Use of integrative bioinformatics to identify targets of sinensetin and its mechanisms to overcome colorectal cancer resistance

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ABSTRACT
It was previously shown that sinensetin increases the effectiveness of 5-fluorouracil (5-FU) in spheroids derived from colorectal cancer (CRC) cells; however, the molecular mechanism by which this occurs remains elusive. This study aims to explore the targets of sinensetin and the molecular mechanisms by which it circumvents CRC resistance. Targets of sinensetin were obtained from the SwissTargetPrediction platform, while regulatory genes of human CRC cells were downloaded from the PubMed database. Venn diagram resulted in 36 potential therapeutic targets of sinensetin against chemoresistance in CRC potential therapeutic target genes of sinensetin (PS). Analyses of gene ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment were conducted with WebGestalt and Database for Annotation, Visualization, and Integrated Discovery, respectively. Protein–protein interaction network and hub gene selection were analyzed by STRING and CytoHubba. Analysis of genetic alterations of PS was carried out using cBioPortal. The analytical results of KEGG pathway enrichment revealed that the CRC and vascular endothelial growth factor signaling pathways, the erbB signaling pathway, and ATP-binding cassette transporters are altered in PS. Moreover, genetic alterations of the query genes affected several pathways, including COADREAD-2012-RTK-RAS-PI(3)K, which regulates proliferation, cell survival, and translation. Sinensetin potentially targets ATP-binding cassette sub-family G member 2, ATP-binding cassette sub-family B member 1, B-Raf proto-oncogene, serine/threonine kinase, MET, platelet-derived growth factor receptor beta, Glycogen synthase kinase-3 beta, and phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit gamma isoform. Moreover, the phosphatidylinositide 3-OH kinase/AKT serine/threonine kinase and mitogen-activated protein kinase signaling pathways are potential target pathways of sinensetin action. The results of the present study await validation by subsequent experiments.

INTRODUCTION
Colorectal cancer (CRC) is one of the most common causes of death due to cancer in the world (Deng, 2017). The incidence and rate of death due to colon cancer have increased in recent decades due to lifestyle changes in modern-day society, including decreased dietary fiber intake and increased processed food consumption (O’Keefe, 2016). In colon cancer treatment, conventional chemotherapy is administered to reduce tumor size and eradicate residual tumor tissue after a curative resection (Polastro et al., 2018). Nevertheless, both intrinsic and acquired resistance can decrease the effectiveness of chemotherapeutics (Hu et al., 2016). Chemoresistance mechanisms arise due to the existence of a minor population called cancer stem cells (Kozovska et al., 2014). Taken together, these lines of evidence indicate the necessity of developing agents that can improve the effectiveness of colon cancer chemotherapy and overcome chemoresistance.

Sinensetin (Fig. 1A), a flavonoid compound found in citrus fruits, is known to have anticancer activity in various cancer cells. Sinensetin exerts cytotoxicity on MDA-MB-468 breast cancer cells (Androutsopoulos et al., 2009), adenocarcinoma gastric cell lines human gastric cancer cells (Dong et al., 2011), and K562 human chronic myeloid leukemia cells (Dansman et al., 2019). An increase

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in cytotoxicity of the tyrosine kinase inhibitor imatinib was observed in a combinatorial study of sinensetin with imatinib in K562 leukemic cells (Dansman et al., 2019). Recent studies have revealed the molecular mechanisms of sinensetin in cancer cells, including induction of cell death by modulating the p53-5’ AMP-activated protein kinase/mTOR signaling pathway in HepG2 liver cancer cells (Kim et al., 2020) and inhibition of migration and invasion of the human gallbladder TJ-GBC2 cell line through inhibition of the phosphatase and tensin homolog (PTEN)/phosphatidylinositol 3-OH kinase (PI3K)/AKT serine/threonine kinase (AKT) signaling pathway (Huang et al., 2020). In addition, another recent study showed that sinensetin increased the effectiveness of 5-fluorouracil (5-FU) in spheroids derived from CRC cells (Pereira et al., 2019); however, the molecular mechanism remains elusive.

In this study, we used an integrated bioinformatics approach to explore the targets of sinensetin and the possible molecular mechanisms by which it acts to circumvent colon cancer resistance to chemotherapy. Sinensetin targets and colon cancer resistance regulatory genes were downloaded from free public databases. Protein–protein interaction (PPI) networks, gene ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses revealed the potential therapeutic targets of sinensetin against chemoresistance in colon cancer potential therapeutic target genes of sinensetin (PS). Further analysis of PS was conducted to explore the genetic alterations in more detail. The present study highlights the importance of sinensetin in overcoming chemoresistance in CRC.

MATERIAL AND METHODS

Data collection and processing

Targets of sinensetin were obtained from SwissTargetPrediction, (http://www.swisstargetprediction.ch) (Gfeller et al., 2014). SwissTargetPrediction is a public database containing updated data and information to effectively and efficiently predict the molecular target (Daina et al., 2019). Briefly, the sinensetin structure was drawn in the form of a simplified molecular-input line-entry system and submitted into the SwissTargetPrediction database. Homo sapiens were selected as target organisms. Predicted targets...
were selected with a cutoff value probability of > 0.1, resulting in 100 targets (Supplementary Table 1). Regulatory genes of human CRC cells were downloaded from PubMed with the keyword “human colorectal cancer cells” and resulted in 1,376 genes (Supplementary Table 2). Venn diagram of CRC regulatory genes and sinensetin targets was generated using Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/index.html), resulting in 36 potential therapeutic targets of sinensetin against chemoresistance in colon cancer (PS) (Fig. 1B and Supplementary Table 3).

**Analysis of GO and KEGG pathway enrichment**

Analysis of GO was carried out to PS by WebGestalt, by using the default settings with a cutoff value of \( p < 0.05 \). KEGG pathway enrichment was conducted by the Database for Annotation, Visualization, and Integrated Discovery v6.7 (Huang da et al., 2009), by using the default settings with a cutoff value of \( p < 0.05 \).

**PPI network and hub gene selection**

PPI network analysis was executed with STRING-DB v11.0 (Szklarczyk et al., 2015). Briefly, PS was submitted into STRING-DB to build a PPI network, with a confidence score > 0.4 as the cutoff value and visualized with Cytoscape software (Shannon et al., 2003). Hub genes were selected from the CytoHubba plugin based on the highest degree score by using the default settings (Chin et al., 2014).

**Analysis of genetic alterations of the PS**

Genetic alteration analysis of the PS was carried out using cBioPortal (http://www.cbioportal.org) (Cerami et al., 2012; Gao et al., 2013). Briefly, selected PS was considered as a query and subjected to genetic alteration among 10 studies of CRC in cBioPortal. Selected CRC study was analyzed for OncoPrint, which depicts genome changes, that is, inframe, missense, and truncating mutation, and was chosen for further connectivity analysis using a one-sided Fisher’s exact test with a cutoff value of \( p < 0.05 \).

**RESULTS AND DISCUSSION**

**GO and KEGG pathway enrichment analysis**

This study aimed to determine the targets of sinensetin action, elucidate its molecular mechanism to overcome chemoresistance in colon cancer (PS), and reveal potential therapeutic targets of sinensetin. To identify gene classes and predict the function of PS, we carried out GO and KEGG pathway enrichment analysis. GO analysis was aimed at checking the role of PS in biological processes, cellular components, and molecular functions. The results of GO analysis revealed the regulation of the biological process response to stimulus and the metabolic process by PS (Fig. 1C). In addition, PS was located in the membrane and nucleus and served as a molecular function in protein, ion, and nucleotide binding. The results of the analysis of KEGG pathway enrichment revealed 21 pathways regulated by PS, including CRC, the vascular endothelial growth factor (VEGF) signaling pathway, the erbB signaling pathway, and the ATP-binding cassette (ABC) transporter (Table 1). Several PS were involved in the CRC pathway, including phosphatidylinositol 3-kinase catalytic subunit gamma isoform (PIK3CG), B-Raf proto-oncogene, serine/threonine kinase (BRAF), Glycogen synthase kinase-3 beta (GSK3B), MET, and platelet-derived growth factor receptor beta (PDGFRB). The results of GO and KEGG pathway enrichment analysis from PS were further investigated for genetic alterations analysis using cBioPortal.

### Table 1. KEGG pathway enrichment analysis of the DEGs.

| No. | Term                                      | \( p \) value | Genes                      |
|-----|-------------------------------------------|---------------|----------------------------|
| 1   | hsa05200:Pathways in cancer               | 1.42E-05      | PIK3CG, BMP4, HIF1A, BRAF, GSK3B, NTRK1, MET, PPARG, PDGFRB, KIT, MMP2 |
| 2   | hsa04510:Focal adhesion                   | 1.87E-05      | PIK3CG, ROCK1, BRAF, ROCK2, GSK3B, MET, PDGFRB, SRC, KDR              |
| 3   | hsa04722:Neurotrophin signaling pathway   | 8.13E-04      | PIK3CG, BRAF, RPS6KA2, MAPK14, GSK3B, NTRK1                             |
| 4   | hsa05210:CRC                             | 0.001552176   | PIK3CG, BRAF, GSK3B, MET, PDGFRB                                       |
| 5   | hsa04914:Progestrone-mediated oocyte maturation | 0.001693817 | PIK3CG, CDK1, BRAF, RPS6KA2, MAPK14                                   |
| 6   | hsa04150:mechanistic target of rapamycin kinase (mTOR) signaling pathway | 0.003705151 | PIK3CG, HIF1A, BRAF, RPS6KA2                                           |
| 7   | hsa04144:Endocytosis                     | 0.004640519   | NTRK1, MET, CXC2, KIT, SRC, KDR                                       |
| 8   | hsa04062:Chemokine signaling pathway      | 0.00497131    | PIK3CG, ROCK1, BRAF, ROCK2, GSK3B, CXC2                                 |
| 9   | hsa04670:Leukocyte transendothelial migration | 0.005342782 | PIK3CG, ROCK1, ROCK2, MAPK14, MMP2                                    |
| 10  | hsa04360:Axon guidance                   | 0.007315417   | ROCK1, ROCK2, GSK3B, MET, CXC2                                       |
| 11  | hsa05120:Epithelial cell signaling in Helicobacter pylori infection | 0.007867395 | MAPK14, MET, CXC2, SRC                                                  |
| 12  | hsa05211:Renal cell carcinoma            | 0.008523033   | PIK3CG, HIF1A, BRAF, MET                                           |
| 13  | hsa05218:Melanoma                        | 0.008862606   | PIK3CG, BRAF, MET, PDGFRB                                        |
| 14  | hsa04370:VEGF signaling pathway          | 0.010006202   | PIK3CG, MAPK14, SRC, KDR                                               |
| 15  | hsa05216:Thyroid cancer                  | 0.013183256   | BRAF, NTRK1, PPARG                                                   |
| 16  | hsa04012:ERBB signaling pathway          | 0.015392691   | PIK3CG, BRAF, GSK3B, SRC                                              |
| 17  | hsa05215:Prostate cancer                 | 0.016357431   | PIK3CG, BRAF, GSK3B, PDGFRB                                         |
| 18  | hsa0210:ABC transporters                 | 0.029030271   | ABCBI, ABCCI, ABCG2                                                 |
| 19  | hsa05213:Endometrial cancer              | 0.039488572   | PIK3CG, BRAF, GSK3B                                                   |
| 20  | hsa04810:Regulation of actin cytoskeleton | 0.039970002   | PIK3CG, ROCK1, BRAF, ROCK2, PDGFRB                                   |
| 21  | hsa05221:Acute myeloid leukemia          | 0.048133595   | PIK3CG, BRAF, KIT                                                   |
Figure 2. (A) PPI network of potential target genes of sinensetin in overcoming CRC resistance, analyzed by STRING. (B) Top 20 hub genes based on highest degree score, analyzed by CytoHubba.
Table 2. Top 20 in the network of sinensetin and CRC resistance, ranked by degree method of CytoHubba.

| Rank | Symbol | Name | Score |
|------|--------|------|-------|
| 1    | SRC    | Proto-oncogene tyrosine-protein kinase Src | 21    |
| 2    | ESR1   | Estrogen receptor | 17    |
| 3    | KDR    | Vascular endothelial growth factor receptor 2 | 15    |
| 4    | MMP2   | 72 kDa type IV collagenase | 12    |
| 5    | ABCG2  | ATP-binding cassette sub-family G member 2 | 12    |
| 6    | HIF1A  | Hypoxia-inducible factor 1-alpha | 11    |
| 7    | MAPK14 | MAPK 14 | 10    |
| 8    | PMARG  | Peroxisome proliferator-activated receptor gamma | 10    |
| 9    | KIT    | Mast/stem cell growth factor receptor Kit | 8     |
| 10   | BMP4   | Bone morphogenetic protein 4 | 8     |
| 11   | ABCB1  | Multidrug resistance protein 1 | 8     |
| 12   | MET    | Hepatocytic growth factor receptor | 8     |
| 13   | CA9    | Carbonic anhydrase 9 | 7     |
| 14   | ESR2   | Estrogen receptor beta | 7     |
| 15   | PDGFRB | Platelet-derived growth factor receptor beta | 7     |
| 16   | CYP1B1 | Cytochrome P450 1B1 | 6     |
| 17   | GSK3B  | Glycogen synthase kinase-3 beta | 6     |
| 18   | PIK3CG | Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit gamma isoform | 6     |
| 19   | CDK1   | Cyclin-dependent kinase 1 | 5     |
| 20   | AURKA  | Aurora kinase A | 5     |

Analysis of the PPI network and hub gene selection

Analysis of the PPI network (confidence level of 0.4) was conducted on PS, which consist of 36 nodes, 113 edges, a PPI enrichment value of < 1.10e–16, and an average local clustering coefficient of 0.542 (Fig. 2A). The top 20 genes with the highest degree scores were identified, including SRC, estrogen receptor (ESR1), kinase insert domain receptor (KDR), matrix metallopeptidase 2 (MMP2), ATP-binding cassette sub-family G member 2 (ABCG2), and PIK3CG (Fig. 2B and Table 2). These results indicated that those genes have a pivotal role in the PPI network, making them strong candidates for target genes. In addition, the results are useful for selecting the candidate of target genes for further genetic alterations analysis using cBioPortal.

Analysis of genetic alterations of potential target genes

Analysis of genetic alterations was carried out to investigate the relationship between the resistance of CRC and sinensetin efficacy. Seven potential therapeutic targets of sinensetin action against chemoresistance in colon cancer (PS), including ABCG2, ABCB1, BRAF, MET, PDGFRB, GSK3B, and PIK3CG, were identified. In the following sections, we will review the role of each gene in CRC chemoresistance. ABCG2, also known as adenosine triphosphate (ATP) binding cassette subfamily G member 2 or breast cancer resistance protein, and ABCB1, also known as ATP binding cassette subfamily B member 1 or P-glycoprotein, are members of the ABC transporter efflux transporter group whose function is to remove or pump drugs from within the cell to outside the cell, thereby reducing the concentration of intracellular drugs (Chen et al., 2016).

B-Raf proto-oncogene (BRAF) encodes a protein that belongs to the RAF proto-oncogene serine/threonine-protein kinase family of serine/threonine protein kinases (Cope et al., 2020). BRAF is one of the protein components of the MAPK pathway, also known as the extracellular-signal-regulated kinase (ERK) signaling pathway (McCain, 2013). The MAPK signaling pathway communicates with other pathways, for example, PI3K/AKT and mTOR (Burotto et al., 2014). MAPK signaling is important for the maintenance of cancer stem cell properties in CRC (Corcoran et al., 2018).
MET, also known as mesenchymal-epithelial transition factor gene or MET protooncogene, encodes a member of the receptor tyrosine kinase family of proteins (Gonzalez-Angulo et al., 2013). Upon binding to its ligand, namely hepatocyte growth factor (HGF), MET induces dimerization leading to activation of intracellular signaling, which is involved in cell proliferation, invasion, and migration (Organ and Tsao, 2011). Moreover, the same author stated that MET signaling also communicates with other intracellular signaling mechanisms, including the PI3K/AKT and MAPK pathways (Organ and Tsao, 2011).

PDGFRB, which is expressed by tumor-associated fibroblasts, is involved in the cellular processes of angiogenesis, tumor growth, and metastasis in colon cancer cells (Takigawa et al., 2016). In addition, PDGFRB is involved in CRC progression via the mechanisms of platelet activation and transforming growth factor beta signaling (Stell et al., 2013). A recent study demonstrated that the platelet-derived growth factor receptor is required for epithelial to mesenchymal transition (EMT), and inhibition of PDGFRB with tyrosine kinase inhibitor effectively inhibits colon cancer progression (Olsen et al., 2019).

Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma (PIK3CG), or p110 gamma, is a class I catalytic subunit of PI3K (Arthur and Uzairu, 2019). PIK3CG, a catalytic subunit of PI3K, is important for regulating PI3K/AKT signaling in the development of colon cancer (Semba et al., 2002).

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**Figure 3.** (A) Overview of genetic changes in ABCG2, ABCB1, BRAF, MET, PDGFRB, GSK3B, and PIK3CG across 10 CRC studies, as analyzed by cBioportal. (B) Summary of alterations in ABCG2, ABCB1, BRAF, MET, PDGFRB, GSK3B, and PIK3CG across CRC patients using a study from Giannakis et al., 2016. (C) Pathway related to genetic alterations of ABCG2, ABCB1, BRAF, MET, PDGFRB, GSK3B, and PIK3CG across CRC patients using a study from Giannakis et al., 2016.
Table 3. Additional mutual exclusivity analysis of selected genes.

| A     | B     | Log2 Odds Ratio | p-value | Tendency     |
|-------|-------|-----------------|---------|--------------|
| BRAF  | MET   | 2.402           | 0.002   | Co-occurrence|
| BRAF  | PIK3CG| 1.53            | 0.002   | Co-occurrence|
| GSK3B | PIK3CG| >3              | 0.003   | Co-occurrence|
| ABCG2 | PIK3CG| >3              | 0.003   | Co-occurrence|
| ABCG2 | ABCB1 | >3              | 0.004   | Co-occurrence|
| BRAF  | GSK3B | >3              | 0.007   | Co-occurrence|

Proposed mechanism of sinensetin action against chemoresistance in colon cancer

In the following section, we discussed the proposed mechanism of sinensetin against colon cancer chemoresistance. ABCB1 and ABCG2 are responsible for chemoresistance to selonsertib (Ji et al., 2019). Inhibition of ABC transporters is a strategy to overcome chemoresistance in colon cancer cells (Wang et al., 2015). A previous study showed that sinensetin overcame doxorubicin resistance in vinceristine-resistant leukemic cells by reversing P-glycoprotein (Choi et al., 2002). Further research on the role of sinensetin in overcoming chemoresistance against colorectal cancer by targeting ABCB2 and ABCB1 is required.

A previous study showed that the upregulation of the MAPK signaling pathway was found in liver metastasis of CRC cells (Tang et al., 2019). Moreover, CRC resistance to BRAF inhibitor is mediated by the activation of Wnt/β-catenin signaling (Chen et al., 2018). The effect of sinensetin on overcoming CRC by targeting BRAF and MAPK signaling remains unclear.

MET not only plays an important role in the progression but is also a potential target for the treatment of CRC (Song et al., 2017). Overexpression of c-MET is an indicator of poor prognosis in patients with CRC (Lee et al., 2018). However, the effect of sinensetin on overcoming CRC by targeting MET remains unclear.

A study showed that activation of the Notch signaling pathway was found in PDGFR-induced EMT and colon cancer progression (Chen et al., 2017b). The role of sinensetin in PDGFRB and its signaling awaits further investigation. A previous study showed that aberrant activation of Wnt/β-catenin signaling was found in CRC cells (He et al., 2016). However, the effect of sinensetin on overcoming CRC by targeting GSK3B remains unclear.

A previous study showed that suppression of PIK3CG gene expression is important for blocking PI3K/AKT signaling, which leads to the inhibition of CRC progression (Semba et al., 2002). Another study showed that sinensetin can overcome chemoresistance in human gallbladder cancer cells by inhibiting the PTEN/PI3K/AKT signaling pathway (Liu et al., 2017). The effect of sinensetin on overcoming CRC chemoresistance by targeting PI3K/AKT awaits further investigation.

The present study revealed potential targets and molecular mechanisms of sinensetin in overcoming chemoresistance in CRC. Since the results of this study were derived from bioinformatic analysis, further in vitro and in vivo studies are required to validate the results. Further research using samples from patients with colorectal cancer is also needed to develop sinensetin as a combinational agent of chemotherapy in colorectal cancer treatment.

CONCLUSION

In conclusion, sinensetin potentially targets ABCG2, ABCB1, BRAF, MET, PDGFRB, GSK3B, and PIK3CG. Moreover, the PI3K/AKT and MAPK signaling pathways are potential target pathways of sinensetin action. The results of the present study await validation in subsequent experiments.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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