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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Lack of Goal Attainment Regarding the Low-density Lipoprotein Cholesterol Level in the Management of Type 2 Diabetes Mellitus

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Abstract

Objective The management of diabetes mellitus includes controlling the blood glucose level, body weight, blood pressure and serum lipid level. The coexistence of diabetes and a high low-density lipoprotein cholesterol (LDL-C) level promotes atherosclerosis of the coronary arteries and increases the risk of coronary artery disease (CAD). We compared the rates of attainment of LDL-C goals in type 2 diabetes patients receiving primary and secondary prevention therapy, the former without a history of CAD and the latter with a history of CAD. Because patients receiving secondary prevention are at greater risk of coronary events, LDL-C management is especially important in this group. This study was designed to determine how frequently diabetic patients attain their LDL-C goals and identify the reasons for the lack of attainment.

Methods The groups were distinguished according to the patients' medical records. Contributory factors for the patients not achieving their goals were recorded in a questionnaire filled out by each patient's physician.

Results The overall attainment rate in both groups was 61%. The most frequent impediment in both groups was "an LDL-C level above or below the goal at every hospital visit" followed by "continuously sufficient effects of dietary therapy only" and the "management of LDL-C by other departments or hospitals," the latter reflecting the increasing problems of polydisease and polypharmacy in diabetes care.

Conclusion Polydisease and polypharmacy issues in diabetes patients with a history of CAD constitute a growing barrier to medication adherence and the attainment of treatment goals.

Key words: type 2 diabetes, LDL cholesterol, coronary artery disease, questionnaire

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Introduction

The coexistence of diabetes and a high low-density lipoprotein cholesterol (LDL-C) level promotes atherosclerosis of the coronary arteries and increases the risk of coronary artery disease (CAD) and its associated morbidity. Treatment for high LDL-C includes dietary intervention, exercise and pharmacological therapy. Representative LDL-C-lowering drugs, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins), reduce the incidence of coronary events and mortality in patients with and without a prior history of CAD (1-7). Recently, the Steno-2 study revealed that the frequency of CAD was markedly reduced by interventions for various risk factors in diabetic patients (8). In that study, the attainment rate for the HbA1c goal was 15% and that for blood pressure was 60%. Strikingly, the attainment rate for the LDL-C goal in the intensive therapy group was 80%.

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Table 1A. Patients' Background in Each Group

	Primary prevention group	Secondary prevention group
Number	499	121
Gender (male / female)	219/280	77/44
Age	65.8 ± 0.5	69.0 ± 0.8
BMI (kg/m^2)	24.1 ± 0.2	24.5 ± 0.3
Systolic BP (mmHg)	127.5 ± 0.7	128.0 ± 1.4
Diastolic BP (mmHg)	73.9 ± 0.4	72.2 ± 1.0
HbA1c (%)	7.7 ± 0.1	7.8 ± 0.1
LDL-C (mg/dL)	112.7 ± 1.0	95.0 ± 2.6
HDL-C (mg/dL)	55.0 ± 0.6	51.9 ± 1.2
Triglyceride (mg/dL)	154.0 ± 5.0	146.5 ± 8.3
Therapeutic modes for hyperglycemia (number of patients)		
Diet alone	92	19
OHA	222	55
Insulin	129	31
Combination of OHA + insulin	56	16

BMI: body mass index, BP: blood pressure, OHA: oral hypoglycemic agent. Data are shown as means ± S.E.M.

Table 1B. Details of Medication of OHA

categories of OHA	number of patients			
	without insulin treatment		with insulin treatment	
	Primary prevention group	Secondary prevention group	Primary prevention group	Secondary prevention group
SU	105	23	15	5
GLN	17	6	4	0
BG	12	1	11	5
α-GI	11	3	11	1
TZD	1	0	1	0
SU+α-GI	11	9	4	2
SU+BG	33	5	4	1
SU+TZD	2	1	0	0
GLN+BG	4	0	0	0
α-GI+TZD	1	0	0	0
BG+TZD	1	0	0	1
GLN+α-GI	0	0	1	1
α-GI+BG	0	0	3	0
GLN+TZD	0	1	0	0
SU+α-GI+BG	16	3	2	0
SU+α-GI+TZD	3	1	0	0
SU+BG+TZD	1	2	0	0
α-GI+BG+TZD	1	0	0	0
SU+α-GI+TZD+BG	3	0	0	0

In the present study, diabetes patients with no prior history of CAD comprised the primary prevention group, while those with a history of CAD comprised the secondary prevention group, the latter of which is more likely to develop new coronary events. According to the guidelines for the prevention of atherosclerosis-associated disease issued by the Japan Atherosclerosis Society in 2007, an LDL-C level less than 120 mg/dL is the goal for diabetes patients treated with primary prevention and an LDL-C level less than 100 mg/dL is the goal for patients treated with secondary prevention (9). In addition, recent randomized trials have demonstrated that intensive statin therapy lowers the LDL-C level more effectively than standard statin therapy (10, 11).

Diabetes patients often have hypertension as well as a high LDL-C level, which can be effectively treated with oral hypoglycemic, antihypertensive and lipid-lowering medications. For secondary prevention, antiplatelet agents and vasodilator agents are additionally added. In such cases, the medication to treat each disease is frequently prescribed separately by different physicians. Such complex polydisease and polypharmacy issues are a growing barrier to medication adherence and the attainment of treatment goals in the US (12).

In Japan, no investigations have addressed the reasons for the lack of attainment of LDL-C goals.

In the present study, we surveyed the attainment rates for LDL-C goals in diabetes patients receiving primary and secondary prevention. In addition, a physician's questionnaire regarding the patients' lack of achievement of the goals was distributed and analyzed. The most frequent response regarding the lack of achievement in secondary prevention was the "management of LDL-C by other departments or hospitals," which reflects the increasing polydisease and polypharmacy issues in current diabetes care.

Table 2. Questionnaires Regarding the Lack of Achievement of Optimum LDL-C Level

	frequency of emergence of answer (%)	
	primary prevention group	secondary prevention group
1) The patient's LDL-C levels are over or under the LDL-C goal at every hospital visit.	47	49
2) Continuously sufficient effect by diet therapy only.	34	11
3) The patient does not accept the prescription of statin despite having its importance as explained by the physician.	6	0
4) The patient takes prescribed drugs contraindicated for co-administration with statins.	3	6
5) The patient has been previously treated with statins and consequently shows moderately elevated serum CPK.	1	0
6) Severe adverse effects of statins on laboratory data besides elevation of serum CPK have been noted in the patient's past history.	1	0
7) Rhabdomyolysis concomitant with severe serum CPK elevation due to treatment with statins has been noted in the patient's past history.	1	4
8) The patient has experienced an adverse effect of statin as a subjective symptom.	4	4
9) Severe chronic renal failure has prohibited prescription of statins for the patient.	1	4
10) Severe muscle disease has prohibited prescription of statins for the patient.	1	0
11) The maximum dose of statin has been prescribed for the patient.	1	2
12) The physician has judged that incremental increases in the dosage of statin would not be helpful for the patient.	3	4
13) Other hospitals or departments have prescribed statins for the patient.	4	45
14) Various other, less common reasons.	13	15

Materials and Methods

Subjects

All patients regularly visited the outpatient clinic of Kyoto University Hospital and received nutritional instruction by dietitians. Because a total cholesterol (TC) level of 200 mg/dL corresponds to an LDL-C level of 120 mg/dL (13), patients with a TC level higher than 200 mg/dL or those previously treated with statins were enrolled in December 2007 (Table 1A, B). The LDL-C level was measured at least once between January and July 2008. Subjects with familial hypercholesterolemia or secondary hypercholesterolemia, such as those with nephrotic syndrome or hypothyroidism, were excluded. Data for the period January 1 to July 31 2008 were prioritized nearest to March 15, 2008.

Methods

The primary and secondary prevention groups were determined according to the patients' medical records. Standard statin therapy included pravastatin, simvastatin or fluvastatin, and intensive statin therapy included atorvastatin, pitavastatin or rosuvastatin. The maximum dose of each statin based on the medical package insert was as follows: pravastatin: 20 mg, simvastatin: 20 mg, fluvastatin: 60 mg, atorvastatin: 20 mg, pitavastatin: 4 mg and rosuvastatin: 10 mg. For several enrolled patients, the following lipid-lowering drugs were additionally prescribed: ethyl icosapentate, probucol, colestimide, fibrate and nicotinate. The LDL-C level was measured according to the selective solubilization method (Determiner L LDL-C test kit, Kyowa Medex Co., Ltd., Tokyo, Japan). The HbA1c level was measured using HPLC (HA-8180; Arcray, Kyoto, Japan). The HbA1c value (%) was estimated as the National Glycohemoglobin Standardization Program equivalent (%) according to the following formula: HbA1c (%) = HbA1c (JDS) (%) + 0.4%, considering the relational expression of HbA1c (JDS) (%) measured according to the previous Japanese standard substance and measurement methods and HbA1c (National Glycohemoglobin Standardization Program) (14).

Questionnaire analysis

The questionnaire consisted of 14 items, as shown in Table 2. The factors contributing to why the patients did not achieve their goals were recorded in the questionnaire by each patient's physician.

Statistical analysis

The data are presented as the mean ± SE. The Chi-square test or Fisher's exact probability test were used to the evaluate results. p values of <0.05 were considered to be statistically significant.

Results

Characteristics of the enrolled patients

Table 1A shows the demographic characteristics of the enrolled patients. The primary prevention group comprised 499 patients (men: 219 and women: 280) 65.8±0.5 years of age, with a body mass index (BMI) of 24.1±0.2 kg/m^2 and a systolic and diastolic blood pressure of 127.5±0.7 mmHg and 73.9±0.4 mmHg, respectively. The HbA1c, LDL-C, high-density lipoprotein cholesterol (HDL-C) and triglyceride levels were 7.7±0.1%, 112.7±1.0 mg/dL, 55.0±0.6 mg/dL and 154.0±5.0 mg/dL, respectively. Regarding treatment for hyperglycemia, 92, 222, 129 and 56 patients were treated with diet alone, OHAs (oral hypoglycemic agents), insulin and insulin plus OHAs, respectively.

Table 3A. Rate of Attainment of LDL-C Goal

		Primary prevention group				Secondary prevention group			
		Total number	Number attaining goal	Number not attaining goal	Rate of attainment (%)	Total number	Number attaining goal	Number not attaining goal	Rate of attainment (%)
Standard statin therapy									
	All dose	116	85	31	73	24	14	10	58
	Max. dose	3	3	0	100	1	0	1	0
	Non-max. dose	113	82	31	73	23	14	9	61
Intensive statin therapy									
	All dose	144	115	29	80	70	52	18	74
	Max. dose	10	9	1	90	10	4	6	40
	Non-max. dose	134	106	28	79	60	48	12	80
No statin		239	100	139	42	27	8	19	30
Total		499	300	199	61	121	74	47	61

Max. dose: Maximum dose, Non-max. dose: Non- maximum dose

Table 3B. Details of Additional Lipid-lowering Medication

additional lipid-lowering drug	number of patients					
	Primary prevention group			Secondary prevention group		
	Standard statin therapy	Intensive statin therapy	No statin	Standard statin therapy	Intensive statin therapy	No statin
ethyl icosapentate	4	0	3	0	2	0
probucol	0	1	0	0	1	0
colestimide	0	1	2	0	0	0
fibrate	0	2	13	1	0	3
nicotinate	8	3	4	0	2	1
ethyl icosapentate + nicotinate	0	1	2	0	0	0
colestimide + fibrate	1	0	0	0	0	0
Total	13	8	24	1	5	4

Table 3C. Rate of Attainment of LDL-C Goal by Additional Lipid-lowering Drug

additional lipid-lowering drug	Rate of attainment of LDL-C goal					
	Primary prevention group			Secondary prevention group		
	Standard statin therapy	Intensive statin therapy	No statin	Standard statin therapy	Intensive statin therapy	No statin
ethyl icosapentate	75%	-	100%	-	50%	-
probucol	-	100%	-	-	0%	-
colestimide	-	100%	50%	-	-	-
fibrate	-	100%	54%	100%	-	0%
nicotinate	75%	100%	50%	-	50%	0%
ethyl icosapentate + nicotinate	-	100%	100%	-	-	-
colestimide + fibrate	100%	-	-	-	-	-
Total	77%	100%	63%	100%	40%	0%

The secondary prevention group consisted of 121 (men: 77 and women: 44) patients 69.0±0.8 years of age, with a BMI of 24.5±0.3 kg/m^2 and a systolic and diastolic blood pressure of 128.0±1.4 mmHg and 72.2±1.0 mmHg, respectively. The HbA1c, LDL-C, HDL-C and triglyceride (TG) levels were 7.8±0.1%, 95.0±2.6 mg/dL, 51.9±1.2 mg/dL and 146.5±8.3 mg/dL, respectively. Regarding treatment for hyperglycemia, 19, 55, 31 and 16 patients were treated with diet alone, OHAs, insulin and insulin plus OHAs, respectively. Table 1B shows the number of patients treated with SUs (sulfonylureas), GLNs (rapid-acting insulin secretagogues), BGs (biguanides), α-GIs (α-glucosidase inhibitors), TZD (thiazolidinedione) and a combination of these drugs in the presence and absence of insulin treatment. The

frequency of SU+α-GI therapy in the secondary prevention group was significantly higher than that observed in the primary prevention group ($p<0.01$). The frequency of other OHAs in the secondary prevention group was indistinguishable from that observed in the primary prevention group.

Attainment rates for the LDL-C goals

Table 3A summarizes the rates of attainment for the LDL-C goals. In the primary prevention group, 300 subjects achieved the LDL-C goal, an achievement of 61%. The patients received standard statin therapy (n=116), intensive statin therapy (n=144) or no statin therapy (n=239). The attainment rates in the patients treated with standard statin therapy, intensive statin therapy and no statin therapy were 73%, 80% and 42%, respectively. We further analyzed the following four subgroups: (1) the maximum dose of standard statins, (2) the non-maximum dose of standard statins, (3) the maximum dose of intensive statins and (4) the non-maximum dose of intensive statins.

In the secondary prevention group, 74 patients achieved the LDL-C goal, an achievement rate of 61% (Table 3A). These patients also received standard statin therapy (n=10), intensive statin therapy (n=18) or no statin therapy (n=19) with attainment rates of 58%, 74% and 30%, respectively. We further analyzed the above-described four subgroups in the secondary prevention group.

Table 3B shows the number of patients treated with other lipid-lowering drugs besides statins (ethyl icosapentate, probucol, colestimide, fibrate and nicotinate). Of those receiving combination therapy with a statin and other lipid-lowering drugs, 0% received the maximum dose of the statin. Table 3C shows the rate of attainment of the LDL-C goal in the patients receiving additional lipid-lowering drugs. In the primary prevention group, the patients treated with combination therapy with a statin and other lipid-lowering drugs showed only a statistically insignificant higher goal attainment rate than those treated with single statin therapy (standard statins: 73% vs. 77%; intensive statins: 79% vs. 100%).

Effectiveness of statin therapy

Table 4A summarizes the details of the statin administration. In the primary prevention group, of the patients attaining the LDL-C goal, 28% received standard statin therapy, 39% received intensive statin therapy and 33% received no statin therapy. Of those not attaining the goal, 16% received standard statin therapy, 14% received intensive statin therapy and 70% received no statin therapy. In the secondary prevention group, of the patients attaining the LDL-C goal, 19% received standard statin therapy, 70% received intensive statin therapy and 11% received no statin therapy. Of those not attaining the goal, 22% received standard statin therapy, 38% received intensive statin therapy and 40% received no statin therapy.

Table 4B summarizes the details of the statin administration associated with additional lipid-lowering drugs in the patients attaining and not attaining the LDL-C goal.

Questionnaire analysis

The background characteristics of the patients who did not achieve their goal were analyzed using a questionnaire completed by each patient's physician. Table 2 shows the questionnaires for the primary and secondary prevention groups.

In the primary prevention group, the three most frequent reasons for the lack of goal attainment were: (1) an LDL-C level above or below the goal at every hospital visit (47%), (2) continuously sufficient effects with dietary therapy only (34%) and (3) low compliance (6%). In the secondary prevention group, the reasons for the lack of goal attainment were: (1) an LDL-C level above or below the goal at every hospital visit (49%), (2) management of the LDL-C level by other departments or hospitals (45%) and (3) continuously sufficient effects with dietary therapy only (11%).

Discussion

In the present study, 61% of the patients in both the primary and secondary prevention groups achieved the LDL-C goal. A study in a university hospital setting in 2001 reported rates of achievement of 56% and 33% in primary and secondary prevention groups, respectively, with a considerably lower rate in the secondary prevention group (15). In the present study, more intensive therapy was associated with an increased LDL-C goal attainment rate; the earlier study did not include intensive statin therapy. Recent randomized controlled trials have demonstrated that intensive statin therapy more effectively lowers the LDL-C level than standard statin therapy (10, 11). In our follow-up study conducted in 2010, the attainment rates for the LDL-C goal reached 71% and 67% in the primary and secondary prevention groups, respectively (unpublished data).

Unexpectedly, 38% of the secondary prevention patients not attaining the LDL-C goal were treated with intensive statin therapy, which indicates a limitation of this treatment. Similarly, 43% of the secondary prevention patients treated with another lipid-lowering drug who did not attain the LDL-C goal also received intensive statin therapy. In fact, in one recent study, cholesterol absorption was reported to be elevated in the secondary prevention group (16), suggesting statin resistance.

Therefore, a high LDL-C level accompanied by statin resistance may be better treated with a combination of statins and cholesterol transporter inhibitors. Very recently, such combination therapy was found to be more effective than statin monotherapy in secondary prevention patients (17). Ezetimibe use was not considered in this study; therefore, further investigation is required.

This is the first study to analyze the background of diabetes patients in Japan who do not attain their LDL-C goal. This is also the first study to use a physician questionnaire to analyze the lack of achievement of LDL-C goals in indi-

Table 4A. Statin Administration in Patients Attaining and not Attaining LDL-C Goal

	Primary prevention group							Secondary prevention group						
	Standard statin therapy			Intensive statin therapy			No statin	Standard statin therapy			Intensive statin therapy			No statin
	All dose	Max. dose	Non-max. dose	All dose	Max. dose	Non-max. dose		All dose	Max. dose	Non-max. dose	All dose	Max. dose	Non-max. dose	
Patients attaining LDL-C goal	28%	1%	27%	39%	3%	36%	33%	19%	0%	19%	70%	5%	65%	11%
Patients not attaining LDL-C goal	16%	0%	16%	14%	0%	14%	70%	22%	2%	20%	38%	13%	25%	40%

Max. dose: Maximum dose, Non-max. dose: Non- maximum dose

Table 4B. Statin Administration with Additional Lipid-lowering Drug in Patients Attaining and not Attaining LDL-C Goal

	Primary prevention group			Secondary prevention group		
	Standard statin therapy with additional lipid-lowering drug	Intensive statin therapy with additional lipid-lowering drug	No statin with additional lipid-lowering drug	Standard statin therapy with additional lipid-lowering drug	Intensive statin therapy with additional lipid-lowering drug	No statin with additional lipid-lowering drug
Patients attaining LDL-C goal	30%	24%	46%	33%	67%	0%
Patients not attaining LDL-C goal	25%	0%	75%	0%	43%	57%

vidual patients. Recently, a physician questionnaire regarding barriers to lipid goal attainment in patients with type 2 diabetes was used in a web-based international survey (18). That study found patient compliance to be the most common impediment, followed by financial restrictions to access to the product, lack of efficacy of available drugs and drug intolerability. Another group reported that the limited amount of time available during clinic visits is a barrier to the ideal management of the LDL-C level in diabetic patients (19). In the present study, statin intolerance and adverse effects were observed in several patients, consistent with previous results. Our questionnaire regarding the lack of achievement of individual patients reflects the details of medical treatment not evaluated using other methods.

In the present study, we found the most frequent reason for the lack of goal attainment in both groups to be a "LDL-C level above or below the goal at every hospital visit." The data for the period January 1 to July 31 2008 were prioritized nearest to March 15, 2008. In several patients, the LDL-C level nearest to March 15 happened to be above the goal, while the levels observed in other months were below the goal. Such occasional fluctuation underlies the "lack of goal attainment of the LDL-C level." This suggests that a certain number of patients may have attained an LDL-C level on the borderline of the goal, indicating that more potent drug therapy may be required.

In the secondary prevention group, the "management of the LDL-C level by other departments or hospitals" was reported with a high frequency. In several patients, particularly those in the secondary prevention group, other departments or hospitals often managed the dyslipidemia therapy and prescribed statins; however, the goal was not attained. In general, the secondary prevention group exhibited several chronic diabetic complications, such as coronary artery disease, retinopathy, nephropathy and neuropathy. In such cases, the medications for each complication may be separately prescribed. Therefore, polydisease might well result in poorer medication adherence. Although the health care system in Japan differs considerably from that observed in the US and elsewhere, polydisease and polypharmacy issues in diabetes care may well become a growing barrier to medication adherence and the attainment of treatment goals. Because patients treated with secondary prevention are more likely to develop new coronary events than those treated with primary prevention, controlling the LDL-C level is especially important in secondary prevention patients. To improve medication adherence, providing team medical care, including the participation of pharmacists and physicians in other fields, is essential.

Several limitations of this study should be considered.

First, the TC level rather than the LDL-C level was used for screening because direct measurement of the LDL-C level was not performed in several patients. Calculating the LDL-C level using Friedewald's equation with the TC, HDL-C and fasting TG levels is another method of determining the LDL-C level. For a considerable number of patients, however, the appointment time in the outpatient department was in the afternoon, when it is difficult to measure the fasting TG level, in which case, Friedewald's equation cannot be used. Strictly speaking, patients administered no statin therapy who attain the LDL-C goal but have a TC level over 200 mg/dL are expected to have a normal LDL-C level. Very few patients with a TC level under 200 mg/dL are expected to have an LDL-C level over 120 mg/dL and not be included. Second, this study was performed in a single university hospital. Further studies with more representative samples are required. In addition, to examine the efficacy of statins, the baseline level of LDL-C should be considered, which was not performed in this study. Due to this limitation of our database, we are unable to show the reduction rate of the LDL-C level in order to evaluate the power of statins in detail. Furthermore, medication adherence was not examined. Further investigations are therefore required.

In conclusion, we surveyed the goal attainment rates for the LDL-C levels in type 2 diabetes mellitus patients. Our analysis of the lack of goal attainment in each prevention group provides useful suggestions for improving LDL-C management in patients with type 2 diabetes.

Author's disclosure of potential Conflicts of Interest (COI).
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In vivo genotoxicity of a novel heterocyclic amine, aminobenzoazepinoquinolinone-derivative (ABAQ), produced by the Maillard reaction between glucose and L-tryptophan

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ABSTRACT

We recently demonstrated that a novel heterocyclic amine, 5-amino-6-hydroxy-8*H*-benzo[6,7]azepino[5,4,3-*de*]quinolin-7-one (ABAQ), is produced from glucose and L-tryptophan by the Maillard reaction at physiological temperature and pH, and that ABAQ was strongly mutagenic for *Salmonella* strains in the presence of S9 mix. Here, we present the results of three *in vivo* genotoxicity assays of ABAQ. The comet assay revealed that DNA damage was significantly increased in the livers, kidneys, lungs, and bone marrows of ICR mice, 3 h after i.p. injection of ABAQ (50 mg/kg body weight (bw)). To evaluate clastogenicity, the peripheral blood micronucleus test was performed, also in ICR mice. ABAQ induced micronucleated reticulocytes (MNRETs) in a dose-dependent manner; the frequency of MNRETs was significantly elevated at all i.p. doses (12.5, 25, and 50 mg/kg bw) after 48 h. To investigate the mutagenicity of ABAQ *in vivo*, *gpt* delta transgenic mice were treated with five consecutive administrations of ABAQ by gavage at doses of 25 or 50 mg/kg per week for 3 weeks. The frequencies of *gpt* mutations (MF) in the liver of mice increased significantly compared with controls, in a dose-dependent manner. No significant increase of *gpt* MF was detected in the kidneys. Base substitutions predominated; both G:C → A:T and A:T → C:G mutations were significantly increased by ABAQ. The Spi$^-$ MF was also significantly increased in the liver after ABAQ treatment. If formed *in vivo*, ABAQ may give rise to adverse genotoxic effects.

1. Introduction

Diabetes mellitus (DM) affects more than 300 million people worldwide and the number is predicted to increase to at least 400 million by 2030 [1]. Epidemiological studies show that diabetes patients have an increased incidence of cancers in certain organs, including liver, pancreas, kidney, and endometrium [2–5]; however, the causal mechanisms are not fully elucidated. A consistent increase in blood sugar levels is characteristic of diabetes, and the Maillard reaction is considered to be implicated in diabetic complications [6]. The Maillard reaction comprises a series of complex non-enzymatic reactions between the carbonyl group of a reducing sugar and the amino groups of amino acids, peptides, or proteins to yield an unstable Schiff base, which then leads to a relatively stable ketoamine known as an Amadori product [7]. The Amadori products react with amino acids or are converted into reactive carbonyl species, such as deoxyglucosone. During the latter stage of the reaction, the carbonyl species react with amino groups in proteins and other molecules.

We recently found that the Maillard reaction of glucose and L-tryptophan at physiological temperature and pH (37 °C and pH 7.4) produces mutagens, and we identified a novel heterocyclic amine,

Abbreviations: ABAQ, 5-amino-6-hydroxy-8*H*-benzo[6,7]azepino[5,4,3-*de*]quinolin-7-one; MNRETs, micronucleated reticulocytes; MF, mutation frequencies; DM, diabetes mellitus; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; *gpt*, guanine phosphoribosyltransferase; LMP, low melting point; NMP, normal melting point; DMSO, dimethyl sulfoxide; 6-TG, 6-thioguanine; SD, standard deviation; HCAs, heterocyclic amines.

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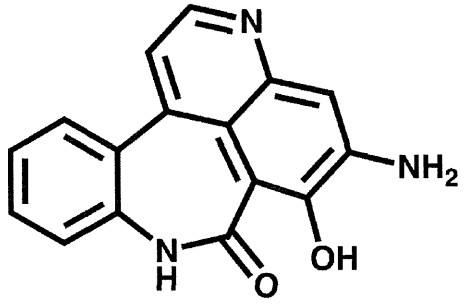

Fig. 1. Chemical structure of 5-amino-6-hydroxy-8*H*-benzo[6,7]azepino[5,4,3-*de*]quinolin-7-one (ABAQ).

5-amino-6-hydroxy-8*H*-benzo[6,7]azepino[5,4,3-*de*]quinolin-7-one (ABAQ, Fig. 1), from the reaction mixture [8]. A plausible mechanism for the formation of ABAQ from glucose and L-tryptophan is also shown in our previous report [8]. ABAQ induced mutations in *Salmonella typhimurium* strains TA98, TA100, YG1024, and YG1029 in the presence of S9 mix, and the mutagenic potency was the highest in YG1024, a derivative of TA98 that overproduces *O*-acetyltransferase. These results suggest that ABAQ mutagenicity depends on metabolic activation catalyzed by cytochrome P450 and *O*-acetyltransferase [8]. These characteristics are very similar to those of a cooked food-derived heterocyclic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), suggested to be formed by the reaction of creatine with Maillard reaction products from glucose and phenylalanine by heating at high temperatures [9]. The mutagenic potencies of ABAQ in TA98 and YG1024 are comparable to those of PhIP [8].

Because ABAQ was discovered only recently, its biological activities have not been determined, aside from *Salmonella* mutagenicity. In the present study, therefore, we have examined the genotoxicity of ABAQ *in vivo*, and compared it to that of PhIP; the acute studies used the micronucleus and comet assays; chronic mutagenicity was evaluated using the *gpt* delta transgenic mouse system, in which point mutations and deletions can be assessed by *gpt* and Spi$^-$ selection, respectively [10,11]. We show here that ABAQ induces acute and chronic genotoxicity, and discuss the possible underlying mechanisms.

2. Materials and methods

2.1. Chemicals

ABAQ (more than 99% pure) was obtained from Hamari Chemicals, Ltd. (Osaka, Japan). Corn oil, low melting point (LMP) and normal melting point (NMP) agarose, dimethyl sulfoxide (DMSO), and Triton X-100 were purchased from Sigma–Aldrich (St. Louis, MO, USA). PhIP·HCl and other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Experimental animals

Male ICR mice (6 weeks old) and guanine phosphoribosyltransferase (*gpt*) delta mice (6 weeks old) were purchased from Japan SLC (Shizuoka, Japan). The *gpt* delta mice carry approximately 80 copies of *lambda* EG10 DNA on chromosome 17 on a C57BL/6J background [12]. The animals were provided with food (CE-2 pellet diet; CLEA Japan, Inc., Tokyo, Japan) and tap water *ad libitum*, and maintained under controlled conditions as follows: a 12-h light/dark cycle, 22 ± 2 °C room temperature, and 55 ± 10% relative humidity. After quarantine for one week, the experiments were conducted according to the "Guidelines for Animal Experiments in the National Cancer Center or Kyoto Pharmaceutical University", and the animal studies were approved by its Experimental Animal Research Committee.

2.3. In vivo comet assay

The alkaline comet assay was performed according to a published method [13]. ABAQ was suspended in olive oil and 25, 50, or 100 mg/kg body weight (bw) doses were injected intraperitoneally (i.p.). PhIP was dissolved in physiological saline and administered i.p. to mice at 12.5, 25, or 50 mg/kg bw. Controls received olive oil or physiological saline i.p. Five ICR mice were used for each group. Liver, kidneys, lungs, and bone marrow were removed 3 h after the injections. Each organ, except for bone marrow, was minced, suspended in chilled homogenizing buffer (pH 7.5, 0.075 M KCl and 0.03 M sodium EDTA), and homogenized gently with a Dounce-type homogenizer. Normal melting point agarose (100 μl) was layered as the first layer on a glass slide; LMP agarose (50 μl) containing 1000 nuclei was layered next; normal melting point agarose (100 μl) was layered last. The slides were immersed in ice-cold lysing solution (pH 10, containing 2.5 M NaCl, 100 mM sodium EDTA, 10 mM Tris–HCl, 1% sodium N-lauryl sarcosinate, 10% DMSO and 1% Triton X-100) for 60 min. The slides were placed on a horizontal gel electrophoresis platform and covered with chilled alkaline solution (containing 300 mM NaOH, 1 mM sodium EDTA) for 20 min to allow for the DNA to unwind and expose alkali-labile sites. The nuclei were electrophoresed at 25 V (1 V/cm) for 20 min. After electrophoresis, the specimens were rinsed twice with 400 mM Tris–HCl (pH 7.5) to neutralize excess alkali, stained with 50 μl ethidium bromide solution, and covered with a cover slip. Nuclei (100 per organ per animal) were inspected using a fluorescence microscope equipped with a CCD camera. The tail moment of the DNA was measured using Komet Assay software (Kinetic Imaging Ltd., Liverpool, UK).

2.4. In vivo micronucleus test

The micronucleus test was carried out according to a published method [14]. ABAQ suspended in olive oil or PhIP dissolved in physiological saline were administered i.p. at 12.5, 25, or 50 mg/kg bw. Control mice were treated with either olive oil or physiological saline. Five ICR mice were used for each group. Peripheral blood (5 μl) was obtained from the ventral tail, spread on an acridine orange-coated glass slide, and covered with a cover slip. Supravitally stained reticulocytes were observed using a fluorescence microscope with a blue excitation and a yellow-to-orange barrier filter. The number of MNRETs per 1000 reticulocytes was scored for each mouse.

2.5. gpt and Spi$^-$ mutation assays

For mutation analysis, each group of five male *gpt* delta mice was orally administered five consecutive doses (25 or 50 mg/kg) of ABAQ per week for 3 weeks. The control mice (n = 4) were treated with solvent (corn oil) alone. The mice were sacrificed at 14 weeks age (5 weeks after ABAQ administration). Liver and kidneys were removed and stored at −80 °C until high-molecular-weight genomic DNA was extracted using a RecoverEase DNA Isolation Kit (Agilent Technology, USA) according to the manufacturer's instructions. *Lambda* EG10 phages were rescued using Transpack Packaging Extract (Stratagene, La Jolla, CA). The *gpt* and Spi$^-$ mutagenesis assays were performed according to published methods [12]. Briefly, *E. coli* YG6020 was infected with the phage and spread on M9 salt plates containing chloramphenicol (Cm) and 6-thioguanine (6-TG) and incubated for 72 h at 37 °C to select for colonies harboring a plasmid carrying the gene encoding chloramphenicol acetyltransferase, as well as a mutated *gpt*. The

6-TG-resistant isolates were cultured overnight at 37 °C in LB broth containing 25 mg/mL Cm, harvested by centrifugation (7000 rpm, 10 min), and stored at −80 °C.

The mutational spectra of 6-TG coding sequences were determined using PCR and direct sequencing, and a 739-bp DNA fragment containing *gpt* was amplified by PCR as described previously [12]. Sequence analysis was performed at Takara Bio Inc. (Mie, Japan). The Spi⁻ assay was performed as described previously [11,12]. The lysates of Spi⁻ mutants were obtained by infection of *E. coli* LE392 with the recovered Spi⁻ mutants.

2.6. Statistical analysis

Comet assay and micronucleus test data are expressed as means ± standard deviation (SD). Student's *t*-test was used to evaluate the significance of differences of DNA tail moment in the comet assay and the frequency of MNRETs in the micronucleus test between groups treated with ABAQ, PhIP, and control groups. The data from the *gpt* and Spi⁻ mutation assays were expressed as means ± SD. The data were compared with the corresponding solvent control using the F test before application of the Student's *t*-test. Mutational spectra were compared using Fisher's exact test [15]. *P* values lower than 0.05 were considered to indicate statistical significance.

3. Results

3.1. Analysis of acute DNA-damaging activity of ABAQ and PhIP in multiple organs (in vivo comet assay)

DNA damage induced by ABAQ and PhIP in multiple organs (liver, kidneys, lungs, and bone marrow) was evaluated as the DNA tail moment, 3 h after i.p. administration, using the comet assay under alkaline conditions. As shown in Fig. 2, the DNA tail moment values increased in a dose-dependent manner following ABAQ treatment, and the values for all organs were significantly higher at 50 and 100 mg/kg bw compared with those of control mice. High mean tail moments were detected in the bone marrow of mice treated with 50 and 100 mg/kg bw and DNA damage values were 1.3- and 1.4-fold higher, respectively compared with the control ($P < 0.01$ at both doses). For PhIP, a significant increase of DNA tail moment values was detected in all organs examined at 12.5, 25, and 50 mg/kg bw. The highest tail moment was detected in the kidneys at 50 mg/kg bw and the DNA damage was 1.3-fold ($P < 0.01$) higher than that of the control group.

3.2. Clastogenicity of ABAQ and PhIP in peripheral blood (in vivo micronucleus test)

The clastogenic activities of ABAQ and PhIP were examined by the micronucleus test. Frequencies of MNRETs were increased dose-dependently, 24 h and 48 h after the administration of ABAQ, and the frequencies were significantly elevated at all doses examined (12.5, 25, and 50 mg/kg bw) at 48 h. The highest frequencies of MNRETs were detected at 48 h, and the frequencies were 3.0-, 4.2-, and 5.8-fold higher than controls at 12.5, 25, and 50 mg/kg bw, respectively ($P < 0.01$ at each dose). Similarly, the frequency of MNRETs was dose-dependently increased 24 and 48 h after injection of PhIP, and was the highest at 50 mg/kg bw at 48 h after administration; the value was 7.4-times higher than that of controls ($P < 0.01$) (Fig. 3).

3.3. gpt and Spi⁻ mutations in the liver and kidneys of gpt transgenic mice treated with ABAQ

3.3.1. General observations of gpt delta transgenic mice administrated ABAQ

Body weights of *gpt* delta mice receiving vehicle control reached 27.5 ± 3.1 g, 30 d after gastric intubation. Values for *gpt* delta mice receiving multiple doses of ABAQ at 25 or 50 mg/kg bw were 28.7 ± 2.3 g and 28.5 ± 1.5 g, respectively, 30 d after administration, and no significant difference was observed compared with the vehicle control group. The average dietary consumption per day per mouse was about 3 g and was not affected by ABAQ.

3.3.2. gpt mutations in the liver and kidneys of gpt transgenic mice following ABAQ treatment

To determine the mutagenic effects of ABAQ in the liver and kidneys, *gpt* delta transgenic mice were treated with low or high doses of ABAQ (five consecutive administrations of ABAQ by gavage of 25 or 50 mg/kg bw per week for 3 weeks). Data are summarized in Table 1 and Fig. 4. MFs in the liver induced by both doses of ABAQ were significantly increased, 3.3- or 3.6-fold compared with the vehicle controls (Fig. 4A). In contrast, there was no increase in the MF in the kidneys following the low dose of ABAQ. The high dose of ABAQ resulted in a slight increase in the MF, but it was not statistically significant (Fig. 4B).

PCR and DNA sequence analyses revealed 42 and 14 independent 6-TG-resistant mutations induced by ABAQ and in the vehicle controls, respectively. The classes of *gpt* mutations are summarized in Table 2. Because our control samples were limited, previously

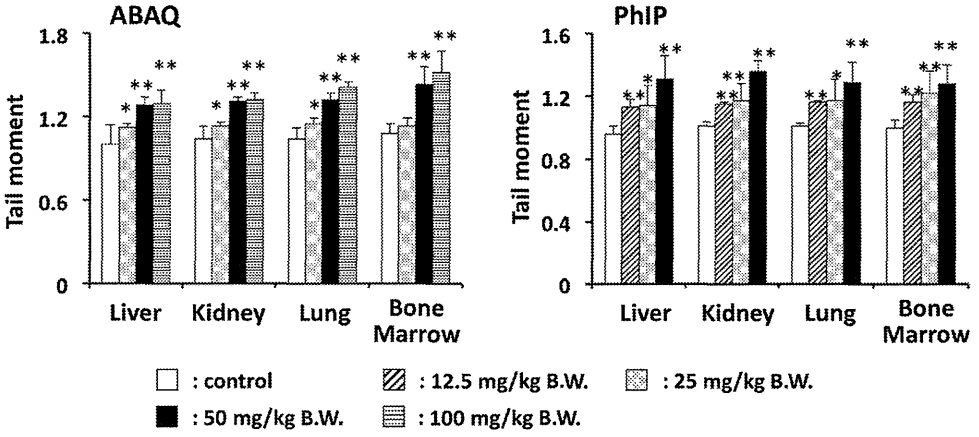

Fig. 2. DNA-damaging activity of ABAQ and PhIP in various organs of mice. Mice were injected i.p. with four doses of either ABAQ or PhIP. Control mice were treated with olive oil or physiological saline. Organs were removed at 3 h after the injection. The values represent the mean of five mice ± SD. Tail moment values of organs untreated mice for ABAQ and PhIP experiments were as follows: liver (1.00 ± 0.14 and 0.96 ± 0.05), kidney (1.04 ± 0.09 and 1.01 ± 0.03), lung (1.04 ± 0.08 and 1.01 ± 0.02), bone marrow (1.08 ± 0.07 and 1.00 ± 0.05). *$P < 0.05$ (*vs.* control), **$P < 0.01$ (*vs.* control).

Table 1
Summary of *gpt* mutation frequency in the liver and kidneys of *gpt* delta mice treated with ABAQ.

	Mouse ID	Number of colonies		MF ($\times 10^{-6}$)	Average MF ($\times 10^{-6}$)[a]
		Mutant	Total		
Liver					
[b]Control	1	5	907,500	5.51	
	2	5	892,500	5.60	
	3	1	862,500	1.16	
	4	3	627,000	4.78	
	Total	14	3,289,500		4.26 ± 2.10
ABAQ					
25 mg/kg × 5	1	8	361,500	22.13	
	2	1	613,500	1.63	
	3	6	625,500	9.59	
	4	10	453,000	22.08	
	5	7	463,500	15.10	
	Total	32	2,517,000		14.11 ± 8.73*
50 mg/kg × 5	1	14	1,129,500	12.39	
	2	10	978,000	10.22	
	3	14	1,003,500	13.95	
	4	27	1,137,000	23.75	
	5	15	922,500	16.26	
	Total	80	5,170,500		15.32 ± 5.20**
Kidneys					
[b]Control	1	4	709,500	5.64	
	2	4	366,000	10.93	
	3	3	384,000	17.81	
	4	5	361,500	13.83	
	Total	16	1,821,000		9.55 ± 3.58
ABAQ					
25 mg/kg × 5	1	8	928,500	6.46	
	2	5	715,500	6.99	
	3	3	519,000	5.78	
	4	14	991,500	14.12	
	5	9	571,500	15.75	
	Total	37	3,726,000		9.82 ± 4.72
50 mg/kg × 5	1	6	702,000	12.39	
	2	5	985,500	10.22	
	3	7	529,500	13.95	
	4	11	492,000	23.75	
	5	12	657,000	16.26	
	Total	41	3,366,000		13.49 ± 7.01

[a] Mean ± SD.
[b] Solvent control (corn oil).
* $P < 0.05$.
** $P < 0.01$ (*vs.* solvent control) by Student's *t*-test.

Table 2
Classification of *gpt* mutations detected in the liver of control and ABAQ-treated mice.

Type of mutation	Control		Control-2[a]		ABAQ		*P* value[b]
	No. of mutations (%)	Specific MF[c] ($\times 10^{-6}$)	No. of mutations (%)	Specific MF[c] ($\times 10^{-6}$)	No. of mutations (%)	Specific MF[c] ($\times 10^{-6}$)	
Base substitution							
Transition							
G:C to A:T	7(46.7)	2.13	21(42.9)	2.78	14(33.3)	5.77	0.02[d], 0.03[e]
A:T to G:C	0(0)	0.00	4(8.2)	0.53	3(7.1)	1.24	0.04[d], 0.25[e]
Transversion							
G:C to T:A	5(33.3)	1.52	5(10.2)	0.66	9(21.4)	3.71	0.10[d], 0.0005[e]
G:C to C:G	0(0)	0.00	2(4.1)	0.26	1(2.4)	0.41	0.24[d], 0.72[e]
A:T to T:A	0(0)	0.00	4(8.2)	0.53	4(9.5)	1.65	0.02[d], 0.09[e]
A:T to C:G	0(0)	0.00	1(2.0)	0.13	3(7.1)	1.25	0.04[d], 0.02[e]
Insertion	1(6.7)	0.30	1(2.0)	0.13	1(2.4)	0.41	0.83[d], 0.40[e]
Deletion	2(13.3)	0.61	6(12.2)	0.79	5(12.0)	2.06	0.12[d], 0.10[e]
Others	0(0)	0.00	5(10.2)	0.66	2(4.8)	0.82	0.10[d], 0.79[e]
Total	15[f](100)	4.56	49(100)	6.48	42(100)	17.30	0.00001[d], 0.00001[e]

[a] Data are from Masumura et al. [15].
[b] *P* values were determined using Fisher's exact test according to Carr and Gorelick [14].
[c] Specific MFs were calculated by multiplying the total mutation frequency by the ratio of each type of mutation to the total mutation.
[d] ABAQ *vs* control.
[e] ABAQ *vs* control-2.
[f] One animal had two mutations.

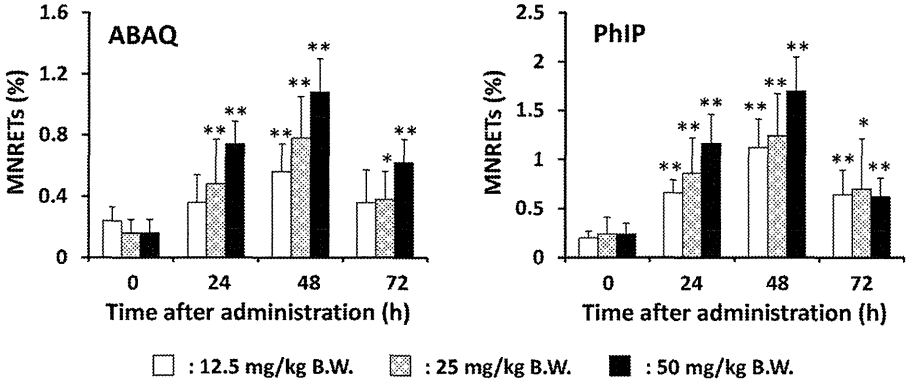

Fig. 3. Clastogenic activity of ABAQ and PhIP in peripheral blood of mice. Mice were injected i.p. with three doses of either ABAQ or PhIP. Control mice were treated with olive oil or physiological saline. One thousand reticulocytes were observed per mouse. The values represent the mean of five mice ± SD. *$P < 0.05$ (*vs.* control), **$P < 0.01$ (*vs.* control).

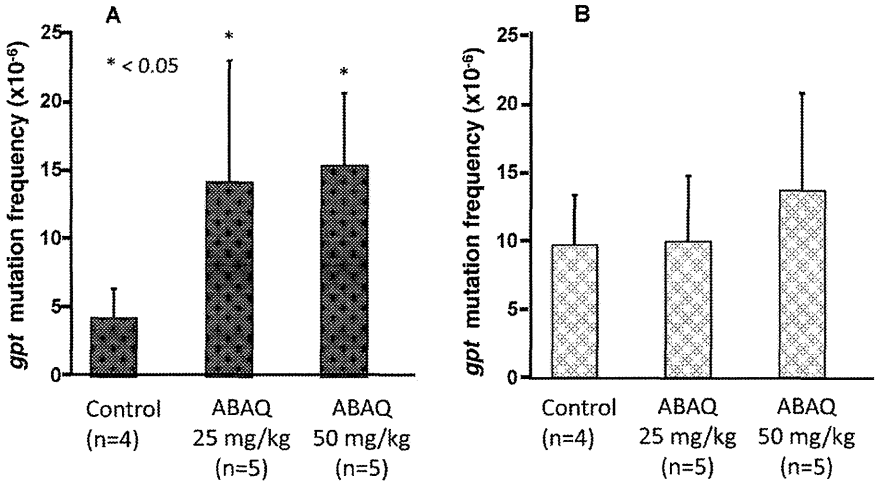

Fig. 4. The *gpt* MFs in the liver (A) and kidneys (B) of mice after multiple administration of ABAQ. Male mice were treated with multiple (25 or 50 mg/kg × 5 times per week for 3 weeks) doses of MGT, and mice were sacrificed 5 weeks after ABAQ administration. The data represent the mean ± SD. *$P < 0.05$, Student's *t*-test *versus* the corresponding vehicle-control mice.

published data [16] are also included in Table 2 (Control-2). Base substitutions predominated in both ABAQ-induced and spontaneous cases. G:C → A:T transitions and A:T → C:G transversions were significantly higher in the ABAQ-treated group.

3.3.3. *Spi⁻ mutations in the liver and kidneys of gpt transgenic mice following ABAQ treatment*

We also measured Spi⁻ MFs in the liver and kidneys of *gpt* delta mice treated with low and high doses of ABAQ (Table 3 and Fig. 5). The mean Spi⁻ MF values in the liver was $1.86 \pm 1.47 \times 10^{-6}$ (control), $6.17 \pm 2.49 \times 10^{-6}$ (low dose) and $6.81 \pm 2.57 \times 10^{-6}$ (high dose), respectively. Spi⁻ MFs in the liver of the both low- and high-dose groups were significantly elevated, up to 2-fold (Fig. 5A). Similar to liver MFs, in the kidneys, Spi⁻ MF showed around 3-fold increase, although this difference was not significant (Fig. 5B).

4. Discussion

The novel heterocyclic amine, ABAQ, identified as a product of the Maillard reaction at physiological temperature and pH, was genotoxic *in vivo*, as revealed by the comet assay, micronucleus test, and *gpt* and Spi⁻ mutation assays. The comet assay is a sensitive method for detecting DNA damages, including double- and single-strand DNA breaks, which are generated indirectly from incomplete excision repair and alkali-labile sites [17]. The DNA tail moment values for liver, kidneys, lungs, and bone marrow in ABAQ-treated (50 mg/kg bw) mice were significantly higher than those of mice treated similarly with PhIP, a representative mutagenic/carcinogenic heterocyclic amine. These results indicate that ABAQ and PhIP have similar levels of DNA-damaging activity in these organs. Under alkaline conditions, not only ABAQ-DNA adducts but also oxidative- and inflammation-related DNA adducts raised by the accompanying immunological response can be measured as DNA damage. ABAQ apparently induced DNA damage in various organs; however, the cause of the damage might be different in each organ. Further studies are required to elucidate this.

The micronucleus test is widely used to detect the clastogenicity of chemicals. Here we show that reticulocytes in the peripheral blood were supravitally stained with acridine orange, and MNRETs can be detected before being trapped and destroyed by the spleen [18]. The frequency of MNRETs was dose-dependently increased at 24 and 48 h after i.p. injection of ABAQ, and the highest frequency was found at 48 h after the injection. Similarly, frequencies of MNRETs increased dose-dependently 24 h and 48 h after PhIP treatment. For ABAQ, the highest frequency of MNRETs was detected 48 h after the injection of 50 mg/kg bw, similar to that observed at 48 h after the injection of 12.5 mg/kg bw PhIP. These results suggest that ABAQ is clastogenic in reticulocytes, with slightly lower potency than that of PhIP.

ABAQ-induced mutations in the liver included base substitutions and deletions. Despite the results of the comet assay, no significant increase of *gpt* and Spi⁻ MF was observed in the kidneys.

Table 3
Summary of Spi⁻ mutant frequency in the liver and kidneys of *gpt* delta mice treated with ABAQ.

	Mouse ID	Number of colonies		MF ($\times 10^{-6}$)	Average MF ($\times 10^{-6}$)[a]
		Mutant	Total		
Liver					
[b]Control	1	9	2,281,500	5.51	
	2	2	2,767,500	5.60	
	3	3	3,115,500	1.16	
	4	7	3,898,500	4.78	
	Total	21	12,063,000		1.86 ± 1.47
ABAQ					
25 mg/kg × 5	1	9	1,135,500	7.93	
	2	6	1,587,000	3.78	
	3	4	1,267,500	3.16	
	4	8	1,041,000	7.68	
	5	11	1,324,500	8.31	
	Total	38	6,355,500		6.17 ± 2.49**
50 mg/kg × 5	1	12	1,299,000	9.24	
	2	8	804,000	9.95	
	3	7	1,293,500	5.41	
	4	5	1,102,500	4.54	
	5	4	811,500	4.93	
	Total	36	5,310,500		6.81 ± 2.57**
Kidneys					
[b]Control	1	3	1,306,500	2.29	
	2	1	745,500	1.34	
	3	0	763,500	0.00	
	4	1	960,000	1.04	
	Total	5	3,775,500		1.17 ± 0.94
ABAQ					
25 mg/kg × 5	1	0	1,945,500	0.00	
	2	1	1,405,500	0.71	
	3	0	1,048,500	0.00	
	4	2	1,501,500	1.33	
	5	2	1,029,000	1.94	
	Total	5	6,930,000		0.80 ± 0.84
50 mg/kg × 5	1	4	867,000	4.61	
	2	1	1,017,000	0.98	
	3	2	700,500	2.86	
	4	5	790,500	6.33	
	5	1	933,000	1.07	
	Total	13	3,366,000		3.17 ± 1.72

[a] Mean ± SD.
[b] Solvent control (corn oil).
** $P < 0.01$ (*vs.* solvent control), Student's *t*-test.

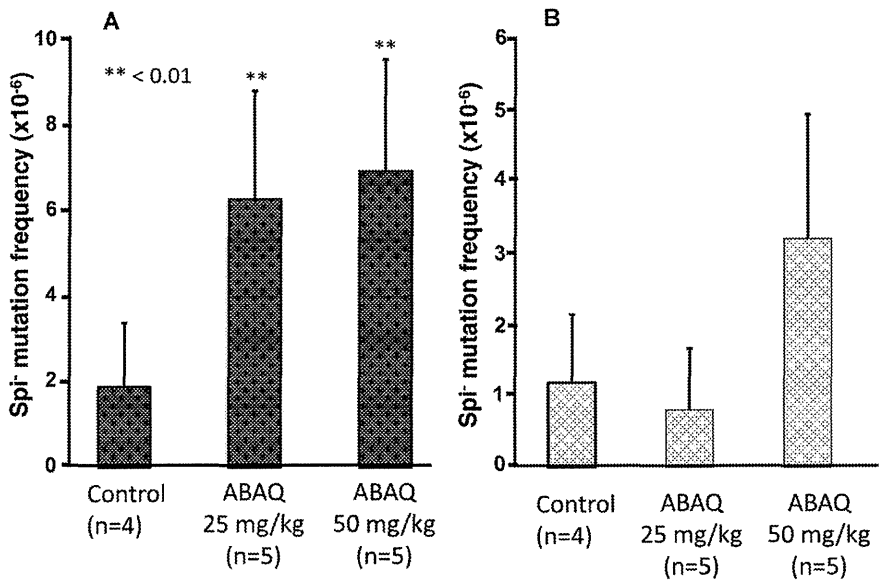

Fig. 5. The Spi⁻ MFs in the liver (A) and kidneys (B) of *gpt* delta mice exposed to multiple doses of ABAQ. An asterisk (*) denotes $P < 0.05$, Student's *t*-test for MFs of ABAQ-treated compared with the corresponding vehicle-control mice.

The reason is not fully understood yet; however, ABAQ administration routes might affect its *in vivo* genotoxicity. In the present study, i.p. injection was used for the comet and micronucleus assays, and i.g. intubation for the *gpt* and Spi$^-$ mutation assays. In general, the absorption rates following i.p. administration were more rapid than i.g. intubation. This may account for the higher genotoxicity of ABAQ observed in the micronucleus and comet assay compared with the results of the mutagenicity test in *gpt* delta mice. Both *gpt* and Spi$^-$ MFs in the kidneys observed in ABAQ-treated mice tended to increase, but this was not statistically significant (Figs. 4 and 5). We suggest, therefore, that ABAQ dosing using i.g. intubation may not be high enough for robust genotoxicity.

The *gpt* mutations induced in the liver are summarized in Table 2. Because our control samples were limited in number, we included and compared the data from a previous report (Control-2 [16]). In the mutation spectrum analysis, the most prominent mutation induced by ABAQ was G:C → A:T ($P < 0.05$) compared with both control groups. A previous study showed that ABAQ is mutagenic for *S. typhimurium* TA98 and YG1024 with S9 mix [8]. The sensitivity was much higher in YG1024 than in TA98, suggesting that *O*-acetyltransferase activity is required to activate ABAQ, as for other food-borne mutagenic compounds, such as heterocyclic amines (HCAs). The exocyclic amino group of HCAs binds guanine bases to form DNA adducts [19–22]. No data are available regarding the chemical structures of ABAQ-DNA adducts, except for the mutational spectral data for ABAQ. Therefore, we may conclude that the guanine base may be involved, to form ABAQ-DNA adducts. In addition, the G:C → A:T transition commonly occurs in spontaneous mutants, and deamination of 5-methylcytosine or alkylation of guanine might be involved in these mutations [23,24]. Moreover, inflammation may be involved [25]. In contrast, the frequencies of A:T → C:G transversions were also significantly different between ABAQ-treated and control groups. Even though its specific MF was low, this type of mutation is rare (almost none in the control cases). Therefore, it might be diagnostic for ABAQ-exposure. Further studies are required to determine the nature of the reactions that produce ABAQ-DNA adducts, and the resulting genotoxic mechanisms.

DM is a risk factor for various types of cancers [2–5], and researchers have focused on the relation between type-2 diabetes and cancer incidence. Evidence indicates that alterations in signal transduction pathways that promote cell proliferation caused by hyperglycemia, or insulin resistance and hyperinsulinemia associated with DM, promote oncogenesis [26–28]. However, whether diabetes initiates tumorigenesis is unknown. *In vivo* Maillard reactions are increased under diabetic conditions [29] and reaction products such as ABAQ may play a role in cancer etiology. A study on *in vivo* formation of ABAQ in diabetic model animals and diabetic patients is in progress in our laboratory. To understand the effect of ABAQ on DM-related cancer, it is important to evaluate the carcinogenicity of ABAQ using animal models. Moreover, epidemiological studies to evaluate the relation between ABAQ and DM-related cancer will also be required.

Conflict of interest statement

None.

Acknowledgments

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AKT is critically involved in cooperation between obesity and the dietary carcinogen amino-1-methyl-6-phenylimidazo [4,5-*b*] (PhIP) toward colon carcinogenesis in rats

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ABSTRACT

Obesity is highly associated with colon cancer development. Whereas it is generally attributed to pro-tumorigenic effects of high fat diet (HFD), we here show that a common genetic basis for predisposition to obesity and colon cancer might also underlie the close association. Comparison across multiple rat strains revealed that strains prone to colon tumorigenesis initiated by a dietary carcinogen amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) tended to develop obesity. Through transcriptome and extensive immunoblotting analyses, we identified the basal level of activated AKT in colonic crypts as a biomarker for the common predisposition. Notably, PhIP induced activation of AKT, which could persist for several weeks under a low fat diet (LFD), but not under HFD. On the other hand, PhIP and HFD independently induced Wnt pathway activation and inhibited apoptosis, through distinct mechanisms involving GSK-3β, caspase 3 and poly-ADP ribose polymerase (PARP). Taken together, these observations provide mechanistic insights into how PhIP-induced activation of AKT might cooperate with HFD at multiple levels toward development of colon cancer.

1. Introduction

Colorectal cancer (CRC) is a leading cause of cancer death worldwide [1]. In the multi-step development of sporadic CRC, Wnt pathway activation is the most frequent initiating event, typically achieved by functional loss of adenomatous polyposis coli (APC) or activating mutation of CTNNB1 encoding β-catenin [2]. Subsequent progression to full-blown tumors is mediated by accumulation of genetic alterations in tumor suppressor genes and oncogenes [3], or by environmental factors, including inflammation. In fact, inflammatory bowel disease is a high-risk condition for CRC in humans [4], and dextran sodium sulfate (DSS)-induced colitis accelerates azoxymethane-induced colon tumorigenesis in mice [5]. Obesity-associated visceral fat or adipocytes have recently emerged as a source of inflammation [6]. Leptin and adiponectin, a class of cytokines secreted by adipocytes, are mediators of inflammation by binding to their specific receptors [7]. Genetic ablation of these pathways in mice indeed affected tumorigenicity under a high fat diet (HFD), confirming the pro-tumorigenic nature of obesity [8,9].

Amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) is a heterocyclic amine (HCA) abundantly contained in cooked meat. It binds to DNA and forms adducts, which could in turn induce mutations, thereby potently inducing tumors in the colon, prostate and mammary glands in rats [10]. Notably, these types of tumors are all closely associated with westernized high-fat diets in humans, and HFD indeed accelerated PhIP-initiated carcinogenesis in these organs in rats [11]. PhIP administration recapitulates multi-step colon tumorigenesis from aberrant crypt foci (ACF), dysplasia, adenoma, and adenocarcinoma [12]. Besides, PhIP-induced tumors frequently harbor mutations in APC and CTNNB1, similar to human CRC [13]. These observations strongly suggested that PhIP might be a major environmental carcinogen for human CRC.

Although ACF are not *bona fide* pre-neoplastic lesions of the colon, susceptibility of strains to chemically-induced tumorigenesis is conveniently estimated by the number of ACF at an early point, largely due to their high correlation, shorter period of time for observation, and higher incidence [14]. The numbers of ACF induced by PhIP vary among inbred strains [12], strongly suggesting that multiple genetic factors determine the susceptibility to colon carcinogenesis. In an effort to identify these loci, we noted that rat strains with more ACF tended to manifest a more severe obese phenotype, which prompted us to investigate the molecular

Abbreviations: PhIP, amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine; HCA, heterocyclic amine; ACF, aberrant crypt foci; GSEA, gene set enrichment analysis.

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basis underlying the common predisposition. We clarified the relevance of AKT in the colonic crypts in linking obesity to PhIP-induced CRC, providing mechanistic insights into the cooperation between obesity and CRC.

2. Materials and methods

2.1. Rats, diet and chemicals

We purchased BUF, F344 and ACI rats from CLEA Japan (Tokyo, Japan), LEW, WKY and BN from Charles River Japan Inc. (Yokohama, Japan), and WKAH, OM, DA and KND from Japan SLC (Hamamatsu, Japan). PVG, DON, LEA, DRH, WF, SDJ, LE and NIG-III were provided from The National BioResource Project (NBRP) for the Rat (Kyoto University, Kyoto, Japan). Animal studies were carried out according to the Guideline for Animal Experiments, drawn up by the Committee for Ethics in Animal Experimentation of the National Cancer Center, which meet the ethical standards required by the law and the guidelines about experimental animals in Japan. Five-week-old male rats were fed a low fat diet (LFD) AIN-93G (Dyets Inc., Bethlehem, PA) for 1 week. To induce ACF, rats were fed LFD containing 400 ppm of amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP) (Nard Institute, Osaka, Japan) for the first 2 weeks, followed by a high fat diet (HFD) containing hydrogenated oil PRIMEX (Dyets) for 4 weeks. To induce tumors, this cycle was repeated three times, and experimental animals were fed a HFD for the rest of the course of experiments, to conduct an intermittent PhIP feeding protocol [11]. N-acetoxy-PhIP (Nard Institute), an active form of PhIP, was used for an *in vitro* experiment.

2.2. Evaluation of obesity and tumorigenicity

After fasting for 16 h, serum and body fat were collected on sacrifice at 12 weeks of age. All the blood biochemistry data were obtained by SRL Inc. (Tokyo, Japan). Body weight and body fat weight were measured at 8, 10, and 12 weeks of age. Visceral fat was harvested from epididymal, mesenteric, perirenal and retroperitoneal fat pads. Subcutaneous fat was collected from the dorsal skin. Total body fat weight was calculated as the sum of visceral and subcutaneous fats. The colons were fixed by 10% neutralized formalin overnight and stained with 0.2% methylene blue for 15 min to count the numbers of ACF, aberrant crypts (ACs), and tumors under a stereoscope. Paraffin-embedded thin sections at 5 μm were subject to hematoxylin and eosin staining for histological analysis.

2.3. Colon crypt isolation

Colonic fragments of 1–2 cm long were washed several times with TBS, and subject to incubation at 37 °C for 30 min in Hanks' balanced salt solution supplemented with 30 mM EDTA, 5 mM PMSF, 40 mM NaF and 5 mM sodium pyrophosphate decahydrate. Isolated crypts were stored at −80 °C until used for further analysis. RNA was extracted with TRIzol reagent (Invitrogen, Tokyo, Japan). Protein was extracted with T-PER Tissue Protein Extraction Reagent (Pierce, Alabama) supplemented with Complete Mini (Roche Diagnostics, Mannheim, Germany) and Halt Phosphatase Inhibitor (Pierce).

2.4. Cell culture

Normal human colon cells FHC were cultured in media containing 10% FBS and supplemented with penicillin and streptomycin. 1 day prior to experiments, the culture supernatant was replaced with serum-free media. N-acetoxy-PhIP, an activated form of PhIP, was dissolved in DMSO and added to the cells at 10 μM.

2.5. Microarray analysis

Labeled cDNA synthesized from 500 ng of total RNA was hybridized with Agilent Whole Rat Genome 4x44K microarrays, G4131F (Agilent Technologies), following the manufacturer's instructions. Hybridization images were scanned by High Resolution Microarray Scanner (Agilent Technologies), and analyzed with Agilent Feature Extraction Software v9.5. Raw data were analyzed by Gene Spring GX 7.3.1. Gene set enrichment analysis (GSEA) was conducted with GSEA software [15].

2.6. Western blotting

The proteins were separated by SDS–PAGE and transferred to PVDF membranes. The primary antibodies against p-AKT (Ser473), AKT, FOXO1, FOXO3a, FOXO4, Bim, Caspase-3, p-GSK3β (Ser9), GSK3β, p-β-catenin (Ser33/37/The41) and non-p-β-catenin (Ser33/37/Thr41) were purchased from Cell Signaling Technology (Danvers, MA), and those against β-catenin and c-myc were purchased from BD Transduction Laboratories (Lexington, KY) and Santa Cruz Biotechnology Inc., (Santa Cruz, CA), respectively. After incubation with HRP-conjugated secondary antibodies, images were visualized by enhanced chemiluminescence (Pierce). Signal intensity for p-AKT and total AKT was quantified by LAS3000 (Fujifilm, Tokyo, Japan).

2.7. Statistical analysis

All data are shown as mean ± SD. Statistical significance was determined by Mann–Whitney's *U*-test with the software JMP 9.0 (SAS Institute Japan, Tokyo, Japan). *p*-values less than 0.05 were considered significant.

3. Results

3.1. F344 rats are more susceptible to PhIP-induced colon tumorigenesis and obesity than ACI rats

Whereas carcinogenicity of chemicals is generally correlated with the number of ACF induced at an early point [16], this relationship remains elusive for PhIP. To address this issue, we chronologically monitored the colons from two rat strains treated with an intermittent PhIP-feeding protocol [11] (Fig. 1A). The numbers of both ACF and aberrant crypts (ACs) were significantly higher in the F344 rats compared to those in ACI rats at 12 weeks of age (Fig. 1B), consistent with a previous report [12]. At later time points, the number of ACs was still significantly higher in the F344 rats than in the ACI rats, but not with ACF. At 38 weeks of age, the number of colon tumors was significantly higher in F344 rats (Fig. 1C). In addition, the total number of dysplastic ACF, adenoma and adenocarcinoma were all higher in F344 rats. These results clearly indicated that the F344 rats more potently develop more advanced lesions than the ACI rats at any time point. Consequently, we reasoned that the number of ACF at 12 weeks of age would in fact serve as a marker to estimate tumor susceptibility of the strain and was used in subsequent analyses.

While examining ACF, we noted that the F344 rats tended to have more fat than the ACI rats. To verify this notion, we strictly quantified the fat weight of both strains at 6–12 weeks of age under LFD. Despite the similar level of body weight, a significantly higher degree of fat deposition was observed in F344 rats (Fig. 1D). This was also the case for visceral and subcutaneous fat. Severe accumulation of visceral fat has been associated with metabolic syndrome, which is characterized by hyperlipidemia, hypercholesterolemia and type II diabetes [17]. We then conducted

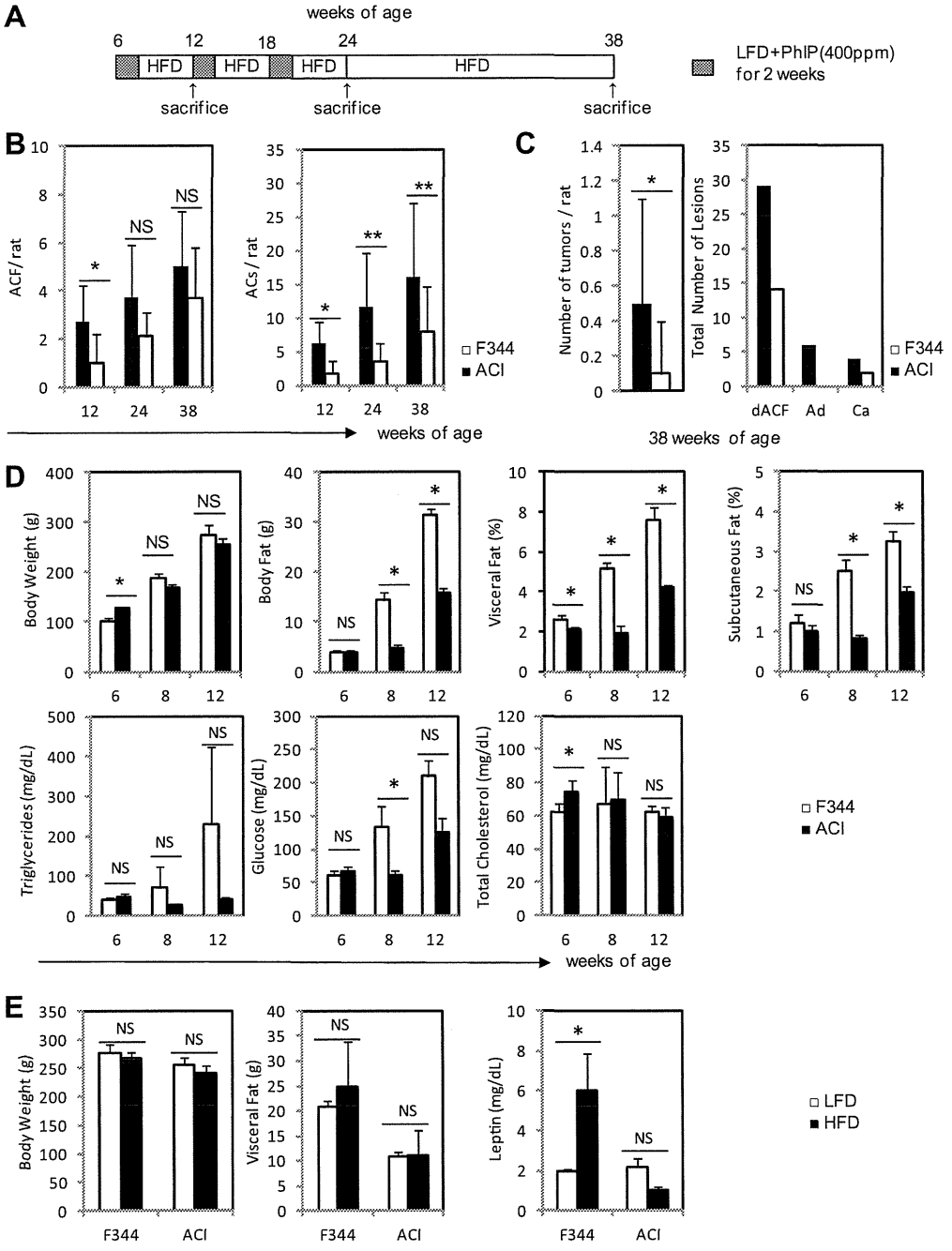

Fig. 1. Higher susceptibility to PhIP-induced colon tumorigenesis and obesity in F344 rats than in ACI rats. (A) A schematic view of the intermittent protocol for PhIP-induced colon carcinogenesis. (B) Time-series analysis of the number of ACF and ACs. The colons were examined at 12 (n = 10 each), 24 (n = 10 each), and 38 (n = 20 each) weeks of age. (C) Total number of tumors at 38 weeks of age. Both adenoma and carcinoma were counted as tumors. dACF, dysplastic ACF. Ad, adenoma. Ca, adenocarcinoma. (D) Time series analysis of body fat weight and blood biochemistry. Rats under LFD were sacrificed at 6 (n = 4 each), 8 (n = 4 each), and 12 (n = 3 each) weeks of age. (E) The effects of HFD on obesity. F344 (n = 4 each) and ACI (n = 5 each) rats under LFD or HFD for 6 weeks were sacrificed at 12 weeks of age. *p < 0.05 NS, not significant.

a blood biochemistry test and found that the level of serum triglycerides (TG) and glucose, but not total cholesterol, tended to be higher in F344 (Fig. 1D). Given that PhIP-induced colon carcinogenesis is promoted by HFD, we examined the effects of 6-week HFD on obesity. During 6–12 weeks of age, neither body weight nor the amount of visceral fat was affected in either strain (Fig. 1E). By contrast, the level of serum leptin significantly increased in F344 rats under HFD (Fig. 1E), in line with increased fat intake and ruling out the possibility that rats were improperly fed. These results indicated that F344 rats are inherently more prone to both CRC and obesity than ACI rats, which could be evaluated by measuring ACF and TG at 12 weeks of age.

3.2. Correlation between the magnitude of obesity and the incidence of ACF across multiple strains

We wondered if the observed correlation between predisposition to obesity and CRC could be more generalized. In an effort to identify genetic determinants of susceptibility to PhIP-induced CRC, we had characterized a total of 18 independent rat strains in terms of incidence of ACF under HFD for 4 weeks and collected blood samples, albeit under non-fasting conditions, from rats under LFD for 4 weeks (Fig. 2A). Although these data and samples may not be ideal for accurate analysis, we took advantage of this situation to gain insights into the common predisposition. Plotting the incidence of ACF (Fig. 2B)

and serum lipid level for each strain revealed a correlation between incidence of ACF and TG, but not cholesterol (Fig. 2C). Out of the 18 strains, we selected six strains, readily available and with relatively strong correlations, for more detailed analyses under strict conditions. Specifically, BUF, LEW, F344, and LEA, ACI, NIG-III, were postulated to constitute a tumor- and obesity-prone subgroup and a resistant subgroup, respectively. Both body fat weight and body fat percentage, with the exception of LEW rats, were indeed high in a tumor- and obesity-prone subgroup (Fig. 2D). Similar results were obtained for TG levels in a fasting state, but not cholesterol or glucose levels (Fig. 2E). These results suggested that predisposition to obesity and PhIP-induced colon tumorigenesis in the five strains might be regulated by a common mechanism.

3.3. Correlation between the magnitude of AKT activation and incidence of ACF

To gain insights into the molecular basis for the common predisposition, we set out to determine genes differentially expressed in the colons between F344 and ACI, under LFD and without PhIP. We performed Gene Set Enrichment Analysis (GSEA), which revealed a number of differentially expressed pathways (Table S1). We focused on the PI3K/Akt pathway (Fig. 3A) on the list, because it has been already implicated in both carcinogenesis and metabolism [18]. As many upstream regulators and downstream effectors of the PI3K/Akt pathway are subject to regulation by phosphorylation, we examined the level of ~30 proteins in the pathway for total protein and phosphorylated protein by Western blot analysis. We eventually found that the magnitude of AKT activation had a good correlation with colon tumor susceptibility among the five selected rat strains (Fig. 3B). In line with this observation, FOXOs and Bim, pro-apoptotic molecules inhibited by AKT, were downregulated in the crypts from the strains with higher tumor susceptibility (Fig. 3C).

3.4. Activation of AKT in the colonic cells by PhIP in vivo and in vitro

Having confirmed the static link between the common predisposition and the level of activated AKT in a basal condition, we next investigated whether PhIP and/or HFD could dynamically regulate the magnitude of AKT activation in the colon. To achieve the highest sensitivity in detecting any alterations, we selected BUF rats, which manifested the most pronounced AKT activation (Fig. 3B). Western blotting revealed that AKT was hyper-activated exclusively in colonic crypts from the subgroup treated by PhIP for 2 weeks, followed by LFD for 4 weeks (Fig. 4A). In line with this

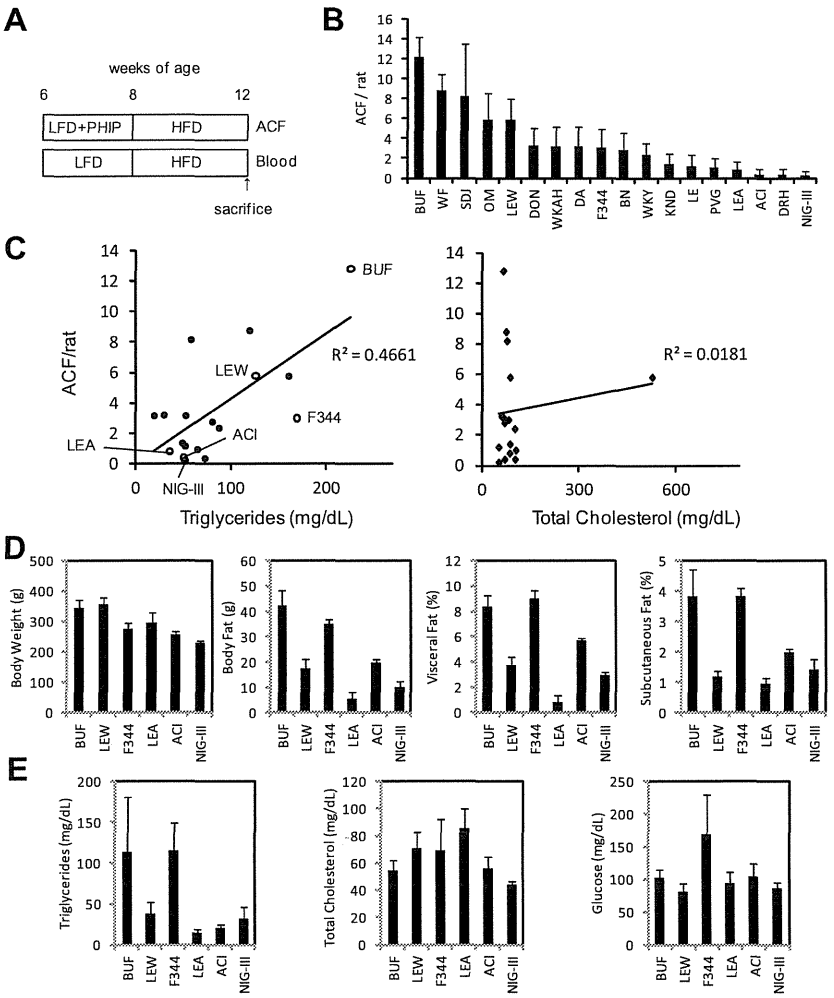

Fig. 2. Correlation between susceptibility to obesity and PhIP-induced colon tumorigenicity. (A) Feeding protocols. (B) The number of PhIP-induced ACF across 18 rat strains ($n = 5$ or 6 each). (C) Correlation between the number of ACF and amount of serum lipid. Triglyceride (*left*), and total cholesterol (*right*). Each circle depicts the mean level of serum lipid taken in a non-fasting condition from 5 individuals of each strain. Open circles labeled by strain name were used in the subsequent analysis D and E. Evaluation of obesity (D) and blood biochemistry (E) in six selected strains. Rats under LFD without PhIP for 2 weeks and subsequently under 4 weeks of HFD were examined ($n = 5$–11 each). BUF and F344, but not LEW, manifested an obesity phenotype, while ACI, LEA, and NIG-III did not (D). Only the level of serum triglyceride exhibited higher in BUF and F344, compared to the others (E).