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Heat shock protein-70 expression in CSCs tumor-associated macrophages induced by *Typhonium flagelliforme* tuber extract

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ABSTRACT

One of the apoptotic resistance mechanisms in breast cancer stem cells (CSCs) is designed by the existence of tumor-associated macrophages (TAMs), type 2 macrophages. TAMs enhance the apoptotic resistance of CSCs by extremely decreasing low levels of inducible nitric oxide (iNO) and a reactive oxygen intermediate (ROI). On the other hand, heat shock protein-70 (Hsp-70) expression may modulate TAMs to upregulate those molecules. *Typhonium flagelliforme* (TF) tuber extract has an apoptotic activity through increasing iNO and ROI levels of cancer cells. However, the role of TF in increasing the Hsp-70 expression of TAMs leading to apoptosis improvement remains unclear. TAMs were produced by coculturing the human breast cancer-derived peripheral blood mononuclear cells with the CSCs. TAMs were assigned into two treatment groups consisting of one treatment group (treated by TF at 50.89 µg/ml) and the control group (medium administration only). The expression of Hsp-70 was analyzed by immunocytochemistry. This study found that Hsp-70 expression was shown to be significantly increased in TAMs. In conclusion, TF may promote the increase of Hsp-70 expression in TAMs. Hsp-70 as a promising drug target in cancer therapy for reducing resistance to cancer therapy has the potential to be developed and investigated further.

INTRODUCTION

Human breast cancer is the most prevalent cancer in the world with the death rate 17.71 per 100,000 women (Bray *et al*., 2018). The current treatment to control the mortality rate of breast cancer has as yet not been optimal, due to the emergence of apoptotic resistance in cancer stem cells (CSCs) to several chemotherapeutic agents (Hermansyah *et al*., 2021). This is supposed to be due to the presence of CSCs within a tumor which have the ability to protect themselves from the apoptotic program by releasing the anti-apoptotic protein (He *et al*., 2014). On the other hand, tumor-associated macrophages (TAMs) as a CSCs niche play a significant role in promoting cancer cell proliferation by constructing immunotolerance and actively enhancing the CSC apoptotic resistance (Ge *et al*., 2020). TAMs enhance the apoptotic resistance of CSCs by extremely decreasing the low levels of inducible nitric oxide and a reactive oxygen intermediate (Perrotta *et al*., 2018). The lower level of reactive oxygen species (ROS) also inhibits the production of extracellular hydrogen peroxide on M2 macrophages leading to decreased signal transducer and activator of transcription 3 activation that inhibits apoptosis in cancer cells (Griess *et al*., 2020). On the other hand, heat shock protein-70 (Hsp-70) expression may modulate TAMs to upregulate those molecules (Gabai *et al*., 2016; Komarova *et al*., 2020). iNO stimulates the expression of Hsp-70, by means of ROI-mediated modification in intracellular antioxidant levels leading to apoptosis inhibition (Manucha and Valles, 2008). Currently, several drugs have

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been developed that target the tumor microenvironment (Bissell *et al*., 2011; Wartha *et al*., 2014), but our options to target TAMs are still limited. Therefore, the target Hsp-70 TAMs leading to induction of the apoptotic program are a promising strategy for the treatment of cancer.

Typhonium flagelliforme (TF) is a herbal medicinal plant widely used in various anticancer studies (Chodidjah *et al*., 2017; Lai *et al*., 2008; Purwaningsih *et al*., 2014). A previous study reported that TF contains saturated hydrocarbons, aliphatic acids, fatty acids, and linoleate acid (LA) (Lai *et al*., 2010; Mankaran *et al*., 2013). A previous study also reported that TF extract has the capability to inhibit cell growth of P388 lymphocytic leukemia cells (Choo *et al*., 2001). TF also induces apoptosis and G0/G1 cycle arrest in cellosaurus cells by upregulating caspase-9 and release of cytochrome c (Mohan *et al*., 2010). Apoptotic inducted LA is thought to be by binding on CSCs-peroxisome proliferator-activated receptors (PPARγ) (Arnett *et al*., 2018). The activated complexes of LA-CSCs-PPARγ initiate the activation of several proapoptotic proteins (Hsu and Ip, 2006). Our previous study also found that TF triggered apoptosis in CSCs by decreasing survivin expression and elevating caspase-3 and caspase-9 levels (Putra *et al*., 2018). However, the effect of TF in increasing Hsp-70 expression of TAMs leading to apoptosis improvement remains unclear. The current study is a continuation of our previous study, which has been previously reported (Putra *et al*., 2018). In the present study, we aimed to identify a possible pathway of Hsp-70-TAM-targeted apoptosis induction in CSCs by TF.

MATERIALS AND METHODS

Extraction of TF

TF (Araceae) herbs were collected from Semarang, Central Java, Indonesia (latitude 7.0051°S and longitude 110.4381°E). Authentication was conducted at the Plant Systematic Laboratory, Faculty of Biology, Universitas Gadjah Mada. 360.4 g of the fresh plant was harvested and washed thoroughly with circulating distilled water, followed by tubers being chopped into small pieces and dried (Memmert, Models 100–800, Schwabach, Germany). The dried tubers were powdered and macerated with hexane for 3 days to remove the nonpolar fractions before being treated with dichloromethanes for 7 days with intermittent shaking (Edmund Buchler Shaker, Model KS-15, Hechingen, Germany) and then filtered and vacuum-evaporated using a rotary evaporator (Buchi, R-210, Postfach, Switzerland) (Suzery *et al*., 2020).

Breast CSCs sorting

CSCs were isolated using magnetic-activated cell sorting (MACS) (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, total populations of MCF-7 breast cancer cells were harvested into a single cell suspension and counted to validate the quantity of the total cells. 300 g of 1 × 107 MCF-7 breast cancer cells was centrifuged for 10 minutes to obtain the pellet. The pellet was incubated with 10 µl microbeads directly conjugated to human monoclonal anti-human CD24-biotin antibody at 2°C–8°C for 15 minutes. Subsequently, the suspended cells were washed by a 1 ml buffer and added to the MACS column that was positioned in a MACS separator's magnetic field. The unlabeled CD24− cells were eluted from the column by a 500 µl buffer and collected as positively selected cells for further investigation. CD24− cell fractions were centrifugated at 300 g for 10 minutes, and then the cell pellet was resuspended in an 80 µl buffer. Finally, 20 µl CD44 microbeads were added to cell suspensions, and these were mixed and then incubated at 2°C–8°C for 15 minutes and then resuspended in a 500 µl buffer. The separation of CD44+ was performed by 500 µl flowed down the column MACS separator, followed by cell suspensions. The labeled cell fractions were flushed out by pushing the plunger into the column rapidly. The CD24−/CD44+ cell fractions were collected into a 15 ml tube.

CSCs validation

To induce mammosphere formation, several isolated CSCs (CD24−/CD44+) were plated in Dulbecco's modified Eagle medium F-12 (DMEM F-12), enriched with 1% bovine serum albumin, 1 µM insulin, 10 ng/ml basic fibroblast growth factor, 20 ng/ml epidermal growth factor, and B-27 supplement, in a low-cell-binding dish. Mammospheres emerged as tight, spherical structures floating in the medium.

Macrophage-polarized TAMs sorting

For the isolation of TAMs using MACS, the peripheral blood mononuclear cells (PBMCs) derived from human breast cancer patients were cocultured with MCF-7 in Roswell Park Memorial Institute media on 72 hours incubation. Human TAMs were purified by MACS with anti-CD163 followed by antiphycoerythrin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's protocol.

Hsp-70 analysis

Briefly, 5 × 104 CSCs and TAM ml−1 were seeded on adhesive slides-coated slides (Matsunami, Japan) and treated by TF concentration at 50.89 µg/ml as IC50 according to our previous study (Putra *et al*., 2018). The cells were incubated at atmospheric pressure of 5% CO2 and temperature 37°C for 24 hours and observed under an inverted microscope. After incubation, cells were fixed with 4% paraformaldehyde for 10 minutes, rinsed with phosphate buffer saline, and permeabilized for 10 minutes with 0.1% Triton-X100. Hsp-70 antigen retrieval was performed in a microwave for 20 minutes with 10 mM citrate buffer (pH 6.0). Endogenous peroxidase was then inactivated for 10 minutes in methanol with 3% H2O2. Ten percent of normal goat serum (Nichirei, Tokyo, Japan) was administered to the cells for 10 minutes and incubated with primary antibodies for Hsp-70 (rabbit polyclonal, Abcam, 1:100) at 4°C overnight. The secondary antibody, biotin-conjugated anti-rabbit IgG (Nichirei, Tokyo, Japan), was used to visualize antibody binding using peroxidase-conjugated streptavidin (Nichirei, Tokyo, Japan), followed by 3,3′-diaminobenzidine (DAB) solution (Metal-Enhanced DAB Substrate Kit, Thermo Scientific, USA). The cells were dehydrated and mounted after being counterstained with new hematoxylin (Muto Pure Chemicals Co., Ltd., Tokyo, Japan). The presence of Hsp-70 staining in TAMs was investigated.

The UALCAN database and the Kaplan-Meier Plotter (KM Plotter)

The UALCAN (http://ualcan.path.uab.edu/) database serves as a portal for tumor gene expression and survival studies (Wu *et al*., 2020). This study's screening conditions are as follows: Gene: HSPA1A; HSP1A1 is Hsp-70 kDa protein 1A that molecular chaperone implicated in a wide variety of cellular processes. The analysis type of this study used was Breast Cancer versus. Normal

Analysis; Cancer Type: Breast Invasive Carcinoma; and Data Type: the Cancer Genome Atlas Project (TCGA) dataset. We also evaluated the survival analysis using the breast cancer dataset of the KM Plotter. The screening conditions are as follows: "Cancer: Breast Cancer"; "Gene symbol: HSPA1A"; "Affy id: 203326_at"; "Survival: Relapse free survival" (Amalina *et al*., 2021; Cahyono *et al*., 2021; Mursiti *et al*., 2021).

Statistical analysis

All studies were performed at least three times, and 200 cells were evaluated for each plot in the immunocytochemical staining analysis. Results are expressed as mean ± SD and analyzed using one-way analysis of variance. Differences were analyzed by the Duncan post hoc test for all groups. $p < 0.05$ was considered to be significant. The statistical analysis was performed with Statistical Package for the Social Sciences version 22.0 (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Cancer cells have high expression of chaperones, particularly Hsp-70, which promote proliferation and escape from programmed cell death (Albakova *et al*., 2020; Komarova *et al*., 2020). Naturally, Hsp-70 can be released into the extracellular milieu by cancer cells without an additional trigger (Seclì *et al*., 2021). In addition, the higher level of Hsp-70 in cancer cells is associated with more chaperone molecules in the microenvironment of CSCs (Kabakov *et al*., 2020; Vega *et al*., 2008). Furthermore, TAMs constitute the tumor microenvironment cellular population which strongly influence cancer cell tumorigenicity including promoting cancer progression, development, metastasis, cancer stemness, and escape immune surveillance (Ge *et al*., 2020). A previous study reported that a pancreas cancer patient with a high TAM density had a lower survival rate (Jung *et al*., 2015). In fact, TAM depletion slows the progression of lung cancer (Fritz *et al*., 2014). In addition, the presence of tumors influences the maintenance of TAM polarization, indicating that tumor cells can maintain TAM polarization. The maintenance of TAM polarization is dependent on the presence of tumors, which suggests that tumor cells can maintain TAM polarization. A previous study also reported that TAMs can promote tumor progression and immune protection through inducing secretion of transforming growth factor-β, vascular endothelial growth factor, fibroblast growth factor, and several matrix metalloproteinases (Ge *et al*., 2020). Therefore, to establish a possible effect of Hsp-70-TAM-targeted apoptosis induction in CSCs by TF tuber extract as a natural chemotherapeutic agent is needed.

CSCs isolation and characterization

MCF-7 cells were used to isolate the CSC population based on the expression of CD44+/CD24− by MACS. The isolated CSC population expressed the highest CD44+/CD24− surface marker up to 93.80% ± 0.50% compared to CD44−/CD24− (3.10%), CD44−/CD24+ (0.10%), and CD44+/CD24+ <AQ> (2.50%) (Fig. 1A and B). High-level CD44 expression has been associated with cancer progression, whereas low-level CD24 expression has been associated with nondifferentiated cells (Crabtree and Miele, 2018). In addition, a high CD44+/CD24− ratio suggests primarily a potential for self-renewal capacity; thus, two markers are assigned different functions during tumor progression (Scioli *et al*., 2019). Furthermore, incubation of CSCs on a low-cell-binding dish with complete DMEM F-12 resulted in the formation of mammospheres (Fig. 1C).

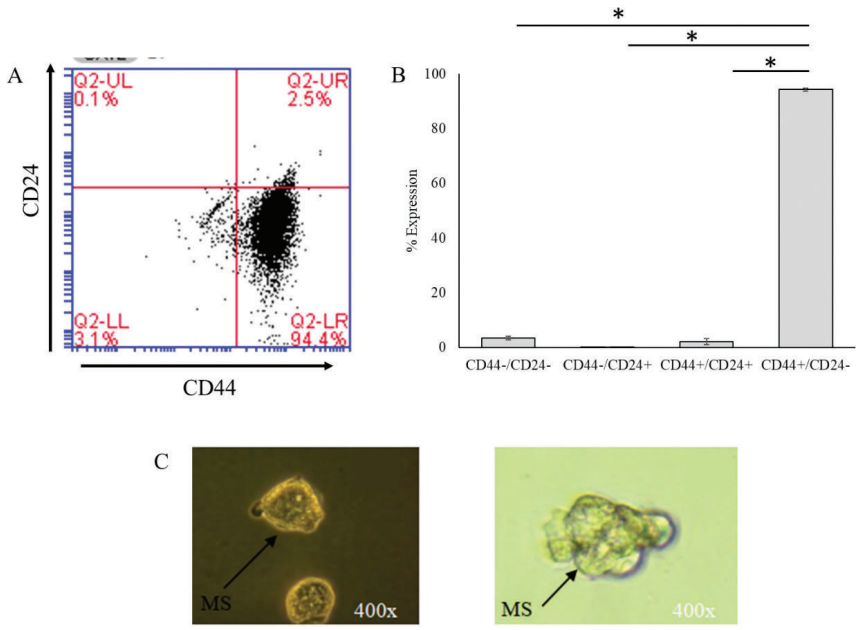

Figure 1. Characterization and validation of clone CSCs. (A) Flow cytometry detection of CD44 and CD24 markers on the surface of CSCs. (B) Percentage of surface marker CSCs, positive CD44, and CD24 negative cells. (C) The formation of mammosphere clones of CSCs. Black arrows indicate the morphology of mammospheres with a size in the range of 80–230 µm (*$p < 0.05$).

MCF-7-induced TAMs

PBMC was cocultured with the MCF-7 cell line to trigger the polarization of macrophages into TAMs. This study showed that the morphological change of macrophages occurred along with the increase of the MCF-7 population (Fig. 2A–F). This finding indicated that the M1 macrophage successfully polarized into the M2 macrophage leading to TAM formation.

TAM isolation and characterization

In order to investigate the effect of TF administration on coculture of a TAM and CSCs, first, we isolated a TAM from the tumor microenvironment using MACS using the CD163 surface marker. The isolated cell expressed a high level of CD163 up to 99.90% on flow cytometry analysis (Fig. 3A) and showed different morphological features compared to the negatively expressed CD163 cell colony (Fig. 3B). TAM colonies were used to analyze the effect of TF on Hsp-70 expression in coculture with a TAM and CSCs.

TF induces the expression of Hsp-70 in TAMs

Our previous study found that 50.89 µg/ml TF was shown to reduce 50% cell viability in CSCs (Putra *et al*., 2018). In order to further evaluate the activity of Hsp-70 on coculture CSCs with a TAM, we performed an immunocytochemistry assay. This study found that TF administration highly induces Hsp-70 expression in TAMs up to 67.67% (Fig. 4). Hsp-70 was localized on the cell membrane surface of cancer cells. Expression of HSPA1A (Hsp-70) across TCGA tumors indicated that the expression level of Hsp-70 was higher in all TCGA tumors than its matched normal tissue in most of the tumors (Fig. 5A), including in breast cancer invasive tumor. We also gathered clinical data for 1,097 cancer patients from the TCGA. Patients with high expression of Hsp-70 had significantly less likelihood of survival than patients with low medium expression of Hsp-70 (Fig. 5B). The results suggest that the presence of Hsp-70 in breast cancer cell predictors can be an indicator of likelihood of survival in breast invasive carcinoma patients.

The positive expression of Hsp-70 expression in TAMs in this study indicated that TF might induce the expression of Hsp-70 TAMs and then is secreted into an extracellular niche which bind to the transmembrane receptor of the TAM itself. This may lead to polarization of the TAM (type 2 macrophage) into a type 1 activated macrophage (M1) (Orecchioni *et al*., 2019). The active macrophages release the TNF-α ligand molecules (proinflammatory cytokines) and may bind to tumor necrosis factor receptor-1 of CSCs, leading to apoptosis of CSCs via an extrinsic apoptotic pathway (Ge *et al*., 2020; Holdbrooks *et al*., 2018). This is supported by a previous study where the increase of intracellular Hsp-70 expression would be secreted in part extracellularly and interacts with the surrounding microenvironment including macrophages that may bind to PPARγ (Wahli and Michalik, 2012). This suggested that TAM polarization to type 1 may strengthen

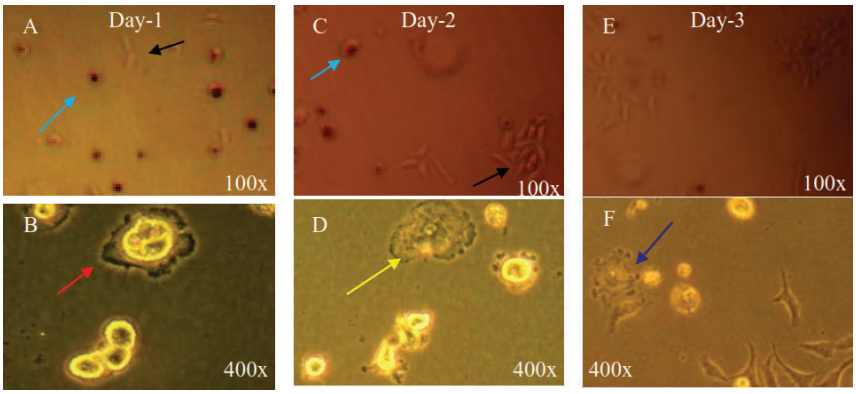

Figure 2. Morphological change in the PBMC cocultured with the MCF-7 cell line. (A) The PBMC on day 1 after coculture (blue arrow) with the MCF-7 cell line (black arrow). (B) Pseudophili formation (red arrow) indicates the polarization of the hematopoietic progenitor into a macrophage. (C–D) The increased number of the MCF-7 population triggers macrophages to undergo the inactive phase (yellow arrow). (E) The number of the MCF-7 population continues to increase leading to macrophage polarization into TAMs (F) (blue arrow).

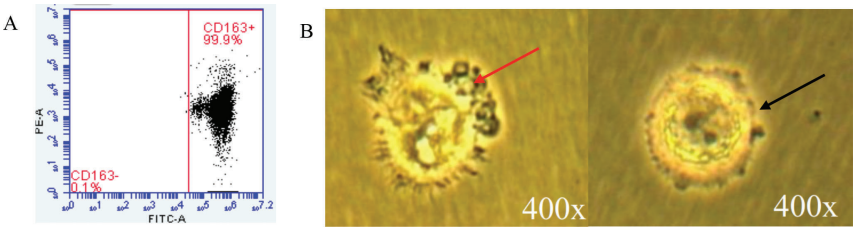

Figure 3. CD163+ on coculture of TAM and CSCs. (A) Flow cytometry detection of CD163+ and (B) morphological features of the coculture population. Pseudophili formation (red arrow) and macrophage polarization into TAMs (black arrow).

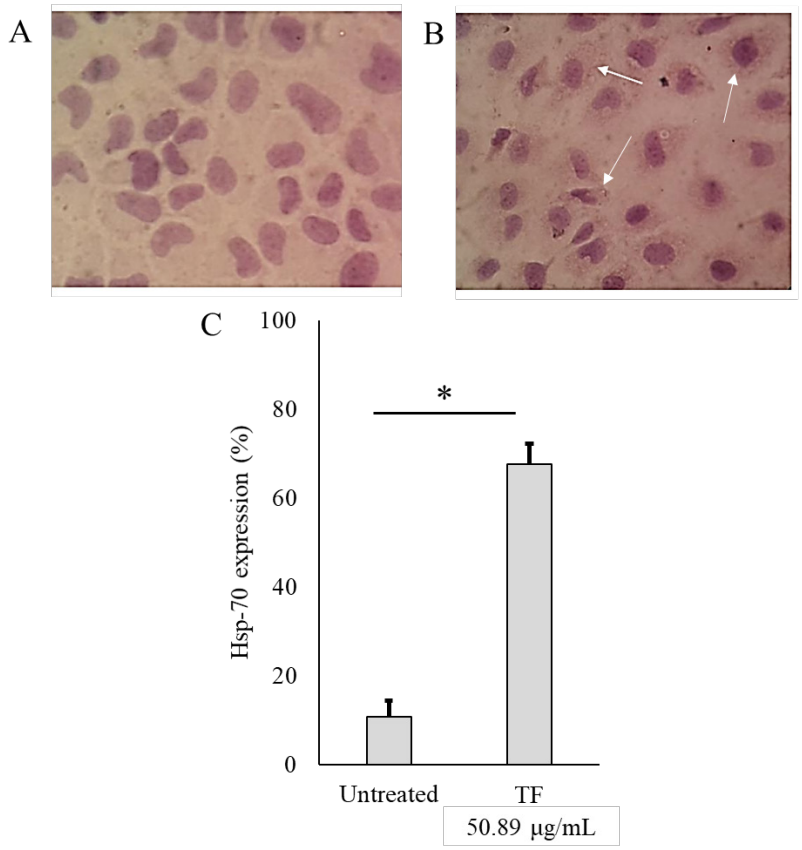

Figure 4. Hsp-70 immunocytochemistry staining, the expression of Hsp-70 determined by brown color on the surface of the cell. (A) Control cells negatively express Hsp-70, (B) highly express Hsp-70 (white arrow), and (C) quantification of Hsp-70 expression. Values in the bar charts are presented as means ± standard deviation. *$p < 0.05$ versus untreated cells.

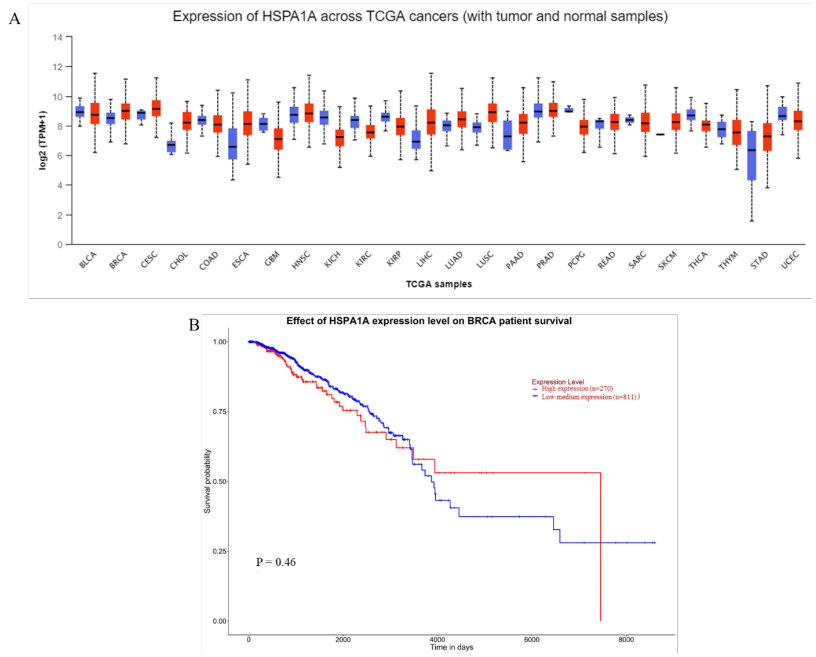

Figure 5. (A) Expression of HSPA1A (Hsp-70) across TCGA cancers versus normal. (B) Survival curve analysis of TCGA breast cancer patients based on HSPA1A (Hsp-70) expression. The patients were divided into two groups. One group had high expression of Hsp-70, and the other group had low medium expression of Hsp-70.

apoptotic induction to CSCs. The limitation of this study is that we did not analyze the polarization of TAMs into type 1 activated macrophages as well as the soluble molecule released. Taken together, our data suggest that TF may enhance the CSCs cell death associated with positive expression of Hsp-70 in TAMs.

CONCLUSION

Based on our findings, we conclude that the TF as a natural chemotherapeutic agent may decrease the CSC population by increasing Hsp-70 expression in TAMs. These findings also indicate that the potential anticancer activities of TF associated with TAM polarization should be further developed for cancer treatment in the future.

AUTHORS' 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.

CONFLICTS OF INTEREST

The authors have declared that no conflicts of interest exist.

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ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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