Protein-Based Biomaterial Marker in Metabolic Syndrome and Colorectal Cancer: A Preliminary Clinical Study of Betatrophin Expression in Javanese Ethnic

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Abstract. The presence of specific and unique chemical structure of a protein with their bioavailability properties provide a hallmark of the essential contribution of the biomaterials. Protein-based biomaterials become a novel trend in the biomedical field, particularly on clinical management and biomarker development. Blinding proteins and other materials in an advanced technological approach is crucial to generating protein-based biomaterials for biomedical. Recently, liver-derived protein, betatrophin/lipasin/ANGPTL-8 showed a potential property as the future biomaterial marker for early detection of gastrointestinal cancer related metabolic syndrome. The betatrophin expression is significantly correlated with cancer incidence and lipid metabolism disorder in a subject with obesity, type 2 diabetes, and insulin resistance. This clinical study aimed to develop betatrophin as the potential protein-based biomaterial marker in colorectal cancer (CRC) and metabolic syndrome, especially among Indonesian subjects. Our preliminary study focuses on Java ethnic as the dominant population with a higher incidence of CRC. The general anthropometric data were collected from CRC patients with different stages and metastasis status. The expression of betatrophin and metastasis related genes were quantified by real-time quantitative PCR (RT-qPCR). Interestingly, the basic profile of betatrophin and genes linked to cancer metastasis showed significant expression in our baseline data. Betatrophin/lipasin/ANGPTL-8 activity correlated to another gene that involved in the progression of cell migration and malignant cell invasion. Thus, we suggested that the alteration of betatrophin and its related genes expression may associate with the progression of CRC incidence in a subject with onset metabolic syndrome. Betatrophin could be proposed as the novel protein-based biomaterial marker in an individual with early stages of colorectal cancer. Therefore, the future expanded clinical study is necessary to well establish the biomaterial property of this liver protein in CRC diagnosis and prognosis.

Keywords: Betatrophin, clinical study, biomaterial, marker, metabolic syndrome, colorectal cancer
1. Introduction
In the global population, the emerging condition related cancer mortality still pay more attention to clinicians and researcher in several countries [1]. The rapid changes in human lifestyle are significantly triggered colorectal cancer (CRC) development in the individual in particular caused by the western diet. As a consequence, CRC was well established as the third silent killer in the community associated cancer [2]. Importantly, the prevalence of colorectal cancer is not only the primary concern for western society but also for Asian countries [3]. Furthermore, CRC incidence has been gradually increasing and categorized as the critical cause of patient deaths in Asia, especially in East, South, and South East Asian countries [4,5]. In addition, there is a lack supporting finding whether the alteration of reported Betatrophin correlated to insulin secretion through PI3K/Akt and insulin resistance in the disorder and suggested related to early stage of cancer. Among them, betatrophi

The development of clinical screening to predict the early symptom of CRCs has been widely used on clinics including stool-based test despite the classical detection by using endoscopy such as colonoscopy and sigmoidoscopy [7]. This method aims to reduce the risk of patients mortality due to CRC. The clinical administration for CRC patients through colonoscopy and sigmoidoscopy were claimed reduced the incidence of colorectal cancer in the European population [8]. This method also proposed as the dominant approach to detect the development of adenoma at the early stage of CRC [9]. However, the major problem remains whereas the participation of patient to involve in the screening process significantly different among region [10]. Thus, an additional alternative protocol to detect the early development of adenoma and less participation of individual suspected CRC is required.

To date, the exploration of sensitive and specific prognostic or predictive biomarker is crucial to provide the essential information for patients, and how the clinicians should decide the appropriate therapy for the individual or personal based on their medical record [11]. The invention of CRC screening and clinical administration related to specific biomaterials-based protein marker may be essential for the future individual based-therapy. The exploration of a novel biomarker can allow the clinicians to use non-invasive methods, sensitive assay, cost-effective procedure for diagnostic and prognosis, and define the dominant biomaterials marker within the human body for available treatment [12,13].

Importantly, the previous findings found that CRC incidence has a significant association with metabolic disturbances [14,15]. Metabolic syndrome is the independent risk factor for colorectal carcinoma compared to another predictor and suggested correlated to recurrence of CRC in the patients [16]. Obesity, diabetes mellitus, and cancer were reported co-interact results in the development the neoplasm by sharing through specific pathway such as insulin-like growth factor-1 (IGF-1), leptin pathway, and proinflammatory signaling pathway related obesity-T2DM [17]. The gradual changes in patient body weight develop to obesity was considered induce colorectal cancer via oxidative stress pathway [18,19]. The first incidence of central obesity caused by rapid overweight status and its screening is crucial since this pathogenic stage play as the inducer of CRC [20,21]. Moreover, the increased serum level of triglyceride induced central obesity significantly triggers the colorectal polyps formation [22]. Therefore, the necessary profiling of patients lipid status and specific potential gene expression related to triglyceride metabolism is crucial.

Recently, the development of clinical biomarker based on the protein-endocrine material has become the primary concern in the clinic. This biomaterials-based protein offers an alternative to predict the early stage of cancer. Among them, betatrophin/leptin/c19orf80/ANGPTL-8/hepatocellular carcinoma-associated gene TD26 is the new liver-derived hormone that strongly associated with lipid metabolism disorder and suggested related to cancer induced by metabolic perturbation [23,24]. Besides the fundamental contribution in lipid metabolism linked triglyceride level, betatrophin also significantly correlated to insulin secretion through PI3K/Akt and insulin resistance in the diabetic subject [25–29]. Betatrophin is the primary regulator of triglyceride level [30,31] and suggested associated with glucose tolerance status in an individual with T2DM and impaired glucose tolerance [32]. Betatrophin was reported as the potential biomarker to tracer the molecular trafficking of triglyceride and lipoprotein lipase activity [33]. However, although several reports have been proposed betatrophin as the pivotal regulator in triglyceride metabolism, there is a lack supporting finding whether the alteration of
betatrophin expression indirectly associated with CRC by regulating the individual lipid status. Hence, the supporting clinical data from different stages of CRC patients for betatrophin expression is necessary.

2. Methods

2.1. The preparation of the sample
The subjects were collected from different stages of CRC based on CT scan data. This study was registered to the ethical committee with registration number KE/FK/1242/EC/2017. The individual with incomplete criteria such as treated by chemotherapy or radiotherapy was excluded from the chosen sample. The participants or patients have filled the informed consent form and purely origin from Javanese ethnic. About 38 subjects were involved in this clinical study, and the medical record of this patients was obtained from the hospital baseline database. The protocol of this research was performed according to the declaration of Helsinki. All the tumor samples were classified based on the tumor-node-metastasis criteria/staging system.

2.2. Betatrophin and cancer metastasis-related gene expression

2.2.1. Total RNA Isolation
The total RNA from colon tissue were performed by using RNA total isolation kit (TRISURE™ BIOLINE, UK). A small piece of fresh tumor samples (100 mg of tissue) were homogenized in 1 mL of TRIsure and incubated room temperature for 5 minutes. The samples were then added 0.2 mL of chloroform per 1 mL of TRIsure and shook vigorously by hand for 15 seconds followed by 3 minutes incubation at room temperature. Next, the mixing samples were homogenized and separated by centrifugation at 12,000 x g for 15 minutes at 4 °C. The aqueous phase was transferred very carefully into a new tube and precipitated by mixing with 0.5 mL cold isopropyl alcohol per 1 mL of TRIsure. In the following step, all samples were incubated at room temperature for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4 °C. The supernatant was removed, and the pellet was washed once time with 1 mL 75% ethanol per 1 mL of TRIsure. The samples were mixed by vortex and centrifugated at 750 x g for 5 minutes at 4 °C. The pellets were dried by the air-drying process and dissolved in in DEPC-treated water by pipetting the solution up and down. At the final step, the RNA samples were incubated for 10 minutes at 60 °C and stored at -20 °C for the next analysis.

2.2.2. Reverse Transcription PCR
The conversion of RNA into cDNA was done by employing a reverse transcription kit (ReverTra Ace® qPCR RT Master Mix with gDNA Remover, TOYOBO, Japan). The premix was prepared by mixing 1 μL of gDNA Remover: 50 μL of 4x DN Master Mix” mixture. The RNA template/samples (6 μL) were placed on the rotor tube, incubated at 65 °C for 5 min and keep on ice for a while. The DNase I reaction solution was prepared on ice by following the formula: 2 μL of 4x DN Master Mix and 6 μL of RNA template. Then, the solution was incubated at 37 °C for 5 minutes. Furthermore, reverse transcription solution was prepared on ice with the specific formula: 8 μL of DNase I reaction and 2 μL of 5x RT Master Mix II. The transcription solution was then incubated at 37 °C for 15 min, at 50 °C for 5 min, and at 98 °C for 5 min. At the final procedure, the reacted solution then was stored at 4 °C or -20 °C.

2.2.3. Real Time-quantitative Polymerase Chain Reaction
The reaction mix solution was set up by the following formula: one reaction volume consisting of 10 μL of 2x SensiFAST SYBR® No-ROX Mix, 1 μL of 10 μM forward primer, 1 μL of 10 μM reverse primer, 1 μL of cDNA template, and 7 μL of sterile distilled water (SensiFAST SYBR No-ROX kit, BIOLINE, UK). The primers sequence was arranged as follow: Betatrophin, forward 5’AGACTCAGATGGAGGAGA 3’ and reverse 5’ ATGCTGCTGTGCCACCATCT 3’; TGFBR1, forward 5’ ACGGCGTTACAGTGGTCTG 3’ and reverse 5’ GCACATAAAACGGCGCTATCTC 3’;
SNAIL, forward 5′ ACTGCAACAAGGAA TACCTCAG 3′ and reverse 5′ GCACCTGGTACTCTTGACATCTG 3′; E-Cadherin, forward 5′ AAAGGCCCA TTTCCTAAAAACCT 3′ and reverse 5′ TCGTTCCTCTATCCAGAGGCT 3′; β Actin, forward 5′ CATGTCGTTTGCTATCCAGGC 3′ and reverse 5′ CTCTTAAATGTCACGCACGAT 3′; GAPDH, forward 5′ ACAACTTTGGTA TCGTGGAAGG 3′ and reverse 5′ GCCATCAC GCCCACAGTTTC 3′. The PCR program was set up as 2-step cycling following: 1 cycle at 95 °C in 2 minutes for polymerase activation then 40 cycles consist by 95 °C in 10 seconds for denaturation step and Tm primer temperature set by in 30 seconds for annealing/extension.

2.3. Data Analysis
All the data were analyzed by using JMP 7 and GrapPadPrism 5 software for the statistical analysis. The data was shown as mean±SD with p-value < 0.05 to indicate a statistically significantly different among groups.

3. Results and Discussion
The basic characteristics of the patient were shown in Table 1. About 41 subjects were enrolled in this clinical case study and separated into two groups. The group of this study was clustered into early stage (stage I and II) and late stage (stage III and IV) based on TNM categories. The early stage group comprised 8 male and 5 female while the last stage group included 13 male and 12 female. Most of the patients were in T3 and T4 classification or categories in both groups. Lymph node metastasis was observed in 7 individuals.

The aim of this study was to develop the early biomarker against cancer in particular betatrophin as the potential protein-based biomaterial marker in colorectal cancer (CRC) onset metabolic syndrome. In this clinical investigation, the comparison of betatrophin and metastasis-related genes was also quantified especially among Javanese and non-Javanese subjects. Interestingly, the betatrophin and some genes related to cancer metastasis expression were significantly different between both ethnics (Figure 1).

The local or tumor tissue expression of lipasin or betatrophin significantly increased in Javanese patients as compared with non-Javanese. Also, the similar pattern was observed in E-cadherin and Snail expression but not in TGF-β1R. In addition, the classical serological marker carcinoembryonic antigen (CEA) level significantly increased in the late stage group (Figure 2).

| Table 1. Baseline characteristics of the study population |
|-----------------------------------------------------------|
| **Early Stage** | **Late Stage** |
| N | 13 | 25 |
| Sex (Male/Female) | (8/5) | (13/12) |
| T stages | | |
| T2 | 4 | 0 |
| T3 | 4 | 6 |
| T4 | 5 | 19 |
| N Stages | | |
| N0 | 13 | 1 |
| N1 | 0 | 8 |
| N2 | 0 | 16 |
| M Stages | | |
| M0 | 13 | 18 |
| M1 | 0 | 7 |

*TNM classification based on the International Union against Cancer (UICC, 2002).*
Figure 1. The expression of betatrophin and metastasis-related genes in CRC patients

Figure 2. The serum level of carcinoembryonic antigen (CEA) in different cancer stages.

Further analysis with linear correlation (Table 2) also showed that betatrophin negatively correlated to age ($r = -0.779$, $p = 0.001$) while CEA circulation negatively associated with sex ($r = -0.454$, $p = 0.013$) and positively correlated to primary tumor (T) development ($r = 0.434$, $p = 0.019$).
Table 2. Univariate correlation of Betatrophin and CEA with CRC predictors

| Parameter                        | Betatrophin | CEA |        |        |
|----------------------------------|-------------|-----|--------|--------|
|                                  |             |     |        |        |
| Sex                              | -0.430      | 0.140 | -0.454 | 0.013* |
| Age                              | -0.778      | 0.001* | 0.266  | 0.163  |
| Primary Tumor (T)                | -0.353      | 0.216 | 0.434  | 0.019* |
| Regional Lymph Node (N)          | -0.331      | 0.247 | 0.140  | 0.478  |
| Distant Metastasis (M)           | -0.430      | 0.125 | 0.295  | 0.121  |

Linear correlation by Spearman’s test. * Significant association between variables with $P < 0.05$.

According to the results of preliminary clinical case study, it is hypothesized that the increasing of betatrophin expression in the tumor site may associated with the progression of cancer cell proliferation and metastasis related lipid metabolism perturbation. Furthermore, the higher level of betatrophin expression may correlated to the lipid accumulation especially triglyceride within the cancer cell to induce anti-apoptosis program. In line with our previous study, increased betatrophin expression positively associated to the cancer progression especially pancreatic ductal adenocarcinoma. The higher level of betatrophin was shown in the ductal of subject with pancreatic cancer and abnormal serum lipid profile linked metabolic syndrome [23]. However, there is no clear information whether the gradual changes of betatrophin expression will associated with cancer cell migration and localization (metastasis). To clarify this discrepancy, here in this study there was an important finding was found whereas the expression of genes related cancer cell metastasis significantly different among different ethnic. Tumor tissue expression of Snail and E-cadherin significantly higher in Javanese compared to non-Javanese subjects. It is suggested that the higher level of betatrophin expression might induced the metabolic perturbation within colorectal epithelial cells to promote cancer cells proliferation and metastasis.

Several previous studies have shown that lipid accumulation has a strong association with anti-apoptosis activity in the cancer cell. The significant changes of lipogenesis and cholesterol synthesis rate in the cancer cell was established as a trigger of proliferation [34–36]. The accumulation of triglyceride within breast cancer cell can protect the cell death program even treated by Fatostatin [37]. The higher amount of lipid droplets in the colon cancer stem cells was predicted as hallmarks and the new regulator of cancer aggressiveness [38]. Moreover, the higher amount of lipid droplets was predicted determine chemotherapy resistance in cancer cells [39]. The inhibition of lipid droplets or lipid accumulation by genistein and daidzein treatment induced colon cancer cell apoptosis [40]. Importantly, triglyceride accumulation in cancer cell is not only provide a hallmark for cell proliferation but also induced epithelial mesenchymal transition (EMT)-linked metastasis program [41]. Hence, it clearly confirmed that the higher expression of betatrophin may strongly associated with the increasing of intracellular triglyceride to promote cell malignancies and develop to the late stage of cancer incidence in CRC patients particularly Javanese individuals.

Based on this study, it is speculated that the different expression of betatrophin and other genes related EMT in Javanese patients as compared with non-Javanese subjects may due to different life style and other risk factors in CRC development. Nevertheless, the limitation of our study is that we cannot fully elucidate the whole possible molecular pathway of betatrophin and associated genes that essentially involved in the progression of CRC in Indonesian ethnic especially Javanese subjects. Thus, the expanded further clinical investigation is necessary to clarify the preliminary hypothesis of this clinical case study.
4. Conclusion
In summary, our study employed fundamental preliminary data for future clinical administration, in particular, the early diagnosis of colorectal cancer progression. Betatrophin may have a significant contribution to the development of CRC by controlling triglyceride level within colon cancer cells. However, the comprehensive investigation is crucial to support our findings. Importantly, the data of this study provided a foundation or hallmark for the molecular approach in CRC patient diagnosis related to a specific potential hormone that contributes to the metabolic syndrome associated cancer development.

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