Reactivation of RASSF1A Protein and Induction Apoptosis in Breast Cancer Cells by Curcumin

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Abstract: Background and objective: Curcumin is one of nontoxic bioactive food component that have reactivation of suppressor genes and anti-oncogenic activity, but the specific mechanism of cytotoxicity of curcumin remains controversial due to the presence of anti-signaling pathway and pro-apoptosis in various cell types. The aim of this study was to examine the mechanism of curcumin in inducing apoptosis through RASSF1A and Bax pathway in the breast cancer cells line. Material and Methods: The breast cancer cells line of CSA03 and MCF-7 were given in a dose and time dependent manner. The anti-proliferative activity of curcumin was determined using MTS cell viability assay and flow cytometry, caspase-3 was used to detect apoptosis for cultured cells. The RASSF1A and bax protein expression in cells was evaluated by ELISA analysis. Results: We found that anti-proliferative effect of curcumin with IC50 values at CSA03 was lower than at MCF-7. The administration curcumin showed a significant increase of expression of RASSF1A and bax protein, and increases caspase-3 in both CSA03 and MCF-7 cells. Conclusion: The results indicate that curcumin mediates anti-proliferative and apoptotic effect through activation of RASSF1A and bax.

Keywords: Apoptosis, Bax, caspase-3, curcumin, CSA03 cells, MCF-7 cells, RASSF1A

1. Introduction

Breast cancer is the most common cancer for women worldwide, and accounts for approximately 50% of breast cancer cases and 58% of deaths occurred in developed countries. Breast cancer is the second leading cause of cancer-related death among females in the world1.

Mechanisms that suppress tumorigenesis often involve modulation of signal transduction pathways, resulting to alterations in gene expression, cell cycle arrest, progression and/or apoptosis. Recent evidence also shows that suppression of apoptosis by tumor-promoting agents in preneoplastic cells is thought to be an important mechanism in tumor promotion. A number of tumor suppressor genes and genes related to apoptosis in breast cancer have been shown to be inactivated in breast cancer. The Ras association gene (RalGDS / AF-6) domain family member 1 isoform A (RASSF1A) is one of the tumor suppressor genes that can regulate proliferation, induce apoptosis, and bind to and stabilise microtubules6,7. Inactivation of RASSF1A is frequently observed in multiple solid tumours and epithelial cancers, including breast cancer4,13.

Several gene products play a significant role in regulating apoptosis. The Bcl-2 gene family is comprised of both pro-apoptotic and anti-apoptotic members, with membership dependent on the presence of at least one Bcl-2 homology domain.6,7 These proteins form homo- and heterodimers, with the ratio between pro- and anti-apoptotic levels an important determinant of cell survival. Bax is a 21 kDa protein that accelerates apoptosis by binding to, and antagonizing the death repressor activity of Bcl-2 in vivo. Bax normally resides in the cytoplasm, but following an apoptotic stimulus, Bax undergoes a conformational change and translocates to mitochondrial membranes, where it inserts and mediates the release of cytochrome c from the intermembrane space into the cytosol, in this manner activating caspase 3 and perpetuating the apoptotic cascade7.

Curcumin, a natural phenolic compound extracted from the root of the Curcuma longa plant, has been shown to have many biological effects, including anti-oncogenic activities, as well as a chemopreventive effect on chemical carcinogen. Recent studies have shown that curcumin is potential in vitro growth inhibitor and has anti-proliferative activity against in vivo tumor cells.6,7 The mechanism of inhibition by curcumin on cancer cell growth has been done on various cancer cell lines and it has been done through modulation of various biological activities including the activation of genetic pathways. Curcumin is known to activate RASSF1A and plays a vital role in the upregulation of different pro-apoptotic genes. Furthermore, curcumin has shown an anti-inflammatory activity and has been involved in the apoptosis...
induction and modulation of key apoptotic proteins such as Bax\(^{10}\).

In this study, we used the human cancer cells line of CSA03 that obtained from Indonesian breast cancer patient and MCF-7 to investigate the effects of the curcumin in the inhibiting of the cell proliferation through the activated RASSF1A and apoptotic pathway by using bax and caspase-3 activities.

2. Material and Methods

Cells and Cell culture: The Cell line CSA03 was obtained from integrated laboratory, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia. The cells was isolated from breast cancer tissue of an Indonesian woman aged 56 years old with a clinical diagnosis of invasive ductal mammary carcinoma T\(_4\)N\(_0\)M\(_0\). Histopathological examination revealed epithelial tumor mass tissue that formed a solid, partially infiltrative structure. Pleomorphic nucleated tumor cells; coarse chromatin and mitosis were found. Lymphatic embolism also appears. On examination of lymph nodes, tumor cells have spread to level II and axillary lymph nodes. On immunohistochemical examination showed ER-positive results (5%, weak), PR-positive (20%, moderate), HER2-negative, and Ki67 positive (20%, weak). Based on the results of the immunohistochemical examination, the CSA03 cells line were included in luminal A subtype breast cancer.

The human breast cancer cells line of MCF-7 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) No: HTB-22. The cells line of MCF-7 has a molecular the luminal A subtype breast cancer with ER-positive, PR-positive, HER2-negative, low Ki67 positive. Cells were maintained in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (Biowest, USA), with 5% of the amphotericin, and 5% of the penicillin-streptomycin (Biosciences). Cells were cultured in a humidified atmosphere with 5% CO\(_2\) at 37\(^\circ\)C.

Proliferation experiments: The antiproliferative activity of curcumin (Sigma Chemical Co., St. Louis, MO, USA) towards CSA03 and MCF-7 human breast cancer cell lines was evaluated by using MTS assay method (promega, Madison, USA). Cells were seeded prior to treatment in a 96-well plate at plating densities 5000 cells/well. Test agents were dissolved in dimethyl sulfoxide (DMSO) 10%. Control culture received the same concentration of solvent alone. Following drug addition, the plate were incubated 48 hours. At the end of the treatment period, 10 \(\mu\)L solution MTS and PMS was added to the wells, which was then incubated at 37\(^\circ\)C with a humidity of 5% CO\(_2\) for 2 hours. The absorbance at 490 nm was read on a spectrophotometric plate reader. The higher the absorbance value, the more converted formazan is present, and so the higher the number of viable cells. This method was carried out in triplicate. Dose-response curves were constructed to obtain the response parameter which were the IC\(_{50}\) Percentage growth inhibition (IC\(_{50}\)) is calculated as\(^{14}\):

\[
\text{Inhibition (\%)} = \frac{\Delta A \text{ control} - \Delta A \text{ treatment}}{\Delta A \text{ control}} \times 100\%.
\]

Where:
- \(\Delta A \text{ control} = A \text{ control} - A \text{ blank}\)
- \(\Delta A \text{ treatment} = A \text{ treatment} - A \text{ blank}\)

Evaluation of RASSF1A and Bax: The RASSF1A assay was obtained from MyBiosource and Bax was obtained from Abcam that examined by ELISA technique. The optical density was determined using a microplate reader set at 450 nm. The test principle applied in the human RASSF1A kit is Sandwich enzyme immunoassay. The microtiter plate provided in this kit has been pre-coated with an antibody specific to Ras Association Domain Containing Protein 1 (RASSF1). Standards or samples are then added to the appropriate microplate well plates with a biotin-conjugated antibody specific to Ras Association Domain Containing Protein 1 (RASSF1). Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain Ras Association Domain Containing Protein 1 (RASSF1), biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of Ras Association Domain Containing Protein 1 (RASSF1) in the samples is then determined by comparing the O.D. of the samples to the standard curve.

A monoclonal antibody specific for Bax has been precoated onto 96-well plates. Standards or test samples are added to the wells, incubated and then washed. A human Bax polyclonal antibody is then added, incubated and washed. A Bax HRP conjugated antibody is then added and incubated. The plate is washed once more and the TRUST substrate is then added which HRP catalyzes, generating a blue coloration after incubation. A stop solution is added which generates conversion tocoloreadat 450 nm which is proportional to the amount ofanalyte bound.

Determination of Caspase-3 activity: The caspase-3 activity was determined using PEG-conjugated monoclonal active caspase-3 antibody apoptosis kit (BD Pharmingen) with intracellular staining (flow cytometry) according to the manufacturer’s instructions. Cisplatin was used as the positive control and the untreated sample was used as the negative control. Briefly, the floating and trypsinized adherent treated and untreated cells were collected and washed twice with cold 1x PBS, then resuspend cells in BD Cytofix/Cytoperm™ solution at a concentration of 1x10^6 cells/0.5 mL. Incubate cells for 20 min on ice. Pellet cells, aspirate, and discard BD Cytofix/Cytoperm™ solution; wash twice with BD Perm/Wash™ buffer (1X) at a volume of 0.5 mL buffer/1x10^6 cells at room temperature. Resuspend cells in the above calculated BD Perm/Wash™ buffer (1X) plus antibody and incubate for 30 min at room temperature. Wash each test in 1.0 mL BD Perm/Wash™ buffer (1X), then resuspend the test in 0.5 mL BD Perm/Wash™ buffer (1X) and analyze by flow cytometry. Fold-increase in caspase activity was determined by comparing the results of treated samples with the level of the untreated control.
**Statistical Analysis:** The all statistical analyses were performed using the SPSS 20.0 statistical software for Windows. Biological activity was examined in three individual experiments, performed in triplicate for each dose. Statistical significance was determined using the one-way ANOVA test followed by the LSD Posthoc for multiple comparisons. Statistical significance was set at $p < 0.05$. The IC$_{50}$ values were calculated from the dose curves by a computer program.

**3. Result**

**Effect of Curcumin on cell proliferation:** We conducted in vitro on human breast cancer cells CSA03 and MCF-7 cell line. The MTS cell viability assay in vitro is the primary stage for screening. To examine the biological effect of curcumin, CSA03 and MCF-7 cells were treated with different concentrations of curcumin at 48 h. The cell proliferation changes were evaluated with a MTS assay. The cell proliferation was inhibited by curcumin in a dose-dependent manner. The IC$_{50}$ values for the CSA03 cells were detected at 39.35 µg/mL and the MCF-7 cells were detected at 75.71 µg/mL. These results indicate that curcumin has potent antiproliferative effect in breast cancer cells. Furthermore, The CSA03 and MCF-7 cells were treated with low and high concentrations based on IC$_{50}$ (40 µg/mL and 50 µg/mL for the CSA03 cells, 70 µg/mL and 80 µg/mL for the MCF-7 cells), and with different time (24 h and 48 h).

**Curcumin Induces Apoptosis in CSA03 and MCF-7:** We used flow cytometry to examine caspase-3 that induced growth inhibition was a result of apoptosis. Our results showed that curcumin in a dose-dependent manner induced apoptosis at 24 h and 48 h. These results were from one representative experiment of three independent trials. As shown in Figure 1, the percentage of caspase-3 at 24 h on cells with a lower dose was increased from 4.21% to 85.57%, from 1.25% to 69.47% in CSA03 and MCF-7 cells. Under the treatment with a higher dose, the caspase-3 on cells increased to 97.74% and 92.84% in CSA03 and MCF-7 cells. The percentage of caspase-3 at 48 h on cells with a lower dose was increased from 2.75% to 45.91%, from 0.34% to 19.9% in CSA03 and MCF-7 cells. Under the treatment with a higher dose, the caspase-3 on cells increased to 43.82% and 72.88% in CSA03 and MCF-7 cells.

![Figure 1: Curcumin induces apoptosis in CSA03 and MCF-7](image)

The cells were treated with curcumin, followed by caspase-3 examination with flow cytometry analysis.

**Curcumin Upregulates the Protein Expression of RASSF1A in CSA03 and MCF-7:** To demonstrate whether suppression of proliferation of breast cancer cells by curcumin is due to downregulation of molecules involved in cell proliferation, we tested the protein levels of RASSF1A in cells. As shown in Fig. 2, at the previously demonstrated pharmacological effective (antiproliferation and apoptosis) concentrations, curcumin can significantly enhance the protein levels of RASSF1A in CSA03 cells (0.6–1.2-fold, $P < 0.05$, $n = 3$) and MCF-7 cells (about 2.3–2.0-fold, $P < 0.05$, $n = 3$). We found that curcumin treatment increases the protein expression of RASSF1A in a dose-dependent and time manner. Compared with CSA03 cells, the MCF-7 cells expressed increasing level of protein in RASSF1A expressed higher levels.
Curcumin increases the protein expression of RASSF1A levels in CSA03 and MCF-7 cells.
* Statistically significant values relative to untreated control (p <0.05)

Curcumin Upregulates the Protein of Bax in CSA03 and MCF-7: We further investigated the mechanisms underlying curcumin-induced apoptosis in breast cancer cells. As shown in Figure 3, Bax levels were significantly upregulated by curcumin in both of cells in a dose-dependent manner, but it was statistically significant on CSA03 cells(1.0–5-fold, p<0.05, n = 3).

Figure 3: Curcumin increases the protein expression of Bax levels in CSA03 and MCF-7 cells.
* Statistically significant values relative to untreated control (p <0.05)

4. Discussion

Nowadays, most cancer in advanced stages is notcurable by chemotherapy and breast cancer arises due to series of frequent recurrent alterations in the genome of normal epithelial cell. Therefore, new drug discovery and development from natural products still continue to play a vital role in the fight against breast cancer. In the present study, we have demonstrated the effects of curcumin on cell proliferation and apoptosis in breast cancer cells. In the literature, multiple mechanisms of action of curcuminrelated in breast cancer have been shown. It is important to clarify the mechanisms of action because it may lead to new drug for intervention in therapy of breast cancer. Our findings show that curcumin exhibited potent antiproliferative action in highly proliferative human breast cancer cell lines. In this result, curcumin showed higher antiproliferative effect in CSA03 cells as compared to MCF-7, where IC_{50} is reached at lower curcumin concentrations. One of the factors that causes differences in the results of the average inhibition of curcumin is individual cell factors. In one average growth study showed that gene modification or the microenvironment often resulted in changes in cell division. Cells from individuals who were often exposed to a compound will produce different results. CSA03 cells from Indonesia can be different from MCF-7 cells that are not from Indonesia because of the difference in consumption of curcumin as natural ingredients that are used daily.

It is generally believed that the balance between proliferation and apoptosis influence the response of tumors to cytotoxic treatment. Apoptosis is considered as a significant form of cancer cell death after treatment with cytotoxic drug and has been recognized as a standard strategy for the selection of anticancer drugs. The caspase...
family of cysteine proteases plays a key role in apoptosis. Caspase-3 is a key protease that is activated during the early stages of apoptosis and responsible for core apoptosis. In the early stages of apoptosis changes occur on the cell surface. One change in the plasma membrane is translocation of phosphatidylserine from the inner side of the plasma membrane to the outer layer, where phosphatidylserine becomes exposed to the outer surface of the cell. Caspase-3 deficiency and down-regulation have been reported to be associated with breast carcinogenesis. This indicates that increased activity of caspase-3 after treatment with curcumin may play an important role in cancer prevention and treatment. In addition, we found that the cytoplectic effect of curcumin on CSA03 cells was more potent than on MCF-7 cells. The percentage of caspase-3 levels decreased in the curcumin treatment after 48 hours. This can occur because at 48 hours there is a possibility that the apoptosis process has reached the final stage of apoptotic so that some have experienced necrosis, while the caspase-3 examination is an early apoptotic examination.

RASSF1A has been reported to be frequently silenced in many cancer types; however, the biologic relevance in clinical standpoint has not yet been clearly characterized. Because there is a probability that unsatisfactory response in breast cancer treatment may in part be due to silencing of gene, the role and clinical relevance of a frequently silenced tumor suppressor gene, RASSF1A, was examined. In this study, qualitative RASSF1A protein was increased in all cells depend on dose and time manner. Increased RASSF1A protein levels indicate that there is a possibility of epigenetic processes in the form of DNA methylation. Hypermethylation of RASSF1A causes suppression of RASSF1A gene function so that it will decrease RASSF1A protein. In contrast to genetic mutation, DNA methylation is susceptible to change and reversible; therefore, it result represents that curcumin was an excellent agent to develop DNA methylation changes for chemotherapy and chemoprevention. Reactivation of RASSF1A by curcumin will result in restoration of their biological functions. Therefore further research is needed to examine the DNA methylation.

The further key cellular process that may be by RASSF1A was the regulation of apoptosis. Curcumin causes DNA damage and endoplasmic reticulum stress and induces apoptosis through mitochondria and activation of caspase-3. Mitochondria play an important role in the process of apoptosis. The intrinsic pathway of apoptosis involves the activation of the proapoptotic members of the Bcl-2 family using its function through mitochondria. Curcumin induces the release of cytochrome c from mitochondria, causing activation of caspase 3 and the breakdown of PARP, which is a feature of caspase-dependent apoptosis. In the study reported that RASSF1A can regulate Bax activity through direct binding to apoptotic modulator (MOAP1), a protein that binds to Bax. Our results demonstrated that the curcumin increase Bax protein levels. An increase in RASSF1A protein levels was followed by an increase in Bax protein levels. These results support the hypothesis that Bax expression precedes the apoptosis.

5. Conclusion
This study concluded that curcumin treatment efficiently enhances the cytotoxic activity leading to suppression of the growth of breast cancer cell in vitro through reactivation of RASSF1A, upregulation of bax, and induction of apoptosis.

Significant Statement
This research investigates the roles of curcumin potentiation effect in vitro. It introduces a key for the researchers to study other molecular targets of curcumin which may be involved in the cytotoxic potentiation of the chemopreventive agents. The finding of the research work encourage the utilization curcumin in many aspect different cancer researches which may provide of progression in the cancer prevention and therapy.

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Competing interest: The authors have declare that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

References
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