The potential role of betatrophin in hepatocellular carcinoma and metabolic syndrome

H Susanto 1*, A Aulanni’am 2*, C H Wang 3, D K Wuragil 2, A Y Handaya 4,5, M P Pertiwi 1, S A Rufiatin Nisa 1

1 Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Malang, Malang, Indonesia
2 Laboratory of Biosains, Faculty of Veterinary Medicine, Universitas Brawijaya, Malang, Indonesia
3 Department of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan ROC
4 Department of Digestive Surgeon Dr. Sardjito Hospital Yogyakarta, Indonesia
5 Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia

*Corresponding author: hendrabio@um.ac.id; aulani@ub.ac.id

Abstract. Metabolic syndrome is a fundamental health problem worldwide induced by obesity and T2DM. Obesity linked long-standing diabetes was established as a crucial event in cancer development. The recent findings improved that betatrophin, regulates lipid and glucose metabolism. However, the potential function of betatrophin activity in the pathogenesis of hepatocellular carcinoma (HCC) due to metabolic syndrome is not fully understood. This preliminary study aimed to elucidate the fundamental role of betatrophin in HCC linked metabolic syndrome. This study was conducted by in vivo model by using miR-122a-/- mouse induced by a high-fat diet. The alteration of liver betatrophin expression was confirmed by RT-qPCR method, while the histological section examination was done to observe the change of liver anatomy. Importantly, our study found that diet-induced obesity (DIO) was able to accelerate metabolic syndrome. The long-term treatment of DIO exacerbate portal inflammation, fatty liver and liver fibrosis accelerate liver tumorigenesis or precancer symptoms. The expression of betatrophin significantly increased in our metabolic syndrome mouse model indicate the involvement of betatrophin in lipid metabolism disorder to induce liver injury and hepatoma. Hence, this preliminary study proves a hallmark of the primary contribution of betatrophin in the early incidence of hepatocellular carcinoma linked metabolic syndrome.

1. Introduction
Metabolic syndrome is a cluster of several metabolic perturbation interconnections, including hyperglycemia, hypertension, hyperlipidemia that observed within the individual. One-fourth of today population has metabolic syndrome [1, 2]. Metabolic syndrome considered as the significant inducer of cardiovascular disease, type 2 diabetes mellitus and other conditions related to metabolic disorder results in the higher rate of mortality in the global population during the last few decades [3]. The primary criteria of metabolic syndrome were proposed by WHO which is the presence of impaired glucose tolerance, and insulin resistance is the essential components in the pathogenesis of metabolic syndrome. It is also established that the increase in body mass index progress to obesity accelerates the progression of metabolic syndrome [2, 4-6]. The gradual changes of daily lifestyle in the modern population suggested the increase in obesity prevalence. Lack of physical activity, sedentary lifestyle, western diet, and genetic predisposition indicated induced obesity. Obesity plays a pivotal role in the progression of vascular disease linked higher mortality and morbidity in patients. The significance alteration of body mass index (BMI) was positively associated with metabolic syndrome. Increased abdominal obesity incidence, circulating lipid disorder, and insulin resistance in the community became the primary concern for clinicians on clinical management of
metabolic diseases. Interestingly, obesity-induced metabolic syndrome tends to become a new trend for metabolic disease in Asian younger population[7]. Moreover, increased BMI in Asian community significantly correlate to risk of type 2 diabetes mellitus improved that future challenge for the Asia Pacific region to combat metabolic syndrome and it risk factor management [8]. Thus, the administration of obesity is through increased physical activity and several drug treatments to reduce body weight [5]. However, the clinical problem remains whereas the discontinuity of physical exercise and drug administration was failed to combat obesity.

The higher prevalence of obesity and T2DM incidence in the East, South East, and South Asia region also has a potential correlation with the development of cancer in those population. Metabolic syndrome associated with obesity can be a trigger for cancer development. Several studies have shown that excess body weight associated with cancer due to lipid metabolism disorder [9, 10]. Obesity linked to type 2 diabetes mellitus have been considered to enhance the risk of cancer in particular gastrointestinal cancer, endometrial cancer, breast cancer and lymphoma [9, 11]. Furthermore, individuals with metabolic syndrome were much more accessible to develop cancer incidence [12]. The significant alteration of hormone homeostasis is correlated with cancer and suggested the raising of obesity-T2DM will stimulate the burden incidence of cancer caused by metabolic syndrome [9, 12, 13]. In addition, hormonal imbalance or abnormalities and increased proinflammatory cytokine expression in a subject with metabolic syndrome are strongly induced cancer incidence [14]. Obesity and hyper nutrition are potentially promoted cancer cell survival and proliferation including the odd-ratio of hepatocellular carcinoma in a patient with metabolic syndrome [15, 16]. Obesity is the critical stimulator of nonalcoholic fatty liver disease (NAFLD) or fatty liver to induce liver carcinoma [17-19]. In NAFLD patients, insulin resistance produced the increase in gene expression related to de novo lipogenesis in the liver [20]. As a consequence, failure of microsomal triglyceride transfer protein (MTTP) activity will exacerbate liver triglyceride accumulation. It is suggested that the up or down-regulation of a liver hormone that controls triglyceride transport contribute to the severity of fatty liver progress to hepatocellular carcinoma. Although several studies have shown that the perturbation of liver lipid metabolism exacerbate dyslipidemia and stimulate the acceleration of T2DM and fatty liver (steatosis) development, however, it is still poorly understood whether the specific hormone derived from liver also play an essential role in the pathogenesis of cancer due to metabolic syndrome.

Nowadays, the exploration and development of protein-based biomaterial paying more attention to engineering, medicine, and applied science [21]. The application of protein-based biomaterial is not only for the diagnostic agent but also on the therapeutical approach in clinical management [22]. Furthermore, protein-based composite materials have been widely explored in the medical field to be applied to drug delivery system, biosensors, and tissue engineering [23]. Importantly, the recent studies have improved that, betatrophin, a liver-derived hormone regulate lipid and glucose homeostasis [24-26]. Betatrophin/lipasin/ANGPTL-8/C19orf80 has a positive association with hyperlipidemia and T2DM [27-30]. In vivo study improved that knockout betatrophin significantly reduced plasma triglyceride levels while overexpression of this hormone results in hypertriglyceridemia [25]. The plasma level of betatrophin is significantly associated with the atherogenic profile in patients with T2DM [31]. Importantly, the level of circulating betatrophin increased in subjects with obesity and metabolic syndrome [28, 32, 33]. The higher level of plasma betatrophin was observed in diabetic subjects with nephropathy and pancreatic ductal adenocarcinoma [34, 35]. Even though previous studies have been done to investigate the primary role of betatrophin in lipid metabolism and metabolic syndrome incidence, however, there is limited information the fundamental contribution of betatrophin in liver carcinoma progression. Hence, further investigation is required to explore the involvement of betatrophin in the early stages of hepatocellular carcinoma pathogenesis caused by metabolic syndrome.

2. Materials and Methods
2.1 Animals
This study was conducted by a guideline for the care and use of laboratory animals of animal center approved by the Animal Ethics Committee of Department Biological Science and Technology, National Chiao Tung University, Taiwan-ROC. Adults mice strain C57BL/6 was obtained from National Yang Ming University, Taiwan. All the animals were housed at room temperature (22 °C ) in the animal center at DBT-NCTU and allowed ad libitum to food and water. The genotyping procedure was done using PCR-Takara kit (Takara, Japan). Small pieces of toes or tail were used to characterize the genotype of the first offspring from the parental/mating cages. Male wild-type and miR-122 knockout mice (n=8-10 per each group) with old 4-6 weeks were kept at 22°C on a 12-h light/dark cycle. The negative control group was treated by standard chow (Biolasco, Taiwan), while the positive control was allowed to a high-fat diet (Research Diet Inc, USA)
for three months. Furthermore, miR-122 KO mice were prepared with the same treatment and regularly monitored during that period. The morphological observation was done during three months exposure to standard chow and high-fat diet. After treatment, all mice were killed using 2% isoflurane inhalation anesthetic protocol (Panion & BF Biotech Inc, Taiwan). The liver organ was harvested and freeze at -80°C. One part of the liver was fixed and embedded within the paraffin block for histology-microscopic analysis.

2.2 Histological Analysis
The liver sample was fixed in 4% paraformaldehyde for at least 24 hours followed by incubation in PBS solution. Then, liver samples were embedded in a paraffin block, and each section of the samples was stained with hematoxylin-eosin. The quantification of histological samples was measured using a binocular light microscope (Leica DM 500, Germany) under the standard protocol. The picture of this sample was captured using forever plus software (Forever plus Inc, Taiwan).

2.3 Real Time Quantitative Polymerase Chain Reaction
In the first step of gene expression measurement, total RNA was extracted from the liver using Rezol™ C & T (PRO tech Technologies Inc, Taiwan). A small piece of liver sample (10-50 mg) was homogenized in 1 ml Rezol solution with power Homogenizer. The cDNA was synthesized using iScript Reverse Transcription Supermix for RT-PCR (BioRad Kit, USA) using 3 µg RNA from each sample. At the final step, the gene expression of betatrophin in the liver was quantified using Real-time PCR with iTaq™ Universal SYBR® Green Supermix (BioRad, USA). The primer for betatrophin was set up as follows: forward (5’-GACGCTTTACACCTTCGAGC-3’) and reverse (5’-GCTGCTGTGTGGAGTCTCTG-3’).

2.4 Statistical Analysis
The differences of betatrophin expression in the liver among groups were assessed by parametric analysis using One-Way ANOVA with Tukey Post Hoc Test. The data were shown as mean ± SEM and P value less than 0.05 was considered statistically significant.

3. Results and Discussion
Our in vivo preliminary investigation has attempted to know whether the progression of hepatocellular carcinoma associated with metabolic syndrome and the alteration of betatrophin/lipasin expression. It was suggested that the increase of fatty liver incidence in our knockout mouse model also related to the betatrophin activity. Interestingly, we found that the expression of liver betatrophin significantly increased in our mutant mouse model compared to the control group (Figure 1). High-fat diet treatment significantly induced the gradual changes of betatrophin expression in the liver of wild-type mice. Furthermore, the higher level of betatrophin expression was observed in miR-122 KO mouse model with standard and high-fat diet. However, there was not significantly different in both groups after the treatment.

Figure 1. Diet-induced obesity in miR-122 KO mice increased betatrophin expression. Normalized betatrophin gene expression in the liver of miR-122 KO mice (n = 6-8 per group). Data are presented as mean ± SEM. * p-value < 0.05 vs WT standard chow; †, p<0.05 vs. WT HFD; ‡, p<0.05 vs. miR-122 KO standard chow.

Importantly, in line with the increasing of betatrophin expression, diet-induced obesity (DIO) was able to trigger the severity of fatty liver/liver steatosis (Figure 2). Diet-induced obesity exacerbated liver inflammation, portal fibrosis, and the structural changes within the parenchymal area of the liver. The
accumulation of liver triglyceride significantly increased in the wild-type group with HFD similar to miR-122 KO mice with the same treatment. Indeed, even the group was treated with regular chow, the progression of liver steatosis, portal inflammation and early stage of liver fibrosis showed in miR-122 KO mice. According to this data, we suggest that failure of triglyceride clearance from the liver, decreased liver fatty oxidation, increased lipogenesis, and decreased lipoprotein lipase activity results in fatty liver in our knockout mouse due to high-fat diet exposure. The long-term treatment of diet-induced obesity (DIO) increased the activation of liver macrophage (Kupfer cell), hepatic stellate cell, and another proinflammatory cell in the portal area (Figure 2).

Figure 2. Histological features of miR-122 KO mice liver post three months NC and HFD exposure by HE stain. (A). Wild-Type with standard chow; (B). Wild-Type with a high-fat diet (HFD); (C). miR-122 KO with standard chow; (D). miR-122 KO with a high-fat diet (HFD). Black arrow shows fatty liver, portal inflammation, and fibrosis. Images are shown at 100x magnification. Scale bar, 20 µm.

The preliminary results of our study demonstrate the potential role of betatrophin as a protein-based biomaterial in metabolic syndrome linked hypertriglyceridemia, obesity progression, and cancer development. The primary finding of our study describes for the first time that increased betatrophin expression significantly exacerbates steatosis and inflammation in the absence of miR-122. As a consequence, it will accelerate fatty liver, fibrosis and a probability develop hepatocellular carcinoma. Also, the significant changes in liver betatrophin expression offer a novel feature of the interconnection between fatty liver incidence, liver fibrosis and hepatocellular carcinoma resulting from the failure of lipid transport. Based on our data, it is suggested that increased betatrophin expression will inhibit the lipoprotein lipase activity (LPL) cause liver triglyceride accumulation.

Previous studies have shown that betatrophin is the potential regulator of triglyceride clearance [29], and was predicted able to block the catalytic site of LPL[36]. The silencing of betatrophin expression was significantly reduced circulating triglyceride and increased LPL activity and expression [25, 37]. Moreover, patients with a higher level of betatrophin significantly correlated with a higher risk of nonalcoholic fatty liver disease (NAFLD) [38] and enhanced ER stress in hepatocyte [39]. Also, the higher expression of betatrophin was reported in patients with liver cirrhosis and has a significant association with the degree of liver fibrosis [40]. Our study was broadly consistent with previous findings that show the fundamental role of betatrophin in the pathogenesis of liver fibrosis related to fatty liver and metabolic syndrome. Thus, the alteration of liver and circulating betatrophin levels may enhance the early stage of hepatocellular carcinoma. According to the preliminary data, here the limitation of our study is that no clear information whether the alteration of betatrophin expression has a direct association with the silencing of miR-122 and early symptom of HCC. Furthermore, we cannot provide a comprehensive data the correlation of betatrophin with tumor growth factor that involved in liver fibrosis-HCC development. The result of this study suggests that this liver-derived hormone would also be beneficial as the potential biomarker for the hepatocellular carcinoma-linked metabolic syndrome. Moreover, these findings can contribute considerably to the
development of early diagnosis of HCC related to hypertriglyceridemia, fatty liver, and fibrosis associated obesity and metabolic perturbation. However, further extensive studies are necessary to support our hypothesis and establish the future contribution of betatrophin-miR122 in the pathogenesis of hepatocellular carcinoma caused by metabolic syndrome. The circulating betatrophin level and liver beta-trophin expression may offer an opportunity for the clinical administration to prevent the severity of liver injury and patient mortality caused by hepatocarcinoma-related obesity.

4. Conclusion

In summary, the silencing of miR-122 and diet-induced obesity enhanced the early symptom of hepatocellular carcinoma. It is suggested that metabolic perturbation resulting from obesity-related insulin resistance might be associated with increased liver betatrophin expression to corroborate liver inflammation and fibrosis. As the final consequence, the higher level of betatrophin may become the early potential biomarker for early stages of hepatocellular carcinoma induced by metabolic syndrome. The further expanded study is necessary to elucidate the primary role of betatrophin in HCC development.

Acknowledgments

I would like to thank my former supervisor, Prof. Dr. dr. M. Rasjad Indra, MS for his support, advice, and guidance. Also thank Directorate General of Human Resource for Science, Technology, and Higher Education (DIKTI) for scholarship support to join the Ph.D. program in the Department of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan ROC.

References

[1] Després JP and Lemieux I 2006 Abdominal obesity and metabolic syndrome Nature 444 881-7
[2] Grundy S M, Brewer H B, Cleeman J I, Smith S C and Lenfant C 2004 Definition of metabolic syndrome report of the National Heart, Lung, and Blood Institute/American Heart Association Conference on scientific issues related to definition Circulation 109 433-8
[3] Katzmarzyk P T, Church T S, Janssen I, Ross R and Blair S N 2005 Metabolic syndrome, obesity, and mortality Diabetes care 28 391-7
[4] Alberti K G M M, Zimmet P and Shaw J 2006 Metabolic syndrome—a new world-wide definition. A consensus statement from the international diabetes federation Diabetic medicine 23 469-80
[5] Eckel R H, Grundy S M and Zimmet P Z 2005 The metabolic syndrome The Lancet 365 1415-28
[6] Kahn R, Buse J, Ferrannini E and Stern M 2005 The metabolic syndrome: time for a critical appraisal Diabetologia 48 1684-99
[7] Nestel P, Lyu R, Low L P, Sheu W H-H, Nitiyanant W, Saito I and Tan C E 2007 Metabolic syndrome: recent prevalence in East and Southeast Asian populations Asia Pacific journal of clinical nutrition 16 362-7
[8] Hsu W C, Araneta M R G, Kanaya A M, Chiang J L and Fujimoto W 2015 BMI cut points to identify at-risk Asian Americans for type 2 diabetes screening Diabetes Care 38 150-8
[9] Cohen D H and LeRoith D 2012 Obesity, type 2 diabetes, and cancer: the insulin and IGF connection Endocrine-related cancer 19 F27-F45
[10] Nguyen N T, Nguyen X-M T, Lane J and Wang P 2011 Relationship between obesity and diabetes in a US adult population: findings from the National Health and Nutrition Examination Survey, 1999–2006 Obesity surgery 21 351-5
[11] Esposito K, Chiodini P, Colao A, Lenzi A and Giugliano D 2012 Metabolic syndrome and risk of cancer: a systematic review and meta-analysis Diabetes Care 35 2402-11
[12] Giovannucci E 2007 Metabolic syndrome, hyperinsulinemia, and colon cancer: a review The American journal of clinical nutrition 86 836S-42S
[13] Xue F and Michels K B 2007 Diabetes, metabolic syndrome, and breast cancer: a review of the current evidence The American journal of clinical nutrition 86 823S-35S
[14] Coller H A 2014 Is cancer a metabolic disease? The American journal of pathology 184 4-17
[15] Argilés J M, Busquets S, Stemmler B and López-Soriano F J 2014 Cancer cachexia: understanding the molecular basis Nature reviews Cancer 14 754-62
[16] Turati F, Talamini R, Pelucchi C, Polese J, Franceschi S, Crispì A, Izzo F, La Vecchia C, Boffetta P and Montella M 2013 Metabolic syndrome and hepatocellular carcinoma risk British journal of cancer 108 222-8
[17] Park E J, Lee J H, Yu G-Y, He G, Ali S R, Holzer R G, Österreichier C H, Takahashi H and Karin M 2010 Dietary and genetic obesity promote liver inflammation and tumorigenesis by enhancing IL-6 and
TNF expression Cell 140 197-208

[18] El-Serag H B and Kanwal F 2014 Obesity and hepatocellular carcinoma: hype and reality Hepatology 60 779-81

[19] Duan X F, Tang P, Li Q and Yu Z T 2013 Obesity, adipokines and hepatocellular carcinoma International journal of cancer 133 1776-83

[20] Kawano Y and Cohen D E 2013 Mechanisms of hepatic triglyceride accumulation in non-alcoholic fatty liver disease Journal of gastroenterology 48 434-41

[21] Harden J L and Li H 2017 Special Issue on Designer Protein Biomaterials, ACS Publications

[22] Gagner J E, Kim W and Chaikof E L 2014 Designing protein-based biomaterials for medical applications Acta biomaterialia 10 1542-57

[23] Hu X, Cebe P, Weiss A S, Omenetto F and Kaplan D L 2012 Protein-based composite materials Materials today 15 208-15

[24] Chen X, Lu P, He W, Zhang J, Liu L, Yang Y, Liu Z, Xie J, Shao S and Du T 2014 Circulating betatrophin levels are increased in patients with type 2 diabetes and associated with insulin resistance The Journal of Clinical Endocrinology & Metabolism 100 E96-E100

[25] Wang Y, Quagliarini F, Gusarova V, Gromada J, Valenzuela D M, Cohen J C and Hobbs H H 2013 Mice lacking ANGPTL8 (Betatrophin) manifest disrupted triglyceride metabolism without impaired glucose homeostasis Proceedings of the National Academy of Sciences of the United States of America 110 16109-14

[26] Yamada H, Saito T, Aoki A, Asano T, Yoshida M, Ikoma A, Kusaka I, Toyoshima H, Kakei M and Ishikawa S-e 2015 Circulating betatrophin is elevated in patients with type 1 and type 2 diabetes Endocrine journal

[27] Fu Z, Abou-Samra A B and Zhang R 2015 A lipasin/ANGPTL8 monoclonal antibody lowers mouse serum triglycerides involving increased postprandial activity of the cardiac lipoprotein lipase Scientific reports 5

[28] Fu Z, Berhane F, Fite A, Seyoum B, Abou-Samra A B and Zhang R 2014 Elevated circulating lipasin/betatrophin in human type 2 diabetes and obesity Scientific reports 4

[29] Zhang R 2012 Lipasin, a novel nutritionally-regulated liver-enriched factor that regulates serum triglyceride levels Biochemical and biophysical research communications 424 786-92

[30] Zhang R and Abou-Samra A B 2013 Emerging roles of Lipasin as a critical lipid regulator Biochemical and biophysical research communications 432 401-5

[31] Fenzl A, Itariu B K, Kosi L, Fritzer-Szekeres M, Kautzky-Willer A, Stulnig T M and Kiefer F W 2014 Circulating betatrophin correlates with atherogenic lipid profiles but not with glucose and insulin levels in insulin-resistant individuals Diabetologia 57 1204-8

[32] Abu-Farha M, Sriraman D, Cherian P, AlKhairi I, Elkum N, Behbehani K and Abubaker J 2016 Circulating ANGPTL8/Betatrophin Is Increased in Obesity and Reduced after Exercise Training PLoS One 11 e0147367

[33] Crujeiras A B, Zulet M, Abete I, Carreira M C, Martinez J A and Casanueva F F 2016 Interplay of atherogenic factors, protein intake and betatrophin levels in obese-metabolic syndrome patients treated with hypocaloric diets International Journal of Obesity 40 403

[34] Chen C-C, Susanto H, Chuang W-H, Liu T-Y and Wang C-H 2016 Higher serum betatrophin level in type 2 diabetes subjects is associated with urinary albumin excretion and renal function Cardiovascular diabetology 15 3

[35] Susanto H, Liu T-Y, Chen C-C, Purnomo J D, Chen S-F and Wang C-H 2016 Increased serum levels of betatrophin in pancreatic cancer-associated diabetes Oncotarget 7 42330

[36] Siddiqa A, Ahmad J, Ali A, Paracha R Z, Bibi Z and Aslam B 2016 Structural characterization of angptl8 (betatrophin) with its interacting partner lipoprotein lipase Computational biology and chemistry 61 210-20

[37] Quagliarini F, Wang Y, Kozlitina J, Grishin N V, Hyde R, Boerwinkle E, Valenzuela D M, Murphy A J, Cohen J C and Hobbs H H 2012 Atypical angiopoietin-like protein that regulates ANGPTL3 Proceedings of the National Academy of Sciences 109 19751-6

[38] Hu W, Shao X, Guo D, Hao H, Zhang Y, Xia M, Gong Y, Zhou H, Fan Y and Yu W 2017 Relationship of Serum Betatrophin with Nonalcoholic Fatty Liver in a Chinese Population PLoS one 12 e0170758

[39] Lee Y H, Lee S G, Lee C J, Kim S H, Song Y M, Yoon M R, Jeon B H, Lee J H, Lee B W, Kang E S, Lee H C and Cha B S 2016 Association between betatrophin/ANGPTL8 and non-alcoholic fatty liver disease: animal and human studies Sci Rep 6 24013

[40] Arias-Loste M T, García-Unzueta M T, Llerena S, Iruzubieta P, Puente A, Cabezas J, Alonso C,
Cuadrado A, Amado J A and Crespo J 2015 Plasma betatrophin levels in patients with liver cirrhosis
*World journal of gastroenterology: WJG* **21** 10662