Indonesian Journal of Pharmacy

VOL 32 (1) 2021: 64–73 | RESEARCH ARTICLE

Integrative Bioinformatics Analysis Reveals Possible Targets and Mechanism of Ellipticine in Inhibiting Breast Cancer Stem Cells

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Info Article

Submitted: 30-12-2020
Revised: 21-02-2021
Accepted: 26-02-2021

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ABSTRACT

Ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole), an alkaloid from *Ochrosia elliptica*, can reduce the growth and inhibit the self-renewal of ALDH1A1-positive breast cancer stem cells (BCSCs); however, the molecular mechanisms of this compound remain unclear. In this study, we use bioinformatics to explore the potential targets and molecular mechanisms of ellipticine in BCSCs. The expression of genes related to the sensitivity of cancer cells toward ellipticine was obtained from COMPARE. A list of genes related to BCSCs was retrieved from Pubmed by using the keywords "*Homo sapiens*, breast cancer stem cells." Protein-protein interaction (PPI) network, Gene Ontology (GO), and KEGG pathway enrichment analyses were performed using STRING-DB. Gene alterations were analyzed using cBioPortal. The prognostic value of the selected genes was evaluated by Kaplan–Meier survival curves. Two genes that are affected by ellipticine and related to BCSCs, namely, *XRCC5* and *CD59*, were selected using a Venn diagram of the COMPARE and PubMed data. Ellipticine may target the complement cascade and DNA repair mechanism in BCSCs. More importantly, *XRCC5* and *CD59* appear to be potential targets and biomarkers for evaluating the bioactivity of ellipticine in BCSCs. The results of this study are useful for elucidating the molecular mechanism and role of ellipticine in BCSCs.

Keywords: Ellipticine, anticancer, bioinformatics, breast cancer stem cells, XRCC5, CD59.

INTRODUCTION

Cancer stem cells (CSCs) represent a minor cell population within a tumor but are considered the major hurdle of successful chemotherapy. These cells often cause tumor relapse, metastasis, and death (Visvader and Lindeman, 2008; Vinogradov and Wei, 2012). Several signaling pathways involved in CSCs, including Notch (Reedijk, 2012; Qiu *et al*., 2013), Wnt/ß-catenin (Jang *et al*., 2015) and Hedgehog Sims-Mourtada *et al*. (2015), have been identified. In breast cancer, CSCs are responsible for tumor progression, metastasis, and chemoresistance (Jonasson *et al*., 2019). Thus, inhibiting the regulatory pathways of CSCs is believed to be essential for achieving successful chemotherapy.

Ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole) is an alkaloid obtained from the plant *Ochrosia elliptica* (Goodwin *et al*., 1959). Stiborova *et al*. demonstrated that ellipticine activation by CYP is essential for DNA adduct formation, which is one of its anticancer mechanisms (Stiborova *et al*., 2001). Ellipticine shows cytotoxicity toward MCF-7 and MDA-MB 231 breast cancer cells (Kuo *et al*., 2005), HepG2 hepatocellular carcinoma cells (Kuo *et al*., 2006), HL-60 and CCRF-CEM leukemia cells (Poljaková *et al*., 2007), U87MG glioblastoma (Martinkova *et al*., 2009), IMR-32 and UKF-NB-4 neuroblastoma cells (Poljaková *et al*., 2009), and RL95-2 endometrial cancer cells (Kim *et al*., 2011) by inhibiting cell growth and triggering apoptosis. A previous study showed that the formation of the ellipticine metabolites 13-hydroxy- and 12-hydroxy is important for its cytotoxicity toward several cancer cells including MCF-7, HL-60, CCRF-CEM, UKF-NB-3, UKF-NB-4, and U87MG (Stiborová *et al*., 2011).

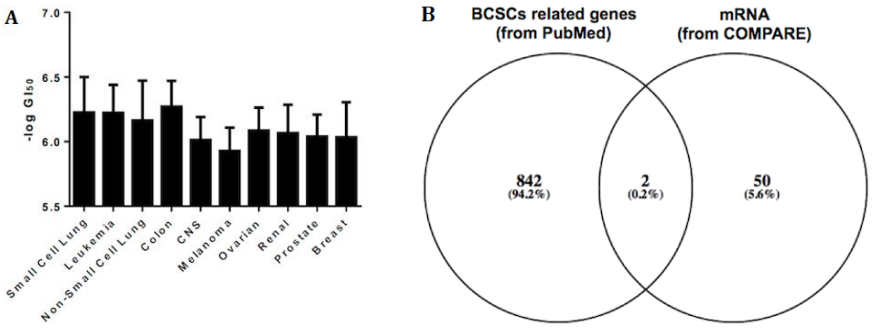

Figure 1. (A) Cytotoxic activity of ellipticine on NCI-60 cancer cells. Data were retrieved from the public library of COMPARE. (B) Venn diagram of BCSC regulatory genes and mRNA array analysis from COMPARE.

Ellipticine inhibits cell proliferation and self-renewal in ALDH1A1-expressing breast CSCs (BCSCs) from MCF-7 (Pandrangi *et al*., 2014). The authors further concluded that ellipticine could potentially be developed as combinatorial chemotherapy for inhibiting BCSCs (Pandrangi *et al*., 2014). However, the molecular mechanism of this compound remains unclear. This study employed a bioinformatics approach to predict the possible mechanisms of ellipticine on BCSCs.

In this study, we conducted an integrated bioinformatics analysis to identify the targets and molecular mechanisms of ellipticine in BCSCs. We used COMPARE's public library to analyze the cytotoxicity and obtain the microarray data of mRNAs known to be influenced by ellipticine. Moreover, protein–protein interaction (PPI) network, Gene Ontology (GO), and KEGG pathway enrichment analyses were conducted using STRING-DB. Finally, genetic alterations and overall survival in breast cancer patients were analyzed using the cBioPortal and KMPlotter databases.

MATERIAL AND METHODS

Data mining and preparation

Cytotoxicity and mRNA expression data were retrieved from the Developmental Therapeutic Program (DTP) of the National Cancer Institute (https://dtp.nci.nih.gov) (Monks *et al*., 1997). COMPARE's public library was analyzed to generate drugs with similar actions based on RNA expression associations, and changes in RNA expression due to ellipticine treatment in NCI 60 cancer cells (Mahmoud *et al*., 2018).

Similarity patterns were depicted using Pearson correlation coefficients of <−0.5 and >0.5. A list of genes related to breast cancer stem cells was retrieved from Pubmed by using the keywords "*Homo sapiens,* breast cancer stem cells", as previously described (Hermawan *et al*., 2020).

PPI network construction, Gene Ontology, and KEGG pathway enrichment analysis

PPI network, GO, and KEGG pathway analyses were carried out using STRING-DB v11.0 as previously described (Szklarczyk *et al*., 2015). Confidence score of >0.4 and FDR < 0.05 were used as inclusion criteria for GO and KEGG pathway enrichment analyses.

Gene expression and genetic alterations among target genes

Gene expression profiles across different breast cancer samples and adjacent tissues in the TCGA study were analyzed by TIMER (http://timer.comp-genomics.org) (Li *et al*., 2020). Alterations in the selected genes were analyzed by cBioPortal (https://www.cbioportal.org) (Cerami *et al*., 2012; Gao *et al*., 2013).

Kaplan–Meier survival analysis

The prognostic value of the target genes was assessed by Kaplan–Meier analysis (http://kmplot.com) (Gyorffy *et al*., 2010) with $p < 0.05$ as the inclusion criterion.

RESULTS AND DISCUSSION

Data mining and preparation

We obtained gene expression profiles affected by ellipticine by analyzing COMPARE's public library. Microarray data were also used to predict the sensitivity of tumor cells to ellipticine. Ellipticine showed anticancer activity toward several cancer cells, with the lowest IC_{50} values for leukemia, lung, and colon cancer cells (Figure 1A).

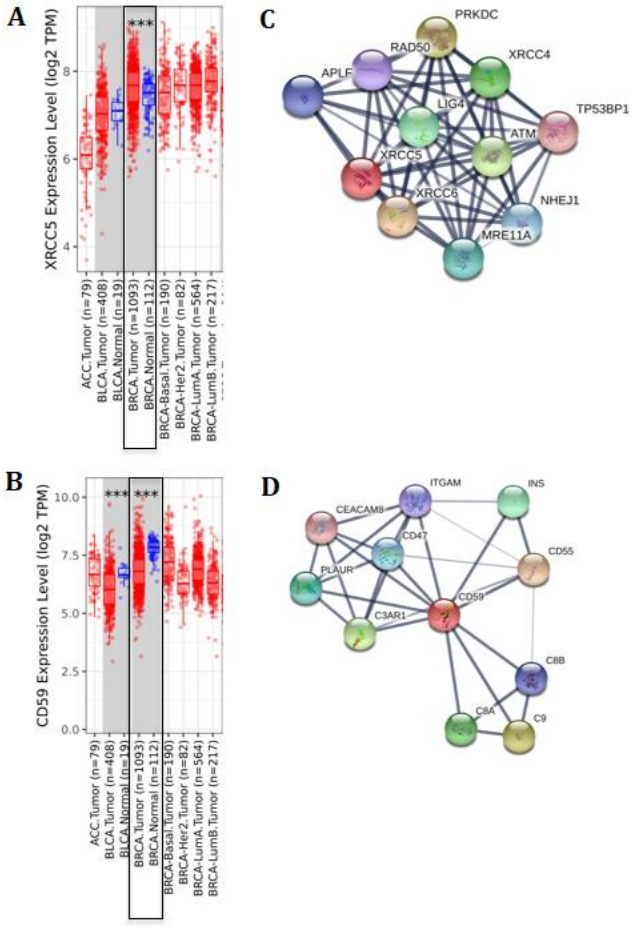

Figure 2. Gene expression of (A) *XRCC5* and (B) *CD59* across different breast cancer samples from TCGA study, as analyzed by TIMER. Statistical significance was calculated using the Wilcoxon test. * or ** or *** indicate $p < 0.05$ or $p < 0.01$ or $p < 0.001$, respectively. Protein–protein interaction networks related to (C) XRCC5 and (D) CD59, as analyzed by STRING

Approximately 52 genes showed direct and inverse correlations with the log IC_{50} of ellipticine (Supplementary Table I). The gene list obtained revealed upregulated and downregulated genes following ellipticine treatment. For instance, *SRSF2*, *XRCC5*, *CDK1*, and *CACYBP* showed direct correlations with ellipticine sensitivity. In contrast, *CREBRF*, *CD59*, *SOX6*, and *SLC27A1* showed inverse correlations with ellipticine sensitivity. A direct correlation means higher mRNA levels increase drug resistance, whereas an inverse correlation means higher mRNA levels reduce drug resistance (Sertel *et al*., 2011).

Data mining from Pubmed resulted in 844 genes related to BCSCs (Supplementary Table II). Two of these genes were retrieved using a Venn diagram of the COMPARE mRNA data and the BCSC regulatory genes obtained from PubMed, namely, *XRCC5* and *CD59* (Figure 1B).

XRCC5 showed direct correlations with ellipticine, with a Pearson correlation coefficient of 0.63. *CD59* showed an inverse correlation with ellipticine, with a Pearson correlation coefficient of −0.571. The gene expression profiles of breast cancer samples (BRCA) across the TCGA study showed that the mRNA expression of *XRCC5* (Figure 2A) and *CD59* (Figure 2B) is significantly higher in BRCA (n=1093) than in adjacent tissues (n=112). This phenomenon is supported by previous studies demonstrating the association between *XRCC5* overexpression and metastatic hepatocellular carcinoma cells (Liu *et al*., 2019). *CD59* overexpression has also been found in breast cancer cells that are resistant to tamoxifen (Xiong *et al*., 2018b).

Table I. Correlation of ellipticine with standard chemotherapeutic agents, as analyzed by COMPARE.

No	Pearson correlation coefficient	NSC Code	Drugs
1	0.676	S143095	pyrazofurin
2	0.656	S332598	rhizoxin
3	0.631	S375575	cyclopentenylcytosine
4	0.615	S268242	"N,N- dibenzyldaunomycin"
5	0.603	S368390	DUP785 (brequinar)
6	0.569	S208734	aclacinomycin A
7	0.561	S366241	bispyridocarbazolium DMS
8	0.551	S368390	DUP785 (brequinar)
9	0.548	S126771	dichloroallyl lawsone
10	0.54	S366140	pyrazoloacridine
11	0.535	S126849	3-deazauridine
12	0.533	S224131	PALA
13	0.531	S163501	AT-125 (acivicin)
14	0.53	S366241	bispyridocarbazolium DMS
15	0.524	S237020	largomycin
16	0.521	S126771	dichloroallyl lawsone
17	0.517	S7365	DON
18	0.516	S7365	DON
19	0.514	S352122	trimetrexate
20	0.513	S740	methotrexate
21	0.509	S268242	"N,N-dibenzyldaunomycin"

Table II. Top 5 results of GO enrichment analysis of the XRCC5 interaction network

GO ID	Term	FDR
Biological Process		
GO:0006303	double-strand break repair via nonhomologous end joining	3.69e-21
GO:0006302	double-strand break repair	1.35e-20
GO:0010212	response to ionizing radiation	2.64e-13
GO:0010165	response to X-ray	1.92e-12
GO:0006310	DNA recombination	2.19e-12
Molecular Function		
GO:0042162	telomeric DNA binding	6.00e-07
GO:0140097	catalytic activity, acting on DNA	1.60e-06
GO:0008022	protein C-terminus binding	1.86e-06
GO:0003677	DNA binding	1.05e-05
GO:0004677	DNA-dependent protein kinase activity	0.00011
Cellular component		
GO:1990391	DNA repair complex	1.96e-18
GO:0070419	nonhomologous end joining complex	1.23e-15
GO:0000784	nuclear chromosome, telomeric region	5.87e-12
GO:0032807	DNA ligase IV complex	4.38e-08
GO:0035861	site of double-strand break	5.47e-08

XRCC5, or X-ray repair cross complementing 5, regulates the DNA repair mechanism. An earlier pharmacogenetics study showed that polymorphisms on *XRCC5* could be a risk factor for the development of gastric cancer in Iranians with a positive family history of cancer (Saadat *et al*., 2015) and is a poor prognostic factor for astrocytoma in Chinese Han patients (He *et al*., 2016). Together with p300, XRCC5 regulates the proliferation of colon cancer through the over-expression of COX-2 (Zhang *et al*., 2017). Over-expression of chloride channel-3 is coordinated by XRCC5 and a poor prognostic marker in patients with gastric cancer (Gu *et al*., 2018). CD59, the membrane complement regulatory protein, stimulates cell proliferation and results in poor prognosis in patients with breast cancer (Ouyang *et al*., 2016). The regulation of CD59 by SOX2 is necessary for stem cells' evasion of complement surveillance and, thus, highlights the pivotal role of complement surveillance in inhibiting CSCs (Chen *et al*., 2017).

The overexpression of the CD59 glycoprotein precursor was recently found in tamoxifen-resistant breast cancer cells; thus, the gene may be a biomarker of the resistance of tamoxifen in luminal breast cancer cells (Xiong *et al*., 2018a). CD59 is a poor prognostic factor for estimating the radio resistance of esophageal squamous cell carcinoma (Zhou *et al*., 2018) and a potential target for cancer immunotherapy (Zhang *et al*., 2018a)

COMPARE analysis

In general, COMPARE analysis showed that high-ranking compounds may have similar mechanisms of action toward the probe compound. COMPARE analysis also revealed that we could predict several anticancer agents with bioactivity similar to that of ellipticine. The results showed 21 anticancer standards with suitable Pearson correlation coefficients (Table I). Ellipticine showed the greatest similarity to pyrazofurin, rhizoxin, *N*,*N*-dibenzyldaunomycin, brequinar, and cyclopentenylcytosine.

A previous study showed that pyrazofurin, an inhibitor of the synthesis of RNA and *N*,*N*-dibenzyldaunomycin, a DNA binder agent, has a mechanism of action similar to that of ellipticine (Huang *et al*., 2005). In addition, brequinar inhibits dihydroorotate dehydrogenase and, thus, stimulates myeloid differentiation in human and mouse models of acute myeloid leukemia (Sykes *et al*., 2016). These results could improve the understanding of the possible mechanisms of ellipticine in comparison with those of existing anticancer agents.

PPI network, GO and KEGG pathway enrichment analyses

We constructed the PPI networks of XRCC5 (Figure 2C) and CD59 (Figure 2D). A protein network of XRCC5 including 11 nodes, 51 edges, average node degree of 9.27, and PPI enrichment p-value of 6.66e-16 was constructed. Moreover, a protein network of CD59 could be constructed with 11 nodes, 29 edges, an average node degree of 5.27, and a PPI enrichment p-value of 0.000212.

The results of GO analysis were divided into biological process, cellular component, and molecular function. A number of genes in the XRCC5 network (Table II) participated in the biological processes of non-homologous end joining, double-strand break DNA repair, and response to ionizing radiation. Some genes were involved in the cellular components of the DNA repair complex and non-homologous end-joining complex. Finally, some genes performed molecular functions in telomeric DNA binding, catalytic activity, acting on DNA, protein C-terminus binding, DNA binding, and DNA-dependent protein kinase activity.

Some of the genes in the CD59 network (Table III) participated in the regulation of inflammatory and acute inflammatory responses, leukocyte-mediated immunity, and regulation of complement activation. Some genes were also involved in the cellular components of specific and secretory granule membranes, extracellular region, and membrane attack complex. Finally, some genes performed molecular functions in complement binding. KEGG pathway enrichment analysis showed the involvement of XRCC5-network genes in several intracellular signaling pathways including non-homologous end-joining, homologous recombination, and regulation of CD59-related genes in the complement and coagulation cascades (Table IV).

Alterations in target genes

Analysis of genetic alterations by cBioPortal showed mutations of 0.4% in *XRCC5* and 1.8% in *CD59* among breast cancer patients (Figur 3A). Genetic alterations in *XRCC5*, including amplification and deep deletion, occurred in invasive breast carcinoma. Many mutations, most of which involve amplification, also occurred in *CD59* in invasive breast carcinoma and breast cancer (Figure 3A).

Table III. Top 5 results of GO enrichment analysis of the CD59 interaction network

GO ID	Term	FDR
Biological Process		
GO:0050727	regulation of inflammatory response	6.44e-12
GO:0002673	regulation of acute inflammatory response	6.44e-12
GO:0002443	leukocyte mediated immunity	6.44e-12
GO:0032101	regulation of response to external stimulus	1.19e-11
GO:0030449	regulation of complement activation	3.67e-11
Molecular Function		
GO:0001848	complement binding	7.43e-06
Cellular component		
GO:0035579	specific granule membrane	6.61e-10
GO:0030667	secretory granule membrane	4.00e-09
GO:0030141	secretory granule	4.84e-08
GO:0005576	extracellular region	2.40e-07
GO:0005579	membrane attack complex	2.78e-07

Table IV. KEGG pathway analysis of genes in the XRCC5 and CD59 interaction networks

GO ID	Term	FDR
XRCC5		
hsa03450	Non-homologous end-joining	8.78e-22
hsa03440	Homologous recombination	1.13e-05
hsa04218	Cellular senescence	0.00039
hsa04110	Cell cycle	0.0075
CD59		
hsa04610	Complement and coagulation cascades	7.62e-16
hsa05146	Amoebiasis	4.46e-06
hsa05020	Prion diseases	1.46e-05
hsa05322	Systemic lupus erythematosus	0.00022
hsa04640	Hematopoietic cell lineage	0.00022

Although the value is usually small, previous studies indicated that the number of genetic alterations in *XRCC5* and *CD59* contributes to the incidence of cancer worldwide. Alterations in *CD59* are responsible for the antiapoptotic mechanism in leukemic cells (Jia *et al*., 2019). Genetic alterations in *XRCC5* are also associated with an increased risk of breast cancer (Cui *et al*., 2016). Thus, genetic alterations in *XRCC5* and *CD59* may affect sensitivity to ellipticine.

Kaplan Meier survival analysis

Breast cancer patients in the high-*XRCC5* mRNA expression group showed no significant difference in overall survival compared with those in the low-level expression group (p = 0.056; Figure 4A). However, the overall survival of breast cancer patients with elevated *CD59* mRNA levels was lower than that of patients with low *CD59* mRNA expression levels (p = 0.032; Figure 4B). This phenomenon is relevant to our COMPARE results, which revealed that tumors with higher expression of *CD59* are more sensitive to ellipticine. Taken together, *XRCC5* and *CD59* appear to be potential targets and biomarkers of ellipticine cytotoxicity in BCSCs.

In the present study, *XRCC5* and *CD59* were found to be key regulatory genes in the toxicity of ellipticine toward BCSCs. Integrated bioinformatics analysis is an extensible approach that can help researchers translate basic research results from bench to clinical applications.

Previous researchers discussed CD59 as a potential target for the development of anticancer therapies, such as breast cancer targets (Li *et al*., 2011) and monoclonal antibodies in immunotherapy (Zhang *et al*., 2018b). The development of CD59 inhibitors has also been reported (You *et al*., 2011).

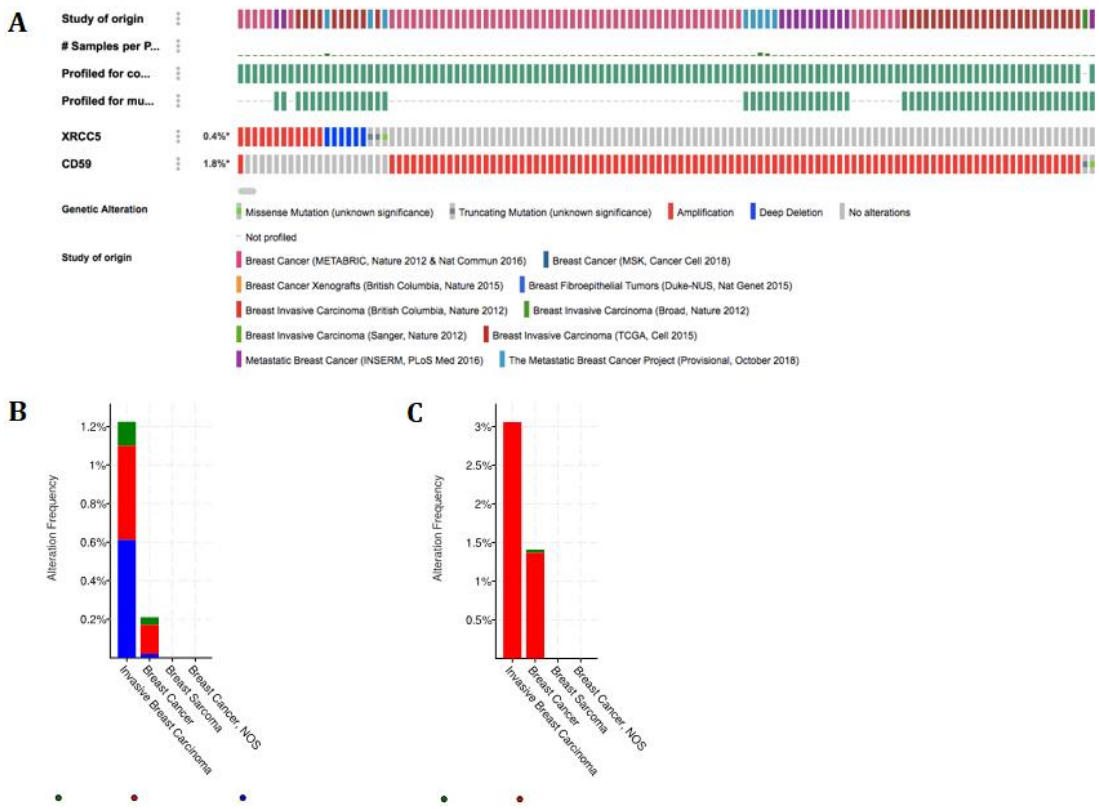

Figure 3. (A) Summary of gene alterations across different breast cancer samples. Summary of genetic alterations in (B) *XRCC5* and (C) *CD59* among different types of breast cancer, as analyzed by cBioPortal.

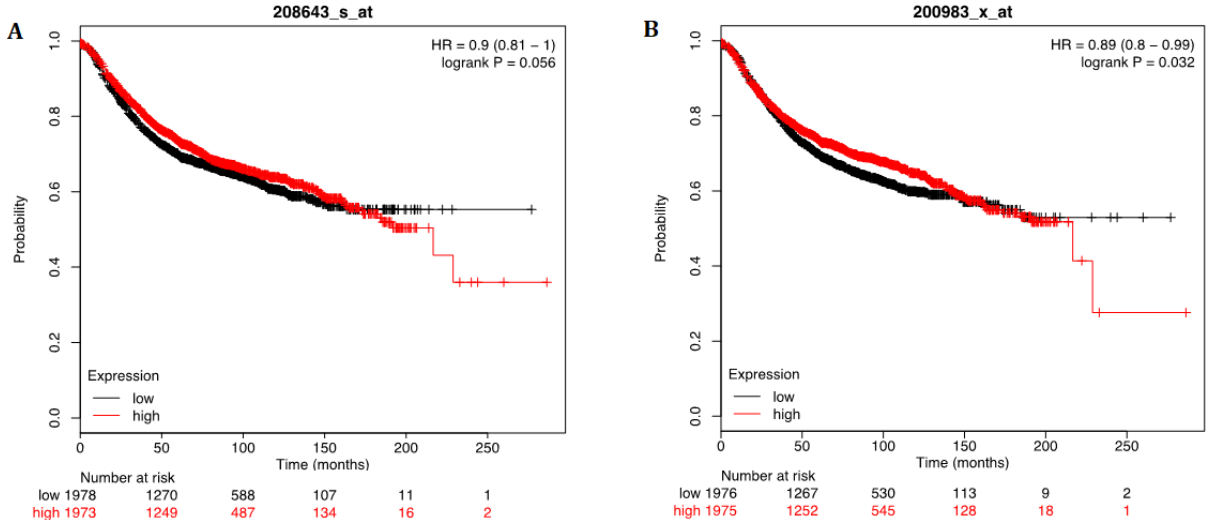

Figure 4. Kaplan–Meier survival curves for (A) *XRCC5* and (B) *CD59*, as analyzed by KMPlotter.

Previous studies showed that several compounds can decrease the expression of CD59 (van Breda *et al*., 2018) and inhibit XRCC5 (Tan *et al*., 2017). Therefore, further research on the mechanisms through which ellipticine targets XRCC5 and CD59 should be conducted.

This study provides a possible mechanism of ellipticine in BCSCs, i.e., the complement cascade and DNA repair mechanism. XRCC5 and CD59 are potential targets and biomarkers for predicting the effectiveness and potential applications of ellipticine in BCSC-targeted therapy. However, the present study was only conducted using *in silico* approaches. Further investigations including *in vitro* and *in vivo* experiments are warranted to verify the mechanisms of ellipticine on BCSCs.

CONCLUSION

In conclusion, ellipticine may target the complement cascade and DNA repair mechanism in BCSCs. More importantly, XRCC5 and CD59 are potential targets and biomarkers for evaluating ellipticine bioactivity in BCSCs. This study provides insights into the molecular mechanism and BCSC-targeting treatment potential of ellipticine. Further studies are needed to corroborate the findings and expand the full therapeutic use of ellipticine against BCSCs.

ACKNOWLEDGEMENT

The authors acknowledge Badan Penerbit dan Publikasi, Universitas Gadjah Mada, for excellent writing assistance.

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