movement and insulin synthesis is comparatively preserved [14].

In contrast, the insulin response to glucose and first-phase insulin secretion were remarkably disordered. The first phase required a rapid and marked elevation of $[Ca^{2+}]_i$ and corresponded to the release of insulin granules from a limited pool [15]. This suggests that glucose stimulation did not increase the intracellular calcium level in pancreatic β cells of this patient. We therefore suspected that glucose metabolism in pancreatic β cells was disordered. However, fasting plasma pyruvate and lactate levels in this patient were within normal limits (data not shown). Further investigation is required to clarify the mechanism of glucose unresponsiveness seen in this patient.

Liraglutide improved glucose tolerance at doses of 0.3 and 0.6 mg. Plasma glucagon levels decreased dramatically with doses of 0.3 and 0.6 mg, whereas the insulin response to glucose on May 25, 2011 (0.3 mg liraglutide) became much higher than that on September 7, 2011 (0.6 mg liraglutide). After the administration of 0.6 mg liraglutide, the patient sometimes experienced gastrointestinal symptoms (soft stool and indigestion). Moreover, the HOMA-R level was lower with 0.6 mg liraglutide than with 0.3 mg liraglutide. These results suggest that the improvement in glucose tolerance with 0.6 mg liraglutide was achieved by delayed gastric emptying, suppression of glucagon secretion, and improvement of insulin sensitivity rather than by augmentation of insulin secretion.

In this patient, postprandial glucose levels were remarkably high, although HbA1c was within normal limits. Consistently, the plasma 1,5-AG level was at the lower end of the normal spectrum, and was improved with liraglutide administration. Thus, in cases of fasting normoglycemia, HbA1c may be readily underestimated. Moreover, postprandial hyperglycemia poses a potential risk for the development of atherosclerosis [16, 17]. In this patient, as the pancreatic β cells would be protected from glucotoxicity because the insulin secretion capacity was very low, normalization of postprandial hyperglycemia and protection against pancreatic β-cell failure and diabetic complications caused by glucose toxicity were crucial.

There are recent reports that fasting CPR, CPR index, and I.I. are useful parameters for predicting the efficacy of liraglutide in patients with type 2 diabetes [18]. In contrast, both the fasting plasma glucose levels and the insulin levels were low in this patient because hepatic insulin sensitivity was preserved. Furthermore, the insulin response to glucagon and the postprandial serum CPR to plasma glucose ratio, rather than the fasting serum CPR to plasma glucose ratio, better reflected islet function in this patient. Consistently, it was reported that the serum CPR to plasma glucose ratio after oral glucose ingestion better predicts β-cell area than fasting measures such as the HOMA-β index [11, 19]. Thus, the insulin response to glucagon and the postprandial serum CPR to plasma glucose ratio are useful tools for estimating pancreatic β-cell function, particularly in lean diabetic patients. However, we should keep in mind that the plasma CPR levels are affected by renal function [20, 21], and that the CPR response to glucagon is affected by the plasma blood glucose levels and may overestimate β-cell function in poorly controlled NIDDM patients with elevated blood glucose levels [22, 23]. Furthermore, in patients with a long-term history of diabetes mellitus, the residual β-cell function and CPR response to glucagon can be expected to be decreased [9]. For these reasons, any estimation of residual β-cell function using a glucagon test is limited to patients with short-duration diabetes mellitus.

Leuprorelin used for the treatment of endometriosis often worsens insulin resistance, subsequently causing postprandial hyperglycemia [24]. In the present case, leuprorelin increased the postprandial glucose level, while alogliptin did not improve glucose tolerance. Similarly, glucocorticoids induce insulin resistance and postprandial hyperglycemia [25], whereas liraglutide improves postprandial hyperglycemia and insulin resistance induced by glucocorticoids. This is due to delayed gastric emptying and suppressed glucagon secretion rather than the potentiation of insulin secretion [26]. Thus, liraglutide may be an effective agent to treat leuprorelin-induced diabetes mellitus.

In conclusion, we found that liraglutide is effective for glycemic control in a lean diabetic patient with an impaired insulin response to glucose. Thus, in patients with a low fasting CPR to glucose ratio, the glucagon test may be appropriate for estimating pancreatic β-cell function when liraglutide is administered.

References

1. Reaven GM. Banting lecture 1988. Role of insulin resistance in human disease. Diabetes. 1998;37:1595–607.
2. Fukushima M, Suzuki H, Seino Y. Insulin secretion capacity in the development from normal glucose tolerance to type 2 diabetes. Diabetes Res Clin Pract. 2004;66:S37–43.
3. Drucker DJ, Sherman SI, Bergenstal RM, Buse JB, Faber OK, Binder C. The safety of incretin-based therapies—review of the scientific evidence. J Clin Endocrinol Metab. 2011;96:2027–31.
4. Holst JJ. The physiology of glucagon-like peptide 1. Physiol Rev. 2007;87:1409–39.
5. Iizuka K, Tomita R, Suwa T, Horikawa Y, Takeda J. A case report: normalization of fasting hyperglycemia is beneficial for successful introduction of small amount of the GLP-1 analog liraglutide in an obese patient with type 2 diabetes mellitus. Diabetol Int. 2011 (in press). doi:10.1007/s13340-011-0052-9.
6. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC. Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes. 2003;52:102–10.

7. Ward WK, Bolgiano DC, McKnight B, Halter JB, Porte D Jr. Diminished B cell secretory capacity in patients with noninsulin-dependent diabetes mellitus. J Clin Invest. 1984;74:1318–28.
8. Clark A, Charge SB, Badman MK, MacArthur DA, de Koning EJ. Islet amyloid polypeptide: actions and role in the pathogenesis of diabetes. Biochem Soc Trans. 1996;24:594–9.
9. Faber OK, Binder C. C-peptide response to glucagon. A test for the residual beta-cell function in diabetes mellitus. Diabetes. 1977;26:605–10.
10. Hendriksen C, Faber OK, Drejer J, Binder C. Prevalence of residual B-cell function in insulin-treated diabetics evaluated by the plasma C-peptide response to intravenous glucagon. Diabetologia. 1977;13:615–9.
11. Meier JJ, Menge BA, Breuer TG, Müller CA, Tannapfel A, Uhl W, Schmidt WE, Schrader H. Functional assessment of pancreatic beta-cell area in humans. Diabetes. 2009;58:1595–603.
12. Goto A, Takaichi M, Kishimoto M, Takahashi Y, Kajio H, Shimbo T, Noda M. Body mass index, fasting plasma glucose levels, and C-peptide levels as predictors of the future insulin use in Japanese type 2 diabetic patients. Endocr J. 2010;57:237–44.
13. Seino Y, Nanjo K, Tajima N, Kadowaki T, Kashiwagi A, Araki E, Ito C, Inagaki N, Iwamoto Y, Kasuga M, Hanafusa T, Haneda M, Ueki K. Report of the Committee on the Classification and Diagnostic Criteria of Diabetes Mellitus. The Committee of the Japan Diabetes Society on the Diagnostic Criteria of Diabetes Mellitus. Diabetol Int. 2010;1:2–20.
14. Seino S, Takahashi H, Fujimoto W, Shibasaki T. Roles of cAMP signaling in insulin granule exocytosis. Diabetes Obes Metab. 2009;11(Suppl 4):180–8.
15. Henquin JC, Ishiyama N, Nenquin M, Ravier MA, Jonas JC. Signals and pools underlying biphasic insulin secretion. Diabetes. 2002;51(Suppl 1):S60–7.
16. Milicevic Z, Raz I, Beattie SD, Campaigne BN, Sarwat S, Gromniak E, Kowalska I, Galic E, Tan M, Hanefeld M. Natural history of cardiovascular disease in patients with diabetes: role of hyperglycemia. Diabetes Care. 2008;31(Suppl 2):S155–60.
17. Ceriello A. Postprandial hyperglycemia and diabetes complications: is it time to treat? Diabetes. 2005;54:1–7.
18. Kozawa J, Inoue K, Iwamoto R, Kurashiki Y, Okauchi Y, Kashine S, Kitamura T, Maeda N, Okita K, Iwahashi H, Funahashi T, Imagawa A, Shimomura I. Liraglutide is effective in type 2 diabetic patients with sustained endogenous insulin-secreting capacity. J Diabetes Invest. 2011 (in press). doi: 10.1111/j.2040-1124.2011.00168.x.
19. Saisho Y, Kou K, Tanaka K, Abe T, Kurosawa H, Shimada A, Meguro S, Kawai T, Itoh H. Postprandial serum C-peptide to plasma glucose ratio as a predictor of subsequent insulin treatment in patients with type 2 diabetes. Endocr J. 2011;58:315–22.
20. Ramirez G, O'Neill WM Jr, Bloomer HA, Jubiz W. Serum C-peptide in renal failure patients following stimulation of pancreatic secretion. Clin Nephrol. 1979;11:297–301.
21. Kajinuma H, Kanazawa Y, Sando H, Hayashi M, Kawazu S, Kosaka K. Human plasma C-peptide immunoreactivity: its correlation with immunoreactive insulin in diabetes, and chronic liver and renal diseases. Endocrinol Jpn. 1979;26:65–73.
22. Nosari I, Lepore G, Maglio ML, Cortinovis F, Pagani G. The effect of various blood glucose levels on post-glucagon C-peptide secretion in type 2 (non insulin-dependent) diabetes. J Endocrinol Invest. 1992;15:143–6.
23. Park SW, Ihm SH, Yoo HJ, Park JY, Lee KU. Differential effects of ambient blood glucose level and degree of obesity on basal serum C-peptide level and the C-peptide response to glucose and glucagon in non-insulin-dependent diabetes mellitus. Diabetes Res Clin Pract. 1997;37:165–71.
24. Coddington CC, Hassiakos DK, Harrison HC, Brzyski RG, Jones GS. Effect of a gonadotropin-releasing hormone analogue on the glucose metabolism in a diabetic patient. Gynecol Obstet Invest. 1990;30:246–8.
25. Rizza RA, Mandarino LJ, Gerich JE. Cortisol-induced insulin resistance in man: impaired suppression of glucose production and stimulation of glucose utilization due to a postreceptor detect of insulin action. J Clin Endocrinol Metab. 1982;54:131–8.
26. van Raalte DH, van Genugten RE, Linssen MM, Ouwens DM, Diamant M. Glucagon-like peptide-1 receptor agonist treatment prevents glucocorticoid-induced glucose intolerance and islet-cell dysfunction in humans. Diabetes Care. 2011;34:412–7.

The Evaluation of Risk Factors Associated with Adverse Drug Reactions by Metformin in Type 2 Diabetes Mellitus

Shinji Okayasu,[a,b] Kiyoyuki Kitaichi,*[,a] Akina Hori,[a] Tetsuya Suwa,[c] Yukio Horikawa,[c] Mayumi Yamamoto,[b,c] Jun Takeda,[c] and Yoshinori Itoh[a]

[a] *Department of Pharmacy, Gifu University Hospital;* [b] *United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University; and* [c] *Department of Diabetes and Endocrinology, Division of Molecule and Structure, School of Medicine, Gifu University; 1–1 Yanagido, Gifu 501–1194, Japan.*
Received January 18, 2012; accepted March 8, 2012

Metformin is a drug to improve glycemic control by reducing insulin resistance and is currently considered to be one of the first-choice drugs for type 2 diabetes mellitus (T2DM). However, during metformin use, adverse drug reactions (ADRs) including gastrointestinal adverse events were frequently observed. Thus, in the present study, we investigated the incidence of ADRs induced by metformin and further analyzed risk factors for ADRs in Japanese patients with type 2 diabetes mellitus who initially administered metformin (500–750 mg). One hundred and one hospitalized patients receiving metformin during September 1, 2009 and August 31, 2010 were studied. The incidence of ADRs and changes in laboratory data including hemoglobin A1c (HbA1c) were monitored retrospectively. The anti-glycemic effect of metformin was successfully observed as indicated by decreased HbA1c. Among ADRs, diarrhea was most frequently occurred during metformin use (26.7% of patients) although the symptom of diarrhea was mild in most cases and disappeared within 3 d after the initial use. A logistic regression analysis showed the existence of six risk factors, including initial dose (750 mg), female, age (≦65), body mass index (≧25), aspartate aminotransferase (≧30 IU/L) and alkaline phosphatase (≧270 IU/L). The incidence of diarrhea increased linearly as the number of risk factors increased. In conclusion, in order to avoid ADRs, especially diarrhea, subsequently improving the quality of life during metformin use, the optimization of the dose of metformin by considering risk factors would be beneficial for patients with T2DM.

Key words metformin; type 2 diabetes mellitus; diarrhea; risk factor; dose optimization

Metformin improves insulin resistance by inhibiting gluconeogenesis and enhancing peripheral glucose uptake through stimulation of AMP-activated kinase.[1] *meta*-Analysis has shown its effectiveness in type 2 diabetes mellitus (T2DM).[2] Metformin has been recommended as a first-choice drug in new-onset type 2 diabetes in a consensus algorithm from the American Diabetes Association and the European Association for the Study of Diabetes.[3] Moreover, recent reports suggest that metformin would be effective 1) in preventing cardiovascular events in type 2 diabetes,[4] 2) in preventing the development of T2DM in nondiabetic subjects,[3,5] 3), in improving impaired glucose tolerance in patients with metabolic syndrome,[6] and 4) limiting weight-gain induced by atypical antipsychotics.[7]

Metformin is generally well tolerated. However, it is well known that metformin frequently causes adverse drug reactions (ADRs) including gastrointestinal adverse events, especially diarrhea.[3] These ADRs occasionally leads to withdrawal of metformin treatment.[8,9] In the present study, we initially clarified the incidence and profiles of ADRs caused by initial treatment of metformin in patients hospitalized with T2DM patients. Secondly, we aimed to identify risk-factors related to ADR(s) to encourage the appropriate use of metformin.

MATERIALS AND METHODS

Patients Our study was carried out in accordance with the guidelines for the care for human study adopted by the ethics committee of the Gifu Graduate School of Medicine and notified by the Japanese government (approved No. 22-174 of the institutional review board). One hundred and one patients admitted to our hospital and started on metformin during September 1, 2009 and August 31, 2010 were enrolled. The enrolled patients possessed normal renal function (serum creatinine <1.2 mg/dL) since in our hospital patients with reduced renal function (serum creatinine ≧1.2 mg/dL) were not given metformin to avoid the risk of metformin-induced lactic acidosis although this complication is rare.[10] Moreover, it should be noted that patients who have any diseases that frequently cause diarrhea (ex. infectious disease, gastrointestinal disease *etc.*)[11–13] or who take any drugs that frequently cause diarrhea (ex. antibiotics, anticancer drugs, non-steroidal anti-inflammatory drugs *etc.*)[14] were eliminated.

Survey of ADRs Associated with Metformin Metformin (Melbin® 250 mg tablet, Dainippon-Sumitomo Pharma Co., Ltd., Osaka, Japan) was administered at a daily dose of 500 mg or 750 mg. Data were retrospectively obtained from the patients' electronic medical records and pharmaceutical records. Briefly, ADRs, assessed by pharmacists, such as diarrhea, nausea, and vomiting during the hospitalization (*i.e.* up to 7 d after starting metformin use) were obtained from pharmaceutical records. The severity of ADRs was assessed by the Common Terminology Criteria for Adverse Events (CTCAE version 4.0).

Monitoring the Laboratory Data and the Patients' Characteristics The laboratory data for alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate aminotransferase (AST), total bilirubin, serum creatinine (Cr) and hemoglobin A1c (HbA1c), as well as the patient's characteristics including age, gender, body mass index (BMI) and prescribed drugs before metformin use were obtained from the medical records. Creatinine clearance (CCr) was obtained

* To whom correspondence should be addressed. e-mail: kitaichi@gifu-u.ac.jp

by the Cockcroft–Gault formula[15] from data on age, sex, body weight and Cr.

Statistical Analyses Data analysis was with the Statistics Program for Social Science for Windows (SPSS II, ver. 11, IBM Co., Somers, NY, U.S.A.). Non-parametric data were analyzed using Fisher's exact probability test or Mann–Whitney *U*-test, while parametric data were analyzed by *t*-test. Risk analyses were carried out by univariate logistic regression analysis.

RESULTS

Patient's Demographics Characteristics of patients were shown in Table 1. Metformin was administered at a dose of 500 mg and 750 mg in 52 and 49 patients, respectively. Before metformin treatment, insulin was administered in 49 patients (48.5%), sulfonylurea in 6 patients (5.9%), α-glucosidase inhibitors in 31 patients (30.7%), and thiazolidines in 12 patients (11.9%).

Hypoglycemic Effect of Metformin As shown in Table 2, HbA1c gradually decreased after treatment with metformin in either group. Significant reduction in HbA1c was observed at 12 weeks in both groups. The extent of the reduction in HbA1c was significantly ($p<0.05$) greater in 750 mg-treated group than in 500 mg-treated group.

Metformin-Induced ADRs As shown in Table 3, the most-frequently observed ADR was diarrhea that occurred in 27 patients of 101 patients (27%). Another ADR was a mild anorexia (*i.e.* G1) which appeared in 3 patients (3%) without diarrhea. Nausea, vomiting, hepatic and renal dysfunctions were not observed. Although it is well known that one of serious ADRs caused by metformin is lactic acidosis,[10] no ADRs related to lactic acidosis (ex. blood pH *etc.*) were observed in the present study (data not shown).

Profiles of Metformin-Induced Diarrhea Profiles of diarrhea were compared between the two initial doses 500 mg and 750 mg. As shown in Table 4, the incidence was significantly higher in 750 mg-treated group than in 500 mg-treated group (36.7% *vs.* 17.3%, $p<0.05$). The onset of diarrhea was in all cases within a day of metformin treatment. The symptom

Table 1. Patients' Demographics

Number of patients (male/female)	101 (62/39)
Age (range)	60.7 (14–86)
HbA1c (%)	9.4±2.2
Initial daily dose	
500 mg	52 patients (51.5%)
750 mg	49 patients (48.5%)
Dose regimen	
Before meal	12 patients (11.9%)
After meal	89 patients (88.1%)
Alanine aminotransferase (ALT, U/L)	30.7±24.2
Aspartate aminotransferase (AST, U/L)	25.2±13.3
Alkaline phosphatase (ALP, IU/L)	233.3±71.4
Total bilirubin (mg/dL)	0.9±0.4
Creatinine clearance (mL/min)	94.7±28.5
Co-administered drugs	
Insulin	49 patients (48.5%)
Sulfonylurea	6 patients (5.9%)
α-Glucosidase inhibitors	31 patients (30.7%)
Thiazolidines	12 patients (11.9%)

Table 2. Effect of Initial Doses of Metformin on the Levels of HbA1c in Patients with T2DM

	Initial daily dose of metformin		Statistical significance[b]
	500 mg ($N=52$)	750 mg ($N=49$)	
Hypoglycemic effect (HbA1c, %)			
Baseline	9.0±2.1	9.8±2.2	0.056
12w	7.1±0.9 ($N=16$[a])*	6.6±0.9 ($N=12$[a])**	0.137
ΔHbA1c	−1.4±0.4 ($N=16$[a])	−3.3±0.7 ($N=12$[a])	0.02

*,**$p<0.05$ and $p<0.01$ *vs.* corresponding baseline. *a*) Patients whose HbA1c was not examined or recorded were excluded from the data. *b*) Unpaired *t*-test.

Table 3. The Incidence of ADRs of Metformin ($N=101$)

ADRs	Number of patients (%)
Diarrhea[a]	27 (26.7%)
Anorexia[a,b]	3 (3.0%)
Nausea [a]	0 (0%)
Vomiting [a]	0 (0%)
Hepatic dysfunctions[c,d]	0 (0%)
Renal dysfunctions[e]	0 (0%)

ADRs were monitored during the hospitalization (up to 7 d after metformin treatment). *a*) We counted the number of patients who developed diarrhea, anorexia, nausea and vomiting as more than G0. *b*) No patients with anorexia occurred diarrhea during metformin use. *c*) Values of AST and ALT 3 times above ULN (*i.e.* G1) considered being hepatic dysfunction after metformin use. *d*) One patient who had high ALT (G1) before metformin use together with unchanged ALT after metformin use was excluded from the analysis. This patient caused diarrhea after metformin use for 3 d. *e*) Values of serum creatinine 1.5 times above ULN (*i.e.* G1) considered being renal dysfunction after metformin use.

Table 4. Effect of Initial Doses of Metformin on the Profiles of Metformin-Induced Diarrhea in Patients with T2DM

	Initial daily dose of metformin		Statistical significance[e]
	500 mg ($N=52$)	750 mg ($N=49$)	
Incidence ($n=27$)[a]	9 (17.3%)	18 (36.7%)	0.042
Gender			
Male	3	9	
Female	6	9	0.681
Severity[b]			
G1 ($n=21$)	6	15	
G2 ($n=6$)	3	3	0.623
Duration[b]			
Within 3 d ($n=18$)	6	12	
Over 3 d ($n=6$)[c]	1	5	0.629
Tolerability[b]			
Treatment continued ($n=24$)	7	17	
Treatment discontinued ($n=3$)[d]	2	1	0.795

Onset, gender, grade, duration and tolerability of diarrhea were monitored during the hospitalization (up to 7 d after metformin treatment). *a*) Diarrhea appeared in all cases within a day of metformin treatment. *b*) There are no statistical differences of gender and age (≦65) between groups in severity, duration and tolerability. *c*) Symptom was G1 at the end of the observation in 5 patients. *d*) All were G2 but the symptom recovered immediately after stopping medication. *e*) Fisher's exact probability test.

Table 5. Comparison of the Characteristics of Patients between Those with and without Diarrhea

	With diarrhea (N=27)	Without diarrhea (N=74)	Statistical significance
Age	56.1±15.2	62.4±13.8	0.028[b]
Gender			
Male	12 (44.4%)	50 (67.6%)	
Female	15 (55.6%)	24 (32.4%)	0.040[a]
Body mass index	26.6±5.6	24.0±3.7	0.036[c]
HbA1c (%)	9.3±2.1	9.4±2.2	0.918[c]
Initial daily dose (mg)	667±120	605±124	0.028[c]
Dose			
500 mg	6 (40.0%)	18 (75.0%)	
750 mg	9 (60.0%)	6 (25.0%)	0.065[a]
Dose regimen			
Before meal	2 (7.4%)	10 (13.5%)	
After meal	25 (92.6%)	64 (86.5%)	0.507[a]
ALT (U/L)	41.9±35.2	25.6±17.3	0.038[c]
AST (U/L)	28.4±15.2	24.0±12.5	0.140[c]
ALP (IU/L)	270.6±98.6	219.1±52.1	0.015[c]
Total bilirubin (mg/dL)	0.94±0.54	0.88±0.41	0.567[c]
Creatinine clearance (mL/min)	103.4±26.5	91.6±28.7	0.066[c]
Co-administered drugs			
Sulfonylurea	1 (3.7%)	5 (6.8%)	1.000[a]
α-Glucosidase inhibitors	8 (29.6%)	23 (31.1%)	1.000[a]
Thiazolidines	1 (3.7%)	11 (14.9%)	0.125[a]
Insulin	12 (44.4%)	37 (50%)	0.659[a]
Laxatives	3 (11.1%)	11 (14.9%)	0.754[a]

a) Fisher's exact probability test. *b*) Mann–Whitney *U*-test. *c*) Student's *t*-test.

of metformin-induced diarrhea was mild (*i.e.* G1) in many cases in either group and most patients recovered from diarrhea within a few days. Three of 27 patients with diarrhea discontinued metformin therapy due to G2 diarrhea, where the symptom disappeared immediately after the cessation of metformin use. There are no significant differences of the severity, duration and tolerability of diarrhea between the initial doses of metformin.

Comparison of the Characteristics between Patients with and without Diarrhea Table 5 shows a comparison of patient characteristics between the two groups with and without diarrhea. Six risk factors identified were age (p=0.028), sex (p=0.04), initial daily dose of metformin (p=0.028), BMI (p=0.036), ALT (p=0.038) and ALP (p=0.015). The odds ratio in a univariate analysis was 2.774 (95% CI, 1.101–6.988, p=0.030) for initial daily dose of 750 mg, 2.604 (1.057–6.416, p=0.037) for female, 3.694 (1.338–10.198, p=0.012) for age ≦65, 2.532 (1.028–6.239, p=0.043) for BMI ≧25, 2.90 (1.13–7.42, p=0.026) for ALT ≧30 IU/L and 3.380 (1.126–9.071, p=0.016) for ALP ≧270 IU/L (Fig. 1).

The Relationship between Risk Factors and the Incidence of Diarrhea It was notable that the incidence of diarrhea was elevated linearly as the number of risk factors increased, when the number of risks exceeded 3 (Fig. 2).

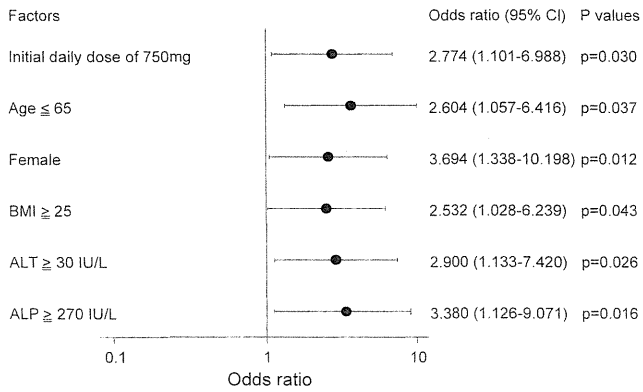
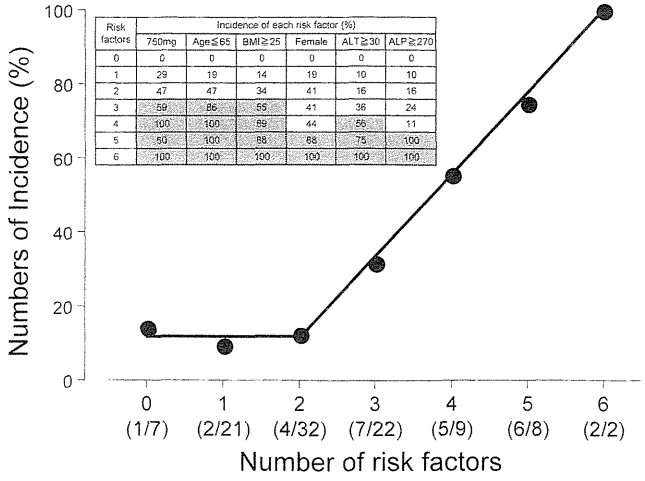


Fig. 1. Risk Factor Analysis for Metformin-Induced Diarrhea
Data were analyzed by univariate logistic regression analyses.

DISCUSSION

The current study revealed that diarrhea is most-frequently occurred ADR during metformin use at an initial dose of 500 mg or 750 mg as shown previously.[3,16] The overall incidence of diarrhea observed in the present study was 26.7% (27 of 101 patients), which was consistent with the data (23.7%) reported in a large clinical trial such as A Diabetes Outcome Progression Trial (ADOPT),[17] although the incident rate was varied among studies.[16] The differences of initial daily doses of metformin failed to affect the severity, duration and tolerability of diarrhea although further large scale study would be needed to analyze the profiles of metformin-induced diarrhea in detail.

In the present study, we determined the risks of metformin-induced diarrhea. An univariate logistic regression analysis indicated that six risk factors for metformin-induced diarrhea was found, including an initial dose of 750 mg, female, age ≦65, BMI ≧25, AST ≧30 IU/L and ALP ≧270 IU/L. It was notable that the incidence of diarrhea was proportionally elevated as the number of these risk factors increased, in which the incidence was dramatically increased when the number of risks exceeded 3. Thus, the number of possessed risk factors



Risk factors	Incidence of each risk factor (%)					
	750mg	Age≦65	BMI≧25	Female	ALT≧30	ALP≧270
0	0	0	0	0	0	0
1	29	19	14	19	10	10
2	47	47	34	41	16	16
3	59	86	55	41	36	24
4	100	100	89	44	56	11
5	50	100	88	88	75	100
6	100	100	100	100	100	100

Fig. 2. Relationship between the Number of Risks and the Incidence of Diarrhea Associated with Metformin Administration

Risk factors for metformin-induced diarrhea included age ≦65, female, body mass index ≧25, initial daily dose of 750 mg, ALT ≧30 IU/L and ALP ≧270 IU/L. Numbers in each parenthesis represent the number of patients with diarrhea per total number of patients. Table in the figure showed the incidence of each risk factor. Shaded column indicated the value over 50%.

would become a good predictor for metformin-induced diarrhea.

Consistent with previous studies,[8,16] we found the importance of dose of metformin since the incidence of diarrhea was significantly higher in patients who received metformin at an initial dose of 750 mg than in those who took at 500 mg (17.3% *vs.* 36.7%). Thus, in order to use a maximal effective dose of metformin without the ADRs as well as the discontinuation, the assessment of risk factors together with appropriate supports such as prophylactic use of antidiarrhoics would be expected to reduce the number of patients who should decrease the dose of metformin due to diarrhea.

The elevation of ALT and ALP also became risk factors. So far, it has been reported that metformin itself induces hepatic dysfunction, including cholestatic damage, as shown by an increase in alkaline phosphatase.[18] Moreover, liver dysfunction by cholestasis decreases the expression of organic cation transporter (OCT) 1 and 3, both of which play a crucial role in the uptake of metformin into liver.[19] Taken together, it is possible that liver damage including cholestasis might modify the incidence of diarrhea.

Several hypotheses underlying metformin-induced gastrointestinal side effects have been postulated although some of them were controversial.[1,20] Although, so far, no patients had a sign of lactic acidosis such as altered blood pH level, it is difficult to exclude out the involvement of lactic acidosis in metformin-induced diarrhea since diarrhea is the most frequent initial symptom of lactic acidosis.[1] The involvement of malabsorption of bile acid from the ilium by metformin in metformin-induced gastrointestinal side effects has also been suggested,[21] although the evidence of the malabsorption of bile salts by metformin was controversial.[21,22] Similarly, the involvement of serotonin and ghrelin in the gastrointestinal side effects of metformin was also conflicting.[20] Other possible mechanism to induce gastrointestinal side effects is the involvement of glucagon-like peptide 1 (GLP-1) since metformin elevated GLP-1 in plasma by inhibiting the degradation of GLP-1[23] and GLP-1 analogs and dipeptidyl peptidase-IV (DPP-IV) inhibitors frequently cause the gastrointestinal side effects.[24,25]

In the present study, age ≦65 became a risk factor although it is well accepted that reduced functions of kidney, liver and other organs in elderly subjects might strengthen the effect of drugs. Furthermore, we also found that female and BMI also became risk factors. It has been reported that genders and body weight failed to affect the pharmacokinetics of metformin.[26] Thus, the further investigation would be needed to understand these data.

Finally, we introduced our preliminary trial that pharmacists recommended three choices to the physicians who underwent the treatment of T2DM by metformin in patients with the risk factors exceeded 3; 1) the use of 500 mg of metformin instead of 750 mg, 2) the prophylactic use of antidiarrhoics, or 3) the discontinuation of currently-used laxatives. Results were likely to be promising since the rate of diarrhea was reduced to 12.0% (3 out of 25 patients) together with the use of 500 mg of metformin in most patients (24 out of 25 patients).

In conclusion, we found six risk factors to cause metformin-induced diarrhea: age ≦65, female, BMI ≧25, initial daily dose of 750 mg, ALT ≧30 IU/L and ALP ≧270 IU/L. The incidence of diarrhea was linearly elevated as the number of these risks increased. Thus, checking the risk factors for metformin-induced diarrhea may aid medical staffs to pre-caution the possible occurrence of metformin-induced diarrhea to the patients together with the use of antidiarrhoics, subsequently expecting to improve the adherence of metformin by reducing the prevalence of metformin-induced diarrhea.

Acknowledgement This work was supported in part by a Grant-in-Aid for Encouragement of Scientists (23929004) from Japan Society for the Promotion of Science.

REFERENCES

1) Scarpello JH, Howlett HC. Metformin therapy and clinical uses. *Diab. Vasc. Dis. Res.*, **5**, 157–167 (2008).
2) Saenz A, Fernandez-Esteban I, Mataix A, Ausejo M, Roque M, Moher D. Metformin monotherapy for type 2 diabetes mellitus. *Cochrane Database Syst. Rev.*, CD002966 (2005).
3) Nathan DM, Buse JB, Davidson MB, Ferrannini E, Holman RR, Sherwin R, Zinman B, American Diabetes Association European Association for Study of Diabetes. Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy: a consensus statement of the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetes Care*, **32**, 193–203 (2009).
4) Schramm TK, Gislason GH, Vaag A, Rasmussen JN, Folke F, Hansen ML, Fosbøl EL, Køber L, Norgaard ML, Madsen M, Hansen PR, Torp-Pedersen C. Mortality and cardiovascular risk associated with different insulin secretagogues compared with metformin in type 2 diabetes, with or without a previous myocardial infarction: a nationwide study. *Eur. Heart J.*, **32**, 1900–1908 (2011).
5) Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, Nathan DM, Diabetes Prevention Program Research Group. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N. Engl. J. Med.*, **346**, 393–403 (2002).
6) Orchard TJ, Temprosa M, Goldberg R, Haffner S, Ratner R, Marcovina S, Fowler S, Diabetes Prevention Program Research Group. The effect of metformin and intensive lifestyle intervention on the metabolic syndrome: the Diabetes Prevention Program randomized

trial. *Ann. Intern. Med.*, **142**, 611–619 (2005).
7) Lee YJ, Jeong JH. A systematic review of metformin to limit weight-gain with atypical antipsychotics. *J. Clin. Pharm. Ther.*, **36**, 537–545 (2011).
8) Garber AJ, Duncan TG, Goodman AM, Mills DJ, Rohlf JL. Efficacy of metformin in type II diabetes: results of a double-blind, placebo-controlled, dose-response trial. *Am. J. Med.*, **103**, 491–497 (1997).
9) Belcher G, Lambert C, Edwards G, Urquhart R, Matthews DR. Safety and tolerability of pioglitazone, metformin, and gliclazide in the treatment of type 2 diabetes. *Diabetes Res. Clin. Pract.*, **70**, 53–62 (2005).
10) Salpeter SR, Greyber E, Pasternak GA, Salpeter EE. Risk of fatal and nonfatal lactic acidosis with metformin use in type 2 diabetes mellitus. *Cochrane Database Syst. Rev.*, CD002967 (2010).
11) Juckett G, Trivedi R. Evaluation of chronic diarrhea. *Am. Fam. Physician*, **84**, 1119–1126 (2011).
12) Manatsathit S, Dupont HL, Farthing M, Kositchaiwat C, Leelakusolvong S, Ramakrishna BS, Sabra A, Speelman P, Surangsrirat S, Working Party of the Program Committ of the Bangkok World Congress of Gastroenterology 2002. Guideline for the management of acute diarrhea in adults. *J. Gastroenterol. Hepatol.*, **17** (Suppl.), S54–S71 (2002).
13) Thomas PD, Forbes A, Green J, Howdle P, Long R, Playford R, Sheridan M, Stevens R, Valori R, Walters J, Addison GM, Hill P, Brydon G. Guidelines for the investigation of chronic diarrhoea, 2nd edition. *Gut*, **52** (Suppl. 5), v1–v15 (2003).
14) Chassany O, Michaux A, Bergmann JF. Drug-induced diarrhoea. *Drug Saf.*, **22**, 53–72 (2000).
15) Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. *Nephron*, **16**, 31–41 (1976).
16) Bennett WL, Wilson LM, Bolen S, Maruthur N, Singh S, Chatterjee R, Marinopoulos SS, Puhan MA, Ranasinghe P, Nicholson WK, Block L, Odelola O, Dalal DS, Ogbeche GE, Chandrasekhar A, Hutfless S, Bass EB, Segal JB. "Oral Diabetes Medications for Adults With Type 2 Diabetes: An Update.": ‹http://www.ncbi.nlm.nih.gov/books/NBK55754›, Rockville MD, Agency for Healthcare Research and Quality, U.S., March, 2011.
17) Kahn SE, Haffner SM, Heise MA, Herman WH, Holman RR, Jones NP, Kravitz BG, Lachin JM, O'Neill MC, Zinman B, Viberti G, ADOPT Study Group. Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy. *N. Engl. J. Med.*, **355**, 2427–2443 (2006).
18) Nammour FE, Fayad NF, Peikin SR. Metformin-induced cholestatic hepatitis. *Endocr. Pract.*, **9**, 307–309 (2003).
19) Nies AT, Koepsell H, Winter S, Burk O, Klein K, Kerb R, Zanger UM, Keppler D, Schwab M, Schaeffeler E. Expression of organic cation transporters OCT1 (SLC22A1) and OCT3 (SLC22A3) is affected by genetic factors and cholestasis in human liver. *Hepatology*, **50**, 1227–1240 (2009).
20) Bouchoucha M, Uzzan B, Cohen R. Metformin and digestive disorders. *Diabetes Metab.*, **37**, 90–96 (2011).
21) Caspary WF, Zavada I, Reimold W, Deuticke U, Emrich D, Willms B. Alteration of bile acid metabolism and vitamin-B12-absorption in diabetics on biguanides. *Diabetologia*, **13**, 187–193 (1977).
22) Scarpello JH, Hodgson E, Howlett HC. Effect of metformin on bile salt circulation and intestinal motility in type 2 diabetes mellitus. *Diabet. Med.*, **15**, 651–656 (1998).
23) Cuthbertson J, Patterson S, O'Harte FP, Bell PM. Addition of metformin to exogenous glucagon-like peptide-1 results in increased serum glucagon-like peptide-1 concentrations and greater glucose lowering in type 2 diabetes mellitus. *Metabolism*, **60**, 52–56 (2011).
24) Bergenstal RM, Wysham C, Macconell L, Malloy J, Walsh B, Yan P, Wilhelm K, Malone J, Porter LE, DURATION-2 Study Group. Efficacy and safety of exenatide once weekly *versus* sitagliptin or pioglitazone as an adjunct to metformin for treatment of type 2 diabetes (DURATION-2): a randomised trial. *Lancet*, **376**, 431–439 (2010).
25) Drucker DJ, Nauck MA. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet*, **368**, 1696–1705 (2006).
26) Sambol NC, Chiang J, O'Conner M, Liu CY, Lin ET, Goodman AM, Benet LZ, Karam JH. Pharmacokinetics and pharmacodynamics of metformin in healthy subjects and patients with noninsulin-dependent diabetes mellitus. *J. Clin. Pharmacol.*, **36**, 1012–1021 (1996).

PLOS | ONE

Hepatic FoxO1 Integrates Glucose Utilization and Lipid Synthesis through Regulation of Chrebp O-Glycosylation

Yukari Ido-Kitamura[1,4], Tsutomu Sasaki[1], Masaki Kobayashi[1], Hye-Jin Kim[1], Yong-Soo Lee[1], Osamu Kikuchi[1], Hiromi Yokota-Hashimoto[1], Katsumi Iizuka[2], Domenico Accili[3], Tadahiro Kitamura[1]*

1 Metabolic Signal Research Center, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Gunma, Japan, **2** Division of Molecule and Structure, Department of Diabetes and Endocrinology, Gifu University School of Medicine, Gifu, Gifu, Japan, **3** Department of Medicine, College of Physicians and Surgeons of Columbia University, New York, New York, United States of America, **4** Department of Internal Medicine, Fujisawa Shounandai Hospital, Fujisawa, Kanagawa, Japan

Abstract

In liver, glucose utilization and lipid synthesis are inextricably intertwined. When glucose availability exceeds its utilization, lipogenesis increases, leading to increased intrahepatic lipid content and lipoprotein secretion. Although the fate of three-carbon metabolites is largely determined by flux rate through the relevant enzymes, insulin plays a permissive role in this process. But the mechanism integrating insulin receptor signaling to glucose utilization with lipogenesis is unknown. Forkhead box O1 (FoxO1), a downstream effector of insulin signaling, plays a central role in hepatic glucose metabolism through the regulation of hepatic glucose production. In this study, we investigated the mechanism by which FoxO1 integrates hepatic glucose utilization with lipid synthesis. We show that FoxO1 overexpression in hepatocytes reduces activity of carbohydrate response element binding protein (Chrebp), a key regulator of lipogenesis, by suppressing O-linked glycosylation and reducing the protein stability. FoxO1 inhibits high glucose- or O-GlcNAc transferase (OGT)-induced liver-pyruvate kinase (L-PK) promoter activity by decreasing Chrebp recruitment to the L-PK promoter. Conversely, FoxO1 ablation in liver leads to the enhanced O-glycosylation and increased protein level of Chrebp owing to decreased its ubiquitination. We propose that FoxO1 regulation of Chrebp O-glycosylation is a mechanism linking hepatic glucose utilization with lipid synthesis.

Citation: Ido-Kitamura Y, Sasaki T, Kobayashi M, Kim H-J, Lee Y-S, et al. (2012) Hepatic FoxO1 Integrates Glucose Utilization and Lipid Synthesis through Regulation of Chrebp O-Glycosylation. PLoS ONE 7(10): e47231. doi:10.1371/journal.pone.0047231

Editor: Rohit Kulkarni, Joslin Diabetes Center - Harvard Medical School, United States of America

Received June 25, 2012; **Accepted** September 10, 2012; **Published** October 8, 2012

Funding: This study was supported by Grants-in-Aid for Scientific Research (Y.I.-K., T.K.), Takeda Science Foundation (Y.I.-K.), Japan Diabetes Foundation (Y.I.-K.), ONO Medical Research Foundation (T.K.), The Cell Science Research Foundation (T.K.) and Global Centers of Excellence program (T.K.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: kitamura@gunma-u.ac.jp

Introduction

The liver plays a central role in integrating glucose and lipid metabolism, effectively exchanging carbons from one energy source to the other for storage and utilization [1]. This process requires both hormone signaling and feedback control by substrate flux. Examples of the latter are the diversion of three-carbon precursors from glycolysis to esterification of FFA to generate triglycerides, and the shunting of citrate from glycolysis to fatty acid synthesis by way of acetyl-CoA carboxylase and malonyl-CoA [2]. Examples of the former are the effects of insulin on expression of genes that rate-control glucose utilization and its conversion into lipids, like glucokinase, glucose-6-phosphatase, pyruvate kinase, and pyruvate dehydrogenase kinase [1]. In physiological situations, the two control mechanisms cohabit peacefully. But in the metabolic syndrome, there is an apparent discrepancy between the inability of insulin to suppress glucose production, and its preserved ability to promote de novo lipogenesis. Various theories have been advanced, but none of them is entirely satisfactory [3].

Key transcriptional mediators of insulin signaling and glucose signaling are FoxO1 and Chrebp. FoxO1 is an Akt substrate and regulates glucose production and bile acid synthesis [4–5]. Chrebp mediates glucose action on glycolysis and lipid synthesis [6]. Among its targets are liver-pyruvate kinase (*Lpk*), one of the rate-limiting enzymes of glycolysis [7], and lipogenic genes, such as acetyl-CoA carboxylase (*Acaca*) and fatty acid synthase (*Fasn*) [8]. Chrebp is activated via protein phosphatase 2A-dependent dephosphorylation in response to xylulose-5-phosphate (Xu-5-P) generated by the pentose monophosphate shunt [9]. Dephosphorylated Chrebp translocates to the nucleus and activates target gene transcription [10].

Impetus for the present studies came from prior observations that genetic ablation of FoxO1 in liver increases systemic insulin sensitivity, and results in lower hepatic glucose production, increased glycogen storage, and increased lipogenesis [11–12]. We reasoned that this model could be deconstructed for the purpose of identifying the physiological mechanism linking glucose with lipid metabolism. We identify a genetic, biochemical, and molecular pathway linking FoxO1 with Chrebp, and propose that it represents the connection between altered glucose and lipid metabolism in type 2 diabetes.

Methods

Antibodies

We purchased antibodies against Chrebp from Novus Biologicals, O-GlcNAc from Covance, OGT (DM-17) from Sigma, FoxO1 (9462) from Cell Signaling, FoxO1 (H-128), Ubiquitin (P4D1), Tubulin (B–7) from Santa-Cruz, HA (12CA5) from Roche. We used these antibodies for immunoprecipitation or immunoblotting according to manufacturer's protocol.

Expression vectors and Adenoviral vectors

We have previously described expression vectors encoding Flag-tagged FoxO1-ADA and His-HA-Ubiquitin [13], and adenoviral vectors encoding HA-tagged wild type and FoxO1-ADA [14]. pcDNA3-HA-OGT and pCMV4-Flag-Chrebp are gifts from Mark Montminy (Salk Institute, La Jolla, CA) and Howard Towle (Univ of Minnesota, Minneapolis, MN), respectively. We generated a synthetic L-PK luciferase vector containing 3x charbohydrate response element in the L-PK promoter (pGL3-3xL-PK-ChoRE).

Cell culture, siRNA transfection, and viral transduction

We purchased primary culture of mouse hepatocyte from Primary Cell Co., Ltd (Sapporo, Japan) and cultured the cells in DMEM supplemented with 10% FCS. The FoxO1-specific siRNA sequence is 5′-ACGGAGGATTGAACCAGTATA-3′. The OGT specific siRNA sequence is 5′-CGACATGCCTTGCGGCTGA -3′. siRNA was transfected using DharmaFECT Duo (Dharmacon). In some experiments, we infected primary hepatocytes with adenovirus at MOI of 10 or 30, 5 hrs before treatments with high glucose. All experiments were repeated at least three times.

Luciferase assays and Chromatin immunoprecipitation assays

We performed luciferase assays as previously described [15] using pGL3-3xL-PK-ChoRE. We performed ChIP assays in mouse primary hepatocytes or mouse liver extracts with primers; 5′-GATTTGAGCCTTTGATCCAGGCTC-3′ and 5′-AAGTTCCCTCCATCTATACAGTGC-3′ according to the previously described methods [13]. All experiments were repeated at least three times.

Immunoprecipitation and Western blotting

We lysed cultured cells in RIPA buffer containing protease inhibitors (Roche). After centrifugation, cell extracts were diluted with Co-IP buffer (50 mM Tris, 150 mM NaCl, 0.1% NP-40, 10% glycerol, 5 mM $MgCl_2$), immunoprecipitated and analyzed by immunoblotting.

mRNA isolation and real-time PCR

We isolated mRNA from primary hepatocytes or mouse liver extracts using the Micro Fast Track 2.0 kit (Invitrogen). We performed real-time RT-PCR using ImProm-II™ Reverse Transcription System (Promega) and LightCycler System (Roche). Primer sequences used for real-time PCR are as follows, for Chrebp; 5′- CTG GGG ACC TAA ACA GGA GC -3′ and 5′- GAA GCC ACC CTA TAG CTC CC −3′, for L-PK; 5′- GGG CCG CAT CTA CAT TGA C −3′ and 5′- GTC CCT CTG GGC CAA TTT T-3′. We carried out each reaction in triplicate, using a standard curve with the relevant cDNA for each primer set.

O-GlcNAc enzymatic labeling

We performed metabolic labeling of Chrebp with tetraacetylated azide-modified N-acetylglucosamine (GlcNAz) in mouse primary hepatocytes. After immunoprecipitation with anti-Chrebp antibody, we detected O-glycosylation modification using biothin-avidin system.

Animal generation and analyses

We generated liver specific FoxO1 knockout mice using FoxO1 flox/flox mice [16] and Albumin-cre transgenic mice (a kind gift from Akihiro Harada, Osaka University). The wild-type, null and $Foxo1^{flox}$ alleles were detected using PCR with primers 5′-GCT TAG AGC AGA GAT GTT CTC ACA TT-3′, 5′-CCA GAG TCT TTG TAT CAG GCA AAT AA-3′ and 5′-CAA GTC CAT TAA TTC AGC ACA TTG A-3′. Individually caged mice were housed in a temperature-controlled facility. All animal care and experimental procedures were approved by the Institutional Animal Care and Experimentation Committee at Gunma University. H-E staining was performed using 4- μm-thick paraffin sections following the standard methods. Hepatic triglyceride (TG) contents were measured as described previously [17].

Results

FoxO1 inhibits Chrebp transcriptional activity by suppressing O-glycosylation and reducing protein stability of Chrebp

Although insulin as well as glucose flux regulate hepatic glucose utilization and lipid synthesis, the underlying molecular mechanisms have not been fully understood. Because FoxO1 is a downstream effector of insulin signaling and Chrebp is a key transcriptional regulator of glycolysis and lipogenesis, we tested whether FoxO1 is involved in the regulation of Chrebp. When we overexpressed constitutively active form of FoxO1, FoxO1-ADA (a mutant FoxO1 with the following amino acid substitutions: T24A, S253D, and S316A) [18], in primary hepatocytes, mRNA level of *Lpk*, a target of Chrebp, was significantly decreased, despite unchanged levels of *Chrebp* mRNA (Fig. 1A). Therefore, we next investigated Chrebp protein levels in these samples. As shown in Figure 1B, FoxO1-ADA expression significantly decreased Chrebp protein level (Fig. 1B, second panel from the top). Because it has been reported that Chrebp protein is stabilized by modification of O-glycosylation [19], we investigated it and found that FoxO1-ADA expression decreased Chrebp O-glycosylation (Fig. 1B, top panel and the bottom graph). Consistent with the previous report [19], O-glycosylation and protein level of Chrebp were increased by high glucose (25mM) in primary hepatocytes (Fig. 1B). Conversely, when we knocked down FoxO1 using an adenovirus expressing FoxO1-specific siRNA in primary hepatocytes, Chrebp O-glycosylation was increased independent of glucose concentrations in the medium (Fig. 1C, top panel and the bottom graph).

Because protein level, but not mRNA level of Chrebp was decreased by FoxO1-ADA, we next checked whether FoxO1-ADA expression affects protein degradation of Chrebp by evaluating its ubiquitination. As shown in Fig. 1D, FoxO1-ADA expression enhanced poly-ubiquitination of both endogenous (left two lanes) and exogenously expressed Chrebp (right two lanes), indicating that changes in Chrebp ubiquitination might explain the decreased protein levels observed in our experiments. Indeed, chromatin immunoprecipitation revealed that overexpression of FoxO1-ADA suppressed high-glucose-induced recruitment of Chrebp to the *Lpk* promoter (Fig. 1E). Furthermore, results of luciferase assays using the *Lpk* promoter indicated that FoxO1-ADA inhibited high-glucose-induced or OGT-expression-induced

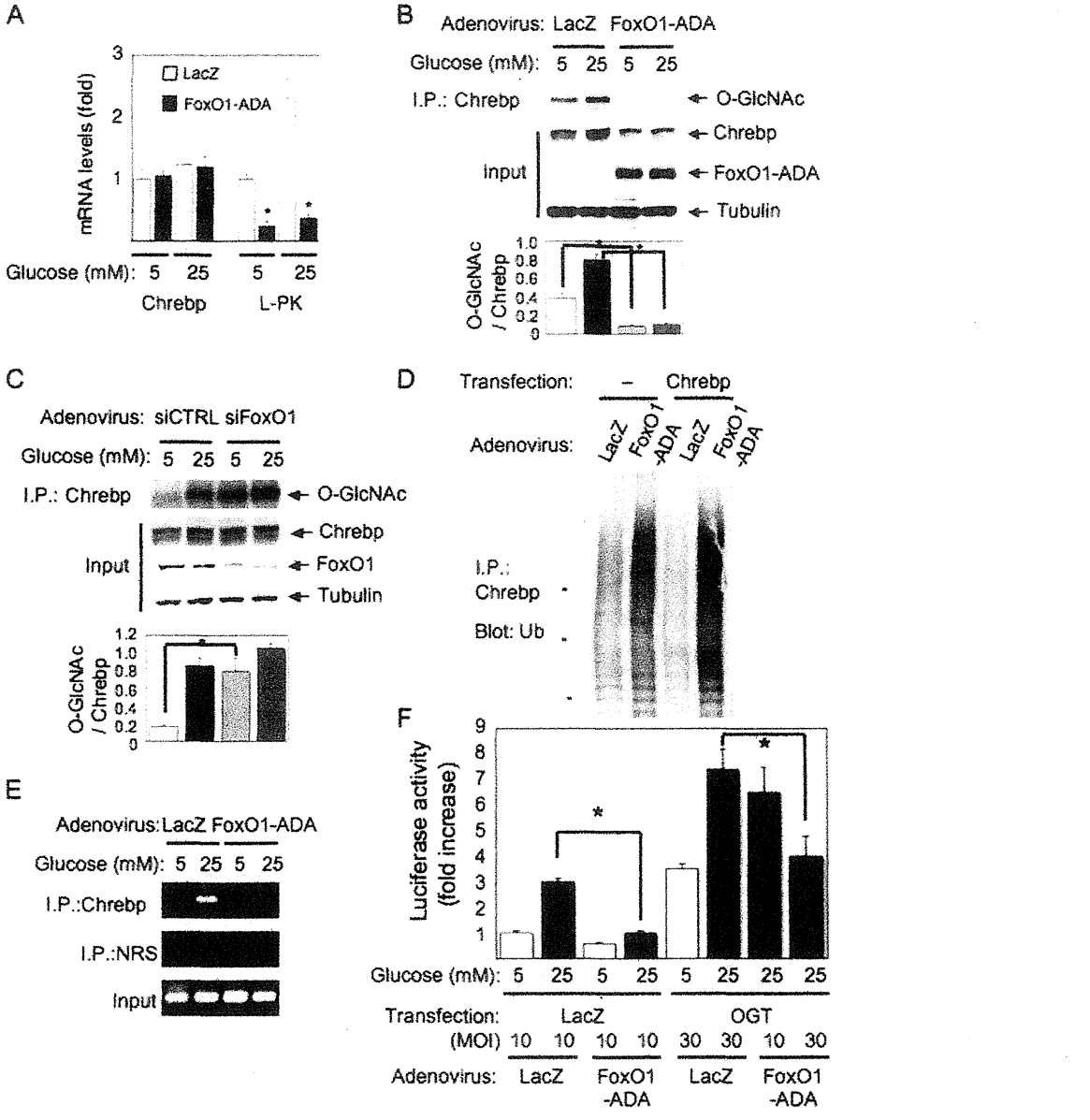


Figure 1. FoxO1-ADA expression and the knockdown of FoxO1 affect Chrebp O-glycosylation, protein stability and transcriptional activity in primary hepatocytes. (A, B, D and E) Mouse primary hepatocytes infected with adenovirus expressing FoxO1-ADA or LacZ were cultured with 5 mM or 25 mM glucose. The cell lysates were subjected to real-time RT-PCR for Chrebp or L-PK (A), immunoprecipitation with anti-Chrebp antibody followed by blotting with anti-O-GlcNAc antibody (B) or anti-ubiquitin antibody (D). The cell lysates were also subjected to chromatin-immunoprecipitation assay using anti-Chrebp antibody and the primers for L-PK promoter (E). (C) Mouse primary hepatocytes infected with adenovirus expressing specific siRNA for FoxO1 or control siRNA were cultured with 5 mM or 25 mM glucose and the cell lysates were immunoprecipitated with anti-Chrebp antibody followed by blotting with anti-O-GlcNAc antibody. Input represents expression levels of Chrebp, FoxO1-ADA, endogenous FoxO1 and Tubulin. Quantitative analyses were performed by assessment of O-glycosylation level compared with the protein level of Chrebp using densitometry, showing as a bar graph below the results of blotting (B and C). (F) Mouse primary hepatocytes co-transfected with pGL3-3XI-PK-ChoRE and OGT or LacZ were infected with adenovirus expressing FoxO1-ADA or LacZ at indicated MOI and cultured with 5 mM or 25 mM glucose for 24 hr. The cell lysates were used for luciferase assays. Experiments were repeated at least three times. Data represent mean ± SEM. *P<0.05.
doi:10.1371/journal.pone.0047231.g001

Chrebp transcriptional activity (Fig. 1F). Taken together, these results show that FoxO1 inhibits Chrebp transcriptional activity by suppressing O-glycosylation and reducing protein stability of Chrebp.

Chrebp O-glycosylation is regulated by OGT

We used western blotting with anti-GlcNAc antibody to show that Chrebp is O-glycosylated in the presence of elevated glucose concentrations (Fig. 1B). To confirm the modification of Chrebp

O-glycosylation, we also used enzymatic labeling with GlcNAz in primary hepatocytes. As shown in Figure 2A, we detected GlcNAz incorporation into Chrebp protein only in the presence of GlcNAz, indicating that Chrebp is directly modified by O-glycosylation. We next investigated whether high glucose-induced Chrebp O-glycosylation is mediated by OGT. When we transfected primary hepatocytes with OGT, Chrebp O-glycosylation was significantly increased even in low glucose condition (Fig. 2B, top panel and bottom graph). It is notable that OGT was coimmunoprecipitated with Chrebp, and that this interaction was enhanced by high glucose (Fig. 2B, second panel from the top). Conversely, when OGT was depleted by specific siRNA for OGT in primary hepatocytes, both high glucose- and glucosamine-induced O-glycosylation of Chrebp were decreased (Fig. 2C, top panel and bottom graph). Thus, Chrebp interacts with OGT in high glucose condition, leading to Chrebp O-glycosylation.

Increased Chrebp O-glycosylation, protein stability, and recruitment to the Lpk promoter in FoxO1 knockout liver

To assess the effects of FoxO1 ablation in liver on Chrebp protein, we generated liver-specific FoxO1 knockout mice (L-FoxO1-KO) by crossing Albumin-Cre mice with FoxO1 flox mice [16]. We isolated livers from L-FoxO1-KO and control mice following a 24-hr fast or 3-hr re-feeding after 24-hr fast, and performed western blotting or ChIP assays using liver lysates. In the liver of L-FoxO1-KO mice, Chrebp O-glycosylation and protein levels were significantly increased, and–more interestingly–OGT interaction with Chrebp was enhanced in both fasted and refed conditions compared with the liver of control mice (Fig. 3A, top two panels). Consistent with the increase in Chrebp protein level, Chrebp poly-ubiquitination were significantly reduced in the liver of L-FoxO1-KO mice in both fasted and refed conditions (Fig. 3B). We also found that Chrebp recruitment to the *Lpk*

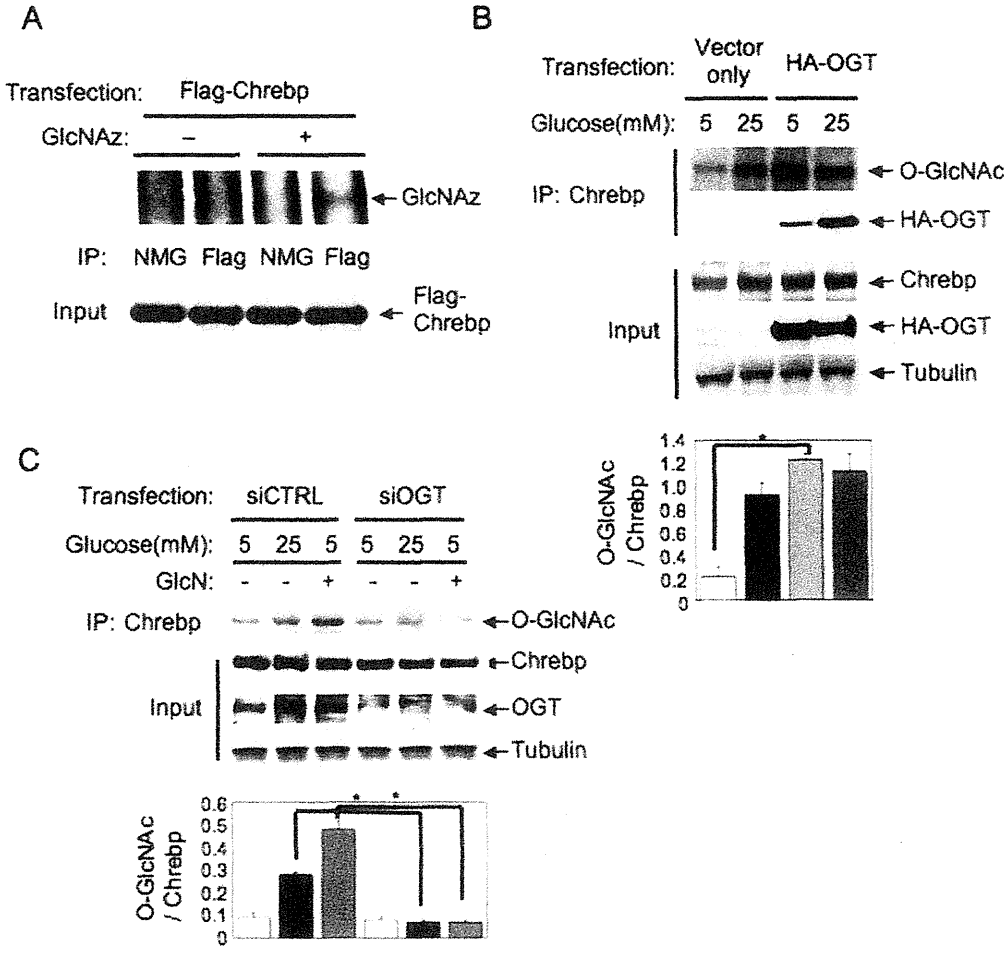


Figure 2. Chrebp O-glycosylation is regulated by OGT. (A) Mouse primary hepatocytes were transfected with Flag-Chrebp and labeled with tetraacetylated azide-modified N-acetylglucosamine (GlcNAz). The cell lysates were immunoprecipitated with anti-Flag antibody or normal mouse globlin (NMG) and subjected to detection of O-glycosylation by biothin-avidin system as described in Materials and Methods. Input indicates the expression level of Flag-Chrebp in each lane. (B and C) Mouse primary hepatocytes were transfected with HA-OGT (B) or siRNA for OGT (C) along with empty vector or control siRNA, respectively and cultured with 5 mM or 25 mM glucose for 24 hr. In some experiments, 10 mM glucosamine (GlcN) was added in the medium. The cell lysates were immunoprecipitated with anti-Chrebp antibody followed by blotting with anti-O-GlcNAc or anti-HA antibody. Input indicates the expression level of Chrebp, HA-OGT, endogenous OGT or tubulin. Quantitative analyses were performed by assessment of O-glycosylation level compared with the protein level of Chrebp using densitometry, showing as a bar graph below the results of blotting (B and C). Experiments were repeated at least three times. Data represent mean ± SEM. *P<0.05.
doi:10.1371/journal.pone.0047231.g002

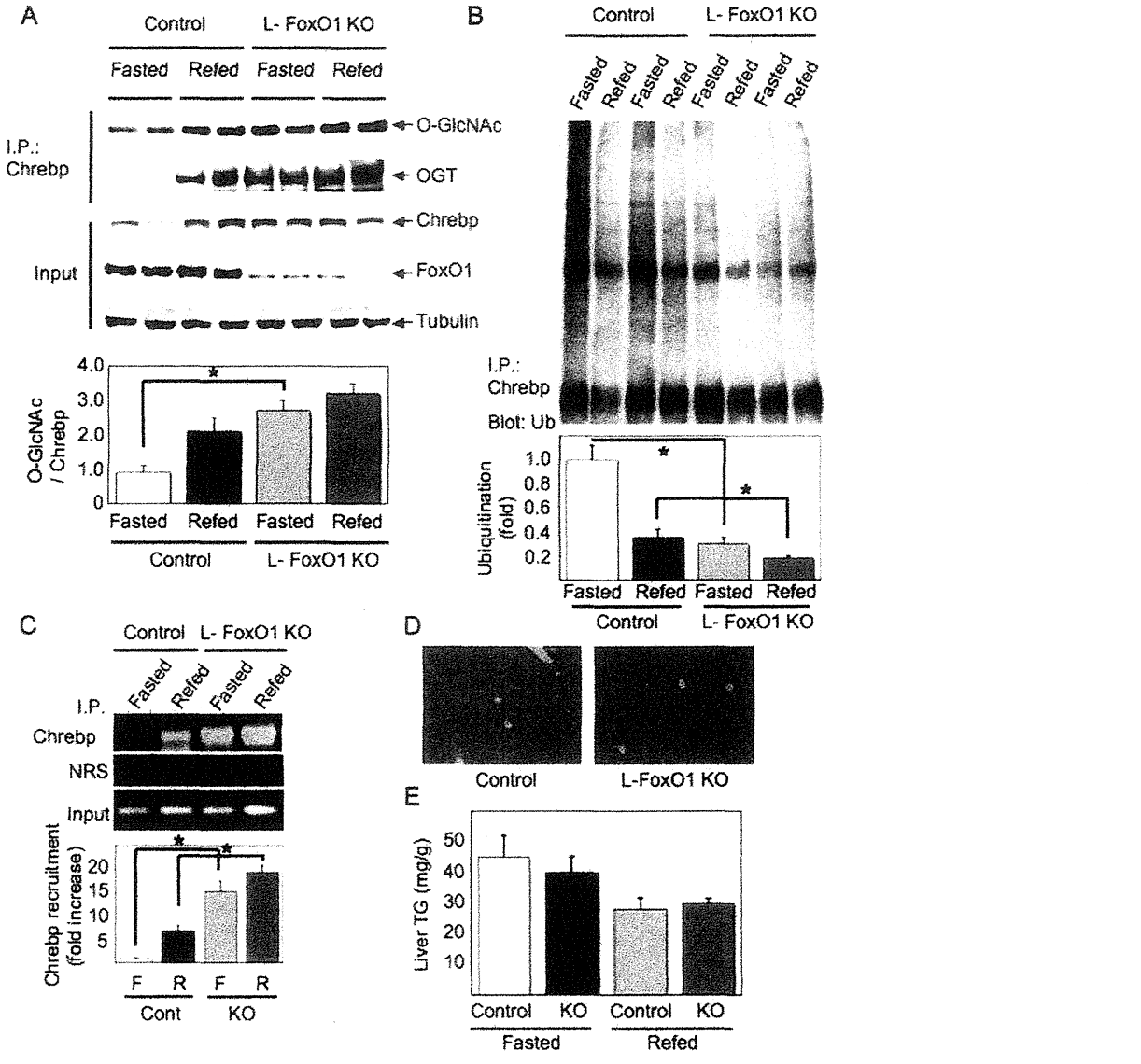


Figure 3. Chrebp O-glycosylation, protein stability, and recruitment to the L-PK promoter are increased in FoxO1 knockout liver. (A and B) The liver samples isolated from L-FoxO1-KO and the control mice at the points of 24 hr fasted or 3 hr refed after 24 hr starvation were subjected to immunoprecipitation with anti-Chrebp antibody followed by blotting with anti-O-GlcNAc, anti-OGT (A) or anti-ubiquitin antibody (B). Input indicates the expression levels of Chrebp, FoxO1 or tubulin. Quantitative analyses were performed by assessment of O-glycosylation (A) or ubiquitination (B) levels compared with the protein level of Chrebp using densitometry, showing as a bar graph below the results of blotting. (C) The liver extracts were also subjected to chromatin immunoprecipitation assay using anti-Chrebp antibody and primers for the L-PK promoter. Quantitative analyses were performed using densitometry. Input indicates extracted DNA prior to immunoprecipitation. Experiments were repeated at least three times. Data represent mean ± SEM. *$P<0.05$. (D) Hematoxylin-and-eosin (H-E) staining of the liver sections from 24 hr fasted L-FoxO1-KO and the control mice. Magnification, x100. (E) Hepatic triglyceride (TG) contents in 24 hr fasted or 3 hr refed (after 24 hr starvation) L-FoxO1-KO and the control mice ($n=6$ for each group). Data represent mean ± SEM.
doi:10.1371/journal.pone.0047231.g003

promoter was enhanced in the liver of L-FoxO1-KO mice compared with controls (Fig. 3C), consistent with our previous results showing that *Lpk* mRNA was increased in liver-specific FoxO1 knockout mice [11].

We next investigated the physiological consequences of FoxO1 ablation in the liver. Because we showed FoxO1 ablation in the liver enhanced Chrebp protein stability and Chrebp recruitment to its target gene promoter, we predicted that hepatic lipid contents should be increased in L-FoxO1-KO mice. However, histological analysis using the liver sections showed no morphological difference between L-FoxO1-KO and control mice (Figure 3D). Furthermore, hepatic triglyceride contents were unchanged in L-FoxO1-KO mice in both fasted and refed conditions (Figure 3E).

Discussion

Our studies identify a direct molecular link between insulin signaling pathways regulating hepatic glucose production and those regulating glycolysis and lipogenesis. In addition to Chrebp, another critical transcription factor for lipogenesis is sterol regulatory element-binding protein 1c (Srebp-1c) [20]. Although the transcriptional activity of Srebp-1c is mainly regulated by the cleavage of its NH_2-terminal domain and nuclear translocation [21], it is also known that Srebp-1c is regulated by insulin at the transcriptional level via liver X receptor (LXR) [22]. Recently, it has been shown that Chrebp, like Srebp-1c, is a direct target of LXR [23], indicating that Chrebp may be also regulated at the transcriptional level by insulin. Our data demonstrate a different mechanism of regulation, as we show that protein, but not mRNA levels of Chrebp are regulated by various metabolic conditions in primary hepatocytes and mouse liver. We also show that Chrebp is O-glycosylated by high glucose (in hepatocytes) and re-feeding (in liver), leading to increased protein level of Chrebp, owing to decreased ubiquitination.

Glucose taken into hepatocytes is mainly converted to pyruvate or glycogen to produce or store energy. However, excess glucose enters into hexosamine biosynthetic pathway (HBP), leading to the production of UDP-N-acetylglucosamine (UDP-GlcNAc). By using UDP-GlcNAc as the donor substrate, O-GlcNAc transferase (OGT) catalyzes O-glycosylation modification of proteins on Ser/Thr residues. Although only ~2–3% of intracellular glucose enters the HBP [24], it is known that hyperglycemia increases glucose flux into HBP and subsequent O-glycosylation of various proteins [25]. Furthermore, transgenic mice overexpressing OGT show diabetic phenotype due to insulin resistance [26]. Taken together, these data suggest that the increase in O-glycosylation is associated with the pathophysiology of diabetes. Recently, three key transcription factors for glucose metabolism, FoxO1, Pgc-1α and Torc2 (Crtc2) have been reported to be regulated by O-glycosylation modification [27–30]. Furthermore, Guinez et al. reported that Chrebp is also regulated by O-glycosylation, leading to the increase in Chrebp protein level and its transcriptional activity [19].

FoxO1 is a member of the forkhead box containing protein of the O subfamily, which regulates metabolism as well as cellular proliferation, apoptosis, differentiation and stress resistance [4]. FoxO1 transcriptional activity is regulated by insulin through phosphorylation by Akt and following nuclear exclusion [31–33]. We previously reported that FoxO1 plays a central role in regulating glucose production in liver through the regulation of gluconeogenic genes, such as glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate-carboxykinase (PEPCK) [34] [12]. We also previously reported that hepatic FoxO1 ablation leads to slight increases in *Fasn* and *Lpk*, two critical Chrebp targets, without affecting *Chrebp* mRNA levels [11]. Thus, in addition to the function of FoxO1 to increase gluconeogenesis, we propose here that FoxO1 also decreases glucose utilization and lipid synthesis by reducing Chrebp activity. Because insulin essentially inhibits FoxO1 transcriptional activity through nuclear exclusion, insulin increases glucose utilization and lipid synthesis as well as decreases glucose production (Fig. 4).

In this study, we showed FoxO1 ablation in the liver enhanced Chrebp protein stability and Chrebp recruitment to its target gene promoter. Therefore we predicted that hepatic lipid synthesis should be increased in L-FoxO1-KO mice. However, hepatic lipid contents were unchanged in these mice (Fig. 3D and 3E). One possible explanation for this phenotype was that because hyperglycemia-induced oxidative stress leads to FoxO1 activation by acetylation-dependent mechanism as we previously reported [13], the effect of FoxO1 on lipid metabolism might only become apparent in hyperglycemic conditions. Another explanation was that because not only Chrebp but also Srebp1c or LXR contribute to the regulation of hepatic lipid metabolism, the effect of FoxO1 ablation might be compensated by the other factors *in vivo*.

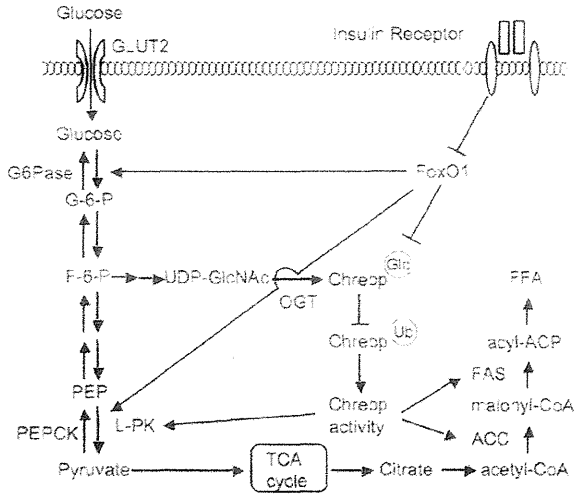


Figure 4. Proposed model for the role of FoxO1 integrating glucose utilization and lipid synthesis through regulation of Chrebp O-glycosylation.
doi:10.1371/journal.pone.0047231.g004

Considering that different amino acid residues are targeted by O-glycosylation (Ser/Thr) *vs.* ubiquitination (Lys), it remains unclear how increased O-glycosylation is associated with decreased ubiquitination of Chrebp. However, one possible mechanism is that O-glycosylation may change protein structure, affecting the susceptibility of ubiquitination and subsequent protein degradation [35]. In future studies, it will be of importance to unveil the mechanism by which FoxO1 inhibits Chrebp O-glycosylation.

Acknowledgments

We thank C. Osawa for excellent technical assistance and members of the Accili and Kitamura laboratories for stimulating discussions. Correspondence and requests for materials should be addressed to T.K.

Author Contributions

Conceived and designed the experiments: YI-K TK. Performed the experiments: YI-K TS MK H-JK Y-SL OK HY-H KI. Analyzed the data: YI-K TS MK DA TK. Contributed reagents/materials/analysis tools: YI-K TS MK HY-H KI. Wrote the paper: DA TK.

References

1. Lin HV, Accili D (2011) Hormonal regulation of hepatic glucose production in health and disease. Cell Metab 14: 9–19.
2. Ferre P, Foufelle F (2010) Hepatic steatosis: a role for de novo lipogenesis and the transcription factor SREBP-1c. Diabetes Obes Metab 12 Suppl 2: 83–92.
3. Haeusler RA, Accili D (2008) The double life of Irs. Cell Metab 8: 7–9.
4. Accili D, Arden KC (2004) FoxOs at the Crossroads of Cellular Metabolism, Differentiation, and Transformation. Cell 117: 421–426.

5. Shin DJ, Osborne TF (2009) FGF15/FGFR4 integrates growth factor signaling with hepatic bile acid metabolism and insulin action. J Biol Chem 284: 11110–11120.
6. Uyeda K, Repa JJ (2006) Carbohydrate response element binding protein, ChREBP, a transcription factor coupling hepatic glucose utilization and lipid synthesis. Cell Metab 4: 107–110.
7. Yamashita H, Takenoshita M, Sakurai M, Bruick RK, Henzel WJ, et al. (2001) A glucose-responsive transcription factor that regulates carbohydrate metabolism in the liver. Proc Natl Acad Sci U S A 98: 9116–9121.
8. Ishii S, Iizuka K, Miller BC, Uyeda K (2004) Carbohydrate response element binding protein directly promotes lipogenic enzyme gene transcription. Proc Natl Acad Sci U S A 101: 15597–15602.
9. Kabashima T, Kawaguchi T, Wadzinski BE, Uyeda K (2003) Xylulose 5-phosphate mediates glucose-induced lipogenesis by xylulose 5-phosphate-activated protein phosphatase in rat liver. Proc Natl Acad Sci U S A 100: 5107–5112.
10. Kawaguchi T, Takenoshita M, Kabashima T, Uyeda K (2001) Glucose and cAMP regulate the L-type pyruvate kinase gene by phosphorylation/dephosphorylation of the carbohydrate response element binding protein. Proc Natl Acad Sci U S A 98: 13710–13715.
11. Haeusler RA, Han S, Accili D (2010) Hepatic FoxO1 ablation exacerbates lipid abnormalities during hyperglycemia. J Biol Chem 285: 26861–26868.
12. Matsumoto M, Pocai A, Rossetti L, Depinho RA, Accili D (2007) Impaired regulation of hepatic glucose production in mice lacking the forkhead transcription factor Foxo1 in liver. Cell Metab 6: 208–216.
13. Kitamura YI, Kitamura T, Kruse JP, Raum JC, Stein R, et al. (2005) FoxO1 protects against pancreatic beta cell failure through NeuroD and MafA induction. Cell Metab 2: 153–163.
14. Nakae J, Kitamura T, Silver DL, Accili D (2001) The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. J Clin Invest 108: 1359–1367.
15. Kitamura T, Nakae J, Kitamura Y, Kido Y, Biggs WH, 3rd et al. (2002) The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. J Clin Invest 110: 1839–1847.
16. Paik JH, Kollipara R, Chu G, Ji H, Xiao Y, et al. (2007) FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell 128: 309–323.
17. Matsumoto M, Han S, Kitamura T, Accili D (2006) Dual role of transcription factor FoxO1 in controlling hepatic insulin sensitivity and lipid metabolism. J Clin Invest 116: 2464–2472.
18. Nakae J, Kitamura T, Kitamura Y, Biggs WH, Arden KC, et al. (2003) The forkhead transcription factor foxo1 regulates adipocyte differentiation. Dev Cell 4: 119–129.
19. Guinez C, Filhoulaud G, Rayah-Benhamed F, Marmier S, Dubuquoy C, et al. (2011) O-GlcNAcylation increases ChREBP protein content and transcriptional activity in the liver. Diabetes 60: 1399–1413.
20. Horton JD, Goldstein JL, Brown MS (2002) SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest 109: 1125–1131.
21. Brown MS, Goldstein JL (1999) A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. Proc Natl Acad Sci U S A 96: 11041–11048.
22. Chen G, Liang G, Ou J, Goldstein JL, Brown MS (2004) Central role for liver X receptor in insulin-mediated activation of Srebp-1c transcription and stimulation of fatty acid synthesis in liver. Proc Natl Acad Sci U S A 101: 11245–11250.
23. Cha JY, Repa JJ (2007) The liver X receptor (LXR) and hepatic lipogenesis. The carbohydrate-response element-binding protein is a target gene of LXR. J Biol Chem 282: 743–751.
24. Bouche C, Serdy S, Kahn CR, Goldfine AB (2004) The cellular fate of glucose and its relevance in type 2 diabetes. Endocr Rev 25: 807–830.
25. Copeland RJ, Bullen JW, Hart GW (2008) Cross-talk between GlcNAcylation and phosphorylation: roles in insulin resistance and glucose toxicity. Am J Physiol Endocrinol Metab 295: E17–28.
26. McClain DA, Lubas WA, Cooksey RC, Hazel M, Parker GJ, et al. (2002) Altered glycan-dependent signaling induces insulin resistance and hyperleptinemia. Proc Natl Acad Sci U S A 99: 10695–10699.
27. Housley MP, Rodgers JT, Udeshi ND, Kelly TJ, Shabanowitz J, et al. (2008) O-GlcNAc regulates FoxO activation in response to glucose. J Biol Chem 283: 16283–16292.
28. Kuo M, Zilberfarb V, Gangneux N, Christeff N, Issad T (2008) O-glycosylation of FoxO1 increases its transcriptional activity towards the glucose 6-phosphatase gene. FEBS Lett 582: 829–834.
29. Housley MP, Udeshi ND, Rodgers JT, Shabanowitz J, Puigserver P, et al. (2009) A PGC-1alpha-O-GlcNAc transferase complex regulates FoxO transcription factor activity in response to glucose. J Biol Chem 284: 5148–5157.
30. Dentin R, Hedrick S, Xie J, Yates J, 3rd Montminy M (2008) Hepatic glucose sensing via the CREB coactivator CRTC2. Science 319: 1402–1405.
31. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, et al. (1999) Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. Cell 96: 857–868.
32. Kops GJ, de Ruiter ND, De Vries-Smits AM, Powell DR, Bos JL, et al. (1999) Direct control of the Forkhead transcription factor AFX by protein kinase B. Nature 398: 630–634.
33. Nakae J, Park BC, Accili D (1999) Insulin stimulates phosphorylation of the forkhead transcription factor FKHR on serine 253 through a Wortmannin-sensitive pathway. J Biol Chem 274: 15982–15985.
34. Nakae J, Biggs WH, Kitamura T, Cavenee WK, Wright CV, et al. (2002) Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. Nat Genet 32: 245–253.
35. Spiriti J, Bogani F, van der Vaart A, Ghirlanda G (2008) Modulation of protein stability by O-glycosylation in a designed Gc-MAF analog. Biophys Chem 134: 157–167.

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i13.1508

World J Gastroenterol 2012 April 7; 18(13): 1508-1516
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Identification of individuals with non-alcoholic fatty liver disease by the diagnostic criteria for the metabolic syndrome

Masahide Hamaguchi, Noriyuki Takeda, Takao Kojima, Akihiro Ohbora, Takahiro Kato, Hiroshi Sarui, Michiaki Fukui, Chisato Nagata, Jun Takeda

Masahide Hamaguchi, Immunology Frontier Research Center at Osaka University, Osaka 5650871, Japan
Takao Kojima, Akihiro Ohbora, Takahiro Kato, Department of Gastroenterology, Murakami Memorial Hospital, Asahi University, Gifu 5008523, Japan
Noriyuki Takeda, Hiroshi Sarui, Department of Endocrinology and Metabo lism, Murakami Memorial Hospital, Asahi University, Gifu 5008523, Japan
Michiaki Fukui, Department of Endocrinology and Metabolism, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto 6028566, Japan
Chisato Nagata, Department of Epidemiology and Preventive Medicine, Graduate School of Medicine, Gifu University, Gifu 5008523, Japan
Jun Takeda, Department of Diabetes and Endocrinology, Division of Molecule and Structure, Graduate School of Medicine, Gifu University, Gifu 5008523, Japan
Author contributions: Hamaguchi M and Takeda N designed research; Hamaguchi M, Kojima T, Takeda N, Ohbora A, Kato T, Sarui H, Fukui M, Nagata C and Takeda J performed research; Hamaguchi M analyzed data; and Hamaguchi M and Takeda N wrote the paper.
Supported by Young Scientists (B) (23790791) from Japan Society for the Promotion of Science
Correspondence to: Masahide Hamaguchi, MD, PhD, Immunology Frontier Research Center at Osaka University, Osaka 5650871, Japan. mhamaguchi@frontier.kyoto-u.ac.jp
Telephone: +81-6-68794275 Fax: +81-6-68794272
Received: June 2, 2011 Revised: January 6, 2012
Accepted: January 18, 2012
Published online: April 7, 2012

Abstract

AIM: To clarify the efficiency of the criterion of metabolic syndrome to detecting non-alcoholic fatty liver disease (NAFLD).

METHODS: Authors performed a cross-sectional study involving participants of a medical health checkup program including abdominal ultrasonography. This study involved 11 714 apparently healthy Japanese men and women, 18 to 83 years of age. NAFLD was defined by abdominal ultrasonography without an alcohol intake of more than 20 g/d, known liver disease, or current use of medication. The revised criteria of the National Cholesterol Education Program Adult Treatment Panel III were used to characterize the metabolic syndrome.

RESULTS: NAFLD was detected in 32.2% (95% CI: 31.0%-33.5%) of men (n = 1874 of 5811) and in 8.7% (95% CI: 8.0%-9.5%) of women (n = 514 of 5903). Among obese people, the prevalence of NAFLD was as high as 67.3% (95% CI: 64.8%-69.7%) in men and 45.8% (95% CI: 41.7%-50.0%) in women. Although NAFLD was thought of as being the liver phenotype of metabolic syndrome, the prevalence of the metabolic syndrome among subjects with NAFLD was low both in men and women. 66.8% of men and 70.4% of women with NAFLD were not diagnosed with the metabolic syndrome. 48.2% of men with NAFLD and 49.8% of women with NAFLD weren't overweight [body mass index (BMI) $\geq$ 25 kg/m^2]. In the same way, 68.6% of men with NAFLD and 37.9% of women with NAFLD weren't satisfied with abdominal classification ($\geq$ 90 cm for men and $\geq$ 80 cm for women). Next, authors defined it as positive at screening for NAFLD when participants satisfied at least one criterion of metabolic syndrome. The sensitivity of the definition "at least 1 criterion" was as good as 84.8% in men and 86.6% in women. Separating subjects by BMI, the sensitivity was higher in obese men and women than in non-obese men and women (92.3% *vs* 76.8% in men, 96.1% *vs* 77.0% in women, respectively).

CONCLUSION: Authors could determine NAFLD effectively in epidemiological study by modifying the usage of the criteria for metabolic syndrome.

Key words: Nonalcoholic fatty liver; Metabolic syndrome; Population based study; Methodology

Peer reviewer: Liqing Yu, Assistant Professor, Pathology-Lipid Sciences, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem 27157-1040, United States

Hamaguchi M, Takeda N, Kojima T, Ohbora A, Kato T, Sarui H, Fukui M, Nagata C, Takeda J. Identification of individuals with non-alcoholic fatty liver disease by the diagnostic criteria for the metabolic syndrome. *World J Gastroenterol* 2012; 18(13): 1508-1516 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i13/1508.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i13.1508

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a common clinical condition with histological features that resemble those of alcohol-induced liver injury, but occurs in patients who do not drink an excessive amount of alcohol (ethanol > 20 g/d)[1,2]. This disease is often associated with obesity[3], type 2 diabetes mellitus[4,5], dyslipidemia[6], and hypertension[7]. Each of these abnormalities carries a cardiovascular disease risk, and together they are often categorized as the insulin resistance syndrome or the metabolic syndrome[8-15].

NAFLD is now considered to be the hepatic representation of the metabolic syndrome[10-15].

Conventional radiology studies used in the diagnosis of fatty liver include ultrasound (US), computed tomography, and magnetic resonance (MR) imaging. Other than these radiological studies, we have no sensitive and low invasive screening method for NAFLD. Alanine aminotransferase (ALT) > 30 IU/L was usually used as the cut off level of screening NAFLD[16,17]. This threshold had a sensitivity of 0.92 for detecting the fatty-fibrotic pattern proven by ultrasound among obese children[18]. However, ALT was within normal levels in 69% of those who had increased liver fat[19]. Similarly, in the Dallas Heart Study, 79% of the subjects with a fatty liver (liver fat content > 5.6%) had normal serum ALT[20]. This implies that a normal ALT does not exclude steatosis. Aspartate aminotransferase (AST) and gamma glutamyltransferase (GGT) also correlate with liver fat content independent of obesity[21], but are even less sensitive than serum ALT.

It was well known that NAFLD was associated with the metabolic syndrome and patients with NAFLD tend to be accompanied with the abnormal component of the metabolic syndrome. However, the efficiency of the criterion of metabolic syndrome for detecting NAFLD has not yet been clarified. We aimed to clarify the efficiency and perform a cross sectional study among apparent healthy Japanese.

MATERIALS AND METHODS

Study design

We performed a cross-sectional study involving participants of a medical health checkup program including abdominal ultrasonography. The program was conducted in the Medical Health Checkup Center at Murakami Memorial Hospital, Gifu, Japan. The purpose of the medical health checkup program is to promote public health through early detection of chronic diseases and the evaluation of their underlying risk factors. Known as a "human dock", medical services of this kind are very popular in Japan.

Study population

All the subjects participating in such health checkup programs at Murakami Memorial Hospital between January 2004 and December 2008 were invited to join this study. The study was approved by the ethics committee of Murakami Memorial Hospital.

Data collection and exclusion criteria were described previously[8]. In short, we collected the data from urinalysis, blood cell counts, blood chemistry and abdominal ultrasonography. The medical history and lifestyle factors were collected by using a self-administered questionnaire. Exclusion criteria were an alcohol intake of more than 20 g/d, known liver disease, or current use of medication which could influence the metabolic syndrome such as anti-diabetic drugs, anti-hypertensive drugs, anti-dyslipidemic drugs, anti-gout drugs, and/or anti-obesity drugs[8,10].

According to the revised National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III)[22] or the new International Diabetes Federation (IDF) definition[23], subjects who had three or more of the following criteria were diagnosed as having the metabolic syndrome. Fatty liver was defined on the basis of ultrasonographic findings[24]. Of 4 known criteria (hepatorenal echo contrast, liver brightness, deep attenuation, and vascular blurring), the participants were required to have hepatorenal contrast and liver brightness to be given a diagnosis of fatty liver[24].

During study period, we invited 20 012 participants in the health checkup program to enroll in the study. Of those, a total of 17 262 Japanese participants (10 329 men and 6933 women) were enrolled after giving informed consent to be included in the study. We excluded 621 participants (420 men and 201 women) who had known liver disease. In addition, 3330 participants (3042 men and 288 women) who consumed more than 20 g of ethanol per day and 1579 participants (1056 men and 541 women) who were currently receiving medication were excluded. As a result, this study ultimately consisted of 11 714 participants (5811 men and 5903 women). The mean ± SD age was 45.5 ± 9.4 years (range: 18 years to 83 years) for men and 44.3 ± 9.3 years (range: 18 years to 79 years) for women, respectively. The mean body mass index (BMI) was 23.2 ± 3.1 kg/m^2 (range: 14.3 to 41.0 kg/m^2) in men and 21.1 ± 3.0 kg/m^2 (range: 14.0 to 58.3 kg/m^2) in women, respectively. The mean abdominal circumference was 81.2 ± 8.1 cm (range: 57.3 cm to 127.5 cm) in men and 71.4 ± 8.2 cm (range: 49.0 cm to 145.0 cm) in women, respectively.

Table 1 The basic characteristics of the study population and the association of nonalcoholic fatty liver disease with gender difference

Men	Total *n* (%)	Obese *n* (%)	Non-obese *n* (%)
Number	5811	1441	4370
NAFLD	1874 (32.2)	970 (67.3)	904 (20.7%)
5 criteria of the metabolic syndrome			
Increased abdominal circumference	791 (13.6)	703 (48.8)	88 (2)
Elevated fasting glucose level	1967 (33.8)	704 (48.9)	1263 (28.9)
Elevated blood pressure	1294 (22.3)	575 (39.9)	719 (16.5)
Decreased HDL cholesterol level	1736 (29.9)	654 (45.4)	1082 (24.8)
Elevated triglyceride level	1063 (18.3)	484 (33.6)	579 (13.2)
ALT > 30	1269 (21.8)	670 (46.5)	599 (13.7)
MS defined by rNCEP-ATP Ⅲ	873 (15)	578 (40.1)	295 (6.8)
MS defined by IDF	479 (8.2)	443 (30.7)	36 (0.8)
At least 1 criterion	3680 (63.3)	1291 (89.6)	2389 (54.7)
At least 2 criteria	1955 (33.6)	957 (66.4)	998 (22.8)
At least 1 criterion or ALT > 30 IU/L	3885 (66.9)	1337 (92.8)	2548 (58.3)
Women			
Number	5903	563	5340
NAFLD	514 (8.7)	258 (45.8)	256 (4.8)
5 criteria of the metabolic syndrome			
Increased abdominal circumference	878 (14.9)	430 (76.4)	448 (8.4)
Elevated fasting glucose level	679 (11.5)	176 (31.3)	503 (9.4)
Elevated blood pressure	578 (9.8)	185 (32.9)	393 (7.4)
Decreased HDL cholesterol level	1320 (22.4)	265 (47.1)	1055 (19.8)
Elevated triglyceride level	195 (3.3)	73 (13)	122 (2.3)
Elevated ALT (ALT > 30 IU/L)	200 (3.4)	78 (13.9)	122 (2.3)
MS defined by rNCEP-ATP Ⅲ	300 (5.1)	174 (30.9)	126 (2.4)
MS defined by IDF	254 (4.3)	162 (28.8)	92 (1.7)
At least 1 criterion	2374 (40.2)	511 (90.8)	1863 (34.9)
At least 2 criteria	853 (14.5)	355 (63.1)	498 (9.3)
At least 1 criterion or elevated ALT	2430 (41.2)	515 (91.5)	1915 (35.9)

NAFLD: Nonalcoholic fatty liver disease; US: Abdominal ultrasonography; BMI: Body mass index; HDL: High density lipoprotein; MS: Metabolic syndrome; rNCEP-ATPⅢ: Revised National Cholesterol Education Program Adult Treatment Panel Ⅲ definition; IDF: International diabetes federation definition; ALT: Alanine aminotransferase.

Statistical analysis

The R version 2.9.0 (available from http://www.r-project.org/) was used for statistical analyses. Two groups of subjects were compared by using the unpaired *t*-test and the chi-square test, and a $P < 0.05$ was accepted as a significant level.

RESULTS

Basic characteristics of study population

The metabolic syndrome defined by revised NCEP-ATPⅢ definition was detected in 15.0% (95% CI: 14.1%-16.0%) of men ($n = 873$ of 5811) and in 5.1% (95% CI: 4.5%-5.7%) of women ($n = 300$ of 5903). The metabolic syndrome defined by IDF definition was detected in 8.2% (95% CI: 7.5%-9.0%) of men ($n = 479$ of 5811) and in 4.3% (95% CI: 3.8%-4.8%) of women ($n = 254$ of 5903) (Table 1). Among obese people, the metabolic syndrome defined by revised NCEP-ATPⅢ definition was detected in 40.1% (95% CI: 37.6%-42.7%) of men and in 30.9% (95% CI: 27.1%-34.9%) of women, and the metabolic syndrome defined by IDF definition was detected in 30.7% (95% CI: 28.4%-33.2%) of men and in 28.8% (95% CI: 25.1%-32.7%) of women, respectively (Table 1).

Association of NAFLD with gender difference, or body fat accumulation

NAFLD was detected in 32.2% (95% CI: 31.0%-33.5%) of men ($n = 1874$ of 5811) and in 8.7% (95% CI: 8.0%-9.5%) of women ($n = 514$ of 5903). The prevalence of NAFLD in men was four times higher than those in women (Table 1). Among obese people, the prevalence of NAFLD was as high as 67.3% (95% CI: 64.8%-69.7%) in men and 45.8% (95% CI: 41.7%-50.0%) in women (Table 1). NAFLD was associated with body fat accumulation strongly both in men and women.

When we separated by quartile the subjects according to their BMI or abdominal circumference, half of NAFLD men and three quarters of NAFLD women were classified in the superior quartile. The prevalence of NAFLD was increased according to the increase of BMI or abdominal circumference (Figure 1A). The role of BMI for NAFLD was equal to that of abdominal circumference both in men and women. The ratio of NAFLD in the superior quartile/total NAFLD was higher in women than in men. The prevalence of individuals who met two or more of the MS criteria other than waist circumference was increased according to the increase of BMI or abdominal circumference (Figure 1B).

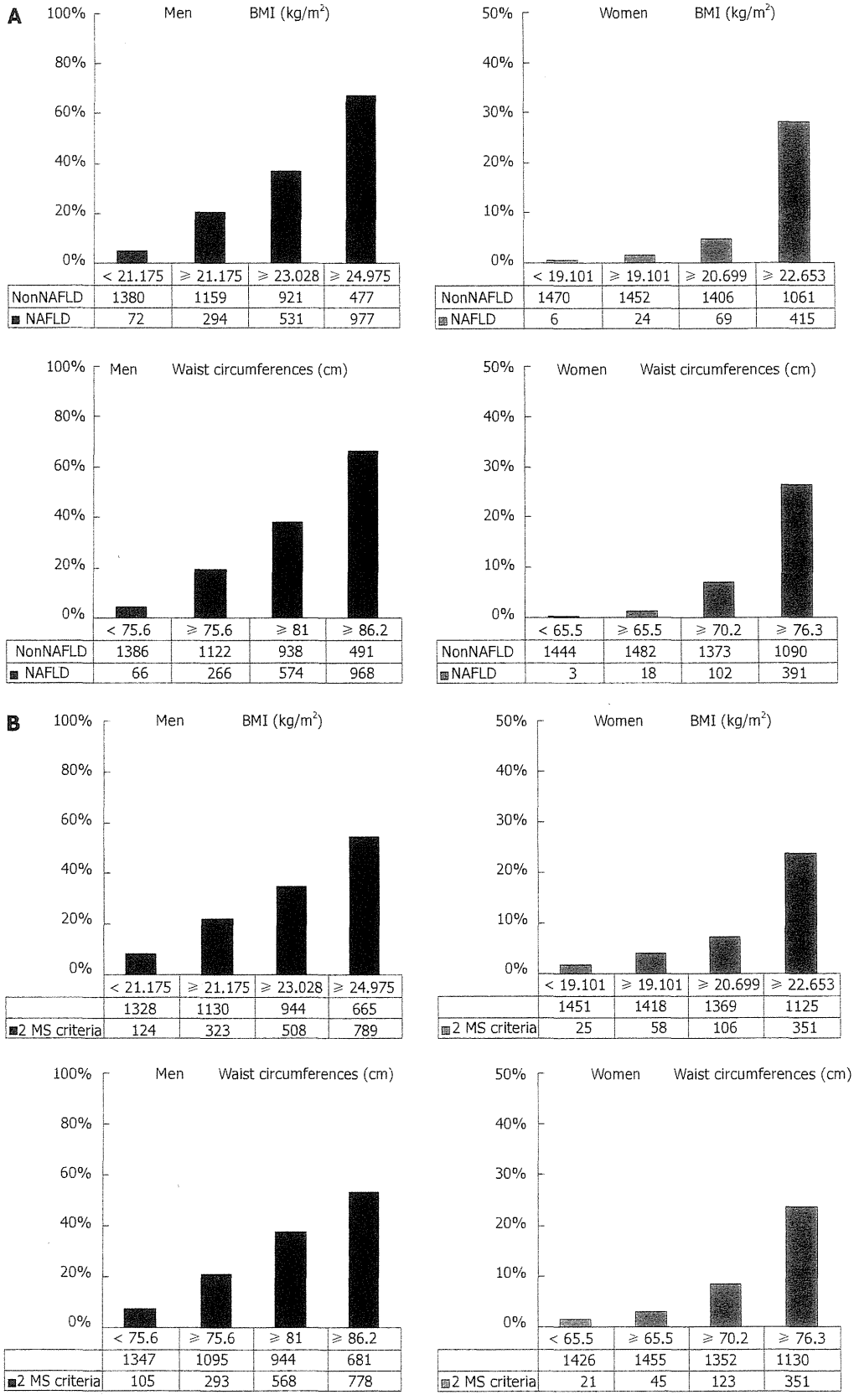


Figure 1 We separated the subjects by quartile according to their body mass index or abdominal circumference. A: The bar indicated the prevalence (%) of individuals with NAFLD; B: Individuals who meet two or more of the MS criteria other than waist circumference according to BMI or waist circumference quartiles. 2 MS criteria means individuals who meets two or more of the MS criteria other than waist circumference. NAFLD: Nonalcoholic fatty liver disease; BMI: Body mass index; MS: Metabolic syndrome.

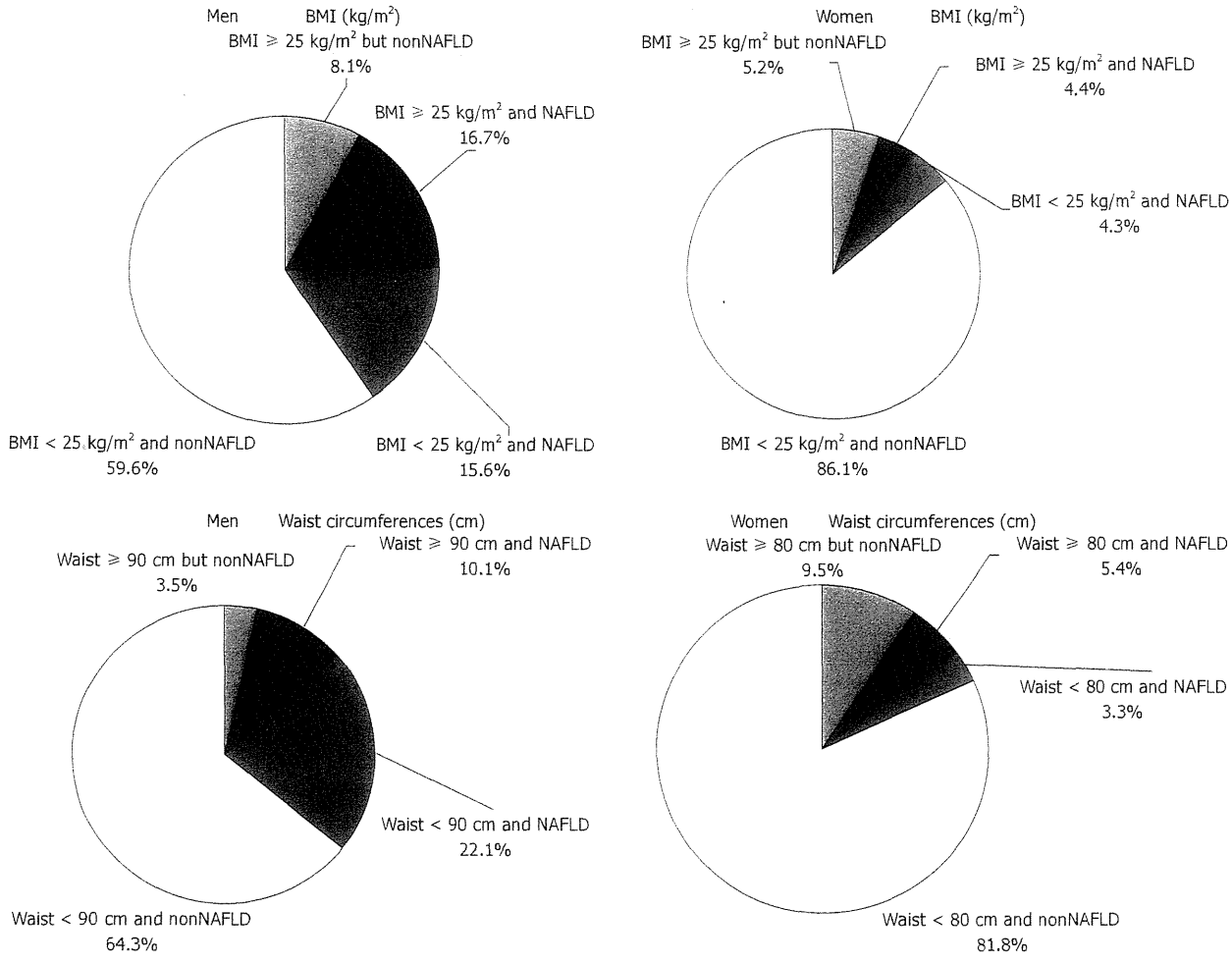


Figure 2 This figure indicates the prevalence of non-alcoholic fatty liver disease and alcoholic fatty liver disease with or without patients being overweight (BMI ≥ 25 kg/m²) or having elevated abdominal circumferences (≥ 90 cm for men and ≥ 80 cm for women). Data was expressed as prevalence (%). NAFLD accompanied with being overweight occurred in 51.8% of NAFLD men (970/1874) and 50.2% of NAFLD women (258/514). NAFLD accompanied by elevated abdominal circumference occurred in 31.4% of NAFLD men (588/1874) and 62.1% of women (319/514). NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index.

Role of the criteria of the metabolic syndrome in detecting or diagnosing NAFLD in obese or non-obese population

Although NAFLD was associated with obesity or body fat accumulation strongly, the population that was neither overweight (BMI ≥ 25 kg/m²) nor had elevated abdominal circumference was not small (Figure 2). Actually, 48.2% of men with NAFLD and 49.8% of women with NAFLD were not overweight (BMI ≥ 25 kg/m²). Similarly, 68.6% of men with NAFLD and 37.9% of women with NAFLD did not satisfy increased abdominal circumference classification. Half of the NAFLD group was classified as non-obese, but the prevalence of NAFLD among the non-obese population was lower. These facts means an effective method is needed to detect NAFLD among the non-obese population. Then, we separated the subjects into two groups, obese group or non-obese group, and investigated the efficacy of the criteria of metabolic syndrome for detecting NAFLD in each group.

Among the criteria for metabolic syndrome, the criterion of abdominal circumferences (≥ 80 cm) had high sensitivity (87.6%) for detecting NAFLD in women who were overweight (BMI ≥ 25 kg/m²) (Table 2). In other words, abdominal circumference was effective for detecting NAFLD in obese women. However, the criterion of abdominal circumference had low sensitivity (36.3%) in non-obese women. The sensitivity of abdominal circumference (≥ 90 cm) was very low (5.8%) in non-obese men. Even in obese men the sensitivity was not high (55.3%). Other criteria for metabolic syndrome had higher sensitivity in obese men and women than in the non-obese population but sensitivity never exceeded 60%.

As a screening tool for NAFLD, the sensitivity of elevated ALT (ALT > 30 IU/L) was 49.7% in men, which exceeded the sensitivity of the criteria of metabolic syndrome, but it was 17.7% in women, which was lower than all metabolic syndrome criteria were. On the other hand, the specificity of elevated ALT was as high as 90.6% in men and 98.0% in women, but the criteria of metabolic syndrome had equally high specificity.

Next, we defined it as positive at screening for NAFLD

Table 2 The role of the criteria of the metabolic syndrome in detecting or diagnosing nonalcoholic fatty liver disease in obese or non-obese population

	Men				Women			
Sensitivity	Total %	Obese %	Non-obese %	*P* value	Total %	Obese %	Non-obese %	*P* value
5 criteria of the metabolic syndrome								
Increased abdominal circumference	31.40	55.30	5.80	< 0.001	62.10	87.60	36.30	< 0.001
Elevated fasting glucose level	49.10	52.10	45.90	0.008	36.80	42.20	31.30	0.013
Elevated blood pressure	34.70	44.10	24.60	< 0.001	31.90	41.50	22.30	< 0.001
Decreased HDL cholesterol level	44.10	49.10	38.80	< 0.001	50.40	56.60	44.10	0.006
Elevated triglyceride level	35.20	41.00	28.90	< 0.001	17.90	20.50	15.20	0.15
Elevated ALT (ALT > 30 IU/L)	47.90	59.20	35.80	< 0.001	17.70	24.00	11.30	< 0.001
MS defined by rNCEP-ATP Ⅲ	33.20	48.60	16.80	< 0.001	32.50	45.00	19.90	< 0.001
MS defined by IDF	21.00	38.10	2.70	< 0.001	29.60	43.40	15.60	< 0.001
At least 1 criterion	84.80	92.30	76.80	< 0.001	86.60	96.10	77.00	< 0.001
At least 2 criteria	61.00	74.20	46.90	< 0.001	61.10	77.50	44.50	< 0.001
At least 1 criterion or elevated ALT	90.40	96.20	84.20	< 0.001	87.40	96.90	79.70	< 0.001
Specificity								
5 criteria of the metabolic syndrome								
Increased abdominal circumference	94.80	64.50	99.00	< 0.001	89.60	33.10	93.00	< 0.001
Elevated fasting glucose level	73.40	57.70	75.50	< 0.001	90.90	78.00	91.70	< 0.001
Elevated blood pressure	83.60	68.80	85.70	< 0.001	92.30	74.40	93.40	< 0.001
Decreased HDL cholesterol level	76.90	62.20	78.90	< 0.001	80.30	61.00	81.50	< 0.001
Elevated triglyceride level	89.70	81.70	90.80	< 0.001	98.10	93.40	98.40	< 0.001
Elevated ALT (ALT > 30 IU/L)	90.60	79.60	92.10	< 0.001	98.00	94.80	98.20	< 0.001
MS defined by rNCEP-ATP Ⅲ	93.60	77.30	95.90	< 0.001	97.50	81.00	98.50	< 0.001
MS defined by IDF	97.80	84.50	99.70	< 0.001	98.10	83.60	99.00	< 0.001
At least 1 criterion	46.90	15.90	51.10	< 0.001	64.20	13.80	67.20	< 0.001
At least 2 criteria	79.40	49.70	83.40	< 0.001	90.00	49.20	92.40	< 0.001
At least 1 criterion or elevated ALT	44.30	14.20	48.40	< 0.001	63.20	13.10	65.40	< 0.001

NAFLD: Nonalcoholic fatty liver disease; US: Abdominal ultrasonography; BMI: Body mass index; HDL: High dense lipoprotein; MS: Metabolic syndrome; rNCEP-ATPⅢ: Revised National Cholesterol Education Program Adult Treatment Panel Ⅲ definition; IDF: International Diabetes Federation definition; ALT: Alanine aminotransferase.

when participants satisfied at least one or two components of metabolic syndrome. The sensitivity of the definition "at least 1 criterion" was 84.8% in men and 86.6% in women. Separating subjects with BMI, the sensitivity was higher in obese men and women than in non-obese men and women (92.3% *vs* 76.8% in men, 96.1% *vs* 77.0% in women, respectively).

The prevalence of subjects with NAFLD who also had the metabolic syndrome is indicated in Figure 3. Although NAFLD was thought of as being the liver phenotype of metabolic syndrome, the prevalence of the metabolic syndrome among subjects with NAFLD was low both in men and women. Among men with NAFLD, 66.8% were not diagnosed with the metabolic syndrome defined by revised NCEP-ATPⅢ definition, and 79.0% were not diagnosed with the metabolic syndrome as defined by revised IDF definition. Even in women, 70.4% and 67.5%, respectively, were not diagnosed withmetabolic syndrome by revised NCEP-ATPⅢ definition and revised IDF definition. These results mean that a large number of participants diagnosed with the metabolic syndrome have NAFLD, but a large number of participants with NAFLD were not diagnosed with the metabolic syndrome, whether we used revised NECP-ATPⅢ criteria or IDF criteria.

DISCUSSION

In this study, we clarified the impact of the criteria of the metabolic syndrome for diagnosing NAFLD in a healthy population. The metabolic syndrome was associated with abdominal obesity and its criteria include waist circumference[22,23,25,26], and NAFLD was reported to be associated with abdominal obesity. However, our results indicated there was no significant difference between BMI and waist circumferences as the strength of association with NAFLD or the accumulation of metabolic syndrome criteria.

The presence of multiple metabolic disorders such as diabetes mellitus, obesity, dyslipidaemia and hypertension is associated with a potentially progressive, severe liver disease[15,27]. Previous reports demonstrated that prevalence of NAFLD increased to 10%-80% in individuals with obesity, 35%-90% in individuals with type 2 diabetes mellitus, 30%-56% in individuals with hypertension, and 26%-58% in individuals with dyslipidemia[9,28-30]. Another study in a Japanese population showed that prevalence of NAFLD increased to 43% in individuals with impaired fasting glucose and 62% in individuals with type 2 diabetes mellitus[28]. Some studies estimate the prevalence of NAFLD be up to 15%-30% of the general population[8,31,32], and the prevalence of metabolic syndrome was estimated to be up to 25% of the general population[33]. In those patients with the metabolic syndrome, liver fat content is significantly increased up to 4-fold higher than those without the metabolic syndrome[34], and the incidence of NAFLD has been shown to be increased 4-fold in men and 11-fold in women with the metabolic syndrome[8].

Our data clearly indicated that 21% to 33% of sub-