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ORIGINAL ARTICLE

The Effect of *Phyllanthus debilis* Methanolic Extract on DNA Methylation of *TAC1* Gene in Colorectal Cancer Cell Line

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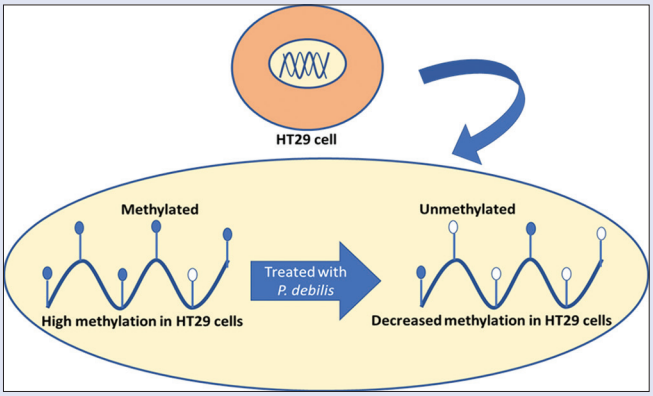
ABSTRACT

Aim/Background: In colorectal cancer, *TAC1* was shown to be highly methylated in early carcinogenesis. Using HT29 cell line as a model of colorectal cancer, we postulated that *Phyllanthus debilis* methanolic extract could regulate DNA methylation in *TAC1* gene promoter region and thus could alter the progression of colorectal cancer. **Methodology:** Cell culture study was done using HT29 cells, which were treated with 0.117 mg/ml *P. debilis* methanolic extract or 0.5 μM 5-Aza-2-Deoxycytidine (5-Aza) for 72 h. Cells were harvested at 72 h and were extracted for DNA. The DNA was bisulfite modified before been PCR and later was pyrosequenced. **Results:** The treatment with *P. debilis* significantly decreased the DNA methylation of *TAC1* gene at site 1 (93.1% ± 2.4% vs. 100% ± 0.1%, $P < 0.05$), site 2 (86.5% ± 1.3% vs. 91.7% ± 0.2%, $P < 0.05$), and site 3 (96.7% ± 0.9% vs. 100% ± 0%, $P < 0.05$); but, no significant changes of DNA methylation were seen at site 4 (96.5% ± 1.9% vs. 93.6% ±0.6%, $P > 0.05$). The average of all Cytosine nucleotide followed by Guanine nucleotide (CpG) sites methylation was reduced, but not statistically significant when compared to the untreated cells (mean methylation 93.2% ± 2.4% vs. 96.3% ± 2.2%, $P > 0.05$). For cells treated with 5-Aza, DNA methylation was significantly decreased only at site 2 (88.6% ±0.8% vs. 91.7% ±0.2%, $P < 0.05$), but there was no significant methylation changes at site 1 (94.8% ± 2.4% vs. 100.0% ±0.06%, $P > 0.05$), site 3 (98.9% ± 1.1% vs. 100% ± 0%, $P > 0.05$), site 4 (90.6% ±1.6% vs. 93.6% ±0.6%, $P > 0.05$), and the average of all CpG sites (mean methylation 93.2% ± 2.3% vs. 96.3% ± 2.2%, $P > 0.05$). **Conclusion:** Treatment of methanolic extract of *P. debilis* reduces the methylation of promoter region of *TAC1* gene, with better effect than low dose 5-Aza at 72 h of treatment. The anticancer effect of *P. debilis* may be partly been regulated through DNA methylation.

Key words: HT29, methylation, *Phyllanthus*, pyrosequencing, *TAC1*

SUMMARY

- The methanolic extract of *Phyllanthus debilis* reduces the methylation of promoter region of *TAC1* gene. The anticancer effect of *P. debilis* may partly been regulated through DNA methylation.



Abbreviations used: °C: Degree celcius; mg/mL: Milligram per milliliter; μL: Microliter; min: Minutes; ng: Nanogram; nM: nanoMolar; mM: MilliMolar; μM: MicroMolar; HT29: Colon cancer cell line; h: Hour; PCR: Polymerase chain reaction.

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INTRODUCTION

Colorectal cancer is one of the most common cancers in Malaysia. It is the second most common cancer affecting males after lung cancer and the third most common cancer affecting females, after breast and uterine cancer.[1] Most of the patients were diagnosed at the late stage, and their 5 years' survival rate was lower than other Asian developed countries.[1] There is no formal/structured national colorectal cancer screening program in Malaysia at the moment. In the developed countries such as in Japan, South Korea, and Singapore, colorectal cancer is one of the most common cancer found in their population.[1] However, the incidence and mortality rates have been stable and are even declining in these countries. This trend may be associated to colorectal cancer screening programs, reduced prevalence of risk factors and/or improved treatments in these countries.[1] Colorectal cancer screening program was shown to reduce colorectal cancer mortality up to 53% in developed countries.[2] Colorectal screening program however was difficult to implement in other parts of the world due to lack of optimal strategy and public acceptance.[2] The most common methods of detection of colorectal cancer are through fecal occult blood test (FOBT) and sigmoidoscopy. However, these two methods had been shown to have poor sensitivity and specificity with the latter at risk of getting gut perforation.[2]

For the last 10 years, DNA methylation-based detection was becoming more common with the availability of sequencing machine. The

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measurement of *SEPT9* gene methylation in peripheral blood had been approved by the United States Food and Drug Administration (FDA) as one of screening methods to detect early colorectal cancer.[3] The monitoring of the changes in the methylation of this gene had shown that it is more accurate and sensitive compared with the current detection of colorectal cancer such as FOBT, carcinoembryonic antigen, and Ca-199.[3,4] Apart from *SEPT9* gene, other gene that is candidate for colorectal cancer biomarker is *TAC1*. DNA hypermethylation of *TAC1* gene was common observation in early colorectal cancer.[5] In this study, we postulated that *Phyllanthus debilis* methanolic extract could regulate the methylation of the *TAC1* gene, in which it was commonly methylated in HT29 cells.

Phyllanthus sp. is one of the herbals that has been used traditionally for treatment of many illnesses such as hepatitis, renal stone, and cancer. *Phyllanthus* sp. has been shown to possess hepatoprotective, anticarcinogenic, antibacterial, antiviral, and anti-inflammatory activity. We had shown in our previous studies that *P. debilis* contained strong antioxidant activity with higher anticancer activity and less toxicity than other *Phyllanthus* species such as *Phyllanthus urinaria* and *Phyllanthus niruri*.[6,7] Based on this result, we had chosen *P. debilis* as our candidate herb to study its mechanism on DNA methylation. Our study is aimed to look at the effect of methanolic extract of *P. debilis* on the methylation of HT29, a colorectal cancer cell line. We compared the effect of *P. debilis* with 5-Aza, a chemotherapeutic drug which the main mechanism as anticancer drug was through regulation of DNA methylation.

METHODOLOGY

Herbal specimen

P. debilis was collected from local collection at Tasek Gelugor, Penang, Malaysia. It was identified and deposited at the Universiti Sains Malaysia herbarium (voucher specimen: 11623).

Methanol extraction

Briefly, whole plant of *P. debilis* was dried in the oven at 50°C for 3 days. Once dried, it was grounded and prepared in the powder form. Five gram of sample was then used to be extracted with 100 mL of methanol (Fisher Chemical) in ultrasonic bath (Power Sonic 405) for 20 min and then filtered. The process was repeated twice with the remaining residual extract. The extracts were dried using rotary evaporator (Buchi Rotary Evaporator RII). Dried extract was kept at −20°C for further use.

Cell culture

HT29 cells were cultured in 24 well plate in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco). Cells were maintained at 37°C in a 5% CO_2 atmosphere.

When the cells reach to 80% confluency, they were treated with 0.117 mg/mL *P. debilis* extract or 0.5 μM of the demethylating agent 5-Aza-2-Deoxycytidine (Acros). Treatments were continued for 3 days (72 h), while replacing RPMI-1640 culture medium (Gibco) plus *P. debilis* or 5-Aza daily. Cells were harvested at 72 h and were pooled for further used.

PCR and Pyrosequencing

HT29 cells were assayed for gene-specific methylation at the promoter of *TAC1* gene. Each of the samples was technically replicates for four times. The promoter of *TAC1* gene (GX_94242 [TAC1/human]) was identified using Genomatix software (Intrexon Bioinformatics Germany).

DNA was extracted from HT29 cells using DNA extraction kit (Promega, USA). The DNA was then bisulfite converted using EZ DNA Methylation Gold™ kit (Zymo Research) according to the manufacturer's protocol. Briefly, 500 ng of DNA was incubated with CT conversion reagent at the following temperatures: 98°C for 10 min, 64°C for 2.5 h, held at 4°C. Once completed, the DNA was transferred to a spin column, washed, and desulphonated. It was further purified using wash buffer before being eluted in 20 μL deionized water.

The bisulfite modified DNA (3 μL) was used in a polymerase chain reaction (PCR) reaction containing 12.5 μL GoTaq Green mastermix (Promega), 400 nM forward primer, and 400 nM biotin-labeled reverse primer in a total volume of 25 μL. PCR amplification was carried out using the following protocol: 95°C 15 min, then 50 cycles of 95°C 15 s, annealing temperature 50°C for 30 s, 72°C for 30 s, followed by 72°C for 5 min. The primer sequences and sequence to analyze were summarized in Table 1.

The PCR product which was biotin-labeled was captured with Streptavidin Sepharose beads (GE Healthcare), and using a Pyrosequencing Vacuum Prep Tool (Qiagen), the PCR product was made into single stranded DNA. The sequencing primer was annealed to this strand by heating to 80°C, followed by slow cooling to the room temperature. Pyrosequencing was then carried out on a Pyromark ID (Qiagen). The methylation at CpG sites was quantified using PyroMark Q96 2.5.8 software. Primer sequences used in the PCR reaction and pyrosequencing and sequence to analyze for *TAC1* assay were shown in Table 1.

Statistical analysis

The differences of mean in treated and untreated cells were analyzed using student *t*-test (GraphPad Prism Version 6.01). All the values are presented as mean ± standard deviation and mean ± standard error of mean.

RESULTS

We measured the methylation changes of *TAC1* DNA methylation at 4 CpG sites, with the treatment of the HT29 cells with *P. debilis* and 5-Aza for 72 h. We compared the methylation changes of the untreated cells with treated cells at each CpG site and overall CpG sites methylation.

On treatment with *P. debilis* methanolic extract at 72 h, DNA methylation showed a significant reduction of DNA methylation at site 1 ($P < 0.05$), site 2 ($P < 0.05$), and site 3 ($P < 0.05$), but not significantly reduced at site 4 ($P > 0.05$) when compared with untreated cells.

When HT29 cells were treated with 0.5 μM 5-Aza, there was a significant decrease of DNA methylation at only 1 CpG site, which was site 2 ($P < 0.05$) when compared to untreated cells. Whereas, no significant DNA methylation changes was observed at site 1, site 3, and site 4 ($P > 0.05$).

For both treatments, the average reduction of DNA methylation at all CpG sites (reduction of 3% when compared with untreated cells) did not show any significant decrease of methylation when compared to the untreated cells ($P > 0.05$). Results of DNA methylation at each CpG site and overall CpG sites for both treatments were shown in Table 2.

DISCUSSION

Methylation at CpG islands, which happened commonly at the promoter region of tumor suppressor genes, can induce gene silences and promote the early changes to cancer.[8] *TAC1* gene encodes 4 products of the tachykinin peptide hormone family, which are mainly substance P and neurokinin A and also neuropeptide K and neuropeptide gamma. They are functioning as neurotransmitter, which interact with nerve receptor and smooth muscle cells and as antimicrobial peptide (substance P), vasodilators, and secretagogues.[9]

Table 1: The primers sequences used in the polymerase chain reaction and pyrosequencing and the sequence to analyze for *TAC1* assay

Assay	Forward primer	Reverse primer	Sequencing primer	Sequence to analyze
TAC1	ATGAGTTATTTTGTTTAAATTTGATGA	ATTTATCCCAACCTCCTTAAAC	TGGGTTTAGATGTTATGG	GTATC/TGAC/ TGAGTTATC/TGTTTC/TG

Table 2: Percentage of *TAC1* DNA methylation at 4 CpG sites and its mean of overall sites

Group	Site 1	Site 2	Site 3	Site 4	Average of all CpG sites
Untreated cells	100.0±0.1	91.67±0.2	100±0	93.6±0.6	96.3±2.2
Treatment with *Phyllanthus debilis*	93.1±2.4*	86.5±1.3*	96.7±0.9*	96.52±1.9	93.2±2.4
Treatment with 5-Aza	94.8±2.4	88.6±0.8*	98.9±1.1	90.6±1.6	93.2±2.3

*Significant different between the two groups measured using student *t*-test. Data presented as mean±SD % for each CpG site and mean±SEM % for all CpG sites. SD: Standard deviation; SEM: Standard error of mean; CpG: Cytosine nucleotide is followed by Guanine nucleotide

TAC1 gene was frequently used as a panel for detection of early cancer, in which the detection of *TAC1* methylation was shown to increase the sensitivity of detection in early lung cancer to up to 93%[8] whereas in colorectal cancer, *TAC1* combined with *SEPT9* methylation can detect early colorectal cancer with 73.1% sensitivity and 92.3% specificity,[5] much higher than current detection methods using FOBT and sigmoidoscopy. Furthermore, *TAC1* methylation can predict the recurrent of colorectal cancer postsurgery.[10]

Our study showed that treatment with *P. debilis* induced small changes in DNA methylation of *TAC1* genes. Although the changes were very small, they did give a statistical significance at 3 out of 4 CpG sites, which were CpG site 1, site 2, and site 3. When compared with 5-Aza-treated cells, the methylation changes were seen at only 1 CpG sites (site 2). For both treatments (*P. debilis* methanolic extract and 5-Aza), the overall changes of methylation were almost similar, even though it was insignificant (both treatments showed 3% reduction of DNA methylation in overall CpG sites). We believed that the effect of *P. debilis* exerted in the DNA methylation at *TAC1* promoter region may be site specific. To our surprise, we did not see the marked changes in methylation in majority of the CpG sites in *TAC1* genes when treated with the lower concentration of 5-Aza (0.5 μM). The dose that we used was probably too low to induce site-specific demethylating changes at *TAC1*-targeted promoter region. The concentration of 5-Aza at 0.5 μM used in this study, however, was shown previously to demethylate genes at genome-wide scale.[11,12]

Result from our study showed that *P. debilis* was able to reduce DNA methylation of *TAC1* gene, a possible mechanism that *P. debelis* exerts as anticancer properties. Previously, we had shown that *P. debilis* methanolic extract had a strong anticancer activity in breast cancer cell line (MCF-7) with less toxicity in the normal breast cells (MCF-10A).[6] *P. debilis* contained high total phenolic and total flavonoid compounds with high antioxidant activity.[7] Analysis of *P. debilis* by high-performance liquid chromatography showed that the extract contains some beneficial compounds such as caffeic acid, p-coumaric acid, myricetin, and kaempferol (data not shown). Natural compounds such as caffeic acid and dietary catechol were shown previously to regulate DNA methylation by demethylating the *RARβ* gene through inhibition of DNA methyltransferase 1 (DNMT1) enzyme.[13] The process was through an increased formation of S-adenosyl-L-homocysteine, a by-product of catalyzed methylation process, which acts as a feedback inhibitor to S-adenosyl-methionine (a methyl donor of methylation reaction)-dependent methylation processes.[13] Figure 1 showed the methylation process, which was catalyzed by the DNMT enzyme family. This process requires S-adenosylmethionine as a cofactor.[14,15]

In cancer, hypermethylation of genes promoter region of tumour suppressor genes were commonly occurred, which silenced its expressions. Reswitching the expression of these genes will exert protective effect to cancer development and will have the possibilities

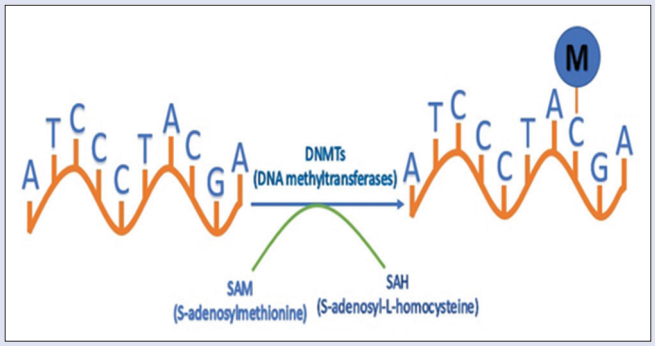

Figure 1: Methylation process catalyzed by the DNA methyltransferases enzyme family. This process requires S-adenosylmethionine as a methyl donor. S-adenosyl-L-homocysteine, a by-product of catalyzed methylation process of SAM. 'M' indicates methyl group

of changing back the cancer phenotype to normal phenotype. We believe that the re-expression of *TAC1* in colorectal cancer cells may have beneficial effect in the long-term by improving the abnormal *TAC1* function (deranged inflammatory and immune) back to normal.

CONCLUSION

Methanolic extract of *P. debilis* reduced the methylation of *TAC1* gene in HT29 cancer cell line. This reduction of methylation may have an effect on the gene functionality. The *P. debilis* methanolic extract may exert its anticancer through regulation of DNA methylation.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Veettil SK, Lim KG, Chaiyakunapruk N, Ching SM, Abu Hassan MR. Colorectal cancer in Malaysia: Its burden and implications for a multiethnic country. Asian J Surg 2017;40:481-9.
2. Ng SC, Wong SH. Colorectal cancer screening in Asia. Br Med Bull 2013;105:29-42.

3. Wang Y, Chen PM, Liu RB. Advance in plasma SEPT9 gene methylation assay for colorectal cancer early detection. World J Gastrointest Oncol 2018;10:15-22.
4. Xie L, Jiang X, Li Q, Sun Z, Quan W, Duan Y, *et al.* Diagnostic value of methylated *Septin9* for colorectal cancer detection. Front Oncol 2018;8:247.
5. Liu Y, Tham CK, Ong SY, Ho KS, Lim JF, Chew MH, *et al.* Serum methylation levels of TAC1. SEPT9 and EYA4 as diagnostic markers for early colorectal cancers: A pilot study. Biomarkers 2013;18:399-405.
6. Omar WA, Zain SN. Therapeutic index of methanolic extracts of three Malaysian *Phyllanthus* species on MCF-7 and MCF-10A cell lines. Pharmacogn J 2018;10.
7. Zain SN, Omar WA. Antioxidant activity, total phenolic content and total flavonoid content of water and methanol extracts of *Phyllanthus* species from Malaysia. Pharmacogn J 2018;10.
8. Powrózek T, Małecka-Massalska T. DNA hypermethylation of tumor suppressor genes as an early lung cancer biomarker. Transl Cancer Res 2016;5:S1531-8.
9. NCBI. TAC1 Tachykinin Precursor 1 [Homo Sapiens (Human)]. USA: NCBI; 2019. Available from: https://www.ncbi.nlm.nih.gov/gene/6863. [Last accessed on 2019, Apr 02].
10. Tham C, Chew M, Soong R, Lim J, Ang M, Tang C, *et al.* Postoperative serum methylation levels of TAC1 and SEPT9 are independent predictors of recurrence and survival of patients with colorectal cancer. Cancer 2014;120:3131-41.
11. Ishiguro M, Iida S, Uetake H, Morita S, Makino H, Kato K, *et al.* Effect of combined therapy with low-dose 5-aza-2′-deoxycytidine and irinotecan on colon cancer cell line HCT-15. Ann Surg Oncol 2007;14:1752-62.
12. Khamas A, Ishikawa T, Shimokawa K, Mogushi K, Iida S, Ishiguro M, *et al.* Screening for epigenetically masked genes in colorectal cancer using 5-Aza-2′-deoxycytidine, microarray and gene expression profile. Cancer Genomics Proteomics 2012;9:67-75.
13. Lee WJ, Zhu BT. Inhibition of DNA methylation by caffeic acid and chlorogenic acid, two common catechol-containing coffee polyphenols. Carcinogenesis 2006;27:269-77.
14. Martin EM, Fry RC. Environmental influences on the epigenome: Exposure- associated DNA methylation in human populations. Annu Rev Public Health 2018;39:309-33.
15. Wang Y, Sun Z, Szyf M. S-adenosyl-methionine (SAM) alters the transcriptome and methylome and specifically blocks growth and invasiveness of liver cancer cells. Oncotarget 2017;8:111866-81.