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Bioinformatics Analysis of Rho GTP-ase Activating Protein 35 (*ARHGAP35*) in Breast Cancer Migration

Dicky Rizky Febrian[1], Joko Setyono[2], Muhamad Salman Fareza[1], Nur Amalia Choironi[1], Arif Fadlan[3], Sarmoko[1*]

[1]Department of Pharmacy, Faculty of Health Sciences, Jenderal Soedirman University, Jl. Dr. Soeparno Karangwangkal, Purwokerto, Central Java, Indonesia 53123
[2]Laboratory of Biochemistry, Faculty of Medicine, Jenderal Soedirman University, Jl. Gumbreg No 1 Purwokerto, Central Java, Indonesia, 53146
[3]Department of Chemistry, Faculty of Science and Data Analytics, Institut Teknologi Sepuluh Nopember, East Java, Indonesia, 60111

Abstract

Breast cancer is a second deadly cancer after lung cancer worldwide. Progression of cancer is driven by mutated cancer drive gene such as *ARHGAP35*. This study aims to analyze the role of *ARHGAP35* in the growth and development of breast cancer cells. *ARHGAP35* expression level was analyzed using Oncomine (*p*-value<1E-4; gene rank top 10%). Overall survival (OS) and disease-free survival (DFS) were evaluated by using GEPIA (median cutoff; HR displayed with 95% CI). STRING was used for analyzing the protein-protein interaction network, while WEBGESTALT for KEGG pathway and gene ontology (GO) of *ARHGAP35* and associated proteins and cBioPortal for gene mutation. *ARHGAP35* was overexpressed in several types of breast cancer, namely invasive ductal breast carcinoma (IDC), invasive ductal and lobular breast carcinoma (IDLC), invasive lobular breast carcinoma (ILC), male breast carcinoma, and mixed ductal and lobular carcinoma (MDLC). High expression of ARHGAP35 had significantly lower OS (*p*=0.045) compared to low expression of ARHGAP35 and the difference in DFS was not significant (*p*=0.98). ARHGAP35 interacted with RHOA, RHOB, RHOC, RHOD, RASA1, RND1, RAC1, CDC42, FYN and SRC. KEGG pathway and GO analysis showed that these proteins are highly involved in actin-based processes through adherent junction, axon guidance, focal adhesion, regulation of actin cytoskeleton, and tight junction. Mutation rate analysis showed 34 missense, 29 truncating, 3 fusion, and 1 in frame on *ARHGAP35*. Taken together, ARHGAP35 may involve in the growth and development of breast cancer through regulation of actin cytoskeleton pathway.

Keywords: *ARHGAP35, breast cancer, KEGG pathway, mutation rate, actin cytoskeleton.*

INTRODUCTION

Breast cancer is the second most common cancer that causes death after lung cancer. In 2004, breast cancer was the most common cancer in women globally. In 2012, 13.8% of all cancer cases were breast cancer. Based on the Global Trends of Breast Cancer Mortality Rate, mortality from breast cancer increases significantly every year, especially

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*Corresponding author: sarmoko@unsoed.ac.id

in developing countries and low-income areas (Azamjah, *et al*., 2019). In addition, breast cancer patients have poor survival and prognosis (Wang, *et al*., 2015).

Breast cancer develops due to DNA damage and genetic mutations. In some cases, DNA defects and oncogenes can be inherited, so family history is a risk factor for breast cancer (Feng, *et al*., 2018). In addition, genetic mutations are also essential factors that can disrupt cell regulatory pathways and promote the initiation and progression of breast cancer. A study states that *ARHGAP35* has the potential as a breast cancer gene driver, which supports breast cancer growth (Rajendran and Deng, 2017).

Small GTPase (guanosine triphosphatase) of Rat sarcoma (Ras) is an essential protein in cell signaling pathways. One of the Ras groups, namely Homologous Ras (Rho), has a vital role in regulating processes involving the actin cytoskeleton involved in adhesion, migration, and cell division. GTPase switch from an active to inactive state or vice versa, which in turn activate downstream signaling (Heraud, *et al*., 2019). GTPase is active when it binds to GTP and is inactive when it binds to GDP. GEF and GAP control GTPase activation regulation. GEF supports the active state of GTP on GTPase, and GAP inhibits GTPase by increasing GTP hydrolysis. *ARHGAP35* acts as a GAP, a negative regulator for Rho GTPase (Heraud, *et al*., 2019; Chen, *et al*., 2019).

The *ARHGAP35* is thought to be involved in the growth and development of cancer cells. It is based on the role of *ARHGAP35* involved in cell cycle events, migration, and invasion (Heraud, *et al*., 2019). The deregulation of *ARHGAP35* in breast cancer can lead to increased Brk/*ARHGAP35* interactions, cell proliferation, migration, and invasion, as well as increased tumorigenicity in mouse models (Shen, *et al*., 2008).

Bioinformatics is an interdisciplinary field that mainly involves molecular biology, computers, mathematics, and statistics (Can, 2014). Chen, *et al*. (2019) conducted a bioinformatics analysis on the ARHGAP gene group in breast cancer, namely *ARHGAP4*, *ARHGAP6*, *ARHGAP7*, *ARHGAP8*, *ARHGAP9*, *ARHGAP10*, *ARHGAP11A*, *ARHGAP14*, *ARHGAP15*, *ARHGAP18*, *ARHGAP19*, *ARHGAP23*, *ARHGAP29*, and *ARHGAP2924,* and *ARHGAP30*. The study used the Oncomine database, Kaplan-Meier Plotter, bcGenExMiner, and cBioportal to determine gene expression levels, patient survival, clinical parameters, and protein-networks. The *ARHGAP9*, *ARHGAP15*, *ARHGAP19*, and *ARHGAP30* were found to be promising targets for breast cancer treatment. According to these data, the ARHGAP gene family impacts breast cancer prognosis (Chen, *et al*, 2019). However, the analysis of *ARHGAP35* on the development of breast cancer has never been carried out, thus prompting us to analyze the role of *ARHGAP35* in the growth and development of breast cancer cells.

MATERIAL AND METHODS

This study was an *in silico* study to reveal the role of *ARHGAP35* on the growth and development of breast cancer cells. The databases used were: Oncomine to analyze the expression level of *ARHGAP35* in breast cancer cells and normal cells, GEPIA to analyze overall survival (OS) and disease-free survival (DFS) due to the effect of *ARHGAP35* expression, STRING to analyze the protein-protein interaction network associated with *ARHGAP35*, WEBGESTALT to analyze GO and KEGG pathway, and cBioPortal to analyze the mutation rate of *ARHGAP35.*

Expression Level of ARHGAP35 in Breast Cancer

The expression of *ARHGAP35* in cancer and normal cells was searched on Oncomine (www.oncomine.org) by using "ARHGAP35" keyword through "cancer vs normal analysis" filter. The threshold used was the *p*-value of 1E-4, the fold change of 2, and the top 10% of gene rank. The

search resulted in a comparison of the expression levels of *ARHGAP35* in normal and cancer cells. The data were taken from the TCGA Breast dataset which analyzed 593 samples and 20,423 genes. The data were exported as box plot data.

Survival Analysis of Breast Cancer Affected by ARHGAP

Survival analysis was performed using GEPIA database (www.gepia.cancer-pku.cn) with the keyword "ARHGAP35". Next, the "survival" and "Survival Plots" tabs were selected in the new page. The "overall survival" to analyze the overall survival and "disease free survival" to analyze DFS were then selected. The intersection point was set in the median, while the hazard ratio was displayed with a 95% confidence interval (CI) along with the log *p* value. The type of cancer was entered in the "datasets" column and the samples were evaluated using the GEPIA survival plot. The data obtained was then exported in pdf file and converted into a jpg format.

Protein-protein Networking Analysis

Protein-protein interaction network associated with *ARHGAP35* was searched using STRING database (www.string-db.org). The keyword "*ARHGAP35*" was applied in the search field and *Homo sapiens* was selected as organism. The results showed a network of protein-protein interactions that have a possible involvement with *ARHGAP35* which was exported as portable network graphic file.

A schematic of the *ARHGAP35* associated protein–protein interaction network consisted of nodules and lines. Each nodule represented an interaction of protein with *ARHGAP35*. Nodules were colored and colorless (white) indicating direct and indirect interaction of protein with *ARHGAP35*. Lines depicted interactions of nodules (proteins) connected by the lines. The light blue and purple lines represent known interactions based on the databases and experimental studies; the green, blue and red lines represent predictable interactions through gene similarity, gene fusion and co-occurrence; and the yellow, black, and white lines depict the interactions obtained through text mining, co-expression and protein similarity.

Gene Ontology dan KEGG Pathway Analysis

Gene ontology and KEGG pathway were analyzed using WEBGESTALT (www.webgestalt.org) by Homo sapiens organisms and the ORA method. In the functional database column, the selected gene ontology included biological processes (BP), cellular components (CC), and molecular functions (MF). In the same column, the KEGG pathway was also selected, and proteins involved with *ARHGAP35* based on protein-protein interaction network analysis was entered in the gene list column. The genome was selected as the reference set and submitted to start the analysis. The analysis produced a bar graph of the enrichment ratio and it was exported in a zip file.

Mutation of *ARHGAP35* Analysis

The *ARHGAP35* mutation rate was analyzed by using cBioPortal (www.cbioportal.org). The "breast cancer" was selected for the "query by genes" analysis and *ARHGAP35* for "gene list". The mutations occurred in *ARHGAP35* was viewed by selecting the "mutation" tab. The analysis resulted in a mutation curve of the *ARHGP35* gene including the type of mutation, the number of mutated samples, and the location of the mutation in the gene. The data was exported as png and tsv files.

RESULTS

The *ARHGAP35* Expression Level in Breast Cancer

The *ARHGAP35* expression level in breast cancer and in normal cells were analyzed using Oncomine. The results showed that *ARHGAP35* was overexpressed in several types of breast cancer, namely invasive ductal breast carcinoma (IDC), in-

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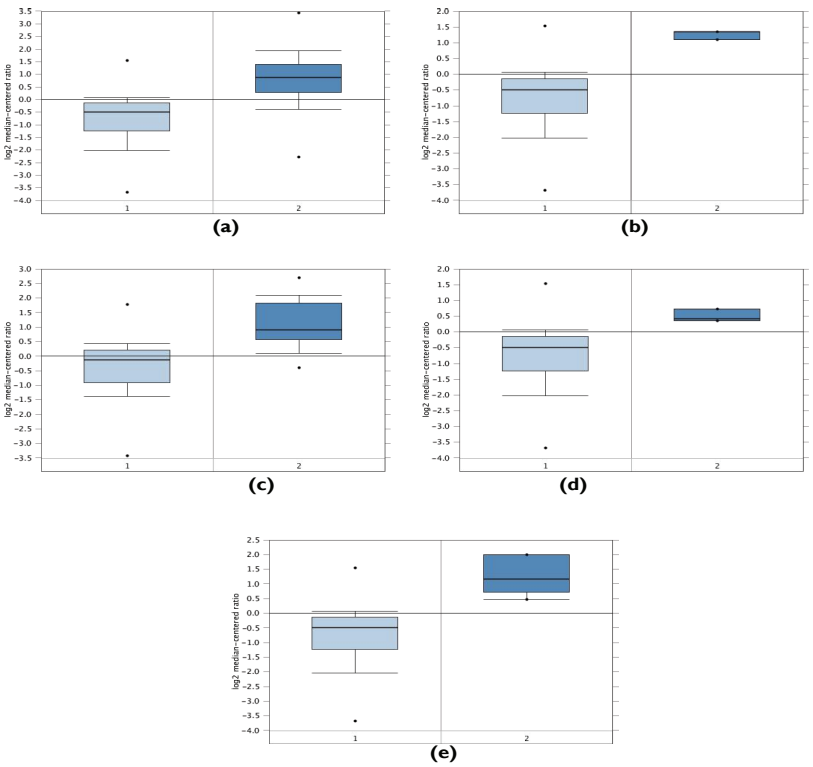

Figure 1. Expression level of *ARHGAP35*. (a) IDC, (b) IDLC, (c) ILC, (d) MBC, (e) MDLC. 1=normal cells, 2=cancer cells.

vasive ductal and lobular breast carcinoma (IDLC), invasive lobular breast carcinoma (ILC), male breast carcinoma, and mixed ductal and lobular carcinoma (MDLC) with fold change values of 2.950, 4.039, 2.889, 2.400, and 4.017 with *p*-value<1E-4 (Figure 1 and Table 1).

Overall Survival (OS) and Disease-Free Survival (DFS) of Breast Cancer

The effect of *ARHGAP35* expression on OS and DFS was analyzed using GEPIA. The OS and DFS analysis used samples on breast invasive carcinoma (BRCA) with a cut point in the median. The analysis produced a Kaplan-Meier graph of survival versus time. The high expression of *ARHGAP35* showed a significantly lower OS (Figure 2). The OS values of BRCA patients decreased significantly at 100 to 150 months. Meanwhile, the DFS analysis showed a non-significant difference before 150 months. It showed a significant decrease in DFS up to <0.4 in high expression of *ARHGAP35* in the mid-month between 150 and 200. The decrease in breast cancer survival percentage may be related to higher *ARHGAP35* expression levels thereby increasing the growth of breast cancer cells.

Protein-protein Interaction Network of *ARHGAP35*

The protein-protein interaction network of *ARHGAP35* was analyzed using the STRING database. *ARHGAP35* interacted with ten proteins namely RHOA, RHOB, RHOC, RHOD, RASA1, RND1, RAC1, CDC42, FYN, and SRC (Figure 3). The analysis of KEGG pathway and GO with the WEBGESTALT database was carried out to determine the involvement and role of these proteins.

KEGG pathway analysis described the interaction pathways that occur from proteins ob-

Table 1. Fold change expression level of *ARHGAP35* in breast cancer cells vs normal cells.

Breast cancer types	Fold change	*p*-value
Invasive ductal breast carcinoma (IDC)	2.950	2.83E-21
Invasive ductal and lobular breast carcinoma (IDLC)	4.039	287E-10
Invasive lobular breast carcinoma (ILC)	2.889	334E-13
Male breast carcinoma (MBC)	2.400	3.42E-5
Mixed ductal and lobular carcinoma (MDLC)	4 .017	4.63E-6

tained by the STRING analysis. The enrichment ratio level represents the evidence related pathways. The KEGG pathway analysis denoted that there are pathways related to actin-based processes, namely adherens junction, axon guidance, focal adhesion, regulation of the actin cytoskeleton, and tight junctions (Figure 4).

The GO analysis described the characteristics of a gene. Gene ontology consists of three categories, namely biological process (BP), cellular component (CC), and molecular function (MF). The BP analysis showed actin-based processes such as the formation or maintenance of the polarity of the actin cytoskeleton, regulation of cell shape, regulation of cell morphogenesis, and the formation of actin filaments. CC analysis describes the localization sites of the analyzed protein. The CC analysis reported that the analyzed proteins were available at the site of cell division, cleavage furrow, cell cortex, ruffle, endosome, and actin cytoskeleton. The MF analysis reflects the molecular functions involving the analyzed protein. The MF analysis pointed out that the molecular functions related to GTPase activity were GTPase activity, GTP binding and amino acids (Figure 5).

Mutation Rates of *ARHGAP35*

Mutation rates in *ARHGAP35* were analyzed using cBioPortal. The number of analyzed samples was 9502 from 16 studies published in Nature, TCGA, Firehose Legacy, PanCancer, PLoS Med, and NPJ Breast Cancer. The results showed that the *ARHGAP35* mutation involved 34 missenses, 29 truncating, 3 fusions, and 1 inframe in breast cancer. The nonsense mutation caused a premature stop codon, namely in R997*, which causes *ARHGAP35* losing its ability to bind to the active RhoA. The nonsense mutation R997* in

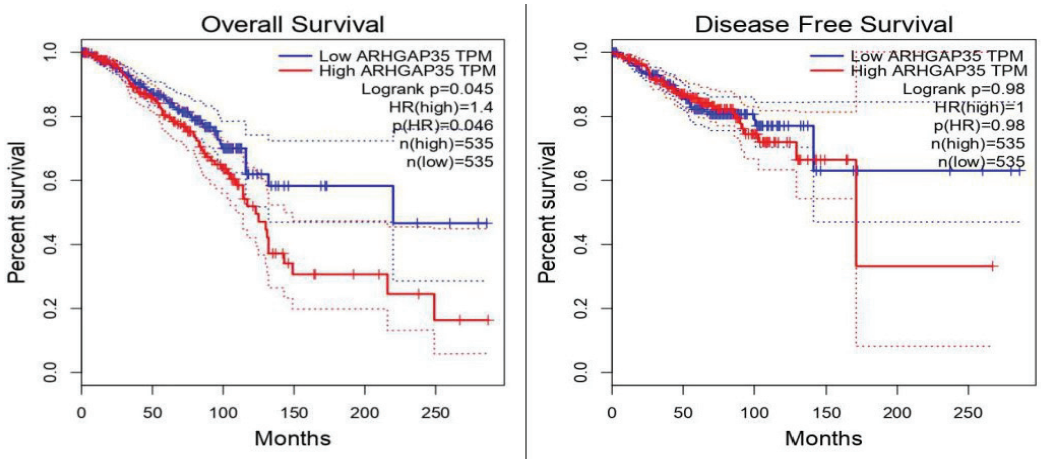

Figure 2. Overall survival (OS) and disease-free survival in breast cancer (BRCA) related to *ARHGAP35* expression.

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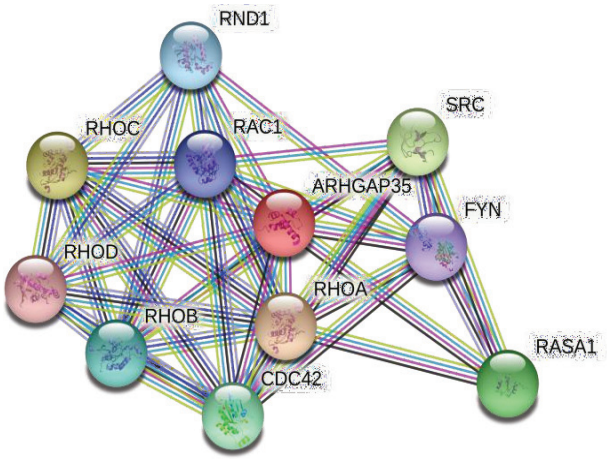

Figure 3. *ARHGAP35*-associated protein-protein interaction network.

ARHGAP35 resulted in the absence of a GAP domain in the *ARHGAP35* structure. In addition, there are also missense mutations such as the Y742H and R832Q that do not have an impact on the protein activity.

Mutations in the pG2 domain showed a strong impact on protein activity. This can be seen from the S866F mutation and in-frame deletion del865-870 which showed an increase in *ARHGAP35* activity. The enhanced function of *ARHGAP35* has been reported to support random cell migration and to reduce the ability of cancer cells to migrate directionally (Binamé, *et al*., 2016). The pG2 domain is located at amino acid number 766-958 (Héraud, *et al.*, 2019). Based on the results of cBioPortal analysis, there are three mutations that occur in the pG2 domain, namely nonsense mutation Q805*, missense mutation R832Q and frame shift deletion mutation K945Rfs*3. The missense mutation R832Q has been shown to have no effect on *ARHGAP35* activity, but no studies of nonsense mutation Q805* and frame shift deletion K945Rfs*3 have reported an effect on *ARHGAP35*.

DISCUSSION

The *ARHGAP35* is a GAP protein coding gene which acts as a Rho GTPase negative regulator. The *ARHGAP35* implicates in the growth and development of breast cancer cells due to its role in cell migration and invasion (Héraud, *et al*., 2019; Chen, *et al*., 2019). The *ARHGAP35* has the potential to be a driver gene that can support cancer growth (Rajendran and Deng, 2017). The *ARHGAP35* is overexpressed in several types of breast cancer, namely IDC, IDLC, ILC, MBC and MDLC. High expression of *ARHGAP35* had a lower survival rate. Protein-protein interaction network analysis showed that *ARHGAP35* interacted with ten proteins, namely RHOA, RHOB, RHOC, RHOD, RASA1, RND1, RAC1, CDC42, FYN and SRC. The results of the KEGG pathway and GO analysis showed that these proteins were widely involved in cell growth and development.

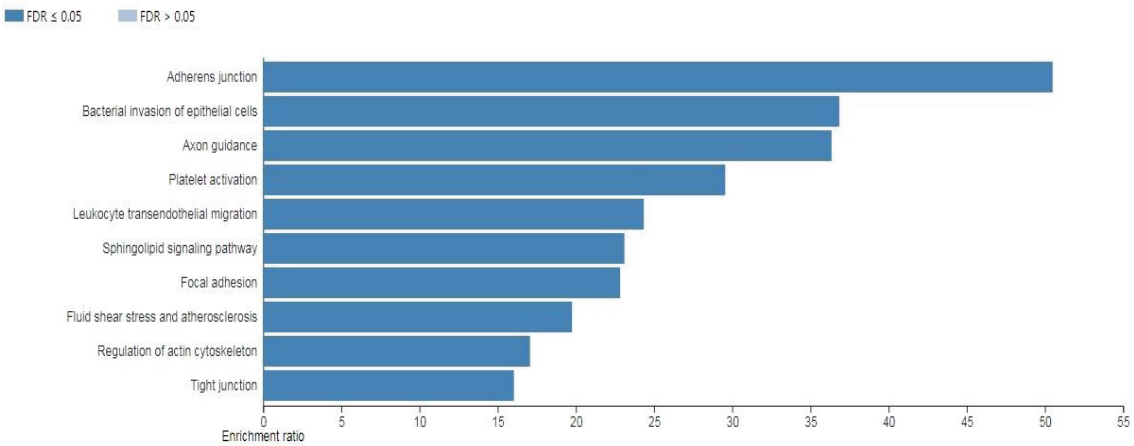

Figure 4. KEGG pathway of *ARHGAP35*.

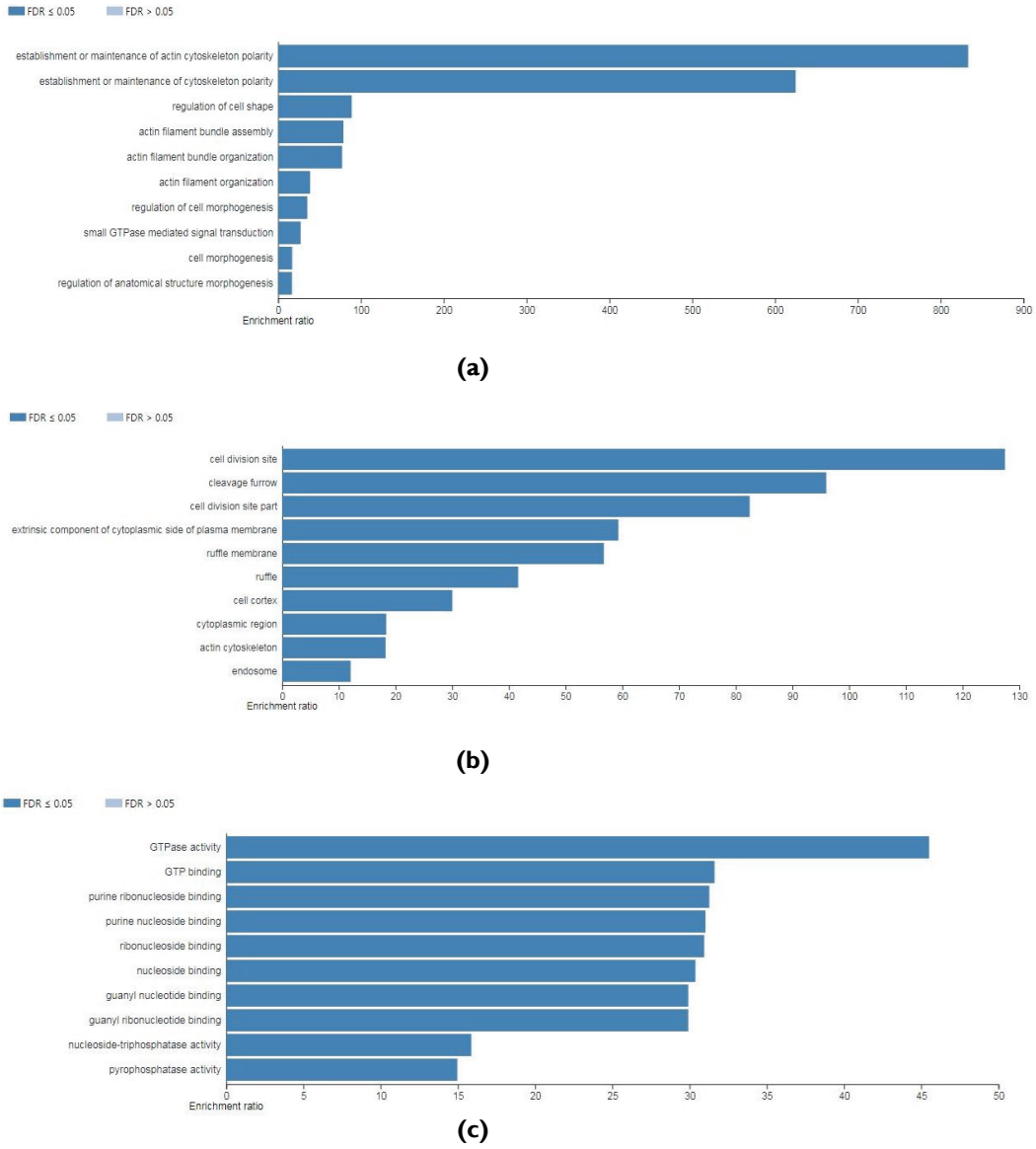

Figure 5. Gene ontology analysis of *ARHGAP35*. (a) biological process (BP), (b) cellular component (CC), and molecular function (MF).

The Oncomine analysis for gene expression level showed increasing *ARHGAP35* expression in IDC, IDLC, ILC, MBC, and MDLC. Overexpression of *ARHGAP35* may be associated with genetic and epigenetic changes (Axelsen, *et al*., 2007). Upregulation of *ARHGAP35* has been linked to the development of breast cancer because it can enhance cell proliferation, migration, and invasion. Regarding the function of *ARHGAP35* as a GAP, phosphorylation of *ARHGAP35* at residue Y1105 by breast tumor kinase (Brk) promotes the formation of the *ARHGAP35*/p120RasGAP complex which can stimulate *ARHGAP35* function. This can result in RhoA inactivation. In addition, disruption of the ARHGAP35/p120RasGAP complex with a dominant-negative *ARHGAP35* moiety

on cells may reduce the stimulatory effect of Brk on cell proliferation, migration, and invasion (Shen, *et al*., 2008).

The survival analysis by using GEPIA indicated that high expression of *ARHGAP35* had a significantly lower OS than low expression of ARHGAP35 (p=0.045). A similar analysis with DFS displayed a non-significant result (p=0.98), but the DFS value dropped drastically in the middle of the 150th to 200th months. These findings showed that a higher *ARHGAP35* expression level has a faster growth of breast cancer cells. Upregulation of *ARHGAP35* has been shown to promote cell proliferation, migration, and invasion, as previously described (Shen, *et al*., 2008).

The KEGG pathway analysis exhibited that *ARHGAP35* was heavily involved in the actin cytoskeleton regulatory pathway required for RhoA regulation (Heraud, *et al*., 2019). RhoA, a Rho GTPase, is a major regulator of the actin cytoskeleton pathway. The active Rho GTPase interacts with effector actin binding protein (ABP) which in turn regulates the rearrangement of actin filament. ABP can be categorized into two, namely G- and F-actin. The former interacts with actin monomers and the latter associates with actin filaments (Lee and Dominguez, 2010).

The *ARHGAP35* as a major RhoA regulator is involved in cell migration, entosis, and cell division. At the cell-cell junction, inhibition of RhoA activity is required to reduce actomyosin activity at cell-cell contacts and facilitate coordinated cell movement. The interaction between claudin-11 and ARHGAP35 in the p120RasGAP domain can reduce the level of RhoA at the cell-to-cell junction so that the stability of cell-to-cell contact is maintained and facilitates the spread of tumor cells (Li, *et al*., 2019).

The *ARHGAP35* is known to be involved in the mechanism of entosis based on its involvement in the attenuation of cell contractility. Entosis is described as a non-apoptotic cell killing program for cancer cells, in which one cell can eat the adjacent cells. This competition between cells results in differences in cell contractility. One of the cells shows high mechanics in deformation, while the other cells accumulate actin and myosin in the cell cortex which generated tension in the cell. The increase of tension in the cell is regulated by Rho/ROCK. Rho regulates cofilin activity via Rho kinase (ROCK) which phosphorylates LIM kinase (LIMK), which in turn phosphorylates cofilin. This signal transduction pathway modulates actin accumulation in response to extracellular stimuli. The localization of *ARHGAP35* in cells that accumulate actin and myosin can polarize RhoA activity and cause propagation of cell tension to promote entosis (Lee and Dominguez, 2010; Sun, *et al.*, 2014).

This *in silico* research was conducted by utilization of the data in the database. Therefore, the *in vivo* and *in vitro* studies is necessarily needed regarding the effect of *ARHGAP35* on breast cancer based on its role as the main regulator of RhoA through the regulatory pathway of the actin cytoskeleton.

CONCLUSION

The *ARHGAP35* may involve in the growth and development of breast cancer through regulation of actin cytoskeleton pathway.

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DECLARATIONS OF INTEREST

None

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