Original article

Association between dietary heterocyclic amine levels, genetic polymorphisms of NAT2, CYP1A1, and CYP1A2 and risk of stomach cancer: a hospital-based case-control study in Japan

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Abstract

Background. Although the associations between grilled (broiled) or barbecued meats or fish intake and stomach cancer risk have been investigated, the evidence implicating heterocyclic amine (HCA) intake as a cause of stomach cancer is limited. We conducted a case-control study to investigate the association between HCA intake and stomach cancer risk. We also investigated the possible effect of genetic polymorphisms of NAT2, CYP1A1, and CYP1A2 on stomach cancer.

Methods. HCA exposure data were assessed using a self-administered food-frequency questionnaire, and estimated HCA intake was verified by measuring 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) values in human hair. A total of 149 cases and 296 controls were included in the analyses. Odds ratios (ORs) were calculated, using conditional logistic regression analysis, to compare intake levels between the first and third tertiles.

Results. Results showed no statistically significant increase in the risk of stomach cancer with respect to total HCA intake (OR, 1.11; 95% confidence interval [CI], 0.36, 3.49), or with respect to the intake of individual HCAs; namely, PhIP, 2-amino-3, 4-dimethylimidazo[4,5-f]quinoline (MeIQ), and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx). Genetic polymorphisms of NAT2, CYP1A1, and CYP1A2 did not influence the association of HCA intake with stomach cancer.

Conclusion. In the present study, with a limited sample size of subjects with low HCA exposure, no association was found between HCA intake and stomach cancer, nor was there any evidence of any influence by genetic polymorphisms of NAT2, CYP1A1, and CYP1A2 on the risk of stomach cancer.

Key words HCA · Stomach cancer · NAT2 · CYP1A1 · CYP1A2

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Received: January 21, 2009 / Accepted: September 1, 2009

Introduction

In November 2007, the World Cancer Research Fund, in collaboration with the American Institute for Cancer Research [1], reported that evidence implicating grilled (broiled) meat as a cause of stomach cancer was limited but suggestive. On reviewing 3 cohort and 12 case-control studies of grilled (broiled) or barbecued meats or fish, the report found that most showed an increased stomach cancer risk with increased intake. However, only two case-control studies investigated heterocyclic amine (HCA) intake and stomach cancer risk, one of which found no relationship [2], while the second did report a relationship [3].

A major reason for the limited number of studies of HCA and stomach cancer risk is the difficulty of assessing human exposure to HCAs. HCA concentrations depend on cooking methods and the “doneness” level of the meat or fish, hampering the development of a complete and standardized database of concentrations; any estimation of dietary intake from food-frequency questionnaires (FFQs) is thus likely to result in misclassification. Among other problems, foods which contribute to HCA intake differ by study area. Further, studies of HCA intake in Japanese populations require original estimation methods due to the considerable HCA intake obtained from the consumption of fish. In this regard, we recently developed a method of estimating HCA intake and we evaluated the validity of HCA intake estimated from an FFQ in a large-scale population-based prospective study Japan Public Health Center (JPHC) study using 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) levels measured in human hair. Although intake in the JPHC study was low compared

with that in other studies, we did identify a positive correlation of HCA intake with PhIP levels in human hair [4, 5].

Like other environmental chemical carcinogens, HCAs require metabolic activation by host enzymes to become genotoxic. Phase I enzymes, including cytochrome P450 (CYP), can metabolically activate carcinogens to form genotoxic electrophilic intermediates [6], while activated metabolites are, in turn, partially detoxified by phase II enzymes such as N-acetyltransferase 2 (NAT2) [7]. The relative activity of these metabolizing enzymes, which is in large part genetically determined, is thought to be an important host determinant of cancer incidence.

Although a number of case-control and prospective epidemiological studies have investigated the association between NAT2, CYP1A1, and CYP1A2 polymorphisms and stomach cancer risk, results have been inconsistent [8]. Moreover, while polymorphisms in these metabolic genes and interaction with tobacco smoke and alcohol consumption and the risk of stomach cancer has been investigated, interaction between metabolic variation and HCAs as potential risk factors for stomach cancer has not.

Here, we report a hospital-based case-control study conducted to investigate the impact of HCA intake on stomach cancer risk, using data from subjects likely to have a low HCA intake. We also investigated the possible effect of genetic polymorphisms of NAT2, CYP1A1, and CYP1A2 on stomach cancer.

Subjects, materials, and methods

Subjects

The study was conducted according to a hospital-based, case-control design, between October 1998 and March 2002 at four hospitals in Nagano Prefecture, Japan. A total of 153 consecutive patients, aged 21 to 76 years and newly diagnosed with stomach cancer at the participating hospitals were enrolled, with no patient refusing to participate. Control subjects were selected from a pool of medical checkup examinees from the four study hospitals, and they were confirmed not to have cancer. Two controls were matched for each case by sex, age (within 3 years), and area of residence during the study period in the same hospitals, although a number of exceptional cases had only one or more than two controls. Of 306 potential controls, 2 duplicate subjects and 1 individual recognized as having cancer after enrollment were excluded, leaving 303 controls participating (99%). Subjects who reported extreme total energy intake levels (upper or lower 2.5% of responses for each sex) were also excluded. Finally,

the study enrolled 149 cases and 296 controls for analysis. Written informed consent was obtained from all case and control subjects, and the study was approved by the Institutional Review Board of the National Cancer Center, Tokyo, Japan.

Exposure assessment

Subjects completed a self-administered questionnaire which inquired about general characteristics, including age, sex, occupation, personal medical history, smoking and drinking habits, vitamin supplement use, and dietary habits, as well as family history of disease, including stomach cancer incidence. We also inquired about membership in the Japan Agricultural (JA) Cooperatives, as most JA members in the study area are engaged in agriculture and are thus eligible to receive financial compensation from JA for health checkups. Food and beverage consumption was assessed with a 141-item semiquantitative food-frequency questionnaire (FFQ) [9], in which subjects reported their average consumption frequency and portion size of the listed items during the past year.

HCA intake from fish was evaluated using a previously described method [4]. Briefly, subjects were presented with nine frequency categories: none, 1–3 times/month, 1–2 times/week, 3–4 times/week, 5–6 times/week, once/day, 2–3 times/day, 4–6 times/day, and 7 times/day. Portion sizes were also described for each fish item. Participants indicated the quantity of grilled skin consumed by selecting one of five categories: almost none, one-third, half, two-thirds, and almost all. HCA intake from fish consumption was then estimated based on the proportion of grilled to total fish consumption, the rate of grilled skin consumption, the ratio of skin to flesh, and data on HCA content in the skin and flesh. Frequency category and portion size for meat consumption were described in the same way as for the fish items, with subjects reporting preferred doneness level (well-done, medium, rare) for pan-fried beef. HCA intake from meat consumption was then estimated based on this information and data on HCA content, and validated using measurements of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) levels in hair from a prior study [5]. Spearman rank correlation coefficients between the FFQ and PhIP level in hair were 0.47 for PhIP, 0.50 for 2-amino-3, 4-dimethylimidazo[4,5-f]quinoline (MeIQ), and 0.51 for total HCA.

Blood samples were obtained from each subject concurrently with the distribution of the questionnaire, and tested for serum pepsinogen I (PGI), pepsinogen II (PGII), and IgG antibody to *Helicobacter pylori* and to the cytotoxin-associated gene A (*CagA*). *H. pylori* infection was defined when one or both serum assays tested positive.

Genotyping and phenotype

The buffy coat of the blood samples was preserved at -80°C until analysis. We determined four single-nucleotide polymorphisms (SNPs) in NAT2 using a MassARRAY system (Sequenom, San Diego, CA, USA) [10, 11]. NAT2 slow acetylator alleles were ascertained as T341→C, NAT2*5; G590→A, NAT2*6; G857→A, NAT2*7; and A803→G, NAT2*13. Previous studies have found that the NAT2*4 allele acts dominantly to produce the fast acetylator phenotype [7]. Individuals with at least two variant alleles were classified as slow acetylators, and those with one variant allele as intermediate acetylators. Genotyping of *CYP1A1* (rs1048943) and *CYP1A2* (rs762551) genes was also performed. Cases and matched controls were analyzed in the same batch by laboratory personnel who did not know the case-control status.

Statistical analysis

Statistical analysis was conducted using conditional logistic regression for the case-control pairs. Intake of HCA and other nutrients and foods was adjusted for total energy intake using the residual model [12] and divided into tertile categories based on the control subjects. The models were adjusted for variables found to be associated with stomach cancer in this study, includ-

ing *H. pylori* status; smoking status; alcohol consumption; family history of stomach cancer; body mass index (BMI); JA membership; salt intake; and total vegetable, meat, and fish intake. To investigate the interaction between HCA intake and genetic polymorphisms, we divided the subjects into individuals with slow or rapid status for NAT2 and with variant alleles and those with two wild alleles for the *CYP1A1* (rs1048943) and *CYP1A2* (rs762551) genes, then we assessed the different effects of HCAs by genotype. The respective different effects by genotype were tested with a log-likelihood ratio test. All statistical analyses were conducted using SAS version 9.1 (SAS Institute, Cary, NC, USA).

Results

No statistically significant differences in PhIP, MeIQ, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), or total HCA intake were found between cases and controls (Table 1). BMI was lower in cancer cases than in cancer-free control cases, while the percentages of current smokers and positivity for *H. pylori* were higher. No statistically significant differences in total energy intake or in the intake of any foods or nutrients were seen between cases and controls, nor were any difference seen in the proportion of those who preferred well-done meat or who ate the largest portion of burned fish.

Table 1. Characteristics of cases and controls

	Cases	Controls	<i>P</i> *
Number	149	296	
Sex (matching factor), men (%)	69.8	71.3	0.745
Age (matching factor), years; mean (SD)	57.9 (9.5)	57.7 (9.4)	0.833
JA membership (%)	57.1	66.9	0.042
Family history of stomach cancer (%)	24.8	17.2	0.058
Smoking, current (%)	32.9	25.7	0.014
Alcohol consumption, g/day; mean (SD)	21.5 (30.9)	17.8 (22.7)	0.155
BMI, kg/m ² ; mean (SD)	23.0 (3.0)	23.7 (2.6)	0.009
<i>Helicobacter pylori</i> , positive (%)	82.6	58.1	<0.0001
Total energy intake, kcal/day; mean (SD)	2160 (633)	2201 (604)	0.515
Meat intake, g/day; mean (SD)	68.7 (45.6)	75.0 (60.8)	0.269
Red meat intake, g/day; mean (SD)	51.6 (37.2)	56.2 (47.2)	0.298
Processed meat intake, g/day; mean (SD)	9.9 (10.3)	10.7 (14.7)	0.584
Fish intake, g/day; mean (SD)	101.5 (67.9)	105.1 (68.0)	0.602
Vegetable intake, g/day; mean (SD)	245.2 (175.4)	249.2 (153.7)	0.806
NaCl intake, g/day; mean (SD)	15.3 (7.0)	15.5 (6.3)	0.818
Total HCA intake, ng/day; mean (SD)	67.6 (50.9)	72.8 (58.0)	0.352
PhIP intake, ng/day; mean (SD)	41.7 (30.2)	44.9 (34.9)	0.336
MeIQ intake, ng/day; mean (SD)	9.1 (7.2)	9.5 (6.6)	0.551
MeIQx intake, ng/day; mean (SD)	10.0 (6.5)	10.6 (7.8)	0.388
Preferable doneness level, well-done (%)	50.7	43.4	0.089
Intake level of grilled skin of fish, almost all (%)	18.9	21.4	0.944

JA, Japan Agricultural (Cooperative); BMI, body mass index; HCA, heterocyclic amine; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; MeIQ, 2-amino-3, 4-dimethylimidazo[4,5-f]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline

* *P* for Mantel-Haenszel test with matched-pair strata

Table 2. Single-nucleotide polymorphisms (SNPs) in *NAT2*, *CYP1A1*, and *CYP1A2* genes, and their allele frequency

Gene	SNP rs number	Amino acid change	Major/minor allele	Major allele frequency in control group
<i>NAT2</i>				
<i>NAT2</i> *5	rs1801280	Ile114Thr	T/C	0.807
<i>NAT2</i> *6	rs1799930	Arg197Gln	G/A	0.975
<i>NAT2</i> *7	rs1799931	Gly286Glu	G/A	0.907
<i>NAT2</i> *13	rs1208	Lys268Arg	A/G	0.984
<i>CYP1A1</i>	rs1048943	Ile462Val	A/G	0.757
<i>CYP1A2</i>	rs762551	5'-UTR	A/C	0.652

Table 3. Odds ratios (ORs) and 95% confidence intervals (CIs) of association of HCA intake with stomach cancer

	Mean (SD)	Case	Control	OR (95% CI) ^a
Total HCAs (ng/day)				
Tertile 1	27.3 (9.3)	53	95	1.00
Tertile 2	57.0 (9.9)	51	98	1.33 (0.59–2.98)
Tertile 3	125.5 (62.6)	45	103	1.11 (0.36–3.49)
Trend P				0.807
PhIP (ng/day)				
Tertile 1	16.8 (5.8)	51	97	1.00
Tertile 2	35.0 (5.7)	52	97	1.75 (0.77–3.99)
Tertile 3	76.3 (36.4)	46	102	1.33 (0.44–4.02)
Trend P				0.556
MeIQ (ng/day)				
Tertile 1	3.2 (1.1)	55	93	1.00
Tertile 2	6.8 (1.2)	52	97	1.23 (0.52–2.89)
Tertile 3	14.5 (7.1)	42	106	1.06 (0.36–3.12)
Trend P				0.922
MeIQx (ng/day)				
Tertile 1	3.8 (1.2)	52	96	1.00
Tertile 2	7.7 (1.2)	51	98	1.06 (0.52–2.14)
Tertile 3	16.4 (7.7)	46	102	0.81 (0.36–1.82)
Trend P				0.248

^aOR (95% CI), adjusted by conditional logistic regression for *H. pylori* status, smoking status, alcohol intake, family history of stomach cancer, body mass index, total vegetable intake, meat intake, fish intake, salt intake, and JA membership

The distribution of SNPs in *NAT2**5 (rs1801280), *NAT2**6 (rs179993), *NAT2**7 (rs1799931), *NAT2**13 (rs1208), *CYP1A1* (rs1048943), and *CYP1A2* (rs762551) is presented in Table 2. Among controls, genotype frequencies of each SNP were consistent with Hardy-Weinberg equilibrium, except for that of *CYP1A2* (rs762551).

Multivariate odds ratios (ORs) for HCA intake are shown in Table 3. No statistically significant increase in the risk of stomach cancer was seen for total HCA (OR, 1.11; 95% confidence interval [CI], 0.36–3.49), or for the individual HCAs PhIP, MeIQ, or MeIQx. We conducted an additional analysis which excluded subjects with cardia cancer ($n = 21$); however, none of the results changed substantially (total HCA: OR, 1.21, 95% CI, 0.29–5.11).

Multivariate ORs for the *NAT2*-imputed phenotype, *CYP1A1* gene, and *CYP1A2* gene are shown in Table 4. The percentage of controls with a rapid *NAT2* phenotype was 49.3%. No significant association was found

between the *NAT2*-imputed phenotype, *CYP1A1* gene (rs1048943), or *CYP1A2* gene (rs762551) and stomach cancer.

Analyses of combinations of HCA intake and *NAT2*-imputed phenotype, *CYP1A1* gene, and *CYP1A2* gene are shown in Table 5. The association between HCA intake and stomach cancer was not affected by *NAT2*, *CYP1A1*, and *CYP1A2* genetic polymorphisms. Further, no statistically significant interactions were seen among intakes of the individual HCAs PhIP, MeIQ, and MeIQx and these genetic polymorphisms (data not shown).

Discussion

In this hospital-based case-control study, we found no evidence of an association between HCA intake and the risk of stomach cancer among 149 subjects with stomach cancer. Further, we saw no influence of genetic poly-

Table 4. Association between NAT2-imputed phenotype, *CYP1A1* and *CYP1A2* genotype, and stomach cancer

	Case	Control	OR (95% CI) ^a
NAT2-imputed phenotype			
Slow acetylator	11	27	1.00
Intermediate acetylator	47	118	0.49 (0.17–1.46)
Rapid acetylator	83	141	0.93 (0.34–2.59)
<i>CYP1A1</i> gene			
AA	91	162	1.00
GA	44	109	0.79 (0.40–1.57)
GG	6	15	2.01 (0.45–9.48)
GA+GG	50	124	0.84 (0.43–1.65)
<i>CYP1A2</i> gene			
AA	56	130	1.00
CA	71	113	0.67 (0.36–1.24)
CC	14	43	0.82 (0.31–2.15)
CA+CC	85	156	0.72 (0.40–1.29)

^a OR (95% CI), adjusted by conditional logistic regression for *H. pylori* status, smoking status, alcohol intake, family history of stomach cancer, body mass index, total vegetable intake, meat intake, fish intake, salt intake, and JA membership

Table 5. Stomach cancer risk and HCA intake stratified by NAT2-imputed phenotype and *CYP1A1* and *CYP1A2* genotype

	HCA intake			<i>P</i> _{interaction}
	Tertile 1	Tertile 2	Tertile 3	
NAT2-imputed phenotype				
Slow or intermediate acetylator				
No.	33/50	18/51	15/54	
OR ^a	1.00	1.12 (0.37–3.33)	1.15 (0.31–4.36)	
Rapid acetylator				
No.	25/50	31/44	27/47	
OR ^a	1.30 (0.46–3.72)	2.17 (0.70–6.71)	1.96 (0.49–7.88)	0.117
<i>CYP1A1</i> gene				
AA				
No.	39/53	35/55	25/64	
OR ^a	1.00	1.54 (0.61–3.88)	1.23 (0.36–4.24)	
GA+GG				
No.	19/47	14/40	17/37	
OR ^a	1.05 (0.35–3.14)	0.96 (0.27–3.41)	1.09 (0.27–4.38)	0.777
<i>CYP1A2</i> gene				
AA				
No.	24/51	22/46	18/43	
OR ^a	1.00	1.35 (0.41–4.44)	1.29 (0.33–5.14)	
CA+CC				
No.	34/49	27/49	24/58	
OR ^a	0.68 (0.24–1.94)	0.95 (0.35–2.58)	0.88 (0.24–3.25)	0.321

^a OR (95% CI), adjusted by conditional logistic regression for *H. pylori* status, smoking status, alcohol intake, family history of stomach cancer, body mass index, total vegetable intake, meat intake, fish intake, salt intake, and JA membership

morphisms in NAT2, CYP1A1, and CYP1A2 on the association of HCA intake with stomach cancer.

At present, more is known about the association between HCAs and colorectal cancer than about such an association with stomach cancer. Nevertheless, in its review of 3 cohort and 12 case-control studies of grilled or barbecued animal-derived foods and stomach cancer, the World Cancer Research Fund identified 2 cohort and 8 case-control studies showing an increased risk for

the highest versus lowest intake groups [1]. One case-control study reported no association between HCAs and the risk of stomach cardia and adenocarcinoma [2], whereas a second study reported a statistically significant increase in stomach cancer risk for the highest versus lowest intake of PhIP [3]. At the same time, a third study showed an association between well-done meat intake and stomach cancer, and stated that consumption was associated with stomach cancer [13].

NAT2 is known to play a role in the metabolism of numerous carcinogens, including the activation of HCA [14]. The NAT2 rapid acetylator phenotype is thought to be indicative of higher risk, because N-acetylation is negligible and O-acetylation is an activation step for PhIP and MeIQx association with several cancers [15, 16]. However, no consistent association between the NAT2 phenotype or genotype and stomach cancer risk has been identified [17–23]. No investigation of the combined effect of well-done meat and HCA intake with the NAT2 phenotype or genotype has appeared.

Genes from the cytochrome P450 family encode enzymes involved in the oxidation of a variety of compounds; among these, CYP1A1 and CYP1A2 play critical roles in the metabolic activation of carcinogenic HCA to electrophilic reactive intermediates, leading to toxicity and cancer [24]. A significant association between CYP1A1, CYP1A2, and the risk of stomach cancer has been reported, as has the interaction of CYP1A1 polymorphisms and smoking in this cancer [25]. However, the interaction of CYP1A1 or CYP1A2 polymorphisms and HCA intake in stomach cancer has not been described.

We previously demonstrated that seropositivity for *H. pylori* was strongly associated with stomach cancer risk in the present case-control study [26]. Although the models were adjusted for *H. pylori* infection, this would not necessarily have removed the influence of confounding by *H. pylori* infection. It has been suggested that *H. pylori* has NAT activity, and acts as a possible promoter of stomach cancer via the bio-activation of food-borne HCAs into genotoxic and carcinogenic products in the stomach [27]. In the present study, however, as only 17.5% of our case subjects were *H. pylori*-negative, we did not examine the interaction between NAT2 phenotype and *H. pylori* infection and stomach cancer risk.

The development of a complete and standardized database of HCA concentrations in meat or fish is hampered by the dependence of the concentration on the cooking method and doneness level. Estimation of dietary HCA intake from a questionnaire is likely to result in misclassification. The validity of estimating PhIP intake against PhIP levels in human hair has been verified [5]. We therefore consider this estimation method to be a more reliable indicator of HCA exposure than those methods using grilled meat intake or the doneness level of meat. Because it was not possible to compare HCA levels in hair for MeIQ, MeIQx, and total HCA, validity was determined by comparison with PhIP levels in the hair. Therefore, as stated above, with methods estimating HCA intake, there is a possibility of misclassification.

One possible reason for our finding of a lack of association between HCA intake and stomach cancer may

have been the low level of HCA exposure in the study subjects; the mean PhIP intake in our control subjects was 44.9 ng/day, versus 78.2 to 170.6 ng/day in other studies that have estimated PhIP intake [28–30]. Nevertheless, one study reported a statistically significant association of PhIP with stomach cancer risk even in subjects with a low PhIP intake [3]. Clarification of this relationship is important for facilitating further study, particularly in view of the increasing consumption of grilled meat among younger generations in Japan. Although our present study did not address Polycyclic Aromatic Hydrocarbons (PAHs) and N-nitroso amines, these entities may also play a role in stomach carcinogenesis. The present finding of the lack of association between meat or fish intake and stomach cancer risk suggests that substances with chemopreventive action, such as the n-3 polyunsaturated fatty acids found in fish, may act to weaken the association between carcinogenic substances such as HCAs, PAHs, and N-nitroso amines and stomach cancer. In this regard, the erythrocyte composition of docosahexaenoic acid has been inversely linked to the risk of stomach cancer [31].

Several limitations of the present study warrant mention. One potential source of bias is differential recall: cancer patients may recall their usual diet differently from controls due to the impact of their diagnosis on dietary habits. A second potential source of error is selection bias, as control subjects were selected from a pool of medical checkup examinees. Against this, the HCA intake of our control subjects was comparable with that in the Nagano-area subjects of the JPHC study (72.8 g for the present study vs 70.4 g for the JPHC study) [4]. Further, given that HCAs are potent mutagens which are thought to play a role in the initiation of stomach tumors, the time lag between actual exposure leading to carcinogenesis and reported HCA intake may be several years. Meat preparation methods may have changed over the years for both patients and controls, and nondifferential misclassification may thus have attenuated the associations under study. The interpretation of our results may also be hampered by the small size of our sample population, which may have been better suited to classification into two categories rather than three. Because the subjects were divided into tertile categories based on estimated HCA intake, we calculated a detectable odds ratio at the number of 100 pairs (two-thirds of the number of the pairs in this study) in the lowest and highest intake groups. Based on this sample size, a statistically significant odds ratio of 2.02 can be detected by a two-tailed test with an alpha value of 0.05 and a beta value of 0.2. Given that the observed odds ratios (shown in Table 3) were less than 2.00, our sample size was considered insufficient to detect the small effect of HCA intake on stomach cancer.

In conclusion, we found that HCA intake was not associated with stomach cancer in a group of subjects with low HCA exposure. Further, we also failed to identify any effect of gene interaction on the relationship between HCA intake and stomach cancer. Future studies may benefit from observing patients with a wider range of HCA exposure.

Acknowledgments The authors gratefully acknowledge the generous assistance of the staff members of each hospital and the Agricultural Technology Institute of the Nagano Farmers' Federation. This study was supported in part by the Agricultural Technology Institute of the Nagano Farmers' Federation; by a Grant-in-Aid for Cancer Research and for the Third-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare of Japan; and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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