

Free fatty acid concentrations in gallbladder bile collected from Chilean patients with gallstones

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Abstract

Objectives: To evaluate the association between gallstones and biliary free fatty acids (FFAs) as the first attempt to clarify whether biliary FFAs relate to developing gallbladder cancer (GBC) in Chile, which has the highest mortality rate in the world.

Design and methods: Gallbladder bile from 21 male and 129 female patients with gallstones from Santiago was collected, and their FFAs were measured. The results were compared with those observed in a similar previous study performed in Niigata and Kochi Prefectures which showed the highest and lowest mortality rates, respectively, for GBC in Japan.

Results: Palmitoleic and linolenic acids compositions in Santiago patients were significantly lower than those in Niigata and Kochi patients, though these compositions in Niigata patients were significantly lower than those in Kochi patients. The levels of these FFAs compositions decreased in the order of Kochi, Niigata, and Santiago patients.

Conclusions: Decreased biliary FFAs compositions may relate to developing gallstones and subsequently GBC.

Keywords: Cholelithiasis; Chile; Bile; Free fatty acids; Palmitoleic acid; Linolenic acid; High-performance liquid chromatography

Introduction

A previous study has demonstrated that the compositions of biliary palmitoleic and linolenic acids in the patients with gallstones were significantly low in the highest risk area (Niigata) for gallbladder cancer (GBC) compared with those in the lowest GBC risk area (Kochi) in Japan [1]. These free fatty acids (FFAs) are referred to as inhibitory free fatty acids (IFFAs) [2], and an inverse correlation between the IFFA concentrations and numbers of revertant colonies by the Ames test (TA98 + S9mix) was observed in both

Niigata and Kochi patients [1]. Since gallstones are accepted as the main risk factor for GBC, this finding suggests that the compositions of these FFAs in the patients who live in high incidence areas for GBC should be low.

On the basis of this evidence, we hypothesized that the compositions of these biliary FFAs would decrease in patients with gallstones in Chile having the highest standardized mortality ratio (SMR) for GBC in the world. However, there is a paucity of studies on the biliary FFAs concentrations or compositions in the patients with or without gallstones in Chile. We therefore examined biliary FFAs concentrations in Chilean patients with gallstones, as the first step to clarify whether biliary FFAs relate to the development of GBC in Chile. The results were compared with those observed in a similar previous study performed in Japan.

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Table 1
Concentrations of biliary free fatty acids (FFAs) in Chilean (Santiago) and Japanese (Niigata and Kochi) patients with gallstones

Location	Cases	Total FFAs	C _{12:0}	C _{14:0}	C _{16:0}	C _{18:0}	C _{16:1}	C _{18:1}	C _{18:2}	C _{18:3}	C _{20:4}
<i>Santiago</i>											
Men	21	5.13 ± 5.82 ^{a,b}	0.07 ± 0.05 ^b	0.19 ± 0.39	1.58 ± 1.56 ^{a,b}	0.42 ± 0.31 ^{a,b}	0.05 ± 0.15 ^c	1.13 ± 1.84 ^b	1.12 ± 1.77	0.12 ± 0.24	0.46 ± 0.99
Women	129	6.72 ± 7.94 ^{a,b}	0.06 ± 0.06	0.39 ± 0.83 ^b	1.84 ± 1.75 ^{a,b}	0.46 ± 0.38 ^{a,b}	0.21 ± 0.53	1.29 ± 1.62 ^{a,b}	2.01 ± 3.74 ^b	0.14 ± 0.29	0.32 ± 0.61
Total	150	6.50 ± 7.69 ^{a,b}	0.07 ± 0.05 ^b	0.36 ± 0.79 ^{a,b}	1.80 ± 1.72 ^{a,b}	0.45 ± 0.37 ^{a,b}	0.19 ± 0.50	1.27 ± 1.65 ^{a,b}	1.86 ± 3.54 ^{a,b}	0.13 ± 0.28	0.34 ± 0.67 ^b
<i>Niigata</i>											
Men	19	0.86 ± 0.79	0.03 ± 0.04 ^d	0.01 ± 0.02	0.26 ± 0.19	0.05 ± 0.03	0.04 ± 0.05	0.16 ± 0.15	0.27 ± 0.27	0.05 ± 0.05	0.06 ± 0.05
Women	27	0.88 ± 0.79	0.04 ± 0.04	0.01 ± 0.02	0.22 ± 0.19	0.04 ± 0.03	0.04 ± 0.05	0.13 ± 0.14	0.25 ± 0.32	0.05 ± 0.05	0.05 ± 0.04
Total	46	0.87 ± 0.78	0.04 ± 0.04	0.01 ± 0.02	0.23 ± 0.19	0.05 ± 0.03	0.04 ± 0.05	0.14 ± 0.14	0.26 ± 0.30	0.05 ± 0.05	0.05 ± 0.05
<i>Kochi</i>											
Men	24	1.73 ± 1.78	0.06 ± 0.04	0.09 ± 0.25	0.46 ± 0.52	0.07 ± 0.05	0.13 ± 0.16	0.35 ± 0.41	0.34 ± 0.56	0.12 ± 0.15	0.11 ± 0.13
Women	19	2.06 ± 3.53	0.06 ± 0.04	0.03 ± 0.06	0.42 ± 0.59	0.07 ± 0.06	0.15 ± 0.25	0.33 ± 0.50	0.68 ± 1.58	0.17 ± 0.35	0.16 ± 0.34
Total	43	1.87 ± 2.66	0.06 ± 0.04	0.07 ± 0.19	0.44 ± 0.55	0.07 ± 0.05	0.13 ± 0.20	0.34 ± 0.46	0.49 ± 1.13	0.15 ± 0.26	0.13 ± 0.24

Biliary FFAs are as follows: C_{12:0}: lauric acid; C_{14:0}: myristic acid; C_{16:0}: palmitic acid; C_{18:0}: stearic acid; C_{16:1}: palmitoleic acid; C_{18:1}: oleic acid; C_{18:2}: linoleic acid; C_{18:3}: linolenic acid; C_{20:4}: arachidonic acid. Each value is represented as mean ± SD (mmol/L).

The results obtained in Niigata and Kochi, which are the highest and lowest incidence areas, respectively, for gallbladder cancer in Japan, were demonstrated by Hori et al. [1] using a similar method as the present study.

^a $P < 0.05$ (comparison between Santiago and Kochi).

^b $P < 0.05$ (comparison between Santiago and Niigata).

^c $P < 0.05$ (comparison between men and women in Chile).

^d $P < 0.05$ (comparison between Niigata and Kochi).

Material and methods

Study subjects

A total of 150 patients [21 men, with a mean age of 54.3 (range, 27 to 80) and 129 women, with a mean age of 48.2 years (range, 23 to 84)] with gallstones who received cholecystectomy between April 2000 and September 2002 at the Sótero del Río Hospital located in the southeastern region of Santiago, the representative capital city of Chile, were included in our study. Written informed consent was obtained from each subject before the start of this study, which was approved by the Ethics Committee at Niigata University School of Medicine.

Bile sampling

Gallbladder bile was obtained by needle aspiration during cholecystectomy and gathered into glass or plastic tubes and stored immediately at -80°C . These frozen samples collected in Chile were sent to Japan and kept at -80°C until analysis of FFAs concentrations.

Measurement of FFAs

A total of 9 FFAs (lauric acid, myristic acid, palmitoleic acid, palmitic acid, linolenic acid, linoleic acid, oleic acid, stearic acid, and arachidonic acid) were detected using a high-performance liquid chromatography system (LC10AS, Shimadzu Corporation, Kyoto, Japan) equipped with a spectrofluorometric detector (RF-550 GLP, Shimadzu Corporation) and an Eclipse XDB-C8 column (dimensions: 4.6×250 mm; particle size: $5 \mu\text{m}$; Rockland Technologies, Inc., USA) following a previously described method [3]. The mean recovery rate for FFAs was 98% (range 92–104%), and the detection limit of the assay was 100 pg. The calibration curves for all FFAs were linear over the range of 0.5–400 ng.

Statistical evaluation

Statistical analyses were performed using Statistical Analysis System software (SAS Institute Inc., Cary, NC, USA). All data were expressed as the mean \pm standard deviation. We used the Student's unpaired *t* test to evaluate the gender differences of the concentrations and compositions in the Chilean patients. All data obtained in Santiago, Niigata, and Kochi were compared by one-way ANOVA using Scheffe's test. *P* values of less than 0.05 were considered to indicate statistical significance.

Results

Table 1 shows the mean concentrations of biliary FFAs in Chilean (Santiago) and Japanese (Niigata and Kochi) patients with gallstones. Previous results in Japan [1] were obtained by similar methods to those used in the present study. The mean age of Niigata and Kochi patients were as follows: men,

Table 2

Mean compositions of biliary free fatty acids in Chilean and Japanese patients with gallstones

	Santiago (150)	Niigata (46)	Kochi (43)
Lauric acid (12:0)	$2.6 \pm 4.0^{\text{a,b}}$	6.0 ± 8.6	7.9 ± 8.2
Myristic acid (14:0)	4.5 ± 6.4	2.5 ± 2.7	3.4 ± 4.2
Palmitic acid (16:0)	$31.8 \pm 13.9^{\text{b}}$	28.9 ± 10.1	26.0 ± 10.3
Stearic acid (18:0)	$10.0 \pm 5.4^{\text{a,b}}$	7.3 ± 3.9	6.8 ± 4.1
Palmitoleic acid (16:1)	$1.9 \pm 3.9^{\text{a,b}}$	$3.9 \pm 2.3^{\text{c}}$	7.3 ± 4.7
Oleic acid (18:1)	19.8 ± 13.6	15.2 ± 4.6	16.7 ± 6.9
Linoleic acid (18:2)	21.3 ± 12.9	24.9 ± 10.7	19.3 ± 9.0
Linolenic acid (18:3)	$1.8 \pm 5.6^{\text{a,b}}$	5.3 ± 2.9	6.9 ± 3.8
Arachidonic acid (20:4)	6.3 ± 6.0	5.8 ± 3.0	5.7 ± 2.9

Each value is represented as mean \pm SD (%) in Chilean (Santiago) and Japanese (Niigata and Kochi) patients with gallstones.

^a *P* < 0.05 (comparison between Santiago and Kochi).

^b *P* < 0.05 (comparison between Santiago and Niigata).

^c *P* < 0.05 (comparison between Niigata and Kochi).

53.5 ± 16.5 and 65.9 ± 15.0 , respectively; women, 53.7 ± 11.5 and 64.9 ± 19.0 , respectively.

In Santiago patients, no significant gender difference was observed in the total FFA concentration, but the mean concentration of palmitoleic acid in men (0.05 mmol/L) was significantly lower than that in women (0.21 mmol/L). The wide standard deviation indicates a considerable inter-individual variability in both men and women.

Although the total FFA concentration in Santiago patients was approximately 3.5-fold higher than in Kochi patients with a low incidence for GBC, and 7.5-fold higher than in Niigata patients with a high incidence for this cancer in Japan, no significant differences in the mean concentrations of palmitoleic and linolenic acids were observed among the three populations. Mean concentrations of myristic, palmitic, stearic, oleic, and linoleic acids in Santiago patients were significantly higher than those in Niigata and Kochi patients.

Table 2 shows the mean compositions of biliary FFAs in Chilean and Japanese patients with gallstones. We also found geographical differences in the compositions of FFAs among the three populations, with no significant differences in myristic, oleic, linoleic, or arachidonic acids. The composition of stearic acid in Santiago patients was significantly higher than that in Niigata and Kochi patients. On the other hand, lauric, palmitoleic, and linolenic acids compositions in Santiago patients were significantly lower than those in Niigata and Kochi patients. Furthermore, palmitoleic acid composition in Niigata patients was significantly lower than that in Kochi patients. The levels of palmitoleic and linolenic acids compositions in the three populations decreased in the order of Kochi, Niigata, and Santiago.

Discussion

This study shows a detailed biliary FFA profile in Chilean patients with gallstones. The results provide evidence of the decreased compositions of palmitoleic and linolenic acids in Chilean patients with gallstones, suggesting that this condition

could be a mechanism through which GBC develops in Chilean patients with gallstones.

Despite the fact that Chile has the highest SMR for GBC in the world [4], the etiology of GBC remains unclear. Our previous study reported the relationship between a high consumption of red chili pepper and the development of GBC in Chile [5]. We think it is interesting to investigate biliary FFAs concentrations in Chilean patients not only with gallstones but also with gallstones and GBC based on the following reasons. First, a previous animal experiment using rats has demonstrated that biliary IFFAs concentrations decreased significantly by treatment with capsaicin, which is the pungent ingredient of chili pepper [6]. These changes observed in rats may also occur in human bile, but the reason is not clear. Second, capsaicin has been reported to be a tumor promoter, carcinogen, and potential mutagen [7]. Moreover, some mutagenic substances and some unidentified substances in gallbladder bile have been reported [8,9]. In addition, a total of 8 FFAs in gallbladder bile have been detected in a previous study [10]. Of these FFAs detected, long-chain unsaturated FFAs, such as palmitoleic, oleic, linoleic, linolenic, and arachidonic acids, have shown an inhibitory effect on the activity of mutagenic substances [2]. These FFAs are referred to as IFFAs, and they are significantly more effective killers of tumor cells *in vitro* than the corresponding saturated FFAs [2]. It is conceivable that the inhibitory effects of IFFAs also act in human gallbladder bile. These findings provide evidence that decreased IFFAs concentrations and mutagenic substances in gallbladder bile may be complementary factors in the development of GBC.

Decreased palmitoleic and linolenic acids compositions in Chilean patients with gallstones are similar to the results obtained in a previous study performed in Japan that found IFFAs compositions significantly lower in Niigata patients compared with Kochi patients. In spite of the genetic and dietary differences in the people that may exist between Niigata and Santiago, it does not appear to be very probable that Kochi and Niigata have the same kind of differences. Although our results raise the possibility that decreased compositions of palmitoleic and linolenic acids and the increased risk of developing GBC are associated in a cause-and-effect manner, at this time, we can only speculate as to the nature of this relationship.

Potential limitations of our study warrant discussion. No patients with GBC have been included in our study. Although well-designed case-control studies based on healthy people, in addition to studying patients with gallstones and GBC would help to clarify the etiologic factors involved in this cancer, we did not collect our bile samples from healthy people for ethical reasons. Also, it is difficult to have a clinical diagnosis of GBC before the cholecystectomy and its pathological examination. As a result, an initial way for us to collect bile samples was from

patients with gallstones who received cholecystectomy. Therefore, further investigations are needed to support our findings. Nevertheless, our results are consistent with the results of a previous study showing that biliary palmitoleic and linolenic acids compositions were also decreased in Niigata patients, which is a very different population compared to patients in Santiago, but which shares a high incidence of GBC [4]. In contrast, patients in Kochi, with a low incidence rate, showed a different profile.

In summary, we observed the lowest compositions of biliary palmitoleic and linolenic acids in Santiago patients among the three populations. Although decreased palmitoleic and linolenic acids compositions may play a role in the development of GBC, the pathophysiological meaning remains unclear. The possibility that the decreased palmitoleic and linolenic acids compositions increase the risk of GBC could not be ruled out because of the lack of inhibitory effects to mutagens in bile and needs further studies. Our results provide preliminary rationale for further studies on the relation between biliary FFAs concentrations and the development of GBC in Chile.

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References

- [1] Hori Y, Nakamura K, Yamamoto M, et al. Geographical variations in the concentration of biliary free fatty acids with anti-mutagenic action. *Mutat Res* 1999;444:41–7.
- [2] Hayatsu H, Arimoto S, Negishi T. Dietary inhibitors of mutagenesis and carcinogenesis. *Mutat Res* 1988;202:429–46.
- [3] Hori Y, Nakamura K, Yamamoto M, et al. Determination of free fatty acids in human bile by high-performance liquid chromatography. *Ann Clin Biochem* 1998;35:279–82.
- [4] Serra I, Garcia V, Yamamoto M, Cavada G. Gallbladder cancer in Chile and selected countries. *Estud Atacam* 1998;15:21–7.
- [5] Serra I, Yamamoto M, Calvo A, et al. Association of chili pepper consumption, low socioeconomic status and longstanding gallstones with gallbladder cancer in a Chilean population. *Int J Cancer* 2002;102:407–11.
- [6] Saito A, Nakamura K, Hori Y, Yamamoto M. Effects of capsaicin on biliary free fatty acids in rats. *Int J Vitam Nutr Res* 2000;70:19–23.
- [7] Zhou S, Koh HL, Gao Y, Gong ZY, Lee EJ. Herbal bioactivation: the good, the bad and the ugly. *Life Sci* 2004;74:935–68.
- [8] Manabe S, Wada O. Identification of carcinogenic tryptophan pyrolysis products in human bile by high-performance liquid chromatography. *Environ Mol Mutagen* 1990;15:229–35.
- [9] Mano H, Roa I, Araya JC, et al. Comparison of mutagenic activity of bile between Chilean and Japanese female patients having cholelithiasis. *Mutat Res* 1996;371:73–7.
- [10] Schone A, Jungst D, Meyer G, Hernandez-Richter T, Fischer S. Effects of phospholipase A2, free fatty acids and 2-lysolecithin on the crystallization of cholesterol in gallbladder bile. *Eur J Clin Invest* 2000;30:715–21.