and lifestyle factors to the risk of colorectal cancer. Cases were patients undergoing surgery for a first diagnosis of colorectal cancer, and controls were selected randomly in the community. The study protocol was approved by the ethical committee of the Faculty of Medical Sciences, Kyushu University and those of all but two of the participating hospitals. The two exceptions had no ethical committees at the time of the survey, so permission was granted by their directors. Details of the methods have been reported previously,[24] and methodological matters relevant to the present analysis are described below.

Subjects. Cases consisted of consecutive patients with histologically confirmed incident colorectal adenocarcinomas who were admitted to two university or six affiliated hospitals for surgical treatment during the period from October 2000 to December 2003. Other eligibility criteria were as follows: age of 20–74 years at the time of diagnosis, residents of the study area (Fukuoka City and three adjacent areas), no prior history of partial or total removal of the colorectum, familial adenomatous polyposis or inflammatory bowel disease, and mental competence to give informed consent and participate in the interview. Of 1053 eligible cases, 840 cases (80%) were actually included.

Eligibility criteria for controls were almost the same as for cases except for two items, that is, having no diagnosis of colorectal cancer and age 20–74 years at the time of selection. Numbers of control candidates by sex and 10-year age class were determined in accordance to sex- and age-specific expected numbers of incident cases of colorectal cancer. A total of 1500 control candidates were selected by the method of two-stage random sampling. First 15 small areas corresponding to primary school zones were selected randomly, and then approximately 100 people were chosen randomly for each small area using the municipal resident registry. At most four letters of invitation were sent to each candidate control. A total of 833 people participated in the survey. The net participation rate was calculated as 60% (833/1382), after exclusion of those who were found to be ineligible for the following reasons: death ($n = 7$), migration from the study area ($n = 22$), undelivered mail ($n = 44$), history of large-bowel resection ($n = 21$), mental incompetence ($n = 19$), and diagnosis of colorectal cancer after the survey ($n = 5$).

In the present analysis, we excluded subjects with an extreme energy intake beyond the range of mean ± 2 SD in the log-transformed energy intake in three age categories (<50, 50–69 and 70 years) for each sex. Finally, 782 cases and 793 controls remained. Numbers of subsite-specific colorectal cancers were as follows: proximal colon 177, distal colon 262, rectum 327, and multiple sites 16. The cecum, ascending colon and transverse colon were combined as the proximal colon, and the distal colon comprised the descending and sigmoid colon.

Lifestyle questionnaire. A questionnaire was used for research nurses to carry out a uniform interview with cases and controls regarding multifaceted lifestyle factors except dietary details. In this study, the referent time for cases was the date of the onset of symptoms or the screening, and that for controls was the time of interview. We attempted to avoid ascertainment of lifestyle factors in the immediate past as much as possible, and different time frames were used for different factors primarily for ease of recalling habitual lifestyles in the past. Anthropometric questions inquired about height (cm), recent bodyweight (kg) and bodyweight at the time 10 years before. Body mass index (kg/m^2) 10 years before was used, because the current body mass index was unrelated to the risk.

Questions on physical activities elicited type of job, activities in commuting, housework and shopping, together with leisure-time activities at the time 5 years previously. As regards type of job, five options were prepared: sedentary or standing work, work with walking, labor work, hard labor work and no job. Regular leisure-time activities were ascertained with regularity defined as at least once per week. As for at most three activities, type of activity, numbers of months and of days per week that individuals participated in each activity, and minutes of participation per occasion were reported. As described in detail previously,[27] physical activity in the leisure time (including activities in commuting, housework and shopping) was expressed as the sum of metabolic equivalents (MET) multiplied by hours of participation in each activity, which was thereafter called MET-hours.

With regard to smoking habit, ever-smokers were asked about years of smoking and numbers of cigarettes smoked per day for each decade of age from the second to eighth decade. We calculated the cumulative exposure to cigarette smoking until the beginning of the previous decade of age. Alcohol use at the time of 5 years prior to the referent time was elicited. Individuals answered open-ended questions regarding the frequency of consumption and habitual amount of alcohol consumed on the day of alcohol drinking. The amount of alcohol was expressed in the conventional unit; one *go* (180 mL) of *sake*, one large bottle (633 mL) of beer, and half a *go* (90 mL) of *shochu* were each expressed as one unit; and one drink (30 mL) of whisky or brandy and one glass (100 mL) of wine were each converted to a half unit.

Dietary assessment. A personal-computer (PC) software program was developed for dietary assessment with support from an external laboratory (Core Create Systems, Kitakyushu, Japan). A total of 148 items of foods and beverage were selected with reference to dietary questionnaires developed previously in Japan.[28–30] Consumption frequencies and portion sizes of these foods and beverages were ascertained by interview using the PC software, which was to help individuals report consumption of specific foods and dishes. The collected information was the same as obtained by the so-called semiquantitative food frequency questionnaire. As for consumption frequency, different numbers of response categories were prepared for rice with each meal, other food items, and non-alcoholic and alcoholic beverages. Typical dishes were shown on the display for each food item, together with typical portion sizes. Options for serving size were 0.5, 1, 1.5 and 2 of the size displayed as a reference for most of the food items. Supplementary questions included an inquiry about consumption of fat portions of beef/pork and skin of chicken at table and others. Three precoded answers were prepared for the consumption of fat portion or skin of chicken (all, half or null consumption), and the corresponding foods, such as beef/pork without fat and chicken with skin, were chosen for calculation of nutrients. Beef and pork were combined for most of the questions. Participants were asked to report the consumption on average over the 1 year before the referent time, and controls answered dietary questions with reference to the past 1 year before the interview. After the episode of bovine spongiform encephalopathy (BSE) in the year 2001,[31] individuals were carefully instructed to provide answers for consumption before change of dietary habits if it had recently occurred.

Based on the interview data, 148 food/dish items in the PC software were collapsed into 211 food items to calculate nutrient intake. Some items in the PC software were dishes and collective foods (such as beef/pork and ham/sausage), and individual foods of such composite items were separated on the basis of typical recipes and market statistics. Of these 211 food items, 202 items corresponded to foods listed in the food composition tables in Japan.[32] An appropriate food could not be assigned to the remaining nine items (five groups of fish for different ways of cooking, miso, vegetables oils, oranges other than mandarin and pickles of non-green vegetables), and composition data were created on the basis of diet records derived from a validation study carried out with 28 subjects aged 41–65 years (13 men and 15 women), who had participated as controls in the present case-control study. Diet records were done over a period of 7 days in four consecutive seasons in accordance with the method used elsewhere.[28] A total of 60 people were asked to participate in

Table 1. Characteristics of cases and controls

Characteristics	Case (n = 782)	Control (n = 793)
Male (%)	60.5	62.4
Age (years), mean (SD)	61 (9)	59 (11)
Residence, rural (%)	39.8	35.9
BMI 10 years before (kg/m^2), mean (SD)	23.3 (3.2)	22.9 (3.1)
Parental colorectal cancer (%)	7.7	5.6
Ever-smoking (%)		
Male	82.5	81.8
Female	15.2	21.8
Alcohol use (%)[†]		
Male	77.6	78.2
Female	28.5	30.2
Non-sedentary job (%)	26.0	30.5
Leisure-time physical activity (%)[‡]	62.7	66.2
Nutrients and foods, median (IQR)[§]		
Total energy (kcal/day)	2185 (1819–2552)	2196 (1814–2606)
Total fat (g/day)	62 (52–70)	62 (54–71)
Red meat (g/day)[¶]	43 (29–61)	45 (29–61)
Fish and fish products (g/day)	77 (54–102)	78 (56–104)
Calcium (mg/day)	647 (519–777)	664 (521–813)
Total fiber (g/day)	14.5 (11.7–17.5)	14.5 (11.9–17.7)

[†]Drinking alcohol at least once per week. [‡]Physical activity of moderate or greater intensities (metabolic equivalent ≥4). [§]Energy-adjusted intake calculated by the regression residual method. [¶]Beef/pork and processed meat combined. BMI, body mass index; IQR, interquartile range; SD, standard deviation.

the validation study. Of them, 35 started to record their diet, and the above 28 people completed the diet records. Details of the validation study will be described elsewhere (in preparation). Pearson correlation coefficients of energy-adjusted intake (see below) of selected nutrients and foods estimated from the two methods were as follows: total fat 0.70, saturated fat 0.72, n-6 PUFA 0.41, n-3 PUFA 0.34, beef/pork 0.70, processed meat 0.57, and fish and fish products 0.21. Beef/pork and processed meat were combined as red meat in the present study.

Statistical analysis. Intakes of foods and nutrients were adjusted for energy intake by the regression residual method with intake values transformed to the natural-log scale.[33] Logistic regression analysis was used to estimate the odds ratio (OR) and 95% confidence intervals (CI) to assess the relationship of fat and meat intake to colorectal cancer risk. Nutrient and food intake were categorized into five levels using the quintiles of each nutrient or food intake in the control group. The trend of the association was assessed with ordinal scores 0–4 assigned to five categories in order. All P-values were two-sided, and considered significant at $P < 0.05$. All analyses were conducted using the Statistical Analysis System (SAS) version 8.2 (SAS Institute, Cary, NC, USA).

Potential confounding factors under consideration were 5-year age class (the lowest class of <40 years), sex, residence area (Fukuoka City or the adjacent areas), body mass index (kg/m^2) 10 years before (<25.0 or ≥25.0 kg/m^2), parental colorectal cancer, smoking (0, 1–399, 400–799 or ≥800 cigarettes/year), alcohol intake (0, 0.1–0.9, 1.0–1.9, or ≥2.0 units/day), type of job (sedentary or non-sedentary), leisure-time physical activity (0, 1–15.9 or ≥16 MET-hours/week) and dietary calcium and fiber (quintiles).

Results

Table 1 summarizes characteristics of cases and controls with respect to potential confounders and some of the factors under study. Occupational and leisure-time physical activity were slightly lower in the cases than in the controls. Body mass index was slightly greater and parental colorectal cancer was more frequent in the cases. Smoking and alcohol use did not vary much according to case-control status in either men or women. Median intake of calcium was lower in the cases, but there was no appreciable difference in total energy intake, total fat, red meat, fish and fish products, and dietary fiber.

Crude and adjusted OR of colorectal cancer did not differ from unity according to consumption levels of beef/pork, processed meat or poultry (Table 2). Total, saturated and monounsaturated fat also showed no clear association with the overall risk (Table 2). Intake of n-6 PUFA seemed to be related to a decreased risk of colorectal cancer, but the decreasing trend was far from statistical significance in the multivariate analysis ($P = 0.17$). As shown in Table 3, the OR decreased progressively with higher intake of n-3 PUFA, and the decrease was almost statistically significant even after full adjustment for the confounding variable ($P = 0.050$). A similar, but less clear, inverse association was noted for the consumption of fish and fish products.

In the subsite-specific analysis, beef/pork, processed meat and poultry showed no clear association with cancer of the proximal colon, distal colon or rectum (Table 4). Again, different types of fatty acids (excluding n-3 PUFA) were unrelated to subsite-specific risks of colorectal cancer. A statistically significant inverse association with n-3 PUFA was observed only for distal colon cancer (Table 5). The subsite-specific analysis also revealed a decreased risk of distal colon cancer associated with the consumption of fish and fish products, showing a nearly significant trend ($P = 0.052$).

Discussion

The present study showed no clear association between red meat or associated fat intake and colorectal cancer in a large case-control study and thus did not provide support for the hypothesis that high consumption of red meat increases the risk of colorectal cancer at any subsite. However, there was an inverse association between n-3 PUFA intake and colorectal cancer, especially distal colon cancer.

High consumption of red meat was shown to be associated with a modest increase in the risk of colorectal cancer in a meta-analysis of studies in Western countries, although findings from

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Table 2. Meat and fat intake and risk of colorectal cancer

Food and nutrient (quintile)[†]	Median (g/day)	No. controls	No. cases	OR[‡]	OR (95% CI)[§]
Beef/pork					
Q1	14.2	158	142	1.00	1.00
Q2	27.3	159	188	1.35	1.35 (0.98–1.85)
Q3	37.4	158	161	1.23	1.28 (0.92–1.79)
Q4	48.6	159	140	1.04	1.03 (0.73–1.44)
Q5	70.1	159	151	1.16	1.13 (0.80–1.61)
Trend		159	151	$P = 0.95$	$P = 0.94$
Processed meat					
Q1	0.4	158	152	1.00	1.00
Q2	2.5	159	149	1.02	1.03 (0.74–1.43)
Q3	4.9	158	160	1.10	1.09 (0.79–1.52)
Q4	8.2	159	151	1.07	1.07 (0.77–1.49)
Q5	14.9	159	170	1.20	1.15 (0.83–1.60)
Trend				$P = 0.25$	$P = 0.40$
Red meat[¶]					
Q1	18.0	158	154	1.00	1.00
Q2	32.3	159	174	1.14	1.14 (0.83–1.57)
Q3	44.5	158	166	1.15	1.13 (0.82–1.57)
Q4	57.4	159	122	0.86	0.84 (0.60–1.19)
Q5	78.9	159	166	1.19	1.14 (0.81–1.62)
Trend				$P = 0.84$	$P = 0.97$
Poultry					
Q1	5.2	158	178	1.00	1.00
Q2	12.0	159	153	0.88	0.88 (0.64–1.21)
Q3	17.0	158	138	0.82	0.79 (0.57–1.09)
Q4	23.5	159	176	0.99	0.92 (0.67–1.26)
Q5	35.0	159	137	0.81	0.75 (0.54–1.05)
Trend				$P = 0.41$	$P = 0.17$
Total fat					
Q1	45.7	158	185	1.00	1.00
Q2	55.6	159	158	0.85	0.91 (0.66–1.26)
Q3	62.0	158	146	0.78	0.86 (0.61–1.20)
Q4	69.6	159	161	0.84	0.97 (0.69–1.37)
Q5	78.2	159	132	0.71	0.77 (0.53–1.13)
Trend				$P = 0.08$	$P = 0.33$
Saturated fatty acids					
Q1	11.39	158	170	1.00	1.00
Q2	14.55	159	186	1.08	1.19 (0.86–1.64)
Q3	16.65	158	144	0.83	0.90 (0.64–1.26)
Q4	19.08	159	133	0.76	0.86 (0.60–1.23)
Q5	22.10	159	149	0.88	1.04 (0.71–1.51)
Trend				$P = 0.12$	$P = 0.52$
Monounsaturated fatty acids					
Q1	15.29	158	180	1.00	1.00
Q2	19.11	159	160	0.90	0.97 (0.70–1.33)
Q3	21.52	158	151	0.85	0.95 (0.68–1.32)
Q4	24.33	159	148	0.82	0.91 (0.65–1.28)
Q5	28.06	159	143	0.83	0.88 (0.62–1.25)
Trend				$P = 0.22$	$P = 0.44$
n-6 PUFA					
Q1	7.98	158	194	1.00	1.00
Q2	10.19	159	152	0.81	0.82 (0.59–1.13)
Q3	11.63	158	150	0.79	0.83 (0.60–1.16)
Q4	13.10	159	145	0.73	0.77 (0.55–1.09)
Q5	15.23	159	141	0.71	0.77 (0.54–1.10)
Trend				$P = 0.04$	$P = 0.17$

[†]Energy-adjusted intake calculated by the regression residual method. [‡]Adjusted for age, sex and residential area. [§]Adjusted for age, sex, residential area, body mass index 10 years before, parental colorectal cancer, smoking, alcohol use, type of job, leisure-time physical activity, dietary calcium and dietary fiber. [¶]Beef/pork and processed meat combined. CI, confidence interval; OR, odds ratio; PUFA, polyunsaturated fatty acids.

Table 3. Fish and fish products, n-3 polyunsaturated fatty acid (PUFA) intake and risk of colorectal cancer

Food and nutrient (quintile)[†]	Median (g/day)	No. controls	No. cases	OR[‡]	OR (95% CI)[§]
Fish and fish products					
Q1	37.4	158	164	1.00	1.00
Q2	61.1	159	159	0.88	0.93 (0.67–1.29)
Q3	77.5	158	168	0.90	0.89 (0.64–1.24)
Q4	98.2	159	143	0.75	0.77 (0.55–1.07)
Q5	138.1	159	148	0.77	0.80 (0.57–1.13)
Trend				$P = 0.07$	$P = 0.11$
n-3 PUFA					
Q1	1.99	158	172	1.00	1.00
Q2	2.55	159	177	0.99	1.02 (0.74–1.40)
Q3	2.92	158	152	0.84	0.89 (0.64–1.23)
Q4	3.29	159	149	0.80	0.84 (0.60–1.18)
Q5	3.94	159	132	0.68	0.74 (0.52–1.06)
Trend				$P = 0.01$	$P = 0.05$

[†]Energy-adjusted intake calculated by the regression residual method. [‡]Adjusted for age, sex and residential area. [§]Adjusted for age, sex, residential area, body mass index 10 years before, parental colorectal cancer, smoking, alcohol use, type of job, leisure-time physical activity, dietary calcium and dietary fiber. CI, confidence interval; OR, odds ratio.

Table 4. Meat and fat intake and subsite-specific risk of colorectal cancer

Food and nutrient consumption[†]	Proximal colon cancer		Distal colon cancer		Rectal cancer	
	n	OR (95% CI)[‡]	*n*	OR (95% CI)	*n*	OR (95% CI)
Beef/pork						
Q1	23	1.00	54	1.00	63	1.00
Q2	48	2.21 (1.26–3.88)	65	1.24 (0.80–1.94)	73	1.18 (0.78–1.79)
Q3	41	2.00 (1.12–3.58)	46	0.94 (0.58–1.52)	70	1.18 (0.77–1.81)
Q4	35	1.67 (0.91–3.06)	41	0.80 (0.49–1.31)	57	0.88 (0.56–1.38)
Q5	30	1.44 (0.76–2.71)	56	1.23 (0.75–2.00)	64	1.01 (0.64–1.60)
Trend		$P = 0.64$		$P = 0.97$		$P = 0.64$
Processed meat						
Q1	40	1.00	48	1.00	59	1.00
Q2	27	0.82 (0.47–1.44)	49	1.10 (0.68–1.78)	70	1.20 (0.78–1.84)
Q3	35	1.12 (0.65–1.92)	57	1.30 (0.81–2.08)	64	1.08 (0.69–1.67)
Q4	33	1.04 (0.60–1.80)	49	1.15 (0.71–1.86)	68	1.21 (0.78–1.87)
Q5	42	1.20 (0.72–2.03)	59	1.32 (0.82–2.11)	66	1.14 (0.73–1.77)
Trend		$P = 0.33$		$P = 0.27$		$P = 0.61$
Red meat[§]						
Q1	27	1.00	56	1.00	69	1.00
Q2	46	1.74 (1.01–3.00)	58	1.03 (0.65–1.61)	68	0.98 (0.65–1.49)
Q3	43	1.69 (0.97–2.95)	48	0.84 (0.53–1.35)	68	1.01 (0.66–1.54)
Q4	29	1.21 (0.66–2.21)	39	0.77 (0.47–1.27)	50	0.70 (0.45–1.11)
Q5	32	1.27 (0.69–2.33)	61	1.28 (0.79–2.07)	72	1.05 (0.67–1.65)
Trend		$P = 0.96$		$P = 0.66$		$P = 0.71$
Poultry						
Q1	39	1.00	65	1.00	71	1.00
Q2	36	0.91 (0.54–1.54)	46	0.71 (0.45–1.12)	66	0.92 (0.60–1.39)
Q3	30	0.83 (0.48–1.45)	51	0.81 (0.51–1.27)	55	0.72 (0.46–1.11)
Q4	40	0.94 (0.56–1.59)	58	0.86 (0.56–1.34)	75	0.94 (0.62–1.42)
Q5	32	0.87 (0.50–1.49)	42	0.65 (0.41–1.05)	60	0.78 (0.51–1.20)
Trend		$P = 0.69$		$P = 0.22$		$P = 0.35$
Total fat						
Q1	34	1.00	74	1.00	74	1.00
Q2	32	1.03 (0.59–1.79)	56	0.86 (0.55–1.35)	67	0.89 (0.58–1.36)
Q3	31	0.94 (0.53–1.68)	41	0.65 (0.40–1.06)	68	0.92 (0.59–1.41)
Q4	50	1.52 (0.88–2.62)	49	0.81 (0.50–1.32)	61	0.89 (0.57–1.40)
Q5	30	0.90 (0.48–1.70)	42	0.70 (0.41–1.20)	57	0.78 (0.48–1.27)
Trend		$P = 0.64$		$P = 0.20$		$P = 0.39$
Saturated fatty acids						
Q1	31	1.00	63	1.00	73	1.00
Q2	36	1.19 (0.68–2.08)	67	1.24 (0.79–1.94)	78	1.06 (0.70–1.60)

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Table 4. *continued*

Food and nutrient consumption†	Proximal colon cancer		Distal colon cancer		Rectal cancer	
	n	OR (95% CI)‡	*n*	OR (95% CI)	*n*	OR (95% CI)
Q3	43	1.45 (0.83–2.55)	44	0.82 (0.50–1.35)	55	0.75 (0.48–1.17)
Q4	36	1.08 (0.60–1.96)	36	0.70 (0.41–1.18)	58	0.85 (0.54–1.35)
Q5	31	0.99 (0.52–1.88)	52	1.17 (0.69–1.99)	63	1.00 (0.62–1.64)
Trend		$P = 0.84$		$P = 0.68$		$P = 0.63$
Monounsaturated fatty acids						
Q1	40	1.00	62	1.00	74	1.00
Q2	28	0.80 (0.46–1.38)	64	1.21 (0.78–1.88)	65	0.87 (0.57–1.32)
Q3	35	0.96 (0.56–1.64)	53	1.09 (0.68–1.74)	59	0.81 (0.53–1.26)
Q4	38	0.98 (0.57–1.66)	40	0.80 (0.48–1.31)	68	0.97 (0.63–1.50)
Q5	36	0.99 (0.56–1.73)	43	0.92 (0.55–1.55)	61	0.84 (0.53–1.34)
Trend		$P = 0.84$		$P = 0.34$		$P = 0.67$
n-6 PUFA						
Q1	32	1.00	77	1.00	81	1.00
Q2	35	1.12 (0.64–1.96)	52	0.71 (0.46–1.11)	60	0.78 (0.51–1.19)
Q3	39	1.29 (0.74–2.24)	41	0.62 (0.39–1.00)	68	0.88 (0.58–1.35)
Q4	33	1.05 (0.58–1.92)	48	0.72 (0.45–1.17)	61	0.77 (0.49–1.19)
Q5	38	1.29 (0.70–2.35)	44	0.67 (0.40–1.12)	57	0.69 (0.43–1.10)
Trend		$P = 0.52$		$P = 0.16$		$P = 0.16$

†Energy-adjusted intake calculated by the regression residual method. ‡Adjusted for age, sex, residential area, body mass index 10 years before, parental colorectal cancer, smoking, alcohol use, type of job, leisure-time physical activity, dietary calcium and dietary fiber. §Beef/pork and processed meat combined. CI, confidence interval; OR, odds ratio; PUFA, polyunsaturated fatty acids.

Table 5. Fish and fish products, n-3 polyunsaturated fatty acid (PUFA) intake and subsite-specific risk of colorectal cancer

Food and nutrient† (quintile)	Proximal colon cancer		Distal colon cancer		Rectal cancer	
	n	OR (95% CI)‡	*n*	OR (95% CI)	*n*	OR (95% CI)
Fish and fish products						
Q1	39	1.00	59	1.00	65	1.00
Q2	32	0.81 (0.47–1.39)	53	0.86 (0.54–1.36)	71	1.03 (0.68–1.58)
Q3	39	0.82 (0.48–1.39)	56	0.84 (0.53–1.33)	70	0.94 (0.62–1.45)
Q4	33	0.71 (0.41–1.23)	48	0.68 (0.42–1.10)	58	0.82 (0.52–1.28)
Q5	34	0.68 (0.38–1.20)	46	0.64 (0.39–1.06)	63	0.91 (0.57–1.43)
Trend		$P = 0.17$		$P = 0.05$		$P = 0.40$
n-3 PUFA						
Q1	31	1.00	74	1.00	65	1.00
Q2	35	1.17 (0.66–2.07)	56	0.79 (0.51–1.23)	82	1.22 (0.80–1.85)
Q3	41	1.28 (0.74–2.24)	41	0.56 (0.35–0.91)	68	1.05 (0.68–1.61)
Q4	41	1.25 (0.71–2.20)	49	0.68 (0.43–1.09)	53	0.76 (0.48–1.20)
Q5	29	0.84 (0.45–1.55)	42	0.56 (0.34–0.92)	59	0.88 (0.56–1.41)
Trend		$P = 0.67$		$P = 0.02$		$P = 0.16$

†Energy-adjusted intake calculated by the regression residual method. ‡Adjusted for age, sex, residential area, body mass index 10 years before, parental colorectal cancer, smoking, alcohol use, type of job, leisure-time physical activity, dietary calcium and dietary fiber. CI, confidence interval; OR, odds ratio.

individual studies were not necessarily consistent.[8,9] Variable results were also reported more recently.[12,34,35] A more evident increase in the risk associated with red meat was noted for distal colon cancer,[26] or rectal cancer,[8,12,25,35] in several studies in Western countries. In Japan, no clear positive association with red meat or individual types of meat was observed for either the overall risk of colorectal cancer or subsite-specific risk.[18,36,37] The present study failed to find any measurable increase in the risk of subsite-specific cancer associated with red meat. However, it should be noted that red meat consumption was relatively low in the present study population (mean intake 50 g/day), which was fairly comparable to that reported in the recent National Nutrition Surveys (mean intake 56 g/day).[38] In Europe and the USA,[12,39,40] the highest consumption levels of red meat ranged from 130 to 160 g/day. In the Japanese population, red meat intake may not have been high or diverse enough to detect a substantive difference in the risk of colorectal cancer.

As mentioned above, epidemiological studies are rather disparate in their findings on the relationship of n-3 PUFA and fish intake to colorectal cancer. The inconsistency may be ascribed to differences in the accuracy of measurement of n-3 PUFA and fish intake. In this context, it is particularly notable that two Japanese studies found a decreased risk of colorectal cancer in those with high levels of n-3 PUFA in sera or erythrocytes.[20,23] The present findings add to evidence that high intake of n-3 PUFA is protective against the occurrence of colorectal cancer. A relatively higher range of fish consumption in the Japanese population compared with Western populations may provide an

advantageous opportunity of addressing the role of n-3 PUFA in colorectal carcinogenesis.

Experimental data are fairly strong regarding the protective role for n-3 PUFA in colorectal carcinogenesis. Fish oil or n-3 PUFA inhibit chemically induced colorectal carcinogenesis in rodents.[41,42] Several mechanisms have been postulated regarding the protective effects of n-3 PUFA.[10,43] High intake of n-3 PUFA suppresses the production of arachidonic acid-derived eicosanoids such as prostaglandin E_2 and leukotriene B_4 due to the change in relative proportions of n-6 and n-3 PUFA in cell membranes.[43] Prostaglandin E_2 increases production of inflammatory cytokines and growth factors that can facilitate tumor growth, invasion and metastasis.[44] Inhibition of cyclooxygenase-2 (COX-2) expression by n-3 PUFA is a mechanistic link of recent interest.[44] Enhanced apoptosis is one of the most important processes linked with the decreased expression of COX-2, although much remains to be clarified with regard to signal transduction downstream of COX-2.[10] It is also suggested that n-3 PUFA suppress the expression of inducible nitric oxide synthetase (NOS) and nuclear transcription factor κB (NF-κB).[43] Inducible NOS is an enzyme involved in the generation of free radicals, and increases DNA damage.[44] NF-κB is deemed to play a crucial role in carcinogenesis by modulating the expression of cell-cycle genes, apoptosis inhibitors and invasive proteases.[45]

In the present study, the intake of n-3 PUFA and fish was most evidently related to a decreased risk of distal colon cancer. Site-specific analysis is of interest because different molecular alterations have been implicated in carcinogenesis of the proximal and distal sites of the colorectum. Genetic alterations such as *K-ras* and *p53* mutations were shown to be more frequent in the distal site, whereas microsatellite instability appears to be almost exclusively associated with proximal colon cancer.[46,47]

The use of community controls, the large size and the high participation rates of both cases and controls are among the advantages of the present study. It is noteworthy that the dietary survey was conducted by in-person interview using PC software. The estimated intake of nutrients and foods seemed to be fairly valid, although caution is needed in extrapolating the results from the validation study, in which the subjects were relatively few and were not selected randomly. Because of the small number of subjects, sex-specific validation was rather difficult. Furthermore, participants completing the diet record may have differed from non-participants in various aspects. A limitation was the retrospective assessment of dietary and other lifestyle factors. Diet in the recent past was used in the present study, but this may not have represented long-term habitual consumption relevant to the development of colorectal cancer. Another concern is the potential effect of the BSE episode on the dietary survey.[31] Beef consumption decreased after the first case of BSE was reported in September 2001 in Japan. Per capita daily intake of beef in the National Nutrition Surveys was 20.5 g/day in the year 2000, 11.3 g/day in 2001 and 14.7 g/day in 2002. However, the consumption of total red meat did not change much due to reciprocal increases of pork and processed meat. The per capita daily intake of pork and processed meat combined for the years 2000, 2001 and 2002 was 36.9 g, 42.0 g and 41.2 g, respectively.[38]

In summary, the Fukuoka Colorectal Cancer Study, a large case-control study in Japan, has provided further evidence that diets high in fish and n-3 PUFA reduce the risk of colorectal cancer, particularly in the distal colon.

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CLINICAL RESEARCH

Surgical anatomy of innervation of the gallbladder in humans and *Suncus murinus* with special reference to morphological understanding of gallstone formation after gastrectomy

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Abstract

AIM: To clarify the innervation of human gallbladder, with special reference to morphological understanding of gallstone formation after gastrectomy.

METHODS: The liver, gallbladder and surrounding structures were immersed in a 10 mg/L solution of alizarin red S in ethanol to stain the peripheral nerves in cadavers (n = 10). Innervation in the areas was completely dissected under a binocular microscope. Similarly, innervation in the same areas of 10 *Suncus murinus* (*S. murinus*) was examined employing whole mount immunohistochemistry.

RESULTS: Innervation of the gallbladder occurred predominantly through two routes. One was from the anterior hepatic plexus, the innervation occurred along the cystic arteries and duct. Invariably this route passed through the hepatoduodenal ligament. The other route was from the posterior hepatic plexus, the innervation occurred along the cystic duct ventrally. This route also passed through the hepatoduodenal ligament dorsally. Similar results were obtained in *S. murinus*.

CONCLUSION: The route from the anterior hepatic plexus via the cystic artery and/or duct is crucial for preserving gallbladder innervation. Lymph node dissection specifically in the hepatoduodenal ligament may affect the incidence of gallstones after gastrectomy. Furthermore, the route from the posterior hepatic plexus via the common bile duct and the cystic duct to the gallbladder should not be disregarded. Preservation of the plexus may attenuate the incidence of gallstone formation after gastrectomy.

Key words: Innervation; Gallstone; Hepatoduodenal ligament; Gastrectomy; Whole mount immunohistochemistry

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INTRODUCTION

Over the last five decades, many reports have shown an at least 10% higher incidence of gallstones in people who have undergone gastrectomy than in the general population[1-9]. With respect to lymph node dissection, the incidence of gallstone formation was 16.3% after D3 and 8.5% after D2-1[8]. More recently, Kobayashi *et al*[10] reported that the incidence of gallstone formation is 27.9% after total gastrectomy and 7.8% after partial gastrectomy, 28.2% *versus* 7.5% for dissection or nondissection in the hepatoduodenal ligament, and 25.1% *versus* 8.2% at 5 years for reconstruction after gastrectomy with duodenal exclusion or non-exclusion, indicating that lymph node dissection in the hepatoduodenal ligament, total gastrectomy and exclusion of the duodenum are risk factors for gallstones after gastrectomy. However, the morphological understanding of the risk factors evaluated remains poor.

Examination of silver-impregnated and methylene blue-stained material has shown that the extrahepatic portion of human biliary tree has a rich nerve supply[11].

Innervation of the gallbladder (GB) and biliary pathways have been studied in men and other species by means of immunohistochemistry and/or histochemical methods (mainly the indirect immunofluorescent technique) using whole mount preparations or sections of the GB. Most neurotransmitters such as vasoactive intestinal peptide (VIP), cholecystokinin (CCK), acetylcholine, serotonin, dopamine, nitric oxide synthase (NOS), calcitonin gene-related peptide (CGRP), galanin, tyrosine hydoxylase (TH), neuropeptide Y (NPY), peptide YY (PYY), pancreatic polypeptide (PP), somatostatin, substance P (SP), and gastric inhibitory peptide (GIP), have been comprehensively discussed[12-27]. However, there has been no detailed description concerning clinico-anatomical and morphologic studies on the innervation, especially the extrinsic neural distribution and spread, of the GB and the biliary pathways.

Therefore, in the present study, the currently used gross anatomical dissection was not employed, but the effective method to label and dissect autonomic nerves of the viscera was employed, as in our previous studies[28-31]. We attempted to clarify the innervation of the GB in humans from a clinico-anatomical point of view, and to obtain a morphological understanding of gallstone formation after gastrectomy. Furthermore, an experimental animal, *Suncus murinus* (*S. murinus*), has been shown to exhibit general morphological characteristics more similar to those of humans than other currently used laboratory animals, such as mouse, rat, rabbit[28,30,31]. Hence, this animal was employed for a comparative study to confirm our morphological observations in humans in the present study.

MARERIALS AND METHODS

Cadavers

The study was performed on 10 cadavers (5 men and 5 women) with a mean age of 79.8 (range 50 to 94) years. All cadavers free from diseases of the abdominal viscera were selected from among bodies used for anatomy research and practice at the Kanazawa University School of Medicine in 1999-2000 (Table 1).

Animals and tissue preparation

Adult laboratory house musk shrews, *S. murinus*, were obtained from a closed breeding colony bred and maintained in the Department of Anatomy and Neuroembryology, Kanazawa University, Japan. The animals were housed and handled in accordance with the Guide for the Care and Use of Laboratory Animals and the Guide for the Care and Use of Experimental Animals of the Canadian Council on Animal Care. Briefly, all shrews were kept individually after weaning (20 d after birth) in plastic cages equipped with a wooden nestbox containing paper strips, in a conventionally conditioned animal room (23℃-27℃ , no humidity control, and 14 L: 10 D light). Commercial trout pellets containing 45.0% protein, 3.5% fat, 3.0% fiber, 13.0% ash and 26.2% complex carbohydrate (Nippon Haigou Shiryou, Tokyo, Japan) and water were supplied *ad libitum*. The mother colony, JIc: CR, was maintained in the Central Institute for Experimental Animals, Kawasaki, Japan[28,30,31]. Adult animals (6 females and 4 males, weighing 45-80 g) were first anesthetized with ether and then given an intraperitoneal injection of a solution of urethrane (sodium ethyl carbamate, 900 mg/kg). After each *S. murinus* was completely narcotized, the abdominal cavity was opened, and a catheter was inserted retrogradely into the abdominal aorta at the level immediately above the bifurcation of this artery into the common iliac arteries. Perfusion was commenced with normal saline containing heparin (10 KU/L), and thereafter with 4% paraformaldehyde buffered with 0.01 M sodium phosphate (PFA, pH 7.4). After perfusion, the animals were injected with neoprene latex to label the blood vessels. Thereafter, the abdominal organs including the liver, GB, stomach, duodenum, common bile duct, and pancreas were extracted *en bloc* with the related nerves and vessels, and then postfixed with 4% PFA at 4℃ overnight for whole mount immunostaining.

Table 1 Cadavers used in this study and GB innervation

Case	Sex	Age	Death	AHPlx[1]	PHPlx[2]	Phrenicus[3]
A	F	94	Pneumonia	O	O	R&L
B	F	81	Cerebral hemorrhage	O	O	R
C	F	83	Myocardial infarction	O	O	R
D	M	87	Myocardial infarction	O	O	-
E	M	86	Pneumonia	O	O	-
F	M	73	Cerebral hemorrhage	O	O	R
G	M	87	Cerebral hemorrhage	O	O	R
H	M	75	Myocardial infarction	O	O	R
I	F	82	Subarachnoid hemorrhage	O	O	R
J	F	50	Pneumonia	O	O	R

[1]Route arising from the anterior hepatic plexus (AHPlx); [2]Route arising from the posterior hepatic plexus (PHPlx); [3]There are offshoots of the right (R) or left (L) phrenic nerve to the hepatic portal vein.

Anatomical procedures under stereoscopic microscopy

Anatomical procedures for the cadavers were in accordance with our previous descriptions[29-31]. The viscera of the upper abdomen (including the liver, GB, pancreas, lower esophagus, stomach, and duodenum) were resected en masse with the abdominal aorta (the region including the celiac artery and superior mesenteric artery), portal system, and nerves (including the vagus nerve, celiac ganglion, and plexus). The resected specimens were immersed in a 10 mg/L solution of alizarin red S (Wako, Osaka, Japan) in ethanol to melt the fat tissue and to stain the peripheral nerves. The solution was changed three times, every 2 to 3 d in principle, but this process was longer depending on the degrees of fat elimination and staining. The area of each sample surrounded by the horizontal plane that passes through the portal region and the lower margin of the horizontal part of the duodenum, and the sagittal plane that passes through the descending part of the duodenum and hilum of the spleen was dissected to the depth of the celiac plexus with the aid of a stereoscopic microscope, keeping the sample completely immersed in 100% ethanol. On dissection, lymphatic vessels and lymph nodes were removed, with particular attention paid to the preservation

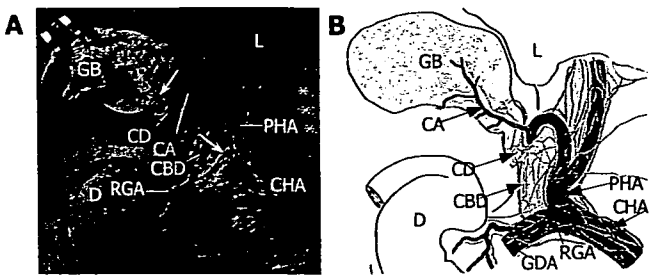

Figure 1 Innervation of the gallbladder (GB) from the ventral aspect (**A**) in a cadaver and a schematic representation of it (**B**). The branches innervating the GB originate from the anterior hepatic plexus, and run along the cystic duct (CD) and the cystic artery (CA). The hepatic divisions (*) of the vagus join in the anterior hepatic plexus in the proper hepatic artery (PHA). Arrows indicate nerve branches. CBD: common bile duct; CHA: common hepatic artery; D: duodenum; GDA: gastroduodenal artery; L: liver; RGA: right gastric artery.

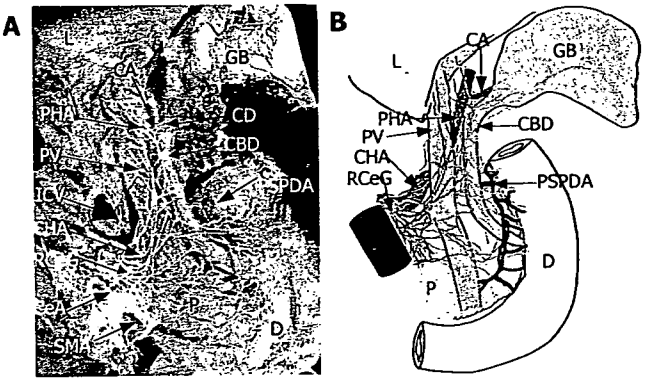

Figure 2 Innervation of the gallbladder (GB) from the dorsal aspect (**A**) in a cadaver and a schematic representation of it (**B**). The branches innervating the GB arise from the posterior hepatic plexus, and run along the cystic duct (CD). Ao: aorta; CA: cystic artery; CBD: common bile duct; CeA: celiac artery; CHA: common hepatic artery; D: duodenum; IVC: inferior vena cave; L: liver; PHA: proper hepatic artery; PSPDA: posterior superior pancreatoduodenal artery; PV: portal vein; RCeG: right celiac ganglion; SMA: superior mesenteric artery.

not only of the nerves but also of the arteries and veins in the areas of the hepatic portal and hepatoduodenal ligament.

Whole mount immunohistochemistry

Whole mount immunostaining procedures for the *S. murinus* were performed as previously described[28,30,31]. Briefly, after rinsing in PBS, the fixed specimens were treated with 10 g/L periodic acid for 20 min to prevent any intrinsic peroxidase reaction. They were then incubated in freshly prepared 5 g/L Papain (Sigma) in 0.025 mol/L Tris-HCl buffer (pH 7.6) for 1 h, and then 25 g/L, 50 g/L, and 100 g/L sucrose in PBS for 30 min, respectively, followed by freezing and thawing three times. The specimens were incubated with the primary antibody (NFP-Ab) in PBS containing 2 g/L bovine serum albumin (BSA), 3 g/L Triton X-100, and 1 g/L sodium azide for 3 d at 4℃. After a thorough wash in PBS, the specimens were incubated with the secondary antibody labeled with peroxidase-conjugated affinity-purified sheep anti-mouse IgG (HRP) in PBS containing 2 g/L BSA and 3 g/L Triton X-100 for 3 d at 4℃. After a thorough wash in PBS, coloration was performed in 0.05 mol/L Tris-HCl buffer containing 20 mg/L 3, 3'-diaminobenzidine (DAB) and 0.1 mL/L H_2O_2 for 1-3 d at 4℃. The stained preparations were then stored in glycerin to obtain transparency. The primary antibody was the anti-neurofilament protein (NFP) antibody, a monoclonal mouse anti-all neurofilament consisting of three subunit proteins: NF-H (200 kDa), NF-M (160 kDa), and NF-L (70 kDa) (M0762, lot 089, clone: 2F11, Dako).

RESULTS

In humans

The innervation of the GB in humans involved three routes: *via* the anterior and posterior hepatic plexus, respectively, and the phrenic nerves.

Via the anterior hepatic plexus

The hepatic division of the vagus, arising from the anterior vagal trunk, ran through the hepatogastric ligament near the edge of the liver (caudal liver), and joined the anterior hepatic plexus in the hepatoduodenal ligament. The plexus, containing parasympathetic and sympathetic fibers, arose from the celiac plexus and wound around the common hepatic artery, i.e., the proper hepatic artery[30], and then sent some branches to the GB via the deep and superficial branches of the cystic artery, which were distributed in the peritoneal aspect of the GB and the site of attachment of this organ in the bed between the GB and the liver (Figure 1). This route was seen in all ten specimens.

On the other hand, the anterior hepatic plexus sent some branches directly to the GB along the cystic duct, i.e., forward to the neck, body, fundus of the GB. However, the route concentrated mainly in the cystic duct and the neck of the GB compared with the body and fundus of the GB (Figure 1). However, it was not observed that the branches arising from the hepatic division of the vagus were sent directly to the GB.

Via the posterior hepatic plexus

The posterior hepatic plexus or the dorsal hepatic plexus[30], arising from the celiac plexus on its right side and running along the dorsal side to the portal vein, was composed of 4-5 nerve fascicles, divided into two groups of nerve bundles, and the thickest branches, about 80% of the nerve fibers, extended along the upper part of the common bile duct and portal vein, joined the liver and the GB, or descended; sending branches to the proximal side of the descending part of the duodenum and the lower common bile duct[30] (Figure 2). Abundant communicating rami behind the common bile duct and portal vein between the ascending and descending plexuses were observed, showing the existence of direct bidirectional neural connections between the duodenal papilla and the biliary tract containing the GB (Figure 2).

As in the case of the anterior hepatic plexus, the branches sent to the GB were distributed mainly in the cystic duct and the neck of the GB (Figure 2). This route was also seen in all ten specimens.

Moreover, abundant communicating rami were seen between the anterior and posterior hepatic plexuses around the cystic duct.

Via the phrenic nerves

In addition, the phrenic nerves sent offshoots forward to

the hepatic portal, which ran along the sagittal sulcus of the liver (data not shown). The offshoots were observed in eight out of ten cases, originating predominantly from the right phrenic nerve in seven and from both the right and left phrenic nerves in one (Table 1).

In S. murinus

In *S. murinus*, the GB was supplied by the cystic artery, a branch of the hepatic artery, corresponded to that in humans. However, there were no anterior and posterior hepatic plexuses in *S. murinus*.

Abundant plexus was found in the common bile duct of *S. murinus*, which arose from the celiac plexus via the common hepatic artery. The plexus ran along the common bile duct, descending to the lower common bile duct and the duodenal papilla, and ascending to the liver and common bile duct (Figure 3). Moreover, innervation of the GB occurred mainly in the cystic duct and the neck of the GB. Namely, it showed the existence of direct bidirectional neural connections between the duodenal papilla and the biliary tract including the GB as in humans (Figure 3).

Similarly, innervation of the GB along the cystic artery was also observed. However, it was much poorer than that in the route starting from the common bile duct, cystic duct (Figure 3).

In addition, communicating rami arising from diaphragm, which were observed running along the ligament between the xiphoid process of the sternum and the GB with an approach to the fundus of the GB, innervated the GB (data not shown).

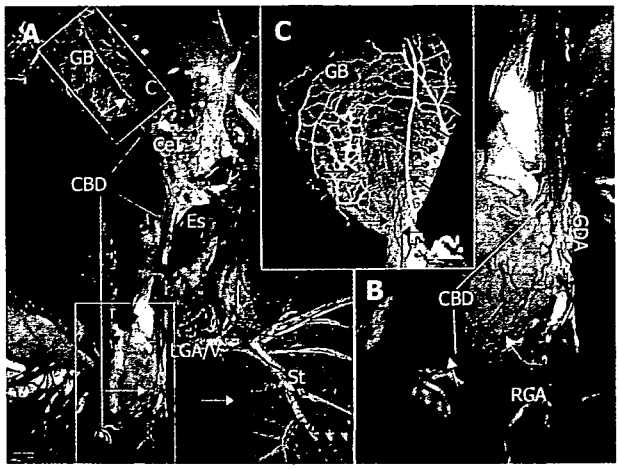

Figure 3 Innervation of the gallbladder (GB) in S. murinus revealed on whole-mount immunostaining (**A**) and high magnification of the boxed areas (**B** and **C**). Arrows indicate the stained/labeled nerve branches or bundles. CBD: common bile duct; CeT: celiac trunk; Es: esophagus; GDA: gastroduodenal artery; LGA/V: left gastric artery/vein; RGA: right gastric artery; St: stomach. Scale bar = 2 mm in **A**.

DISCUSSION

The present paper concerns detailed observation of the innervation of the GB and biliary pathways, with the aim of providing an anatomical basis for understanding the surgical clinical phenomenon of gallstone formation after gastrectomy.

Functionally, innervation of the GB in humans involves both sympathetic postganglionic nerve fibers and parasympathetic fibers. Morphologically, innervation of the GB occurred predominantly through two routes. One was from the anterior hepatic plexus containing the branches arising from the hepatic division of vagal nerves and the celiac plexus, the innervation occurred along the cystic arteries and the cystic duct. Invariably this route passed through the hepatoduodenal ligament. The other route was from the posterior hepatic plexus, containing the branches originating from the celiac branches of the posterior vagal trunk and the celiac plexus, the innervation occurred along the cystic duct ventrally. This route also passed through the hepatoduodenal ligament dorsally. Furthermore, nerve offshoots arising from the phrenic nerves were observed, and communicating twigs between the GB and the duodenal papilla were identified. Although there were no anterior and posterior hepatic plexuses, similar results were obtained for a laboratory animal, *S. murinus*.

The incidence of gallstone formation is higher in patients after gastrectomy than in the general population. Kobayashi *et al*[10] identified lymph node dissection specifically in the hepatoduodenal ligament as a significant risk factor for gallstone formation, suggesting that dissection of nerve fibres near the GB might be important. This viewpoint is consistent with our morphological observations, i.e., innervation of the GB, either *via* the anterior or posterior hepatic plexus, occurred through the hepatoduodenal ligament. However, innervation of the GB arising from the anterior hepatic plexus surrounded the common hepatic artery, i.e., the proper hepatic artery to the cystic artery. If skeletonization of the hepatoduodenal ligament, especially the common and proper hepatic arteries, can be avoided in dissection, preservation of innervation of the GB originating from the hepatoduodenal ligament is possible. Virtually, the entire idea of radical resections for gastric cancer remains somewhat controversial, with different results obtained in Asia *vs* the US. It is not necessary to perform skeletonization of the hepatoduodenal ligament for all cancer resections of stomach, although it is general maneuver to excise lymph nodes in the hepatoduodenal ligament. Both potential risk of metastasis and quality of life after operation should be taken into consideration. It is important to have a suitable excision of lymph nodes and to avoid skeletonization of the hepatoduodenal ligament for quality of life and the formation of cholelithiasis after gastrectomy.

Although the degree of lymph node dissection in D2 affects the incidence of gallstone formation as it has been shown that there is an at least% higher incidence of gallstone formation in people who have undergone gastrectomy than in the general population[10], we cannot agree with the idea that prophylactic cholecystectomy should be performed during radical gastrectomy, even if the GB is normal, in order to prevent the complications of acute cholecystitis and cholelithiasis[7]. Kobayashi *et al*[10] reported that most patients with gallstone formation after gastrectomy are asymptomatic and less than 0.5% of them require cholecystectomy, suggesting that preventive cholecystectomy appears to be unnecessary.

However, besides complete abolition of the innervation of the GB, gallstone development after gastrectomy also depends on the extent of gastrectomy (total or partial gastrectomy), on reconstruction after gastrectomy and exclusion or non-exclusion of the duodenum[10].

Furthermore, studies on other species have shown the existence of direct bidirectional neural connections between the GB and duodenum containing the sphincter of Oddi, indicating that local reflux between the GB and the sphincter of Oddi might be important in regulation of the pressure within the bile ducts and the flow across the sphincter[14,16,24,27,32,33], demonstrating the presence of this intrinsic neural pathway from the duodenum to the GB in these species. Our data also showed the presence of extrinsic neural connections between the duodenum and the GB in humans and *S. murinus*, and supported that local reflux between the GB and the duodenum, the sphincter of Oddi, might be important in the regulation of the flow in the GB and duodenum[31]. The integrity of these extrinsic neural connections may play a role both in maintaining the GB motor activity and in preventing gallstone formation after distal gastrectomy[34].

Whether the cystic duct plays any regulatory role in the process of GB filling and emptying remains unknown. The cystic duct clearly possesses contractile muscle and is the narrowest part of the extrahepatic biliary tree. Poiseuille's law dictates that only minor changes in the caliber of the duct could greatly influence the resistance to the flow of bile[35]. In dogs, the innervation density parallels the thickness of the musculature, which is the greatest toward the neck of GB[36]. Our data supported this viewpoint. In our study, abundant neuron fibers were distributed in the neck of GB and the cystic duct both in humans and *S. murinus*. This may be important for the emptying of GB and the cystic duct.

In addition, horseradish peroxidase is utilized to study the distribution of afferent fibers from the GB in cats. Afferent cell bodies have been found in the nodose ganglion and the T4 to L1 dorsal root ganglia[37,38]. Iwahashi *et al*[39] also reported that the GB of cats was innervated by bilateral phrenic nerves, the nodose ganglia and dorsal root ganglia T2-L3. The phrenic nerves were concerned in about 33% of the subjects, the right side predominating over the left. In our study, the human subjects with some twigs to the GB from the phrenic nerves accounted for 80% (eight of 10 cases). In one case both the right and left phrenic nerves were the origin of the twigs, the right phrenic nerve was predominant in the other seven cases. Iwahashi *et al*[39] and Inomata[38] showed that some afferent fibers from the GB traveling via the phrenic nerves, particularly on the right side, and entering the cervical segments may be a supplementary mechanism as to the generation of the referred pain in GB disease.

In conclusion, we revealed the details of innervation of the GB from morphologic and clinico-anatomical perspectives in this study. The whole mount immunostaining with a peripheral neuron marker for *S. murinus*, and the alizarin red S staining technique for humans are effective for peripheral nerve labeling. There are three routes for GB innervation in humans, of which the route from the anterior hepatic plexus via the cystic artery and/or duct is crucial for preserving GB innervation. Lymph node dissection specifically in the hepatoduodenal ligament may affect the incidence of gallstone formation after gastrectomy. However, the route from the posterior hepatic plexus via the common bile duct and the cystic duct to the GB should not be disregarded. Preservation of the plexus may attenuate the incidence of gallstone formation after gastrectomy. However, in the present study, we did not examine the intrinsic neurons system in the biliary duct (containing GB) or the upper gastrointestinal tract. Both extrinsic nerves and intrinsic neurons containing abundant neurotransmitters, participate in the regulation of GB motion.

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Ribonucleotide reductase subunit M2 mRNA expression in pretreatment biopsies obtained from unresectable pancreatic carcinomas

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Background. Gemcitabine is an efficacious cytotoxic agent used in the treatment of unresectable pancreatic carcinoma (PC). Recently, gemcitabine resistance has been associated with the ribonucleotide reductase subunit M2 (*RRM2*). In this prospective study, we hypothesized that *RRM2* expression in PC biopsy specimens would be a significant predictor of outcome. ***Methods.*** *RRM2* mRNA expression in 35 endoscopic ultrasonography-guided fine needle aspiration biopsy (EUS-FNAB) samples was quantified using real-time quantitative reverse transcription-polymerase chain reaction. ***Results.*** Thirty-one of 35 biopsy specimens could be assessed for *RRM2* expression levels. The mean *RRM2* expression relative to glyceraldehyde-3-phosphate dehydrogenase was 0.248 (range, 0.00739 to 0.858). Eighteen patients (64.5%) had low *RRM2* levels, and 13 patients (35.5%) had high *RRM2* levels with a cutoff of 0.1. The median survival was 8.8 months for patients with low *RRM2* levels and 5.0 months for patients with high levels ($P < 0.05$). In the low *RRM2* expression group, a complete response (CR) was observed in one patient, and a partial response (PR) was observed in eight patients. In contrast, in the high *RRM2* expression group, PR was observed in one patient, and CR was not observed. The overall response rate between the high and low expression groups was significantly different (50.0% vs. 7.7%, $P < 0.05$). ***Conclusions.*** *RRM2* mRNA expression of EUS-FNAB specimens is a key predictive marker of survival in gemcitabine-treated patients with PC.

Key words: pancreatic cancer, *RRM2*, EUS-FNA, predictive marker

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Introduction

Pancreatic carcinoma is the fifth leading cause of cancer death in Japan[1] and the fourth in the United States,[2] and is usually unresectable (80%–90%) at the time of diagnosis despite recent progress in imaging modalities. The prognosis of advanced and metastatic pancreatic carcinoma is dismal, and chemoresistance is a major cause of pancreatic carcinoma treatment failure. Gemcitabine, like S-1, is an efficacious cytotoxic agent[3] that currently has marketing approval in Japan for the treatment of pancreatic carcinoma. However, it is difficult to distinguish between patients who are sensitive or resistant to gemcitabine before chemotherapy.

Recently, the expression and activity of ribonucleotide reductase have been reported to be determinants of gemcitabine chemoresistance in human tumor cells.[4] Ribonucleotide reductase mediates the rate-limiting step in DNA synthesis because it is the only known enzyme that converts ribonucleotides to deoxynucleotides, which are required for DNA polymerization and repair. The ribonucleotide reductase holoenzyme consists of dimerized subunits M1 and 2 (*RRM1* and *RRM2*).[5] Ribonucleotide reductase enzymatic activity is modulated by the levels of *RRM2*.[6] Moreover, overexpression of *RRM2* is associated with resistance to gemcitabine in patients with pancreatic cancers.[7]

Endoscopic ultrasonography-guided fine-needle aspiration biopsy (EUS-FNAB) has been established as a safe and precise procedure for the diagnosis of pancreatic masses.[8,9] Several studies have reported the diagnosis by genetic analysis of samples obtained by EUS-FNAB in patients with pancreatic carcinoma,[10,11] but no reports of the use of this technique for the assessment of treatment options have been published. In this first prospective report, we describe the utility of analyzing the expression of *RRM2* mRNA obtained from pretreatment EUS-FNAB specimens of unresectable pancreatic carcinoma.

Materials and methods

Patients

Biopsy specimens from 35 patients with unresectable pancreatic carcinomas (23 patients with stage III and 12 patients with stage IV disease)[12] were obtained at the Fourth Department of Internal Medicine of Tokyo Medical University between December 2003 and October 2005. The eligibility criteria included histologically confirmed, locally advanced, or metastatic pancreatic carcinoma with no prior chemotherapy; clinically measurable or evaluable disease; Southwest Oncology Group scale performance status of 0–2; a life expectancy of greater than 12 weeks; and adequate bone marrow and hepatic and renal function. The EUS-FNAB procedure has been described in previous reports.[11] The first specimen was used for standard histological examination by hematoxylin-eosin staining (Fig. 1), and the second specimen was used for detecting *RRM2* mRNA. The final diagnosis of unresectable pancreatic carcinoma was based on ultrasonography, EUS, and computed tomography imaging studies. All EUS-FNAB procedures were performed by the same investigator (T.I.). This study was carried out in accordance with the institutional review board guidelines, and written informed consent was obtained from all of the patients. All patients were treated with gemcitabine (1000 mg/m2) administered intravenously over 30 min on days 1, 8, and 21 on a 28-day cycle.

Laboratory methods

Total RNA was isolated using the RNeasy Kit (Qiagen, Chatsworth, CA, USA), and DNase treatment was performed using the RNase-Free DNase set (Qiagen), according the manufacturer's instructions. cDNAs were generated using the Superscript II First Strand cDNA Synthesis kit (Invitrogen, Carlsbad, CA, USA). Quantification of *RRM2* cDNA and an internal reference gene (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) was conducted using a fluorescence-based real-time polymerase chain reaction (PCR) method (Taq-Man PCR using an ABI PRISM 7700 Sequence Detection System, Applied Biosystems, Foster City, CA, USA). The following primers were used for real-time PCR. *RRM2*; forward primer, 5′-CTATGGT GAACGTGTTGTAGCCTT-3′; reverse primer, 5′-GTCCTCGTTTCTTGAGCCAGA-3′; TaqMan probe, 5′-FAM-CTGCAGTGGAAGGCATTTTCTTTT CCG-TAMRA-3′ GAPDH; Predeveloped TaqMan Assay Reagents human GAPDH (Applied Biosystems). In brief, cDNA was added into a reaction mixture containing 1 × TaqMan buffer A, 150 nM each primer, 100 nM TaqMan probe, 200 μM dNTP, 3 μM MgCl2, and 0.625 units of AmpliTaqGold, in a final volume of 25 μl. The PCR conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Quantification was performed using the relative standard curve method. The standard curve was created automatically by the ABI PRISM 7700 by plotting the threshold cycle (Ct) against each input amount (containing 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 copies) of standard plasmid DNA. The correlation coefficient determined by linear regression (r) for each standard curve was greater than 0.990. The relative amount of each unknown sample was calculated using linear regression analysis from the respective standard curve. A relative target gene expression value for GAPDH as an internal reference gene was used.

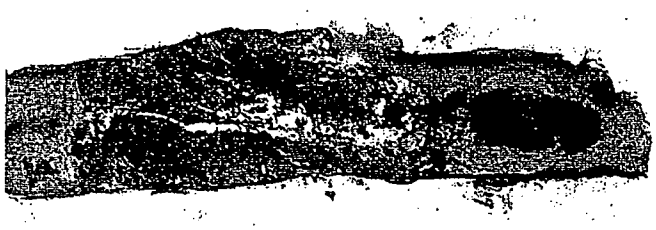

Fig. 1. Photomicrograph of representative biopsy specimen samples obtained by endoscopic ultrasonography-guided fine-needle aspiration biopsy using a 19-gauge needle (×20)

Efficacy assessment

Standard tumor response criteria were used to determine an objective tumor response. A complete response (CR) was defined as the disappearance of all measurable and evaluable disease for at least 4 weeks without the appearance of any new lesions. A partial response (PR) indicated a reduction of >50% in the sum of the products of the greatest perpendicular dimensions of all measurable lesions for at least 4 weeks without the appearance of any new lesions. Stable disease (SD) corresponded to a decrease of <50% in the sum of the product of the greatest perpendicular dimensions of measurable lesions or an increase of <25% in the sum of the products of the greatest perpendicular dimensions of measurable disease for a minimum of 3 months. Progressive disease (PD) was defined as an increase of >25% in the sum of the products of measurable lesions, the appearance of new lesions, or deterioration of any evaluable disease. Survival was measured from the time of initiation of therapy until death. Response duration was defined as the time from documentation of response to the first observation of progressive disease. Patients were treated until disease progression or the occurrence of unacceptable toxicity without second-line chemotherapy for gemcitabine.

Statistical analysis

Quantitative PCR analyses yield values that are expressed as ratios between two absolute measurements (gene of interest: internal reference gene). We used the maximal χ-squared method to determine a cutoff value to segregate patients into groups with low or high transcript levels. To determine the P value, we used bootstrap-like simulations to estimate the distribution of a maximal χ-squared statistic. The Kaplan-Meier test for survival and time to progression was used. The log-rank test was applied to compare survival and time to progression between subgroups. A value of $P < 0.05$ was considered to indicate statistical significance. All analyses were performed with the SPSS software package, version 10.0.5 (SPSS, Chicago, IL, USA).

Results

Patient characteristics

A total of 31 out of 35 pancreatic carcinomas could be assessed for *RRM2* expression levels. Two cases were not quantifiable because of fibrotic or necrotic tumor tissue, and two cases were omitted owing to the presence of renal cell carcinoma and malignant lymphoma. The clinical characteristics of the 31 patients are shown in Table 1 (20 patients with stage III and 11 patients with stage IV). Median patient age was 66 years; 16 patients were male; and 93.5% of patients had a performance status of 0 or 1. The pancreatic masses were located in the head of the pancreas in 15 patients and in the body/tail in 16 patients. The 12 patients with masses in the head of the pancreas had obstructive jaundice. The EUS-FNAB procedure was performed without any procedure-related complication.

Table 1. Clinical patient and gene characteristics[a]

No. of patients	31
Age, years (range)	66.0 (30–81)
Sex	
Male	16 (51.6%)
Female	15 (48.4%)
Performance status	
0–1	29 (93.5%)
2	2 (6.5%)
Icterus	12 (38.7%)
Location	
Head	15 (48.4%)
Body/Tail	16 (51.6%)
Histology	
Adenocarcinoma	30 (96.8%)
Adenosquomous carcinoma	1 (3.2%)
TNM cancer staging	
Stage III	20 (64.5%)
Stage IV	11 (35.5%)
RRM2[b] mRNA, mean (range)	0.248 (0.00739–0.858)
RRM2 mRNA	
≤ 0.1	18 (58.1%)
>0.1	13 (41.9%)
No. of chemotherapy cycles, median (range)	3.8 (2–11)

[a] Numbers in parentheses indicate percentages except when specified as a range

[b] *RRM2*, ribonucleotide reductase subunit M2

Patients received a median of 3.8 cycles (range, 2–11) of chemotherapy. The mean total dose of gemcitabine used was 16530 mg (range, 7800–52800 mg). CR was observed in one patient (3.2%), PR was observed in nine patients (29.3%), and SD in 11 patients (35.5%), whereas ten patients (32.3%) developed PD, resulting in an overall response rate of 32.3% [95% confidence interval (CI) 0.17–0.51]. Twenty-one of the 31 patients studied had died and ten were alive at the completion of the study. The overall survival time for all 31 patients was 7.8 months (95% CI, 4.3–10.2 months) (Fig. 2). The therapy was well tolerated; grade 3–4 neutropenia (no grade 3–4 infection), thrombocytopenia (no bleeding), nausea, asthenia, and alopecia were seen in 20%, 6.7%, 13.3%, 6.7%, and 6.7% of patients, respectively.

RRM2 *mRNA expression and clinical outcome*

The mean *RRM2* expression relative to the GAPDH internal reference gene was 0.248 (range, 0.00739 to 0.858) (Table 1) (Fig. 3A, B). The median *RRM2* expression was 0.101. Eighteen patients (58.1%) had low *RRM2* levels, and 13 patients (41.9%) had high *RRM2* levels, using a cutoff of 0.1. The median survival time was 8.8 months for the 18 patients with low *RRM2* levels and 5.0 months for the 13 patients with high levels ($P = 0.0104$) (Fig. 4). Thirteen stage III cases and five stage IV cases were included in the group showing low *RRM2* expression. In contrast, seven stage III cases and six stage IV cases were included in the group showing high *RRM2* expression. There was no statistical significance in the proportion of Stage III to Stage IV disease between low and high *RRM2* expression ($P = 0.499$). Response to chemotherapy between high and low *RRM2* expression is shown in Table 2. In the low *RRM2* expression group, CR was observed in one patient, PR was observed in eight patients, and SD in eight patients, whereas one patient developed PD. In contrast, in the high *RRM2* expression group, PR was observed in one, CR in none, and SD in three patients, and PD was observed in nine patients. The overall response rate between high and low expression groups was significantly different (50.0% vs. 7.7%, $P = 0.013$).

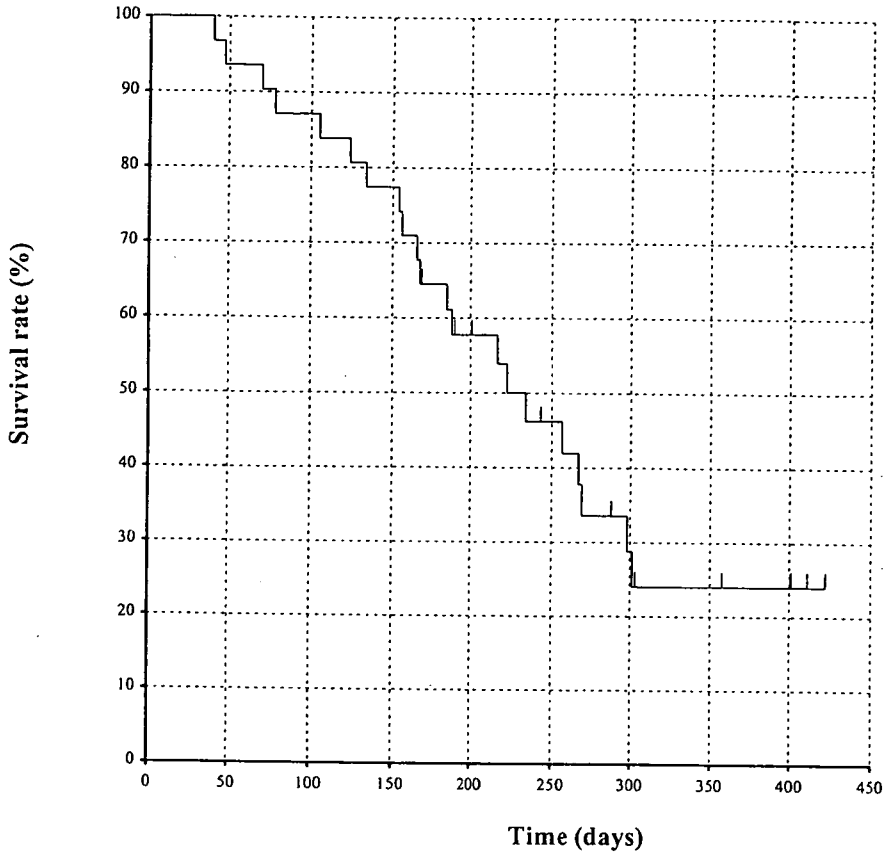

Fig. 2. Kaplan-Meier estimates of overall survival. Median survival for 31 patients was 7.8 months

Table 2. Response to gemcitabine between the high and low *RRM2* expression groups

	RRM2 > 0.1 (n = 13)	*RRM2* ≤ 0.1 (n = 18)
Complete response	0	1
Partial response	1	8
Stable disease	3	8
Progressive disease	9	1

RRM2, ribonucleotide reductase subunit 2

Discussion

In this prospective study, we investigated the utility of analyzing the expression of the *RRM2* gene to predict chemosensitivity to gemcitabine in patients with pancreatic carcinoma. This study showed that *RRM2* mRNA levels may affect survival. The median survival of the patients with low *RRM2* expression was significantly longer than those with high *RRM2* expression. Interestingly, CR could be achieved only in patients with low *RRM2* expression, and the overall response rate was significantly higher also in these patients than in the patients with high *RRM2* expression. These data suggest that it is worthwhile evaluating pretreatment *RRM2* expression in unresectable pancreatic carcinoma patients. Previous reports on gemcitabine monotherapy described survival times similar to the present data.[3,13–18] Although *RRM2* expression was not examined, these reports may contain cases of both high and low *RRM2* expression, leading to variations in chemosensitivity to gemcitabine.

In this study, we measured *RRM2* expression, although the ribonucleotide reductase holoenzyme consists of dimerized *RRM1* and *RRM2*. Duxbury et al.[7] reported that *RRM2* enhanced pancreatic adenocarcinoma chemoresistance to gemcitabine in vitro. We planned this prospective study on the basis of their data. There have not been any clinical studies on ribonucleotide reductase overexpression in conjunction with gemcitabine-based chemotherapy for pancreatic carcinoma. To the best of our knowledge, although the current study is preliminary, this is the first clinical trial of *RRM2* expression in patients with pancreatic carcinoma using EUS-FNAB pretreatment biopsy specimens. Although there is no report on *RRM1* expression in patients with pancreatic cancer, *RRM1* gene expression was described as a crucial predictive marker of survival in non-small cell lung cancer patients receiving gemcitabine-based chemotherapy.[19–21] In particular, *RRM1* levels influenced time to progression and survival in the

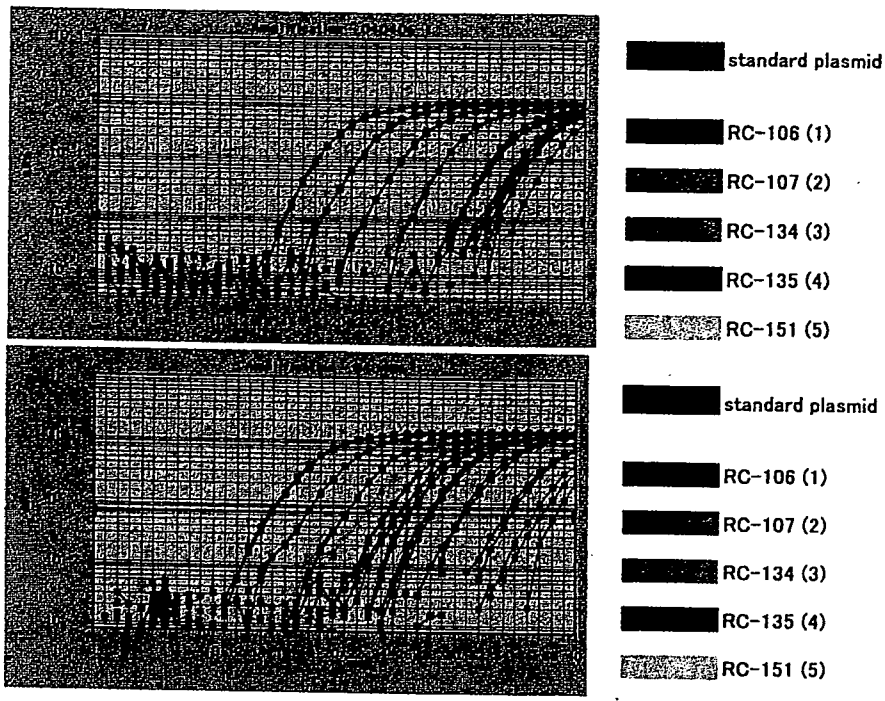

Fig. 3A,B. Ribonucleotide reductase subunit M2 (*RRM2*) mRNA quantification was performed using the relative standard curve method. The standard curve was created automatically by the ABI PRISM 7700 by plotting the threshold cycle against each input amount (containing 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 copies) of standard plasmid DNA. A *RRM2* mRNA expression value (**A**) with glyceraldehyde-3-phosphate dehydrogenase (**B**) as an internal reference gene was used

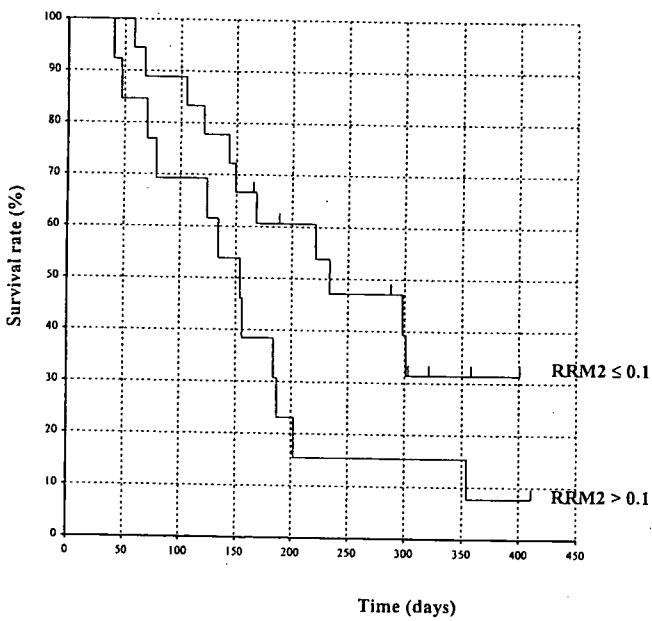

Fig. 4. Kaplan-Meier estimates of time to survival according to *RRM2* mRNA expression. The median survival was 8.8 months for the 18 patients with low *RRM2* levels (>0.1) and 5.0 months for the 13 patients with high levels (≤0.1)

gemcitabine/cisplatin arm (8.4 months vs. 2.7 months, $P = 0.009$; 13.7 months vs. 3.6 months, $P = 0.02$, respectively).[20] These data suggest that genetic testing of *RRM1* mRNA expression levels can and should be used to personalize chemotherapy in patients with non-small cell lung cancer. We should examine not only *RRM2* expression but also *RRM1* expression to clarify chemosensitivity to gemcitabine in the future.

To date, several gemcitabine-based combination chemotherapy regimens have undergone considerable testing for advanced pancreatic carcinoma[14–18] and it seems at present that gemcitabine-based combination chemotherapy may become the first-line treatment for pancreatic carcinoma. Thus, it is very important to predict chemosensitivity to gemcitabine prior to treatment.

Currently, with the progress of EUS-FNAB procedures, the necessary samples can be obtained easily, and the diagnostic accuracy for pancreatic solid masses is at least 85%.[8,9] One of the important aspects of this study is the genetic analysis of EUS-FNAB specimens in combination with routine diagnosis. Interestingly, in this study, two samples were omitted owing to the presence of renal cell carcinoma and malignant lymphoma. These data suggest that pretreatment biopsy specimens by EUS-FNAB in patients with pancreatic masses should be obtained, not only to test for gemcitabine chemoresistance but also to confirm a diagnosis of adenocarcinoma to ensure that the appropriate treatment is administered. In the present study, we examined only *RRM2* expression, but in the near future it may be necessary to examine the chemosensitivity of a range of drugs using the EUS-FNAB procedure.

In this study, there were some cases in which survival time did not correlate with *RRM2* expression. Gemcitabine chemosensitivity depends not only on *RRM2* expression but also on several other factors. Recently,

3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP, Triapine; Vion Pharmaceuticals, New Haven, CT, USA) was described as a new inhibitor of *RRM2*.[22] A Phase I trial of 3-AP in combination with gemcitabine for advanced cancers, including pancreatic carcinoma, is underway.[23,24] A preliminary study suggested that there was prolonged stabilization of disease or decreases in serum tumor markers associated with stable disease though there were no objective responses.

In conclusion, the results from this prospective study demonstrate that *RRM2* mRNA expression levels may correlate with gemcitabine sensitivity. In the near future, a large number of prospective randomized chemotherapy trials will be scheduled. These data suggest that *RRM2* is a novel, informative biomarker for predicting and monitoring the responses of pancreatic adenocarcinoma patients to gemcitabine.

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Serum albumin-associated peptides of patients with uterine endometrial cancer

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The incidence of endometrial cancer is predicted to increase in developed countries. Because of the relatively high incidence of complications and low diagnostic sensitivity associated with endometrial tissue sampling, there is an urgent need for the development of a safe and non-invasive diagnostic method. The proteomic spectrum of albumin-associated peptides was obtained from a total of 125 serum samples (92 from endometrial cancer patients and 33 from controls) by matrix-assisted laser desorption/ionization hybrid quadrupole time-of-flight mass spectrometry, and the candidate markers were selected by the Mann–Whitney *U*-test and receiver operator characteristics analysis. We selected three mass peaks at 4769, 6254 and 11 792 m/z from a total of 507 peaks as distinguishing cancer patients from controls ($P < 0.00001$ and area under curve of over 0.8). When the cut-off points were defined as the averages of the values in the controls + 2 SD, the combination of the three peptides detected endometrial cancer with a sensitivity of 65.2% (60/92). Even stage I early endometrial cancers were detected with a sensitivity of 60.3% (38/63). Unfortunately, the three peptides were also detected in 44.6% (33/74) of myoma uteri patients, indicating that they are not specific to endometrial cancer. Although a large-scale study is necessary to confirm the clinical significance of the peptide biomarkers identified in this study, direct profiling of serum-albumin-bound peptides by high-resolution mass spectrometry was proven to have potential as a means of identifying biomarkers for a variety of diseases. (*Cancer Sci* 2007; 98: 822–829)

The incidence of endometrial cancer is high in developed countries, and its morbidity has increased in recent years. In 1970, endometrial cancer constituted only 3% of all uterine cancers in Japan, but the proportion had increased to 40% in 1998.[1] Excessive fat consumption, overweight, physical inactivity, high energy intake, hypertension and a high serum glucose concentration have been identified as risk factors for endometrial cancer.[2–6] A medical history of breast cancer and use of tamoxifen to treat breast cancer increase the risk of endometrial cancer,[7] but pregnancy reduces it.[8,9] Because most of the above are characteristic of the lifestyle in developed countries, the incidence of endometrial cancer is predicted to continue to increase in the future, and thus the development of an effective mass screening method is needed urgently.

Abnormal uterine bleeding is the most frequent initial symptom of endometrial cancer, but many other disorders also give rise to this symptom. Endometrial cancer is usually diagnosed by histological examination of endometrial tissue obtained with miniature endometrial biopsy devices. However, endometrial biopsy cannot be carried out in postmenopausal patients with a closed external os of the uterus, and endometrial biopsy is often associated with complications, such as infection, bleeding and perforation of the uterus. Transvaginal ultrasonography has been used as an alternative non-invasive diagnostic method for the diagnosis of endometrial diseases, but its diagnostic accuracy is not satisfactory.[10]

The circulating serum proteome holds great promise as a reservoir of information that will be useful for the diagnosis of various diseases. A large variety of low molecular weight protein fragments and peptides are known to be produced as a consequence of the proteolytic processes occurring in the microenvironment of diseased tissues,[11] and these protein fragments are released into the blood circulation and become bound to high-abundance proteins, such as serum albumin.[12,13] Serum albumin-associated peptides are protected from renal clearance and may be concentrated over time during the course of chronic diseases, such as cancer.[14] However, it has never been determined whether disease-related peptides actually accumulate in the serum of cancer patients and whether detection of such peptides can be applied to cancer diagnosis.

Mass spectrometry-based quantitative proteomics approaches have gained considerable attention as effective modalities for identifying new biomarkers of various diseases.[15–17] To answer the above questions we directly quantified the serum albumin-associated peptides of a large number of endometrial cancer patients with a high-resolution mass spectrometer. In this paper we report that a certain set of peptides accumulate in the serum of endometrial cancer patients and that quantification of these peptides has diagnostic significance.

Materials and Methods

Subjects and serum samples. The serum samples ($n = 199$) used in this study were collected at the National Hospital Organization Hokkaido Cancer Center Hospital (Sapporo, Japan) between 2000 and 2004 with the informed consent of all donors. The 199 subjects consisted of patients with untreated endometrial cancer ($n = 92$), metroptosis patients ($n = 16$), myoma uteri patients ($n = 74$), and healthy volunteers ($n = 17$) (Table 1). The samples were collected in glass tubes, and after allowing them to clot, the serum was separated and cryopreserved at −80°C until analyzed. The protocol of the study was reviewed and approved by the ethics committees of the National Hospital Organization Hokkaido Cancer Center and National Cancer Center (Tokyo Japan). The characteristics of the subjects are summarized in Table 1. The endometrial cancer patients were classified as surgical stage 0, I, II, III or IV.[18]

Purification of serum albumin-associated peptides. Native albumin-associated peptides were separated and concentrated from the serum samples with a proXPRESSION kit (PerkinElmer, Boston, MA, USA) according to the instructions provided by the supplier (Suppl. Fig. S1). Briefly, a 40-μL sample of serum was diluted 1:10 with Biomarker Enrichment Binding Buffer

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Abbreviations: AUC, area under the curve; CC, correlation coefficient; CHCA, cyano-4-hydroxycinnamic acid; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; MALDI QqTOF-MS, matrix-assisted laser desorption/ionization hybrid quadrupole time-of-flight mass spectrometry; ROC, receiver operator characteristics.

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