



Identification of lapatinib sensitivity-related genes by integrative functional module analysis

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Background: Globally, gastric carcinoma (GC) is one of the most commonly encountered malignancies and is the second highest contributor to cancer mortality. Lapatinib is a potent, orally-bioavailable small-molecule inhibitor of both epidermal growth factor receptor and human epidermal growth factor receptor-2 tyrosine kinases, and is administered to treat GC. However, a large proportion of patients either develop resistance to or do not respond to lapatinib, often because the treatment activates alternative signaling pathways. It is, therefore, vital to identify the key pathways which mediate resistance to lapatinib treatment.

Methods: The lapatinib sensitivity-related genes were extracted from the CellMiner database (version 2.2) using “NCI-60 Analysis Tools”. The differentially expressed genes (DEGs) in gastric cancer were derived from The Cancer Genome Atlas (TCGA) database, the protein-protein interaction (PPI) network was derived from the Human Protein Reference Database (HPRD), and the Database for Annotation, Visualization and Integrated Discovery (DAVID) facilitated the functional analysis. The cell function was tested by CCK-8 cell viability assay, colony formation assay, acridine orange/ethidium bromide (AO/EB) staining, and Transwell assay.

Results: The functional linkage networks of lapatinib sensitivity were constructed. Two modules were identified, and pathway analysis indicated that these modules were involved in several pathways, including the neuroactive ligand-receptor interaction network and the Rap1 signaling pathway. Finally, the breast cancer anti-estrogen resistance 1 (*BCAR1*) gene was selected for further study with lapatinib-resistant SUN216 cells (SUN216/LR). We found the expression of *BCAR1* was upregulated in SUN216/LR cells compared to SUN216 cells. The IC₅₀ of lapatinib in SUN216/LR cells was reduced upon *BCAR1* knockdown, as measured by a CCK-8 assay. A clonogenic assay showed fewer SUN216/LR colonies with *BCAR1* knockdown and lapatinib treatment.

Conclusions: In brief, we efficiently identified those crucial modules highly related to lapatinib sensitivity in GC by using a topological network method. *BCAR1* was identified as a potentially critical gene that plays a role in lapatinib sensitivity, and experiments confirmed that *BCAR1* might contribute to lapatinib resistance in GC. These results provide further insight into the molecular basis of lapatinib sensitivity and may offer novel strategies for the future treatment of GC.

Keywords: Gastric carcinoma (GC); functional linkage network; apoptosis; sensitivity

Submitted Sep 20, 2019. Accepted for publication Dec 24, 2019.

doi: 10.21037/tcr.2020.01.30

View this article at: <http://dx.doi.org/10.21037/tcr.2020.01.30>

Introduction

Globally, gastric carcinoma (GC) is one of the most commonly encountered malignancies and is the second highest contributor to cancer mortality (1-3). Although therapy for GC has seen progressive developments in the last decade, especially with chemotherapeutic drugs, the survival rate of GC patients remains unsatisfactory (4,5). Therefore, methods to enhance the efficacy of chemotherapeutic drugs for GC patients are needed.

Lapatinib, which is orally bioavailable, is a small-molecule reversible inhibitor of both epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor-2 (HER2) tyrosine kinases (6). Although lapatinib has found a purpose as a treatment of trastuzumab-resistant, HER2-positive advanced GC, many patients eventually develop resistance to or do not respond to the drugs (7,8), often as a consequence of the activation of alternative signaling pathways (9,10).

In view of this, it is critical to be able to predict which signaling pathways could be activated to mediate resistance to lapatinib treatment, which could help identify combination or secondary treatments to mitigate this resistance (11,12). However, it has been difficult to identify the key pathways and proteins involved in lapatinib resistance. Park *et al.* found that FOXO1, through negative crosstalk, acts as a bridge between HER2 and MET signaling pathways, and plays a core role in the regulation of acquired lapatinib resistance (13). Meanwhile, inhibition of Hes1 enhances lapatinib sensitivity in GC sphere-forming cells (14), and Kim *et al.* demonstrated that testican-1-mediated epithelial-mesenchymal transition signaling results in acquired resistance to lapatinib in GC (15).

However, these pathways and proteins linked to lapatinib resistance were identified by previous publications and experiments, and a comprehensive method to investigate the pathways or signaling networks involved in lapatinib resistance is needed. Fortunately, these limitations can be alleviated by the advancements that have been made with high-throughput experimental and bioinformatics databases. Exploration of the relationship between drug sensitivity and genomic data was facilitated by NCI-60 cell line panel and associated drug screens (16). Meanwhile, the furtherance of CellMiner has enabled the efficient retrieval of both genomic data and the activity reports of ~20,000 chemical compounds across the NCI-60 cell line (17).

In this study, we sought to explore the relationship between sensitivity genes and lapatinib response in GC

by integrating genomic, drug sensitivity, and tumor transcriptomic data. We constructed a lapatinib sensitivity negative network (LSN) and a lapatinib sensitivity positive network (LSP), and identified modules within each of these networks. We then evaluated these modules using the extent of enrichment of dysregulated genes in GC. *BCAR1* gene, which was present in module 4 and was enriched in the Rap1 signaling pathway, was selected for further analyses. Our data indicated that knockdown of *BCAR1* can reverse lapatinib resistance in SUN216/LR cells.

Methods

Network construction

Construction of a functional linkage network for lapatinib sensitivity was performed with 164 positively related genes and 117 negatively related genes, which were extracted from the CellMiner database (version 2.2) using “NCI-60 Analysis Tools”. The potential positively and negatively related genes involved in lapatinib sensitivity were obtained by calculating the Pearson’s correlation coefficient between the mRNA expression profiles of the NCI-60 cell lines and the 50% growth inhibitory concentration values. The values were determined by the developmental therapeutics program (DTP) drug screen with CellMiner, which is a platform which facilitates the efficient retrieval of gene transcript data and the activity reports for chemical compounds (17).

The protein-protein interaction (PPI) network was derived from The Human Protein Reference Database (HPRD), which contains information manually harvested by expert biologists who read, interpret, and analyze data from published literature (18). For the current study, HPRD release 9, which contains 37,039 PPIs among 9,465 proteins found in humans, was downloaded. Negatively related genes and positively related genes were projected into the HPRD PPI network and then incorporated with their first neighbors to construct the LSN and LSP networks, respectively. Visualizations were performed using Cytoscape software (19).

Identification of differentially expressed genes (DEGs) in gastric cancer

RNA-seq v2 datasets derived from GC were obtained from The Cancer Genome Atlas (TCGA) (<http://tcga-data.nci.nih.gov/>). The raw read counts were extracted for every exon from exon quantification file in the TCGA level 3 dataset. The

reads per kilobase of transcript per million read mapped (RPKM) value of each gene could then be calculated by applying the formula as follows:

$$\text{RPKM} = \frac{\text{EC} \times 10^9}{\text{SC} \times \text{L}} \quad [1]$$

EC constitutes the mapped read counts of all exons of 1 gene, while SC equates to the mapped read counts of all exons in a sample. The number “L” is the total length of all exons in a gene. GENCODE (V14) was used to obtain the exon structures of genes via download. We identified the up- and down-regulated genes using the fold-change method. Genes with fold change value greater than 2 or less than one-half were considered up- or down-regulated, respectively.

Identification and evaluation of functional modules

Network modules were identified using the MCODE plug-in in Cytoscape. In the lapatinib sensitivity negatively correlated LSN and LSP networks, we identified 2 and 9 modules, respectively. Up-regulated genes in cancers tend to inhibit anti-cancer drug sensitivity while down-regulated genes show the opposite effect. To identify the functional modules of lapatinib sensitivity, we assessed the significance of the overlap between up-regulated genes in GC and each module in the LSN network using a hypergeometric test. Modules were considered significant with a p-value of less than 0.05. Similarly, we also tested the significance of the overlap between down-regulated genes in GC and each module in the LSP network. The hypergeometric test used assumes that if the entire genome has a total of m genes, in which t is part of a functional module, and up-regulated or down-regulated genes in GC are a total of n genes, in which r is part of the functional modules in LSN or LSP, then the p-value can be worked out to assess that drug's enrichment significance using the following formula:

$$p = 1 - \sum_{x=0}^{r-1} \frac{\binom{t}{x} \binom{m-t}{n-x}}{\binom{m}{n}} \quad [2]$$

Using the above-described method, we calculated the enrichment significance between up- and down-regulated genes and each functional module.

Lastly, we identified 2 significant modules in the LSP: module 4 (P=0.024) and module 9 (P=0.019). To investigate the functions of these modules, we worked to enrich

pathways using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) tool (20).

Cell culture

The GC cell line, SUN216, was purchased from the American Type Culture Collection (Manassas, VA, USA). Culturing of the cells took place in Dulbecco's Modified Eagle medium (DMEM, Invitrogen, USA) with 10% fetal bovine serum (FBS; Hyclone, USA), 50 U/mL penicillin, and 50 µg/mL streptomycin (Invitrogen, CA, USA). All cells were kept at 37 °C in a humidified incubator at 5% CO₂.

Establishment of lapatinib-resistant SUN216 cell lines

Increasing doses of lapatinib (Shanghai Aladdin Biochemical Technology Co., Ltd., Shanghai, China) were consistently applied to the SUN216 cell line for about 12 weeks. The dosing began at 5 µM and increased to 10 µM after 4 weeks, 15 µM after another 4 weeks, and remained at 15 µM for the last 4 weeks. The resistant SUN216 cell line that we established was then maintained in DMEM medium with 10% (v/v) FBS and 10 µM lapatinib.

Cell transfection

The sequence of the *BCAR1*-siRNA (stQ0002578-1) was obtained from RiboBio (Guangzhou, China). SUN216 cells were seeded in 60-mm dishes 24 hours before to transfection. *BCAR1*-siRNA or control-siRNA was transfected into cells with Lipofectamine 3000 (Invitrogen) in serum-free media according to experiments request. Five hours following the procedure, the media was switched to complete media. Harvesting of cell lysates was carried out 48 hours after transfection.

CCK-8 cell viability assay

SUN216 cells transfected with control-siRNA or *BCAR1*-siRNA were seeded into 96-well plates at a density of 5×10^3 cells/per well and culturing took place for 4 days with varying concentrations of lapatinib (0, 5, 10, 20, 50 µg/mL). The viability of cells was determined with the Cell Counting Kit-8 (CCK-8, Promega, Kumamoto, Japan) at days 0, 2, and 4.

Colony formation assay

Approximately 8×10^2 SUN216 cells transfected with

control-siRNA or *BCAR1*-siRNA were placed in 60 mm dishes. After 10 days had passed, colonies were stained with 0.1% crystal violet in 20% methanol for 15 min, before photographs were taken and the number of visible colonies was counted.

Acridine orange/ethidium bromide (AO/EB) staining

Acridine orange (20 μ L) and ethidium bromide (20 μ L) mixing solution were placed for 5 minutes (Solarbio of Biotechnology, Beijing, China) in 1 well with 2 mL PBS at room temperature. Fluorescence microscopy ($\times 200$) allowed cellular morphological changes to be studied. The following formula was used to figure out the percentage of apoptotic cells: apoptotic rate (%) = apoptotic cells/all cells counted.

Transwell assay

Transwell chambers were used to test the migration (Cat No. 3422, Corning Costar, Cambridge, MA, USA) and invasion (Cat No. 356234, BD Biosciences, San Jose, CA, USA). All procedures were conducted, as described by Zhang *et al.* (21).

Western blotting

As described previously, Western blotting was implemented (22). Protein (60–80 μ g) was separated by 10% SDS-PAGE and then transfected to membranes for 1 hour. Membrane blocking was achieved with 5% dry nonfat milk for 2 hours and subsequent incubation with BCAR1 (Cat: ab3183, Abcom, USA) overnight at 4 $^{\circ}$ C. An electrochemiluminescence detection system (Thermo Fisher Scientific, CA, USA) was employed to detect signals.

Data analysis

Data are expressed as mean \pm SD. Student's non-paired *t*-test or a one-way analysis of variance (ANOVA), followed by Tukey's test for multiple group comparisons, was applied for statistical analysis. $P < 0.05$ signified a significant difference. Graph Pad Prism 5.0 and SPSS 19.0 were used to analyze data.

Results

Construction of lapatinib sensitivity-related functional linkage networks

To identify the positively and negatively related genes

involved in lapatinib sensitivity, we obtained lapatinib sensitivity-related genes from the CellMiner database by using the default cutoff. A total of 164 positively related genes and 117 negatively related genes were obtained and considered as core genes. LSP and LSN networks were constructed with the proteins encoded by these genes. Linkages between proteins were extracted from the HPRD database. Finally, the LSP and LSN networks were constructed using core genes and their first neighbors. There were 581 nodes and 574 edges in the LSP (Figure 1A), and 438 nodes and 414 edges in the LSN (Figure 1B).

Evaluation of functional modules in the LSP and LSN networks

Genes in similar biological processes, such as signaling pathways, tend to carry out similar functions, and therefore interact with each other as groups with high density in interaction networks (22). These entities are classed as functional modules, in which members are functionally linked to each other. In the LSP and LSN networks, we identified 2 and 9 of these modules, respectively. We then identified the significant modules in the LSP and LSN networks and evaluated these modules by the extent of enrichment of dysregulated genes in GC. We finally identified 2 significant modules in the LSN: module 4 ($P = 0.024$) and module 8 ($P = 0.019$) (Figure 2). There were no significant modules in the LSP network. Pathway enrichment analysis revealed the involvement of module 4 genes in the neuroactive ligand-receptor interaction network (hsa04080), the cAMP signaling pathway (hsa04024), and the Rap1 signaling pathway (hsa04015) (Figure 3A). Module 8 was involved in the TNF signaling pathway (hsa04668) and the MAPK signaling pathway (hsa04010) (Figure 3B). Among these, we chose BCAR1 in the Rap1 signaling pathway in module 4 for further investigation, because of its higher degree in module 4 (Figure 2A).

Knockdown of BCAR1 sensitizes GC cells to lapatinib treatment

We investigated whether knockdown of *BCAR1* could sensitize GC cells to lapatinib treatment. We first established lapatinib-resistant SUN216 cells (SUN216/LR) after 4 months of culturing with lapatinib. SUN216/LR cells had a similar cell morphology as SUN216 cells (Figure 4A). Using the CCK-8 assay, we determined the IC50 value of SUN216 cells to be 0.24 nM. However,

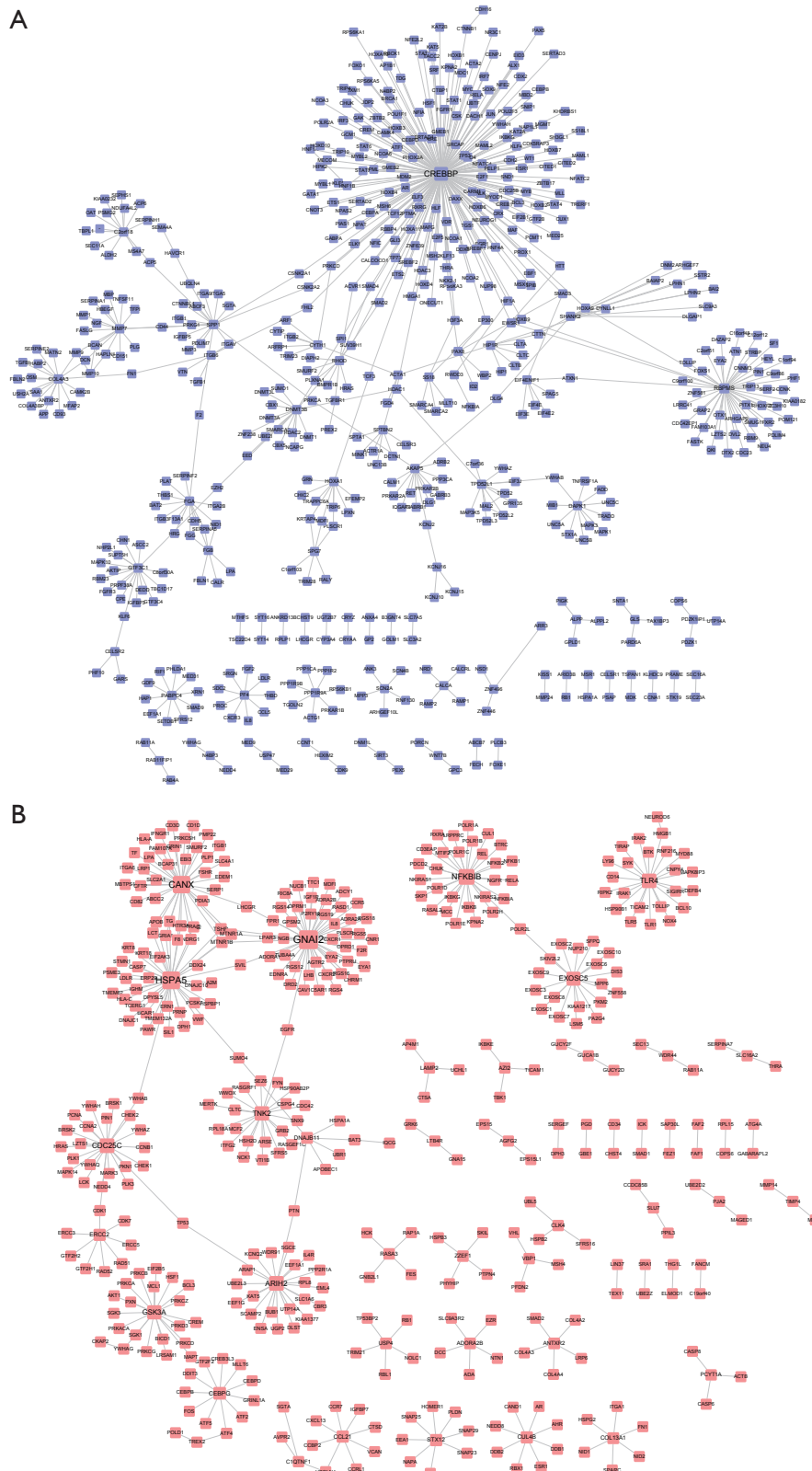


Figure 1 Lapatinib sensitivity-related network. (A) The positive network contains 581 nodes and 574 edges; (B) the negative network contains 438 nodes and 414 edges.

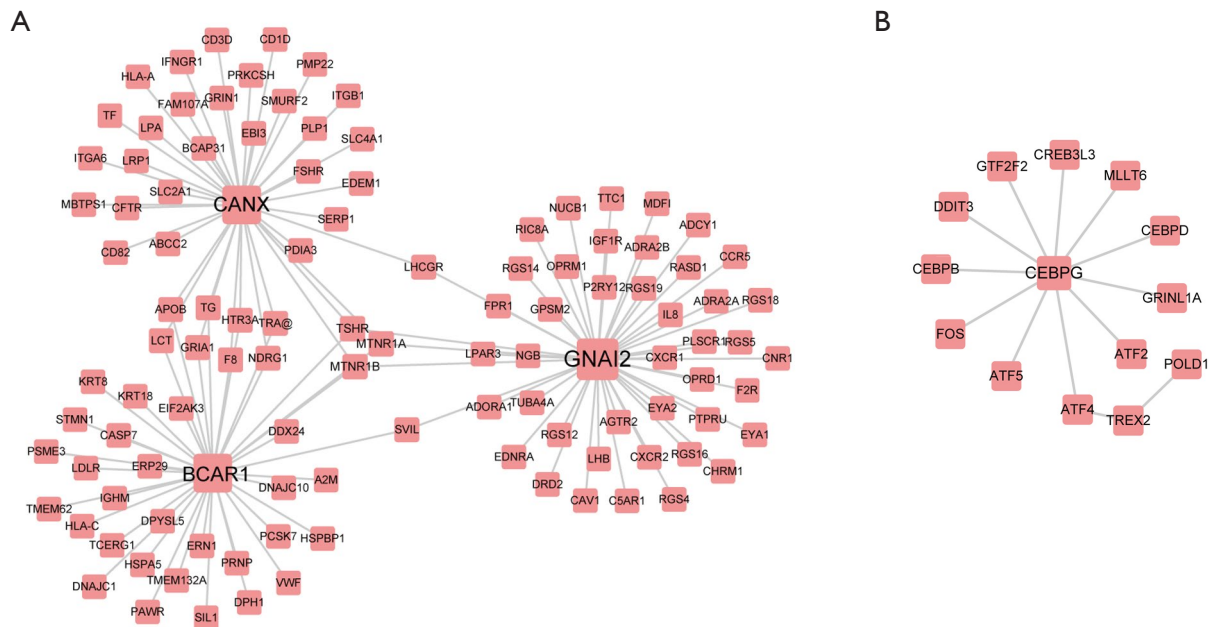


Figure 2 Two significant modules in the LSN. (A) Module 4 contains 112 nodes and 125 edges; (B) module 8 contains 14 nodes and 13 edges.

a much higher IC₅₀ value (>10 nM) was determined for SUN216/LR cells (Figure 4B). We examined the levels of BCAR1 in SUN216/LR cells. Compared with normal SUN216 cells, SUN216/LR cells had significantly higher BCAR1 levels (Figure 4C). To investigate whether BCAR1 regulates lapatinib resistance, we first tested the expression after transfection with BCAR1-siRNA by Western blotting. Our data showed that the expression of BCAR1 was down-regulated after the transfection with BCAR1-siRNA (Figure 4D). BCAR1 was knocked down in SUN216 cells using BCAR1-siRNA. Using the CCK-8 assay, we demonstrated that the IC₅₀ value of lapatinib in SUN216/LR cells was markedly reduced upon BCAR1 knockdown (Figure 4E). A clonogenic assay exhibited fewer colonies of SUN216/LR cells after BCAR1 knockdown and lapatinib treatment (Figure 4F). Next, using acridine orange/ethidium bromide (AO/EB) staining, we tested the cell apoptosis after transfection with BCAR1-siRNA and found the cell apoptosis rate to be more up-regulated in the BCAR1-si group than in the control group (Figure 4G). We also tested migration and invasion (Figure S1), and our data showed that the silencing of BCAR1 could inhibit cell migration and invasion. These data indicate that knockdown of BCAR1 reverses lapatinib resistance in SUN216/LR cells.

Discussion

Acquired resistance is a major cause of poor clinical outcomes in cancer patients undergoing chemotherapy (23). Identification of genes of sensitivity to anti-cancer drugs would help to optimize the anti-tumor efficacy and lower toxicity of chemotherapeutic drugs like (24). Herein, we present a computational procedure for identifying sensitivity genes for lapatinib treatment based on multiple functional gene modules, which we verified using an *in vitro* model.

We first constructed 2 functional linkage networks, LSN and LSP, and mined the functional modules from the 2 networks. We then investigated the significance of the modules in the 2 networks. Finally, 2 modules in the LSP network, module 4 and module 8 were identified. A Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed the involvement of genes in module 4 in pathways, including the neuroactive ligand-receptor interaction network (hsa04080) and protein processing in the endoplasmic reticulum (hsa04141). Some tyrosine kinase inhibitors, such as lapatinib, produce reactive oxygen species-dependent endoplasmic reticulum stress and rapid cytosolic calcium mobilization from endoplasmic reticulum stores (25-27). Genes in module 8 were involved in the TNF signaling pathway (hsa04668), the estrogen signaling pathway (hsa04915), and the MAP kinase signaling pathway

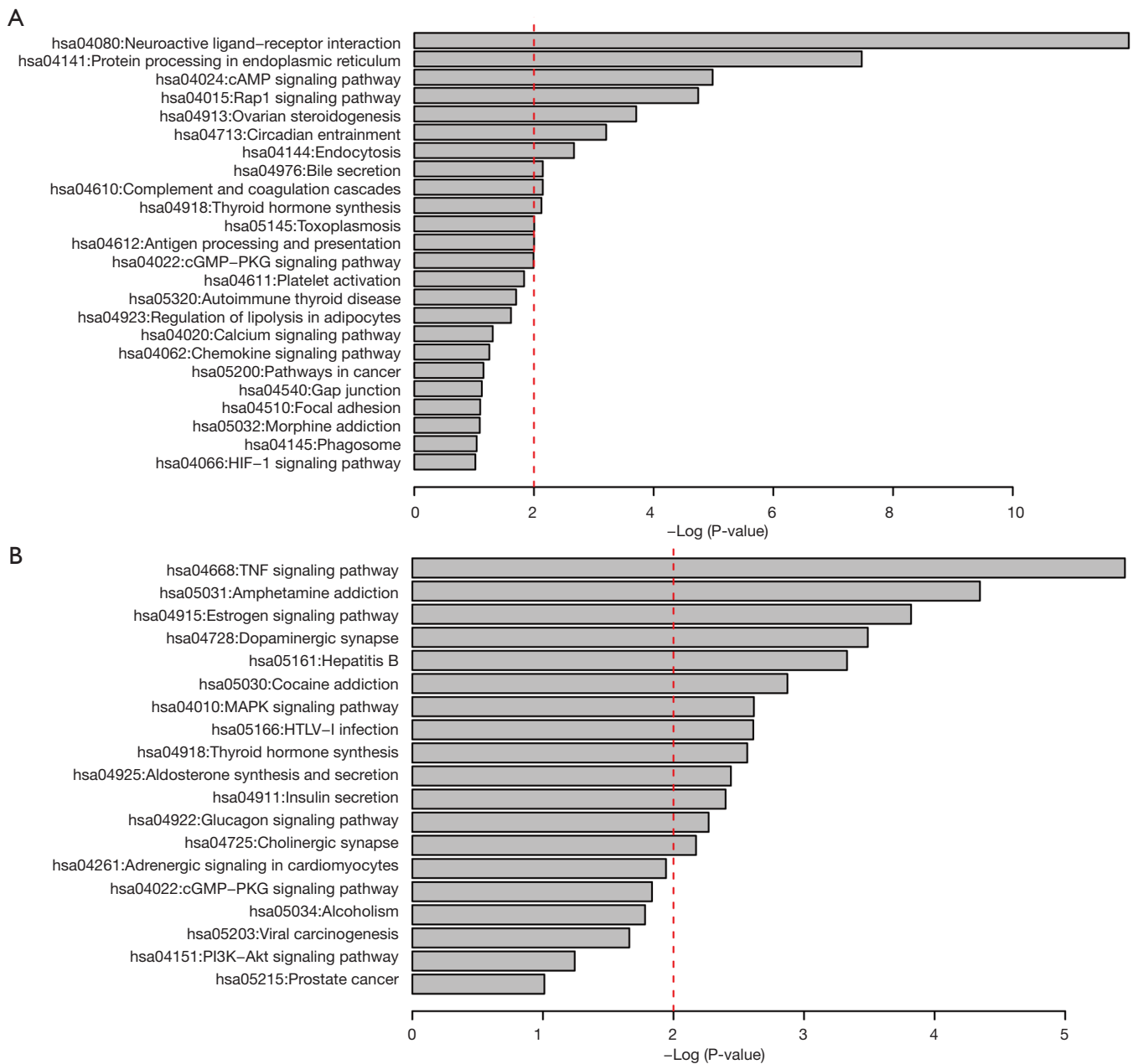


Figure 3 The pathway enrichment analysis results of (A) module 4 and (B) module 8. Pathway enrichment was conducted using the online web tools of DAVID. The X-axis represents $-\log(P \text{ value})$ of enrichment significance, and the Y-axis represents enriched pathways

(hsa04010).

Among these pathways, the role the Rap1 signaling pathway takes on in cancer was recently noted (28,29). Rap1 signaling is a major player in the control of cell-cell and cell-matrix interactions through its regulation in the function of integrins and other adhesion molecules in a variety of cell types (30,31). Rap1 is also a regulator of

MAP kinase activity in a fashion which is highly conditional on cell type context (32). In this pathway, we found that one of the annotated genes, BCAR1, encodes a scaffold protein that acts as a central pivot in cellular signaling. It paves the way for multi-protein complexes that regulate diverse cellular processes, such as migration, invasion, proliferation, and survival, to assemble. BCAR1 takes part

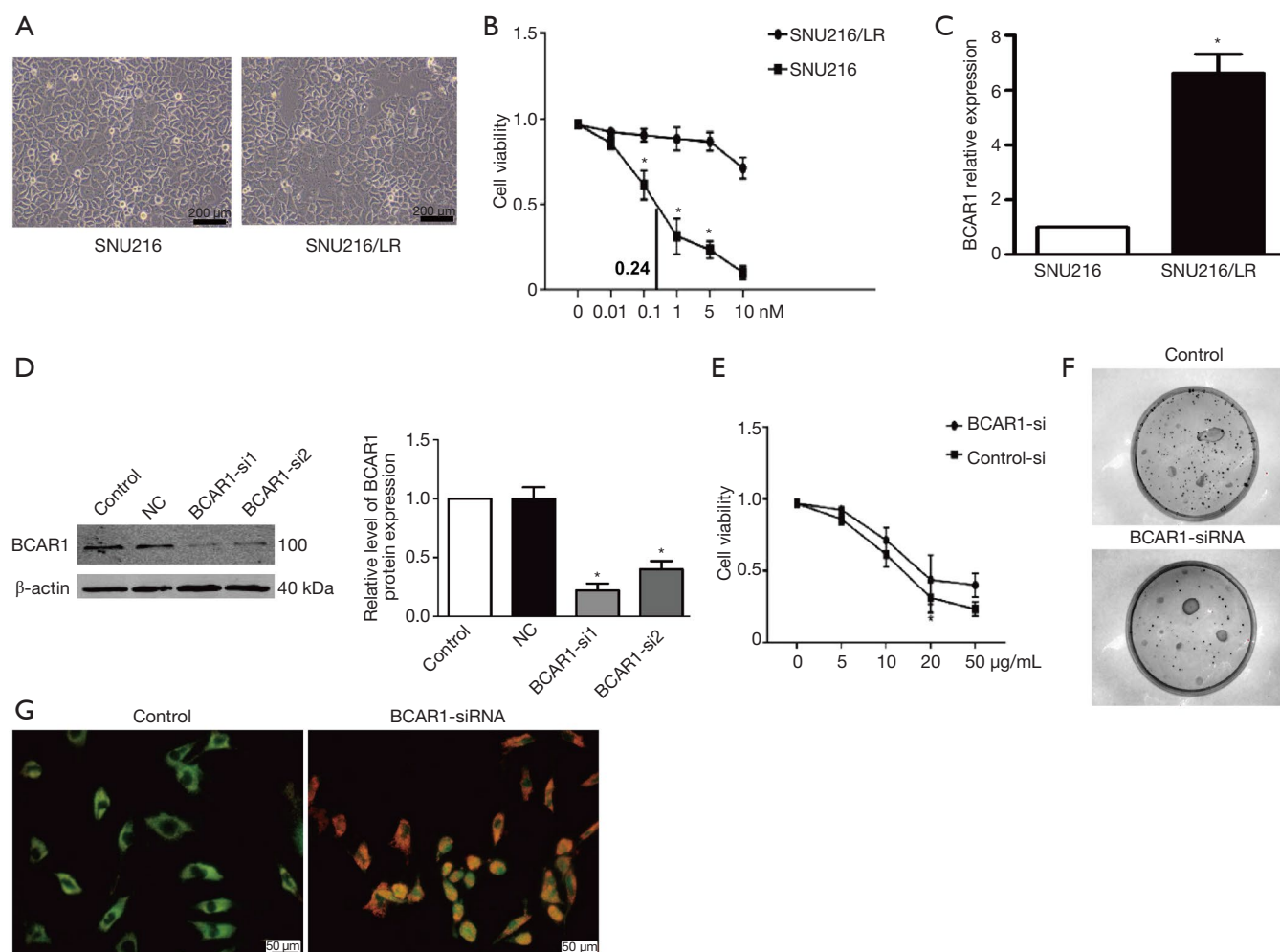


Figure 4 Knockdown of BCAR1 sensitizes GC cells to lapatinib treatment. (A) Morphology of sunitinib-resistant cells and related control cells; (B) BCAR1-siRNA decreased IC₅₀ of lapatinib treatment in SGC7901/LAP cells; (C) the expression of BCAR1 in SGC7901/LAP and SGC7901; (D) the expression of BCAR1 after knockdown; (E,F) knockdown of BCAR1 suppressed colony formation ability of SGC7901/LAP cells upon lapatinib treatment; (G) AO/EB staining after transfection with BCAR1-siRNA. SGC7901-S, SGC7901-sensitive or SGC7901; SGC7901-R, SGC7901-resistant or SGC7901-LAP. *P<0.05.

in the signal transduction of major oncogenic kinases such as Abl, FAK, and Src. Consequently, the overexpression of BCAR1 in a diverse range of malignancies, including cancer of the breast, lung, liver, and brain, has been demonstrated, and a link between BCAR1 overexpression and adverse features in these organs had been made (33,34). Earlier evidence also suggested that BCAR1 is a factor in prostate cancer progression (35), as its overexpression was linked to an adverse tumor phenotype and biochemical relapse in 3 studies analyzing 110, 130, and 242 prostate cancer specimens (36). However, there are no studies concerning the relationship between BCAR1 and lapatinib in GC.

Therefore, we assessed the role of BCAR1 in lapatinib sensitivity by using lapatinib-resistant SUN216 cells *in vitro*. We found that BCAR1 was up-regulated in SUN216/LR cells compared to SUN216 cells. Interestingly, knockdown of BCAR1 dramatically reduced the IC₅₀ of lapatinib in SUN216/LR cells. Thus, we discovered that BCAR1 might contribute to lapatinib resistance in GC.

In this study, we efficiently identified the crucial modules that were highly related to lapatinib sensitivity in GC by using a topological network method. BCAR1 was identified as a potentially critical gene playing a role in lapatinib sensitivity, and experiments confirmed that BCAR1 might

contribute to lapatinib resistance in GC. These results provide further insight into the molecular basis of lapatinib sensitivity and could offer novel strategies for the future treatment of GC. However, different GC cell lines, as well as *in vivo* experiments, are required to confirm the function of BCAR1 in the lapatinib resistance in GC.

Acknowledgments

Funding: None.

Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2020.01.30>). 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. The data comes from databases, so ethical reviews and informed consent are exempt. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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Cite this article as: Yuan QH, Liu G, Hu Q, Wang J, Leng K. Identification of lapatinib sensitivity-related genes by integrative functional module analysis. *Transl Cancer Res* 2020;9(3):1351-1360. doi: 10.21037/tcr.2020.01.30

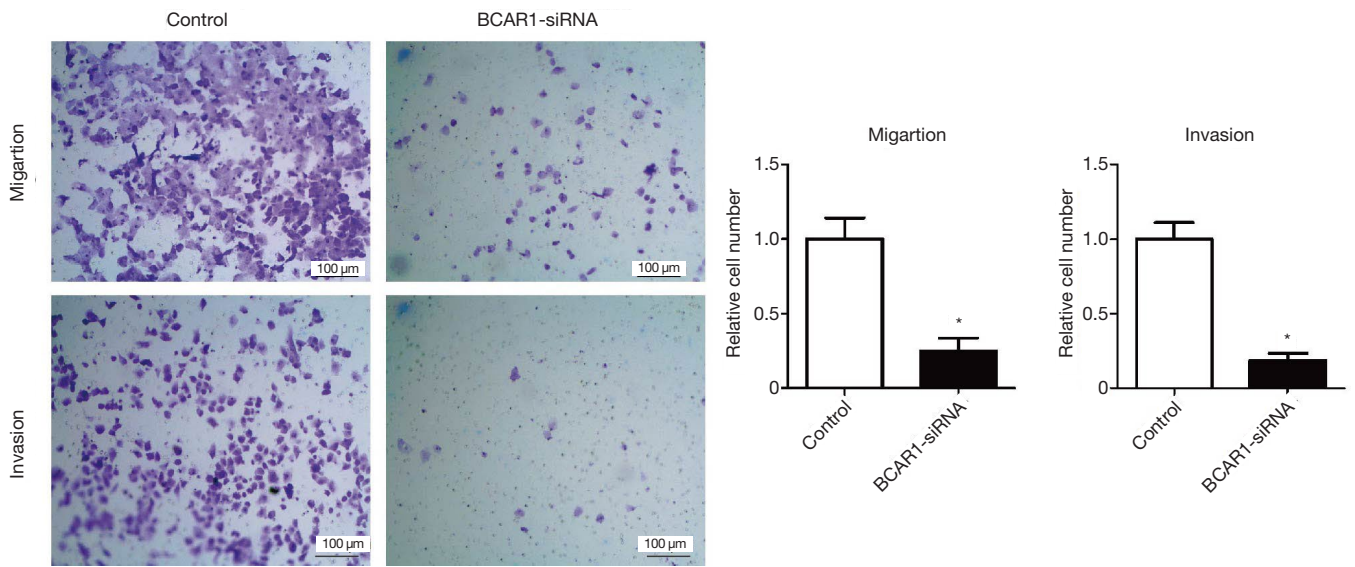


Figure S1 Knockdown of BCAR1 inhibits migration and invasion. To detect the migration and invasion of SUN216/LR cells after treatment with BCAR1-siRNA, SUN216/LR cells under the surface were treated with MA (methyl alcohol) and stained with 0.1% crystal violet for 20 min. *P<0.05.