile, previous studies showed that RT is an effective way to treat BC patients especially those with BLBC (7), but the local recurrence (LR) was still high (8) and overall survival (OS) was poor (9). With the development of the therapy technique, targeted therapy shows preferable clinical effects for BC patients. PARP inhibitors, one of the targeted therapy drugs, were approved to apply to BRCA-mutant triple-negative breast cancer (TNBC) patients (10). The targeted treatments for TNBC are still at the early stage (11) and it was urgently needed to determine the newly-finding effective drugs targeting BLBC which can improve the LR and OS in clinical practice.

The proteasome is found in all eukaryotic cells and is responsible for degenerating the intracellular proteins as well as determining cell growth and apoptosis. Nowadays, proteasome inhibitors have been set as the important drugs for several diseases including multiple myeloma, mantle cell lymphoma, etc. (12). The main function of proteasome inhibitors was to obstruct pathways for protein degradation and disturb regulatory networks which can significantly influence the cell growth and apoptosis (10). The clinical research showed that bortezomib, one of the proteasome inhibitors, showed preferable clinical effects to liquid tumors. Furthermore, researchers tried to promote the studies in the next generation of proteasome inhibitors which were expected to show more effective effects on cancer patients and less pharmaco-resistance and cytotoxicity (13). MLN2238 (ixazomib) was set as one of the next-generation reversible proteasome inhibitors with a similar efficacy to bortezomib, and has been given great promise (14).

MLN2238, as an orally bio-available, small molecule,

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potent, reversible agent, mainly provides clinical effects by selectively inhibiting the β5 site of the 20S proteasome. The research showed that the structure of MLN2238 is different from bortezomib (15). MLN2238 (ixazomib) has been given great promise because of the proven clinical effects of bortezomib which was another proteasome inhibitor (16,17). Moreover, the disassociation half-life of ixazomib was six-fold faster than bortezomib which made ixazomib fast dissociate from red blood cells and rapidly entered into the tumor cells (18). The comparisons between bortezomib and ixazomib showed that ixazomib own better clinical efficacy in tumor suppression (19). Furthermore, MLN2238 has already shown significant inhibitory effects on tumor growth in melanoma, colorectal cancer and so on (20,21). And the main function of MLN2238 was to regulate target gene MCL-1, NOXA expression (22,23). However, few studies concentrated on the effects of MLN2238 on BLBC.

In this research, bioinformatics analysis was used to clarify the functional mechanism of small molecule antitumor drugs and potential targeted therapy. Our results suggest that MLN2238 is a potential BLBC-targeted drug, and PLK1 is the target gene of MLN2238 to inhibit BLBC.

Materials and Methods

Data collection

The information on small-molecule drugs was downloaded from the GDSC database, which is the most comprehensive public resource for information on drug sensitivity in cancer cells and drug response molecular markers (13). The target genes of the small-molecule drugs were obtained from the SWISS website. Furthermore, the RNA-Seq data of BRCA samples and adjacent samples were obtained from the TCGA database which can screen the differentially expressed genes in a variety of malignant tumor tissues and their matched normal tissue samples.

Differentially expressed genes (DEGs) screening

DEGs were screened between cancer and normal samples using the "Limma" package from R 4.1.1 software. Genes with $|\log_2 FC|>1$ and the P.val < 0.05 were set as DEGs.

Anti-tumor drugs screening

GSEA analysis was performed based on the genome-wide rank of the *t*-value from DEGs screening and the target genes of the small-molecule drugs. The NES scores were set as the intermediary between the DEGs data and the small-molecule drugs.

Cell culture

TNBC cell line HCC38 (ATCC, USA, CRL-2314D) was cultivated in RPMI-1640 medium (Hyclone, USA, SH30809.01B) complemented by 10% fetal bovine serum (FBS, Hyclone, USA, SH30070.03), and 1% streptomycin-penicillin at 37°C, 5% incubate in CO_2 gas phase.

Cell Counting Kit-8 test

The Cell Counting Kit-8 (CCK-8, Dojindo, Tokyo, Japan, CK04-100T) kit was used to detect cell proliferation in accordance with the manufacturer's instructions. The HCC38 cells were seeded in 96-well plates with a cell density of 1000 cells per well and cultivated at the cell incubator with a stable atmosphere. 10 μL CCK-8 solution combined with serum-free medium was added at 24 hours, 48 hours and 72 hours after cultivation. We utilized a microplate reader to detect the absorbency at a test wavelength of 450 nm and a reference wavelength of 630 nm after a 2-hour incubation period.

Enzyme-linked immunosorbent assay (ELISA)

The proliferation ability of HCC38 cells was evaluated using the ELISA-BrdU Kit (Roche, Basel, Switzerland, 11647229001). HCC38 cells were seeded at a density of 5×10^4 per well into 12-well plates. After 48 hours of cultivation, 20 μL BrdU solution was added to each well. After 40 min incubation, the optical density (OD) value of each well was tested and recorded at the 450 nm wavelength.

Cell cycle analysis

HCC38 cells were planted in 6-well plates at a density of 1×10^5 cells/well and cultured in a cell incubator with a stable atmosphere. After 3 days of cultivation, the cells were obtained for further detection. Firstly, cells were washed with PBS and fixed with 70% ethanol at -20°C for 30 min. Then, the cells were stained with 50 μg/ml PI (Sigma Chemicals, St. Louis, MO, USA) after being incubated with 100 μg/ml RNase for 30 min (Sigma Chemicals, St. Louis, MO, USA). And finally, FACSC-Calibur (BD Biosciences, Franklin Lakes, NJ, USA) and FCS express software (DeNovo Software, Los Angeles, CA, USA) were used to analyze DNA contents by flow cytometry (17).

Western-blot

Cells were lysed with 40 μL RIPA ice-cold lysis buffer (Rockford, IL, USA) supplemented with protease inhibitor (Vazyme Biotech, Nanjing, China) for 30 min at indicated time points. The protein concentrations in the whole cell lysates were determined using the BCA (Rockford, IL, USA) method after centrifugation at 7000 g for 10 min at 4°C. The protein samples were separated depending on their molecular weight on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by a transfer to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). 5% nonfat powdered milk in Tris-buffered saline was used to block the membranes, which were stained with primary antibodies overnight at 4°C. After that, the membranes were probed with a 1:10,000 dilution of a peroxidase-labeled secondary antibody. An ECL reagent (Rockford, IL, USA) was used to detect the antigen-antibody complexes. Primary antibodies against β-actin were obtained from SIGMA St. (Louis, MO, USA), p21Waf1/Cip1, and p27 from Cell Signaling Technologies (Beverly, MA, USA). The ratio of drug-treated samples to control samples was used to calculate the relative expressions, which were normalized by the quantified level of β-actin expression.

PPI network construction and KEGG pathways analysis

The Protein-Protein interaction (PPI) network of the significantly upregulated target genes (P < 0.01) was constructed at the Search Tool for the Retrieval of Interacting Genes (STRING) database (https://string-db.org/). Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (http://www.genome.jp/kegg/pathway.

html) on the WebGestalt website (http://www.webgestalt.org/), the functional pathways of target genes connected to BLBC were studied.

Cell transfections

The siPLK1 and negative control siRNA (NC siRNA) were synthesized by Suzhou GenePharma Co., Ltd. (Suzhou, China). Following is the list of the sequences: si-PLK1-501 sense, 5′-GCACCGAAACCGAGUUAUUTT-3′; siPLK1-501 antisense, 5′-AAUAACUCGGUUUCGGUGCTT-3′; NC siRNA sense, 5′-UUCUCCGAACGUGUCACGUTT-3′; NC siRNA antisense, 5′-ACGUGACACGUUCGGAGAATT-3′ (19). The PLK1 overexpressing recombinant vector pcDNA3.1-PLK1 and empty plasmid as negative control (NC) were synthesized by GenePharma (Shanghai, China).

Statistical analysis

All analysis was performed based on Statistical Product and Service Solutions (SPSS) 24.0 (SPSS, Chicago, CA, USA) software. In this research, all experiments were carried out in triplicate in dependently. Data were expressed as the mean ± standard deviation. Student's two-tailed t-test was used to determine statistical significance for comparisons between two groups, and Dunnett's test was used when the data involved three or more groups. $P < 0.05$ was defined as the difference was significant.

Results

Selection of potential drugs for the treatment of breast cancer

365 drugs were obtained from the GDSC database. The GSEA analysis based on the rank of the t-value of DEGs screening showed the NES scores of each drug (Figure 1A). From the GSEA analysis, the top three drugs (paclitaxel, thalidomide and MLN2238) were obtained as potential candidates for BC treatment (Figure 1B). The target genes of the top three small molecule drugs were enriched in the top rank of t value from BRCA ($P < 0.05$). Since there are a lot of literatures about paclitaxel and thalidomide, MLN2238 can be a preferable choice for further study. Furthermore, MLN2238 showed higher sensitivity to BC than other tumors ($P = 0.013$, Figure 1C). Therefore, MLN2238 could be the potential drug for BC therapy.

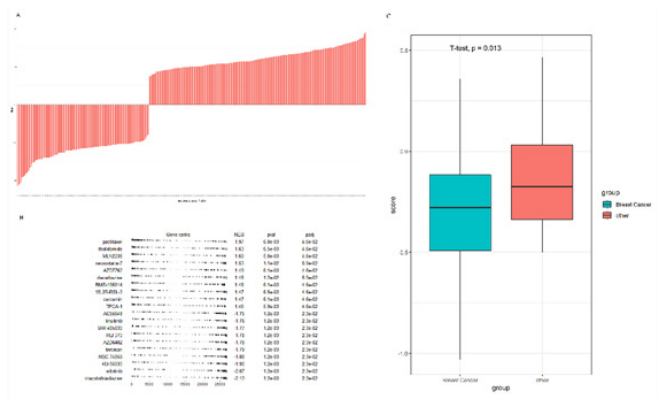

Figure 1. MLN2238 is a potential drug for breast cancer. (A) The NES score of 365 small molecule drugs of BRCA by GSEA method; (B) The NES rank of small molecule drugs; (C) Comparison of NES scores between BC and other tumors.

BLBC is sensitive to MLN2238

The GSEA analysis based on the four subtypes (luminal A, luminal B, HER2-enriched (HER2E) and basal-like) of BRCA indicated that the BLBC subtype is most sensitive to MLN2238 compared to the other three subtypes. (Figure 2A-D). The IC50 value of MLN2238 to BC cell lines was obtained from the GDSC database. A total of 35 IC50 values including 17 values to Basal-like breast cancer subtype cell lines and 16 values to other breast cancer subtype cell lines were obtained (Figure 2E). And the result manifested that IC50 of the HCC38 cell line was at the least value (Figure 2E). Therefore, the HCC38 cell line was chosen for further cell verification. The CCK-8 analysis indicated that MLN2238 owed certain inhibitory effects to HCC38 cells. And the survival rate of HCC38 decreased as the concentration of MLN2238 increased. When the concentration of MLN2238 reached 250 nM, the survival rate of HCC38 fell to 50% (Figure 2F). ELISA-BrdU test showed that MNL2238 significantly decreased the cell proliferation ability of HCC38 cells (Figure 2G). Therefore, MLN2238 can be an effective indicating medication to treat HCC38 cells.

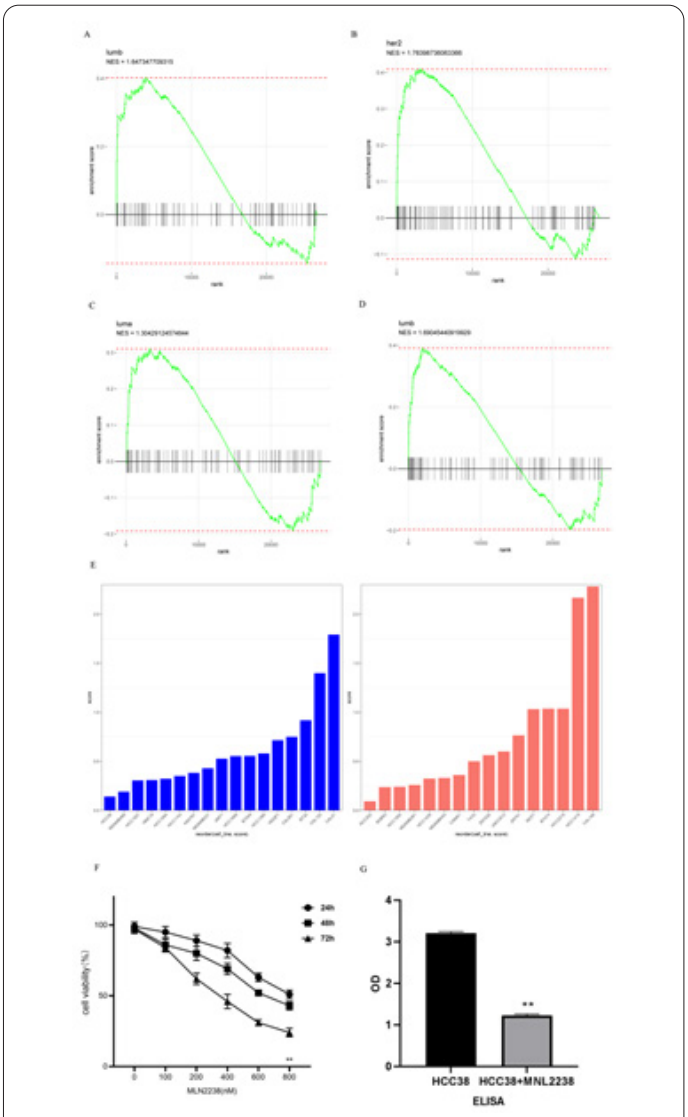

Figure 2. BLBC is sensitive to MLN2238. (A-D) The NGSEA analysis of MLN2238 in the four subtypes of BRCA; (E) The IC50 values of MLN2238 from the GDSC database; (F) CCK-8 analysis at 24, 48, 72 hours after different concentrations of MLN2238 in HCC38 cells; G. ELISA-BrdU test to determine the cell proliferation ability of HCC38 cells (** P<0.01).

The targeted genes of MLN2238 in BLBC were enriched in the cell cycle

Take the intersection of upregulated DEGs and MLN2238 target genes, 47 genes were selected as the significantly upregulated target genes ($P < 0.01$). The PPI network was constructed based on the 47 significantly upregulated target genes ($P < 0.01$) and shown in Figure 3A, the target genes were exhibited as the node and the interaction between the proteins was indicated by the edge. From the PPI network analysis, PLK1 is at the core and had a higher degree. Therefore, PLK1 was considered to be the core gene during MLN2238 treated with BLBC. KEGG pathway enrichment of the 47 significantly upregulated genes was executed to explore the molecular mechanism of MLN2238 on BLBC cells. The KEGG analysis showed that the significantly upregulated genes were enriched in the cell cycle, P53 signaling pathway, etc. (Figure 3B). The results indicated that PLK1 may be the core gene of MLN2238 targeted genes, and MLN2238 targeted genes in BLBC were enriched in the cell cycle.

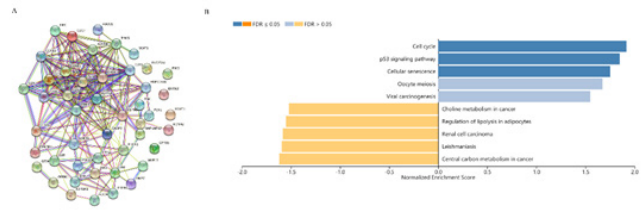

Figure 3. Target genes of MLN2238 are related to the cell cycle. (A) Protein–protein interaction (PPI) networks of significantly upregulated target genes; (B) KEGG pathways of significantly upregulated target genes.

MLN2238 induced the G2/M cell cycle arrest in BLBC cells

The cell cycle phase distributions of HCC38 cells were examined by flow cytometry analysis to clarify the inhibitory effect of MLN2238 on BLBC cell proliferation. As shown in Figure 4A, B, MLN2238 treatment (250 nM) for 24 h induced a higher cell population in the G2/M phase, and a lower population in the G0/G1 phase than those in the control. The Western-blot experiment demonstrated that the expression of p21 and p27 was significantly lower in the 0 nM group compared to the 250 nM NML2238 group. (Figure 4C, D). The experiments above manifested that MLN2238 induced G2/M cell cycle arrested and elevated the expression of p21 and p27 in HCC38 cells.

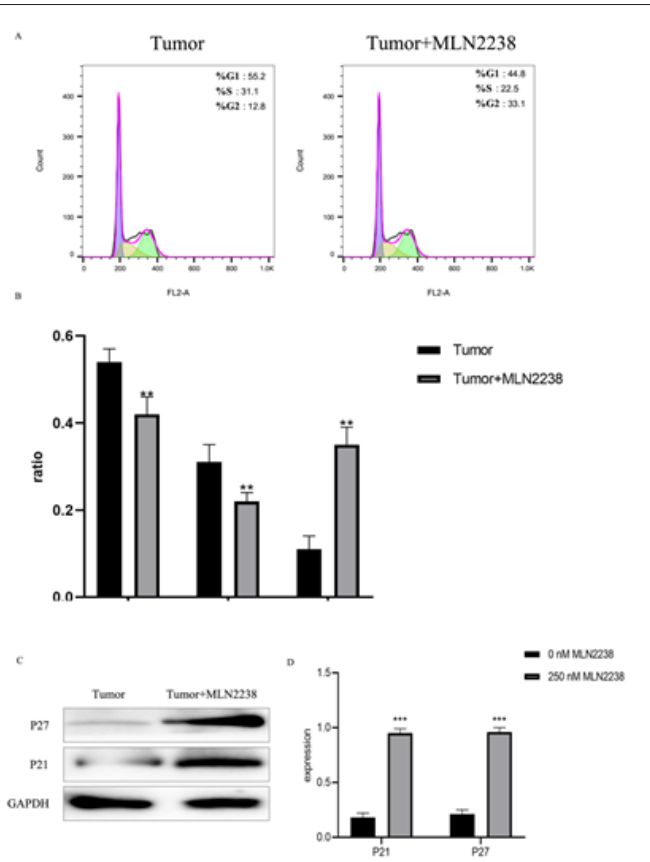

Figure 4. MLN2238 treatment induces cell cycle arrest in HCC38 cells. (A) Flow cytometry to determine the cell cycle distribution in cells with 0 and 250 nM MLN2238 for 24 h; (B) The proportion of each phase (G2/M, S and G0/G1) of the cell cycle in HCC38 cells with 0 and 250 nM MLN2238 for 24 h; (C-D) Western-blot analysis of p21 and p27 in HCC cells after treatment with 0 and 250 nM of MLN2238 for 24 h. (***P<0.001)

MLN2238 inhibits BLBC proliferation by upregulating PLK1

RNA-seq profile and clinical information based on the BLBC patients and control group were downloaded from the TCGA database. Based on the DEGs threshold, 2403 DGEs were found, with 996 up-regulated DEGs and 1407 down-regulated DEGs. (Figure 5A). The significantly upregulated genes (including AURKA, PLK1, AURKB, CCNA2, etc.) from targets of MLN2238 were screened as candidates for further study. The survival heat map of candidates manifested that PLK1 has the most significant positive impact on the survival of breast cancer (Figure 5B). The mRNA level of PLK1 was up-regulated in BRCA tissues compared to normal tissues with significance (Figure 5C). The K-M curves showed that BCRA with high PLK1 expression owned lower OS than BCRA with low PLK1 expression (Figure 5D, P<0.05).

RT-PCR showed that PLK1 expression was significantly decreased after siPLK1 transfection (Figure 5E, P<0.01). To further evaluate the effect of PLK1 expression on HCC38 cell viability, the CCK-8 analysis manifested the proliferation ability of cells was dramatically inhibited in the siPLK1 group (Figure 5F, P<0.05) which indicated that high expression of PLK1 was correlated to better cell proliferation ability. Meanwhile, the ELISA-BrdU experiment showed that PLK1 knockdown significantly decreased the cell proliferation ability and PLK1 overexpression increased the cell proliferation ability (Figure 5G, P<0.01). When NML2238 was added into the cells, the proliferation ability in the cell proliferation ability group and PLK1 knockdown group were dramatically decreased (Figure 5G, P<0.01). Based on the experimental analysis, NML2238 may inhibit BLBC proliferation by upregulating PLK1.

Discussion

The results verified that MLN2238 can be a sensitive drug to treat BLBC and PLK1 is the key gene in BLBC proliferation. MLN2238 decreased the cell proliferation ability induced by upregulating PLK1 in HCC38 cells.

The ixazomib has been approved for the clinical treatment of multiple myeloma by the FDA based on the studies that conducted the antitumor effects of ixazomib (24-26).

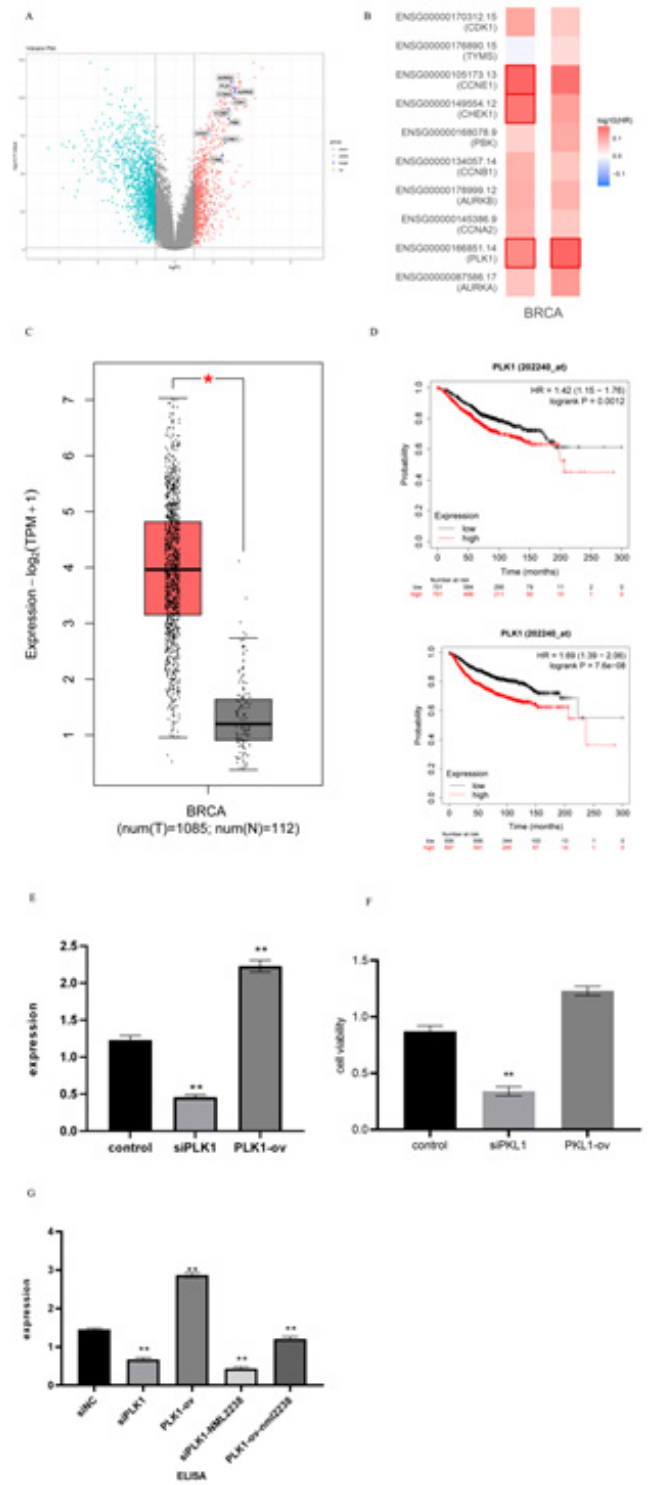

Figure 5.MLN2238 inhibits cell proliferation induced by PLK1 overexpression. (A) Volcano plot of DEGs related to BLBC; (B) The survival heat map of significantly upregulated targets of MLN2238; (C) TCGA database analysis of the relatively differential expression level (log2) of PLK1 in BRCA ($P < 0.05$); (D) K-M curves comparison between BRCA patients with PLK1 high-expression and PLK1 low-expression ($P < 0.05$, HR>1); (E) RT-PCR analysis of PLK1 expression in siPLK1 and PLK1-ov groups; (F) CCK-8 analysis in siPLK1 and PLK1-ov groups; G. ELISA-BrdU analysis in HCC38 cell with siPLK1, PLK1-ov, siPLK1-MLN2238 and PLK1-ov-MNL2238. (**P<0.01)

Furthermore, MLN2238 has been subsequently proven to be an effective method for other tumors such as colon adenocarcinoma (27), osteosarcoma (28), melanoma (29), and neuroblastoma cells (30). In this study, we analyzed the NES scores of small molecule antitumor drugs by GSEA in BC patients. And the results showed that MLN2238 was the most sensitive to BLBC. Based on the bioinformatics analysis, we further verified the effects of MLN2238 on BLBC by experiments. The CCK-8 experiment revealed that MLN2238 could significantly inhibit the proliferation of the BLBC cell lines HCC38.

To further determine the main function of MLN2238 to BLBC, Bayesian analysis was performed and finally, 47 significantly up-regulated target genes of MLN2238 were obtained and a gene-interaction network was constructed. Through the enrichment analysis of MLN2238 target genes, it is suggested that MLN2238 affects the cell cycle process. Previous studies showed that in tumor cells, the proliferation ability is mainly controlled by genetic and epigenetic mechanisms. Meanwhile, the intervention to cell proliferation abilities is mainly by causing an incompetent checkpoint control or affecting the expression of cell cycle regulatory proteins. And finally, abnormal responses to the cellular injury occurred, resulting in cell death (31). In vitro experiments revealed that MLN2238 can inhibit tumor cell proliferation, and arrest the cell cycle in the G2/M phase combined with upregulated p21 and p27 proteins.

The survival heat map of significantly upregulated genes ($P < 0.01$) found that PLK1 has the most significant impact on the survival of BRCA. Polo-like kinases (PLK), one of the members of cell cycle protein kinases, have been proven to be critical roles in regulating the process of mitotic entry and chromosome segregation. PLK1 has been proven to over-express in a wide range of tumors (32,33) and can promote the proliferation of tumor cells (34). The main function of PLK was to ensure the correct formation of daughter cells (35,36). There are 5 paralogues of PLK in cells which were named from PLK-1 to PLK-5. Among them, PLK1 was the most important in regulating cell division (37). Studies had shown that PLK-1 can ensure the effectiveness and efficiency of cell division(38) and is expressed during the G2/M phases of the cell cycle (39). Based on these backgrounds, PLK1 has been suggested as one of the most potent cancer therapeutic markers (40). Meanwhile, PLK1 expression was considerably higher in BRCA tissues than in normal tissues, according to RT-PCR results. K-M curves between BRCA patients with PLK1 high-expression and PLK1 low-expression showed that the high-expression of PLK1 owned low overall survival. To further determine the mechanism of PLK1 in HCC38 cells, in vitro experiments were performed and the CCK-8 results showed that the HCC38 cell proliferation was significantly inhibited after PLK1 knockdown. The ELISA-BrdU test showed the proliferation ability of cells was dramatically decreased in the PLK1 knockdown group and increased in the PLK1 overexpression group. Furthermore, when NML2238 was added into each group, the proliferation ability in PLK1 knockdown group and PLK1 overexpression group were significantly decreased.

This is, as far as we know, the first study to evaluate the efficacy of ixazomib in BLBC. And the results showed that MLN2238 can inhibit the proliferation of the BLBC cell line HCC38, arrest the cell cycle in the G2/M phase and rescue the cell proliferation caused by overexpression of PLK1.

These results imply that MLN2238 restrained the proliferation of HCC38 and induced the cell cycle arrest

in HCC38 by upregulating the PLK1 expression. While further in vivo studies are needed, targeting PLK1 with a selective inhibitor like MLN2238 could be an appealing molecular-targeted therapy for BLBC.

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Conflicts of interest
The authors have no conflicts of interest to declare.

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

Contributions
(I) Conception and design: Dapeng Wang; (II) Administrative support: Yunyan Li; (III) Provision of study materials or patients: Fushen Luo, Shuang Wu, Na Zhang; (IV) Collection and assembly of data: Ying Chen; (V) Data analysis and interpretation: Dapeng Wang, Xue Song; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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