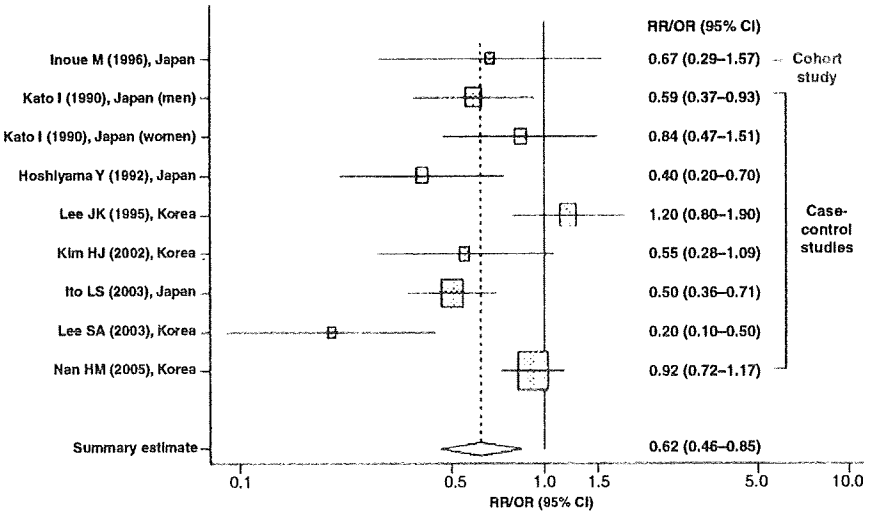

Fig. 1. Summary estimate of the relationships between fresh vegetable intake and gastric cancer risk in Japanese and Korean populations. CI, confidence interval; OR, odds ratio; RR, relative risk. Shaded box, point estimate of each study; horizontal line, 95% CI of each study; diamond, summary point estimate and its 95% CI of studies.

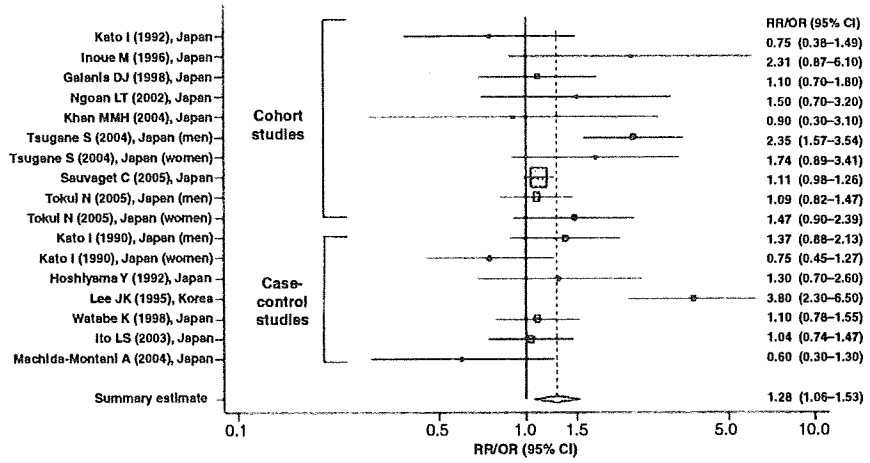

Fig. 2. Summary estimate of the relationships between pickled vegetable intake and gastric cancer risk in Japanese and Korean populations. CI, confidence interval; OR, odds ratio; RR, relative risk. Shaded box, point estimate of each study; horizontal line, 95% CI of each study; diamond, summary point estimate and its 95% CI of studies.

Discussion

The American Institute for Cancer Research reported that the summary relative risks of GC comparing high to low categories for total vegetable consumption were 0.50 (95% CI = 0.38–0.65) for 14 case–control studies and 0.80 (95% CI = 0.54–1.18) for 4 cohort studies through meta-analysis.[16] In a meta-analysis of 8 cohort studies, the summary relative risk of GC in high *versus* low categories for total vegetable intake was 0.88 (95% CI = 0.69–1.13).[24] Similarly, two large European cohort studies[25,26] reported that total vegetable intake was not associated with GC risk, regardless of the anatomic site. Although the protective effects of vegetable consumption on GC risk is widely accepted,[1–6] the results of the above meta-analyses indicate that the evidence from cohort studies does not support the protective effects of total vegetable intake on GC risk.[16,24–26]

Japanese and Korean populations have higher rates of GC incidence,[12] despite the fact that total vegetable consumption is higher in Japan and Korea,[7,8] than those in other countries with a lower intake of vegetables.[9,10] There is a possibility that a higher incidence of GC in Japan and Korea is partly due to the low consumption of fruits in these areas. However, the total consumption of vegetables and fruits is also higher in Korea (414.4 g/day)[7] and Japan (373.1 g/day)[8] than in the USA (358 g/day)[9] or northern Europe (278–288.5).[10] Moreover, Japanese and Korean people tend to consume more cooked, salted, or pickled vegetables than do people from North America or Europe.[7,10,60] Based on this observation, we inferred that the effects of vegetable consumption on GC risk may be different according to the preparation of the vegetables.

In the present meta-analysis, we observed significant inverse associations between a high intake of fresh vegetables and GC risk (overall summary OR = 0.62, 95% CI = 0.46–0.85). It has been suggested that the anticarcinogenic effect of vegetables is attributed in part to the effect of antioxidant vitamins, especially vitamin C and β-carotene, which inhibit the intragastric

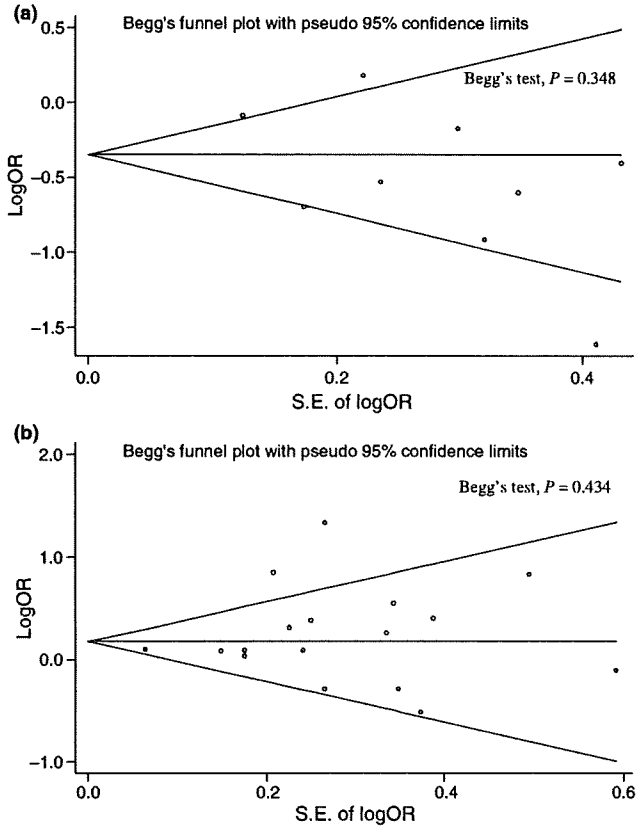

Fig. 3. Begg's funnel plot for publication bias in our overall meta-analysis of published epidemiological reports regarding fresh vegetable intake (a) and pickled vegetable intake (b) and gastric cancer risk. SE of logOR, standard error of log odds ratio.

formation of carcinogens such as *N*-nitroso compounds from secondary amines and nitrite. This inhibition might be caused by the reduction of nitrites into nitric oxide in the presence of reducing equivalents, such as vitamin C, or the combination of antioxidant vitamins with amines.[4,61,62] Another possible mechanism for the anticarcinogenic effects of antioxidants is the neutralization of reactive oxygen free radicals that can damage DNA.[63,64] Fresh vegetables contain a larger amount of antioxidant vitamins, such as vitamin C and β-carotene, than processed vegetables.[20,21,65] As well as antioxidant vitamins, vegetables contain various phytochemicals that act as antioxidants and scavenge free radicals, which could help to prevent cancer that occurs as a result of oxidative stress.[15]

We observed that a high intake of pickled vegetables was significantly associated with an increased risk of GC (overall summary OR = 1.28, 95% CI = 1.06–1.53). Examples of pickled vegetables include Japanese *tsukemono* and Korean *Jangajji*. Japanese *tsukemono* includes *takuan* (daikon), *umeboshi* (ume plum), ginger, turnip, cucumber, and Chinese cabbage.[18] Korean *Jangajji* is a pickled vegetable made by pickling or marinating garlic, daikon, cucumber, chili pepper leaves, and perilla leaves in soy sauce, chili pepper paste, soybean paste, or diluted vinegar.[66] Because they are preserved in brine (a solution of salt in water) or marinated and stored in an acid solution, pickled vegetables contain a substantial amount of salt. Salt is not a directly acting carcinogen, but consumption of salt and salt-preserved foods may cause atrophic gastritis by directly damaging the gastric mucosa, which could induce DNA synthesis and cell proliferation that contributes to stomach carcinogenesis[67] or enhance the penetration of carcinogens.[68] In addition, it has been reported that a high-salt diet enhances *H. pylori* colonization in the stomach.[69] *Helicobacter pylori* infection may increase the endogenous synthesis of nitrate in the stomach and decrease gastric vitamin C concentrations,[70] thereby increasing endogenous *N*-nitroso compound formation.[16] For these reasons, a high intake of salt and salt-preserved foods has been considered a probable cause of GC in many studies.[16,36,40,51,54,71,72] The loss of antioxidants in fresh vegetables as a consequence of processing and storage under acid and oxygen might partially explain the harmful effects of consumption of pickled vegetables on GC risk.[15,20,21] Another possible explanation is that pickled vegetables are a possible food source of nitroso compounds, thereby contributing to gastric carcinogenesis.[22,23]

There are several limitations concerning the interpretation of this meta-analysis. We selected a random-effect model to ameliorate the effect of large heterogeneity between studies in this meta-analysis, but this model has a typical limitation in that it does not strictly rule out the effects of heterogeneity; moreover, the relative weighting of the larger studies becomes reduced, whereas the weighting of the smaller studies is increased.[73] In this meta-analysis, the statistical significance of the results based on a fixed-effect model and random-effect model were not changed (OR = 0.71, 95% CI = 0.61–0.82 in fixed-effect model for fresh vegetables; OR = 1.19, 95% CI = 1.09–1.30 in fixed-effect model for pickled vegetables; data not shown). To explore the possible variables that explain the heterogeneity between studies, we carried out a meta-regression analysis that included nationality, study design, sex, and the year the study started. As a result, only nationality was observed as a source of heterogeneity between studies. Although we carried out a meta-analysis using adjusted RR/OR in order to consider several confounders, a residual confounding effect could remain because the variables included in the multivariate model were different from study to study.

In addition to the above limitations, various types of bias could occur in this meta-analysis. Publication bias is a typical one involved in finding published studies that may lead researchers to draw incorrect conclusions from their meta-analysis, because studies with statistically significant results are more likely to be published.[73] The results of Begg's test suggest that publication bias did not exist in this meta-analysis, but the possibility of publication bias, which is a characteristic inherent to meta-analyses, could still be present. In addition, because most studies were not designed to determine the effects of consumption of fresh or pickled vegetables on GC risk, there is a possibility that an outcome-reporting bias may have influenced the validity of our meta-analysis.[74] That is, non-significant associations between the consumption of fresh or pickled vegetables and GC risk may not have been presented in the results and, therefore, cannot be detected for meta-analysis. The application of strict inclusion criteria for the selection of studies also introduces inclusion criteria bias.[74] However, as the results with the same population can lead to overestimation due to duplication, we excluded these studies. We also excluded one case–control study using death cases,[57] which are more prone to various types of bias in the case–control design than incidence cases. However, even if we include this study of death cases in our meta-analysis, the significance of the overall summary estimate does not change (overall summary OR = 1.26, 95% CI = 1.05–1.50; data not shown). The interpretation and conclusions made from the results of this meta-analysis should be regarded cautiously due to the above limitations and bias.

In conclusion, the results of this meta-analysis provide evidence that high intake of pickled vegetables was associated with an increased GC risk, whereas high intake of fresh vegetables was associated with a decreased GC risk. These

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results may explain why the GC incidence rates in Japan and Korea remain high despite a high consumption of vegetables in these countries. A high consumption of fresh vegetables, rather than the total amount of vegetables, which includes pickled vegetables, should be promoted to reduce GC rates in Japan and Korea.

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Risk factors for intrahepatic cholangiocarcinoma: a possible role of hepatitis B virus

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SUMMARY. There are several established risk factors for intrahepatic cholangiocarcinoma (ICC), namely primary sclerosing cholangitis, fibropolycystic liver disease, parasitic infection, intrahepatic biliary stones and chemical carcinogen exposure. However, the majority of patients with ICC do not have any of these risk factors. Therefore, identification of other risk factors is warranted for the prevention and early detection of ICC. We evaluated the risk factors for ICC in a large-scale cohort study in the province of Osaka, Japan. This retrospective cohort study included 154,814 apparently healthy individual blood donors, aged 40–64 years at the time of blood donation in the period 1991–1993. The average observation period was 7.6 years, resulting in 1.25 million person-years of observation. Incident ICC cases were identified by linking the blood-donor database to the records in the population-based cancer registry for the province. There were 11 incident ICC cases during follow-up, with an incidence rate of 0.88 per 100 000 person-years. Compared with subjects aged 40–49 years, the subjects aged 50–54 years and 55–59 years had a significantly higher risk for ICC (hazard ratio [HR] = 5.90; 95%CI:1.08–32.31 and 11.07; 95%CI:1.98–61.79, respectively). Compared with those with ALT level of 19 Karmen Units (KU) or less, subjects with ALT level of 40 KU or higher had a significantly higher risk for ICC (HR: 8.30; 95%CI:1.47–46.83). Compared with those who tested negative for both HBsAg and anti-HCV, those who tested HBsAg-positive had a significantly higher risk for ICC (HR: 8.56; 95%CI: 1.33–55.20). Our results suggest that HBV infection and liver inflammation are independently associated with ICC development. These findings need to be verified by further large cohort studies.

Keywords: aetiology, cholangiocarcinoma, cohort studies, hepatitis, inflammation, liver.

INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver cancer, accounting for approximately 10–20% of liver cancers [1]. Worldwide, it is estimated to account for 3% of all gastrointestinal cancers [2]. Advanced ICC has a very poor prognosis with a median survival of less than 24 months [3]. There is extensive variation among the incidence rates of ICC in different parts of the world, and the incidence is reported to be higher in East Asia [1]. The reported incidence of ICC in several developed countries has been increasing in recent years [1,4,5].

Abbreviations: HBsAg, Hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; anti-HCV, antibody to HCV; HR, hazard ratio; HTLV-1, human T-cell lymphotropic virus type 1; ICC, intrahepatic cholangiocarcinoma; KU, Karmen Unit; OCR, Osaka Cancer Registry.

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There are several established risk factors for ICC [1,4], namely primary sclerosing cholangitis, fibropolycystic liver disease, parasitic infection, intrahepatic biliary stones and chemical carcinogen exposure, but the majority of patients with ICC do not have any of these risk factors. Therefore, identification of other risk factors is warranted for the prevention and early detection of ICC. In addition to the established risk factors mentioned earlier, some other potential risk factors for ICC have been suggested, such as infection with hepatitis B virus (HBV) [6–9], hepatitis C virus (HCV) [9–14] or liver cirrhosis [7,11,12]. Almost all of the studies that suggested these potential risk factors, however, were case–control studies. Also, half of the reported studies were conducted in the USA or Italy, and their results may not be applicable for populations in other countries or areas, partly because the relative importance of risk factors may vary by country or by area. To our knowledge, no cohort study has been reported from a non-Western country on the risk factors for ICC. In this background, we conducted a retrospective cohort study in Japan, using a large group of

apparently healthy blood donors, to assess the incidence and risk factors for ICC.

MATERIALS AND METHODS

Subjects

The subjects were those who were involved in our previous study on the risk of developing hepatocellular carcinoma, which was reported elsewhere [15]. They were selected from voluntary blood donors who gave 1 235 926 allogeneic blood donations at the Osaka Red Cross Blood Center between 1991 and 1993. The blood centre is in charge of managing volunteer blood donations in the province of Osaka, which had a population of approximately 8.6 million during this period. Conditions required for potential blood donors in Japan during the study period were described elsewhere [16]. In brief, they had to be aged between 16 and 64 years and have a haemoglobin level of 12 g/dL or higher. Potential blood donors were preliminarily screened by a self-administered questionnaire with items regarding past and present illnesses, history of blood transfusion, illegal drug use and high-risk sexual behaviours. Major exclusion criteria in the preliminary screening included: (i) known or potential infection with HBV, HCV, human immunodeficiency virus (HIV) or human T-cell lymphotropic virus type 1 (HTLV-1); (ii) presence or history of chronic liver disorders as well as malignant, allergic or autoimmune disorders; (iii) history of illegal drug use or high-risk sexual behaviour. Those who did not meet any of the exclusion criteria in the preliminary screening donated blood without monetary incentive. The donated blood of these donors was tested for the earlier-described viruses, and, if tested positive, the blood was discarded, but the information on the positive-tested donors was kept in the blood-donor database as was the information on the negative-tested donors. Using this database containing information on all of the donors and the results of the serologic screening test, we identified 667 461 individual donors by donor birthdate, sex, first name, family name and ABO blood type. From these individuals, we selected residents in Osaka province, aged 40 years or older at the time of blood donation, who were negative for antibodies to HIV and HTLV-1. Those who were 39 years or younger were excluded because the incidence of ICC in this age-group in the Japanese population was negligible. Those infected with HCV or HBV were first-time blood donors who claimed to be asymptomatic at the time of blood donation. Those who were infected with both HBV and HCV (25 subjects) were also excluded. As a result, we identified 154 814 persons to be included in this cohort study.

Blood screening tests

The serum alanine aminotransferase (ALT) level expressed in Karmen Units (KU) and serum total cholesterol level (mg/dL) were obtained from the database. The ALT value in KU tested by the blood centre can be translated into a value in International Units by multiplying with 1.5 [17]. An individual was defined as having HCV infection if the titre of anti-HCV in a second-generation passive hemagglutination assay (PHA) (Dainabot Co., Ltd, Tokyo, Japan) was 2^{12} or higher, because the positive predictive value for being HCV-RNA-positive using this cut-off point in Japanese blood donors was guaranteed [18]. An individual was considered to have HBV infection if he/she was positive for HBsAg in a reverse passive hemagglutination assay (Japan Red Cross, Tokyo, Japan).

Follow-up

The subjects were followed-up by record linkage between the blood-donor database and the database of the Osaka Cancer Registry (OCR) [19]. The records in the two databases were linked by the parameters of sex, date of birth, address and the first character of the family name in Chinese letters. The OCR is a population-based cancer registry that covers all of the population in Osaka province. The OCR registers all incident cancer cases using reports from health care facilities in the province as well as death certificate information provided by the Osaka Provincial Government [19]. In the OCR database, ICC cases were identified by the ICD-10 code (C22.1). The diagnosis of ICC was based on histological examination and/or combined clinical, radiological (echography, computed tomography and endoscopic retrograde cholangio-pancreatography) and laboratory findings. Subjects who remained unaffected by ICC were censored at the last date of follow-up, 31 December 2000, as were the subjects in the previous study [15]. The study protocol was approved by both the Ethical Committee of Osaka Medical Center for Cancer and Cardiovascular Diseases, and the Ethical Committee of the Osaka Red Cross Blood Center.

Statistical analyses

The number of person-years of observation of the subjects was determined, and the incidence rate of ICC per 100 000 person-years was calculated by the strata of age-group, sex, ALT level, cholesterol level and HBV/HCV infection status. Ninety-five per cent confidence interval (95%CI) for the rate was calculated using Byar's approximation of the exact Poisson test. Independent factors associated with the development of ICC were analysed by Cox proportional hazards model, and hazard ratios were calculated with 95%CI. In the model, age-group, sex, ALT level, cholesterol level, and HBV/HCV infection were included as independent variables. Data analyses were performed with the SAS/PC statistical package (SAS Institute, Cary, NC, USA).

RESULTS

The baseline characteristics of the 154 814 study subjects and incidence rates of ICC stratified by different characteristics

are shown in Table 1. The proportions of those with ALT level of 40 KU or higher, with positive HBsAg test, and with positive anti-HCV test were 2.2%, 1.6% and 1.2%, respectively. The average observation period was 7.6 years, resulting in 12.50×10^5 person-years. There were 11 incident ICC cases, with an incidence rate of 0.88 per 100 000 person-years (95%CI: 0.44–1.58). By strata, the point-estimate incidence rate was higher among those aged 55–59 years, those with ALT level of 40 KU or higher, or those who tested positive for HBsAg or anti-HCV.

Factors associated with the development of ICC in blood donors are shown in Table 2. Compared with subjects aged 40–49 years, the subjects aged 50–54 years and 55–59 years had a significantly higher risk for ICC. Compared with those with ALT level of 19 KU or lower, subjects with ALT level of 40 KU or higher had a significantly higher risk for ICC. Compared with those who tested negative for both HBsAg and anti-HCV, those who tested positive for HBsAg had a significantly higher risk for ICC. The hazard ratio for anti-HCV positivity was 2.63, although it was not significant.

The characteristics of the 11 ICC cases identified during the follow-up period are summarized in Fig. 1. The observation period from the date of blood donation to the date of diagnosis of ICC ranged from 14 to 90 months among the eight cases not infected with HBV or HCV, while it was '41', '77' and '108' months in the one HCV- and two HBV-infected cases.

DISCUSSION

To our knowledge, this is the first cohort study that investigated the risk factors for ICC in East Asia, where the incidence of primary liver cancer is high [20]. A literature search in Medline from January 1992 up to May 2009 revealed that all of the analytical studies on the risk factors for ICC in the past employed the case–control design, except for one study (Table 3). The only cohort study in the past was reported from the USA in early 2009 and focused on the risk of HCV infection for ICC and other hepatobiliary carcinomas [10]. The present study assessed whether HBV infection or liver inflammation as expressed by the ALT level was independently associated with ICC, which was not scrutinized in the cohort study from the USA.

We found that the incidence rate of ICC among the apparently healthy population aged 40–64 years was

Table 1 Baseline characteristics of the study subjects of blood donors aged 40–64 years and incidence rates of intrahepatic cholangiocarcinoma.

	N	(%)	ICC incident cases	Person-years ($\times 10^5$)	Incidence rate of ICC (per 10^5 person-years)	95% Confidence interval* of ICC incidence rate (per 10^5 person-years)
All cases	154 814	100.0	11	12.50	0.88	0.44–1.58
Age at blood donation (years)						
40–49	90 223	58.3	2	7.29	0.27	0.03–0.99
50–54	35 308	22.8	4	2.85	1.41	0.38–3.59
55–59	20 668	13.4	4	1.67	2.40	0.64–6.13
60–64	8615	5.6	1	0.69	1.44	0.02–8.06
Sex						
Male	84 205	54.4	7	6.81	1.03	0.41–2.12
Female	70 609	45.6	4	5.68	0.70	0.19–1.80
ALT (KU)						
19 or lower	127 757	82.5	7	10.32	0.68	0.27–1.40
20–39	23 666	15.3	2	1.91	1.05	0.12–3.78
40 or over	3391	2.2	2	0.27	7.36	0.83–26.74
Cholesterol (mg/dL)						
139 or lower	4533	2.9	0	0.37	0.00	0.00–12.60
140–199	82 575	53.3	8	6.67	1.20	0.52–2.36
200 or over	67 706	43.7	3	5.46	0.55	0.11–1.61
Hepatitis B/C virus infection						
HBsAg+	2519	1.6	2	0.22	9.08	1.02–32.82
anti-HCV+	1927	1.2	1	0.16	6.34	0.08–34.77
All negative	150 368	97.1	8	12.12	0.66	0.28–1.30

None of the subjects was positive for human immunodeficiency virus or human T-cell lymphotropic virus type 1. All negative: tested negative for both anti-HCV and HBsAg. ALT, alanine aminotransferase; HBsAg+, tested positive for Hepatitis B surface antigen and negative for Hepatitis C virus antibody; anti-HCV+, tested positive for anti Hepatitis C virus antibody and negative for Hepatitis B surface antigen. *95% confidence interval was calculated by Byar's approximation of the exact Poisson test.

Variable	*n*	ICC	Hazard ratio	95%CI
Age at blood donation (years)				
40–49	90 223	2	1.00	
50–54	35 308	4	5.90	1.08–32.31
55–59	20 668	4	11.07	1.98–61.79
60–64	8615	1	6.61	0.59–74.59
Sex				
Male	84 205	7	1.00	
Female	70 609	4	0.79	0.22–2.82
ALT level (KU) at blood donation				
19 or lower	127 757	7	1.00	
20–39	23 666	2	1.47	0.29–7.36
40 or over	3391	2	8.30	1.47–46.83
Cholesterol level at blood donation*				
200 mg/dL or higher	67 706	3	1.00	
140–199	82 575	8	2.36	0.60–9.26
139 or lower	4533	0	–	–
Hepatitis B/C virus infection				
All negative	150 368	8	1.00	
HCV-Ab +	1927	1	2.63	0.25–27.73
HBs Ag+	2519	2	8.56	1.33–55.20

Table 2 Factors associated with the development of intrahepatic cholangiocarcinoma in blood donors according to Cox proportional hazard analysis

None of the subjects was positive for human immunodeficiency virus or human T-cell lymphotropic virus type 1. Age (4 categories), sex, serum ALT level at blood donation (3 categories), and serum cholesterol level at blood donation (3 categories) were included as independent variables in the Cox proportinal hazard analysis. ALT, alanine aminotransferase; CI, confidence interval; HBsAg+, tested positive for Hepatitis B surface antigen and negative for Hepatitis C virus antibody; HCV-Ab+, tested positive for anti Hepatiis C virus antibody and negative for Hepatitis B surface antigen; ICC, intrahepatic cholangiocarcinoma; KU, Karmen Unit.

roughly 1 ± 0.5 per 100 000 person-years in Osaka where the HCV carrier rate is relatively high [21]. This figure is higher than the estimated incidence rate of 0.037 in the population aged 40–69 years reported in another Japanese study [22], of which the rate was underestimated because the numerator was derived from a multi-centre survey of primary liver cancer across Japan [23,24] with limited population coverage. The point-estimate incidence rate of ICC among anti-HCV positive subjects in our study (6.34 per 100 000 person years) was not very different from the estimate in the cohort study from the USA (4.0 per 100 000 person years) [10], even though our confidence interval was quite large.

Our results suggest that HBV infection is likely to be an independent risk factor for ICC in Japan. Studies in the past, in aggregate, suggested that HBV and HCV infection are potential risk factors for ICC, but their impact might be different across different countries or areas (Table 3). Several studies from the USA and Italy [9–12,14] consistently found that HCV infection was significantly associated with ICC, while the association with HBV infection was inconsistent or not assessed. On the other hand, recent hospital-based case–control studies from Korea and Shanghai, China [7,8] found that not HCV but HBV infection was significantly associated with ICC. The most recent study from Taiwan found that both HBV and HCV are significantly associated with ICC [6]. To our knowledge, our study is the first cohort study to demonstrate a significant association between HBV infection and ICC. This higher level of evidence supports prior observations of the association in East Asia. Our finding is also supported by the results of the earlier mentioned multi-centre primary liver cancer survey in Japan, which consistently showed a high prevalence of HBsAg among ICC cases (4–9%) since 1990 [24][detailed data since 1990 available in reports by the The Japan Society of Hepatology (Japanese only)].

The mechanism of carcinogenesis by HBV in intrahepatic bile ducts has not yet been elucidated, but HBV infection is an established risk factor for hepatocellular carcinoma. Because both hepatocytes and cholangiocytes differentiate from the same progenitor cells, HBV might induce carcinogenesis in both cell types through the same mechanism. The HBV gene has been detected in cholangiocarcinoma tissue in some studies [25,26], and its presence has been associated with the potential of carcinogenesis in human cholangiocytes [27]. Alternatively, hepatitis-associated ICC may arise from hepatic progenitor cells, as suggested by Lee *et al.* [6].

In our study, the association between ICC and HCV infection was not significant, even though its hazard ratio

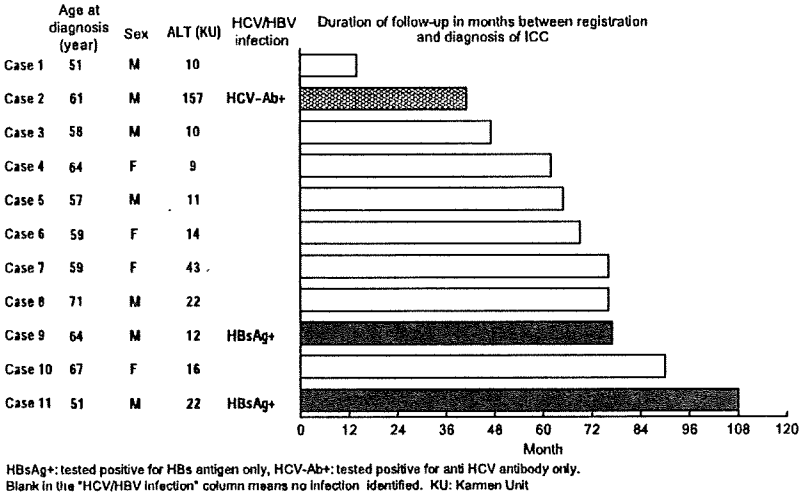

Fig. 1 Characteristics of the 11 intrahepatic cholangiocarcinoma (ICC) cases identified among the cohort of blood donors during the follow-up period.

was greater than unity. We might have seen a significant association if our cohort had been even larger. In the multicentre survey in Japan, the prevalence of anti-HCV among ICC cases (20–30%) has consistently been high since 1990 [24]. [detailed data since 1990 available in reports by The Japan Society of Hepatology (Japanese only)].

Our findings also suggest that liver inflammation as expressed by ALT level ≥40 KU may be an independent risk factor for ICC after adjustment for HCV and HBV infection status. In our previous study using the same cohort, we also found that liver inflammation is an independent risk factor for hepatocellular carcinoma [15]. The only study in the past that assessed the impact of ALT level on the development of ICC was performed in a case–control study [13], and found a significant association between ICC and ALT level of ≥40 IU, independent of HBV or HCV infection status. In addition to viral hepatitis, fatty liver disease is likely to be a major cause of liver inflammation in the subject population. Here, inflammation of hepatocytes may connote inflammation of intrahepatic bile ducts. Epidemiologic and experimental evidence shows that inflammation of the bile duct from various aetiologies induces carcinogenesis in cholangiocytes [1,4,28]. Alternatively, inflammation of hepatic progenitor cells may result in cholangiocarcinoma [6].

Liver cirrhosis has been suggested as an independent risk factor for ICC in some case–control studies where liver inflammation was not included as a covariate [7,11,12]. We were not able to assess the risk of liver cirrhosis for ICC because of lack of this information in the blood-donor database, but patients with known liver cirrhosis were excluded in our study upon screening before blood donation, minimizing the effect of a potential confounding factor. In part, the presence of liver cirrhosis among ICC cases in the case–control studies might be a proxy of present or past liver inflammation. If so, ICC might be associated with liver inflammation in cohort studies using subjects in a pre-cirrhosis state, and with liver cirrhosis in case–control studies. The only case–control study that included both liver cirrhosis and inflammation as covariates demonstrated that ICC was significantly associated with the presence of inflammation but not with liver cirrhosis [13]. Further studies on this issue are necessary.

Our study has some potential limitations. First, information on the presence of established major risk factors for ICC, namely infection with liver fluke, intrahepatic biliary stones, fibropolycystic liver disease and primary sclerosing cholangitis, was not available in the blood-donor database, and we were not able to adjust the hazard ratios for them. However, liver fluke (*Clonorchis sinensis*) infection was already rare in Osaka and surrounding areas by the 1960s [29]. Also, patients with chronic liver or autoimmune diseases were excluded from blood donors upon their claim in the screening questionnaire. Therefore, influence from known confounding factors should be minimal in our study. Second, the number of ICC cases in our study was fairly limited because of the relatively low incidence of ICC, resulting in a wide confidence interval in the estimated incidence rates or relative risks. Our findings on the risk of viral infection and inflammation, however, were consistent with observations in past studies, and it should, at least in part, warrant the validity of our findings. Third, we may have underestimated the incidence rates of ICC in our study, because the number of person-years of observation described in the Results section was not adjusted by the number of subjects who might have moved out of the province or died, before the final date of follow-up. The estimated incidence rates, after adjustment using the revised values for these parameters, would be approximately 25% higher than the figures described earlier.

Table 3 Results of analytical studies on the association of intrahepatic cholangiocarcinoma (ICC) and hepatits B and C virus

Authors and year	Study area	Type of study	No. of ICC case	RR (95% CI) for HBV infection*	RR (95% CI) for HCV infection*
Shin *et al.* 1996 [30]	Pusan, Korea	Case–control, hospital- based	41	OR = 1.3 (0.3–5.3)	OR = 3.9 (0.9–17.1)
Donato *et al.* 2001 [14]	Italy	Case–control, hospital- based	26	OR = 2.7 (0.4–18.5)	OR = 9.7 (1.6–58.9)
Yamamoto *et al.* 2004 [13]	Osaka, Japan	Case–control, hospital- based	50	OR = 1.8 (0.3–10.1)	OR = 16.8 (5.7–50.0)
Shaib *et al.* 2005 [12]	TX, USA	Case–control, Medicare- beneficiaries	625	OR = 0.8 (0.1–5.9)	OR = 6.1 (4.3–8.6)
Welzel *et al.* 2007 [11]	USA	Case–control, population-based	535	–	OR = 5.4 (2.9–10.2)
Shaib *et al.* 2007 [9]	TX, USA	Case–control, hospital-based	83	OR = 28.6 (3.9–1268.1)†	OR = 7.9 (1.3–84.5)
Lee *et al.* 2008 [7]	Korea	Case–control, hospital-based	622	OR = 2.3 (1.6–3.3)	OR = 1.0 (0.5–1.9)
Zhou *et al.* 2008 [8]	Shanghai, China	Case–control, hospital-based	312	OR = 8.8 (5.9–13.1)	OR = 0.9 (0.3–3.1)
Lee *et al.* 2009 [6]	Taiwan	Case–control, hospital-based	160	OR = 5.0 (2.8–9.0)	OR = 2.7 (1.2–6.3)
El-Serag *et al.* 2009 [10]	USA	Cohort, veterans population	37	–	HR = 2.6 (1.3–5.0)
Tanaka *et al.* (present)	Osaka, Japan	Cohort, blood donor population	11	HR = 8.6 (1.3–55.2)	HR = 2.6 (0.3–27.7)

Note: Hepatitis B virus infection status was identified by the presence of HBs antigen except the study by Shaib *et al.* 2007. (–): not asssessed as a covariate. CI, confidence interval; HBV, hepatitis B virus; HCV, hepatitis C virus; RR, relative risk of develping intrahepatic cholangiocarcinoma; OR, adjusted odds ratio by multiple logistic regression analysis; HR, adjusted hazard ratio by Cox proportional hazard regression analysis. *The original figures reported in each study were rounded up to the first decimal place. †HBV infection status verified by the antiHBc+ and HBsAg–; Odds ratio for HBsAg+/antiHBc– was shown to be 2.9 (95%CI: 0.1–236.9).

Lastly, coverage of the cancer registry in Osaka was not perfect, potentially reducing the estimated incidence of ICC. Nevertheless, we assume that the coverage for cancers with poor prognosis, like ICC, was sensitive enough because of the use of death certificate information. Also, the imperfect coverage of the registry should not affect the relative risk for HBV/HCV infection or liver inflammation because the registry coverage was independent of the presence of these risk factors.

In conclusion, our results suggest that HBV infection and liver inflammation are independently associated with ICC development. These findings as well as their association with liver cirrhosis and other potential risk factors need to be verified by further large cohort studies.

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STATEMENT OF INTERESTS

Author's declaration of personal interests: None of the authors claim potential conflict of interest that pertains to this manuscript.

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EML4-ALK fusion transcripts in immunohistochemically ALK-positive non-small cell lung carcinomas

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Abstract. EML4-ALK fusion transcripts have been found in a subset of non-small cell lung carcinomas (NSCLCs); however, their protein expression status has not yet been fully elucidated. In this study we investigated ALK protein expression in 302 NSCLCs and 291 gastric carcinomas by means of immunohistochemical analysis. Twelve (4.0%) NSCLCs, but none of the gastric carcinomas, were found to be positive for ALK. The ALK signal was detected in the cytoplasm of cancer cells. Subsequent RNA analysis of 10 RNA-available, immunohistochemically ALK-positive tumors revealed that three tumors had EML4-ALK variant 1, three tumors had variant 2, three tumors had variants 3a and 3b, and one tumor had a novel variant in which exon 14 of EML4 is connected to the nucleotide at position 53 of exon 20 of ALK by a 2-bp insertion. These results suggest that immunohistochemical ALK detection is a useful way to screen NSCLCs for tumors containing ALK fusions.

Introduction

Structural chromosome aberrations that result in the production of fusion oncogenes are one of the most common causes of oncogenesis. In the past they have been reported in many classes of hematological malignancies and mesenchymal tumors (1,2), and recently in a few types of epithelial carcinomas (3-5). A fusion gene comprising portions of the *EML4* gene and the *ALK* gene that resulted from a small inversion in chromosome 2p was recently discovered in a subset of non-small cell lung carcinomas (NSCLCs) (4). The fused mRNA based on the gene fusion encodes the N-terminal portion of EML4 ligated to the intracellular region of the receptor-type protein tyrosine kinase ALK. EML4-ALK oligomerizes constitutively in cells through the coiled-coil domain within the EML4 region and becomes activated to exert oncogenicity both *in vitro* and *in vivo* (4,6). Several types of EML4-ALK variants have been found in NSCLCs (4,6-18), and although one NSCLC containing KIF5B-ALK and another NSCLC containing TFG-ALK have been found (13,15), all of the other ALK fusions detected in NSCLCs have been EML4-ALK fusions.

Notably, recent studies have shown that ALK inhibitors have potential therapeutic efficacy for NSCLCs that are positive for ALK fusion proteins (4,6,16,19). Thus, the development of a diagnostic system for NSCLCs expressing ALK fusion proteins will be essential to identifying subgroups of NSCLC patients for treatment with ALK inhibitors. Immunohistochemical analysis of paraffin-embedded sections during routine pathologic diagnosis is a convenient means of examining the level of protein expression when the analytical condition is determined. Takeuchi *et al* recently reported an effective means of immunohistochemical detection of EML4-ALK by the intercalated antibody-enhanced polymer (iAEP) method (13). However, another group reported difficulty detecting EML4-ALK immunohistochemically (14), and it is speculated that the low expression level of EML4-ALK protein may be attributable to a low level of EML4 transcriptional activity or to instability of EML4-ALK in cells (13). Moreover, based on the results of a fluorescence *in situ* hybridization (FISH) analysis, Perner *et al* reported finding that only a subset of tumor cells contains the 2p rearrangement that leads to the formation of EML4-ALK (10). Thus, a system for immunohistochemical detection of ALK in NSCLCs would need to be established in order to diagnose tumors containing ALK fusions and elucidate the expression status of ALK fusion proteins. We also believe that immunohistochemical screening for ALK fusions may lead to the identification of novel EML4-ALK variants or novel fusions with ALK in addition to known EML4-ALK variants. Moreover, although the only carcinomas in

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Key words: EML4, ALK, non-small cell lung carcinoma, fusion transcript, immunohistochemistry

which ALK fusions have been found thus far are NSCLCs, ALK fusions may be present in other types of carcinomas. However, no studies using the iAEP method, except a study by Takeuchi *et al* (13), have been published. Therefore, in the present study, we immunohistochemically evaluated a total of 302 NSCLCs and 291 gastric carcinomas for ALK expression using the iAEP method and then investigated RNA-available, immunohistochemically ALK-positive tumors for expression of EML4-, KIF5B- and TFG-ALK fusions.

Materials and methods

Surgical specimens. Samples of surgical specimens from 302 NSCLC and 291 gastric carcinoma patients who underwent surgery for their cancer at Hamamatsu University School of Medicine, University Hospital or Mikatahara Seirei General Hospital were obtained. The mean age of the 302 NSCLC patients was 63.9 years [standard deviation (SD) 10.7], and they consisted of 168 men and 134 women. The NSCLC tumors were histologically classified as adenocarcinoma in 184 cases, squamous cell carcinoma in 98 cases, large-cell carcinoma in 9 cases and adenosquamous carcinoma in 11 cases. The mean age of the 291 gastric carcinoma patients was 65.4 years (SD 11.8), and they consisted of 206 men and 85 women. The gastric tumors were histologically classified as intestinal-type adenocarcinoma in 151 cases, diffuse-type adenocarcinoma in 138 cases and adenosquamous carcinoma in 2 cases. This study was approved by the Institutional Review Board (IRB) of Hamamatsu University School of Medicine and the IRB of Mikatahara Seirei General Hospital.

Immunohistochemical staining. Immunostaining for ALK using the iAEP method was performed as described previously (13) with slight modifications. In brief, paraffin-embedded tissue sections were deparaffinized, rehydrated and boiled at 96°C for 40 min in Target Retrieval Solution (pH 9.0) (Dako, Kyoto, Japan) for antigen retrieval. Endogenous peroxidase activity was blocked by incubation for 5 min in a 3% hydrogen peroxide solution. Next, the sections were incubated with a Protein Block, Serum-free (Dako) for 10 min at room temperature (RT) and then with a mouse anti-ALK monoclonal antibody (clone 5A4; Abcam, Cambridge, UK) at a dilution of 1:50 for 30 min at RT. To increase the sensitivity of detection, the sections were incubated with polyclonal rabbit anti-mouse immunoglobulin at a dilution of 1:500 for 15 min at RT. After washing, the sections were incubated for 30 min at RT with an amino acid polymer conjugated with goat anti-rabbit IgG and horseradish peroxidase (Histofine Simple Stain MAX-PO Kit; Nichirei, Tokyo, Japan). The antigen-antibody complex was visualized with 3,3'-diaminobenzidine tetrahydrochloride, and the sections were counterstained with hematoxylin. The staining was performed with a Dako autostainer (Dako) (20).

Reverse transcription (RT)-polymerase chain reaction (PCR). Total RNA was extracted from lung tissue samples with an RNeasy Kit (Qiagen, Valencia, CA, USA) and converted to first-strand cDNA with a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) by following the supplier's protocol. PCR was performed in 20-μl reaction mixtures containing HotStarTaq DNA polymerase (Qiagen) under the following conditions: 30 sec at 94°C, 30 sec at 61°C and 90 sec at 72°C for 45 cycles. A total of five different PCR primer pairs for EML4-ALK, three PCR primer pairs for KIF5B-ALK and one PCR primer pair for TFG-ALK were used for the RT-PCR. The forward PCR primers were: 5'-GCC TCA GTG AAA AAA TCA GTC TCA AG-3' for the sequence on exon 2 of EML4, 5'-ACA AAT TCG AGC ATC ACC TTC TCC-3' for the sequence on exon 4 of EML4, 5'-GTG CAG TGT TTA GCA TTC TTG GGG-3' for the sequence on exon 13 of EML4, 5'-CTG TGG GAT CAT GAT CTG AAT CCT G-3' for the sequence on exon 14 of EML4, 5'-CTT CCT GGC TGT AGG ATC TCA TGA C-3' for the sequence on exon 19 of EML4, 5'-CAC TAT TGT AAT TTG CTG CTC TCC ATC ATC-3' for the sequence on exon 10 of KIF5B, 5'-AAT CTG TCG ATG CCC TCA GTG AAG-3' for the sequence on exon 17 of KIF5B, 5'-TGA TCG CAA ACG CTA TCA GCA AG-3' for the sequence on exon 24 of KIF5B and 5'-TCG TTT ATT GGA TAG CTT GGA ACC AC-3' for the sequence on exon 4 of TFG. The reverse PCR primer used was the same, i.e., 5'-GAG GTC TTG CCA GCA AAG CAG TAG-3' for the sequence on exon 20 of ALK. The PCR products were fractionated by electrophoresis on an agarose gel and stained with ethidium bromide. The PCR-amplified products were purified with a PCR purification kit (Qiagen) and directly sequenced with a BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Tokyo, Japan) and the ABI 3100 Genetic Analyzer (Applied Biosystems) as described previously (7). The reference sequences for the *ALK*, *EML4*, *KIF5B* and *TFG* genes are accession numbers NM_004304, NM_019063, NM_004521 and NM_006070, respectively.

Statistical analysis. Statistical comparisons were performed by the two-tailed Student's t-test with Excel software (Microsoft Corp., Redmond, WA, USA).

Results

Immunohistochemical detection of ALK-positive NSCLCs. Samples of 302 NSCLCs and 291 gastric carcinomas were immunohistochemically stained for ALK with 5A4 anti-ALK monoclonal antibody using the iAEP method, and 12 (4.0%) of the NSCLCs and none (0%) of the gastric carcinomas were positive for ALK expression (Table I). ALK staining was observed in the cytoplasm of the cancer cells in all 12 NSCLCs, but not in any of the non-cancerous cells (Fig. 1). The mean age of the NSCLC patients whose tumors were positive for ALK was 57.3 years (SD 15.7) and significantly lower than the mean age of the NSCLC patients whose tumors were negative for ALK (64.2 years of age, SD 10.4) ($p=0.027$). The NSCLC patients whose tumors were positive for ALK consisted of 6 men and 6 women, and the ALK-positive NSCLC tumors were classified histologically as adenocarcinoma in 10 cases, adenosquamous carcinoma in 1 case and squamous cell carcinoma in 1 case (Table I).

Detection of various EML4-ALK fusion transcripts in NSCLCs. Next, 10 RNA-available, ALK-positive NSCLCs were investigated for expression of EML4-, KIF5B- and TFG-ALK fusion transcripts by RT-PCR and subsequent sequencing

Table I. Clinicopathological information and EML4-ALK fusions detected in immunohistochemically ALK-positive NSCLCs.

No.	Age	Gender	Histopathological diagnosis	EML4-ALK transcript
1	48	Female	Adenocarcinoma	Variant 1
2	49	Male	Adenocarcinoma	Variant 1
3	66	Male	Adenocarcinoma	Variant 1
4	46	Female	Adenocarcinoma	Variant 2
5	57	Male	Adenocarcinoma	Variant 2
6	79	Male	Adenocarcinoma	Variant 2
7	33	Female	Adenosquamous carcinoma	Variants 3a and 3b
8	63	Female	Adenocarcinoma	Variants 3a and 3b
9	83	Male	Adenocarcinoma	Variants 3a and 3b
10	58	Male	Adenocarcinoma	A novel variant[a]
11	36	Female	Adenocarcinoma	Not examined[b]
12	69	Female	Squamous cell carcinoma	Not examined[b]

[a]Exon 14 of EML4 is connected to a 2-bp fragment that in turn is ligated to the nucleotide at position 53 of exon 20 of ALK. [b]Due to the absence of an RNA sample, RT-PCR analysis for ALK fusions was not performed.

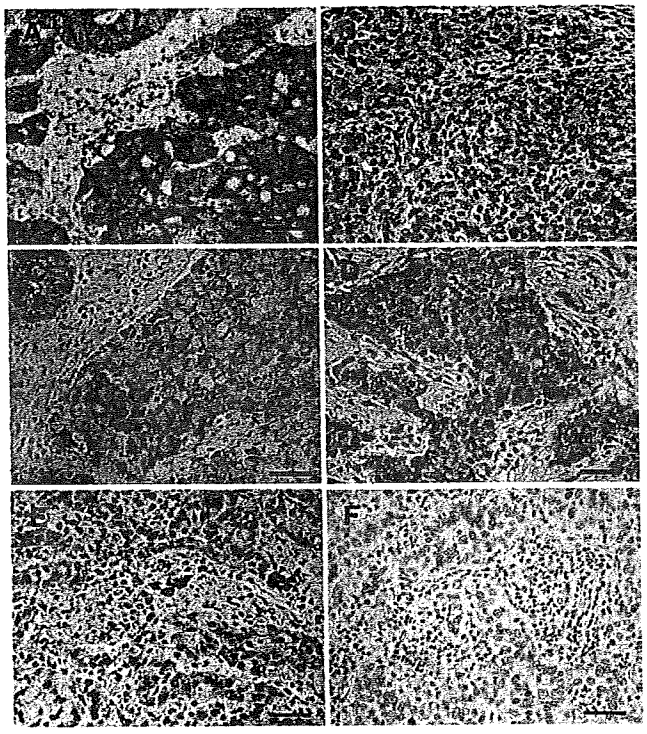

Figure 1. Representative results of immunohistochemical staining for ALK in non-small cell lung carcinomas. ALK protein expression was detected with 5A4 anti-ALK monoclonal antibody by the intercalated antibody-enhanced polymer method. A, B, C, D and E are the adenocarcinomas in cases No. 2, 5, 6, 8 and 10, respectively. F is the adenocarcinoma which showed no ALK expression. Bar, 50 μm.

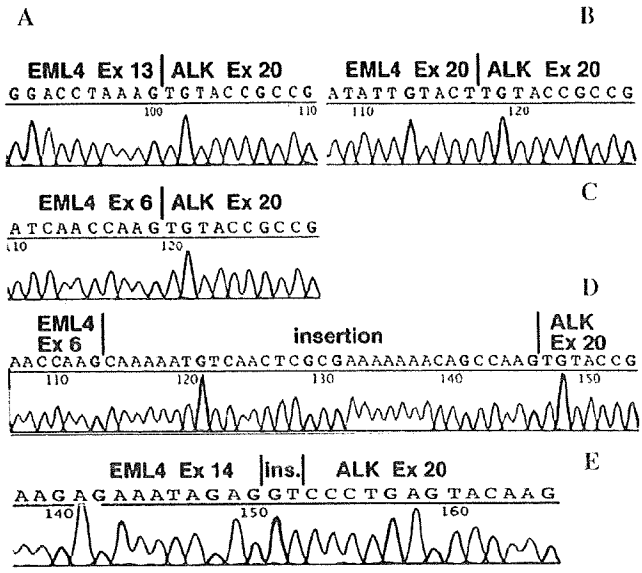

Figure 2. Detection of EML4-ALK fusion transcripts in non-small cell lung carcinomas. (A) EML4-ALK variant 1 transcript detected in case No. 1. (B) EML4-ALK variant 2 transcript detected in case No. 4. (C and D) EML4-ALK variants 3a (C) and 3b (D) transcripts detected in case No. 7. (E) Novel EML4-ALK transcript variant detected in case No. 10.

analyses. As a negative control, we also performed an RT-PCR analysis of 30 randomly selected, immunohistochemically ALK-negative NSCLCs. No expression of KIF5B-ALK or TFG-ALK fusion transcripts was detected in any of the carcinomas, but EML4-ALK fusion transcripts were detected in all 10 RNA-available, ALK-positive NSCLCs (Table I). As expected, no RT-PCR products were detected in any of the 30 immunohistochemically ALK-negative NSCLCs. Regarding the type of the EML4-ALK transcript, in 3 cases (No. 1-3) the fusion was variant 1, a fusion between exon 13 of EML4 and exon 20 of ALK (Fig. 2A), and in 3 cases (No. 4-6) it was variant 2, a fusion between exon 20 of EML4 and exon 20 of ALK (Fig. 2B). In 3 other cases (No. 7-9) the RT-PCR analysis yielded two bands, and they corresponded to variant 3a, a fusion between exon 6 of EML4 and exon 20 of ALK (Fig. 2C), and variant 3b, a fusion containing an additional 33-bp sequence derived from intron 6 of EML4 between exon 6 of EML4 and exon 20 of ALK (Fig. 2D). Notably, in case No. 10,

sequencing of the RT-PCR product revealed that exon 14 of EML4 was connected to an unidentified 2-bp fragment that was in turn ligated to the nucleotide at position 53 of exon 20 of ALK (Fig. 2E). The EML4-ALK sequence detected in case No. 10 allows an in-frame connection between the two genes and is a novel variant. The mean age of the 10 NSCLC patients whose tumors contained EML4-ALK transcripts was 58.2 years (SD 15.3), and they consisted of 6 men and 4 women. The NSCLC tumors containing EML4-ALK transcripts were histologically classified as adenocarcinoma in 9 cases and adenosquamous carcinoma in 1 case. These findings suggest that the iAEP method is useful for screening paraffin-embedded tissue sections for NSCLCs containing ALK fusion transcripts.

Discussion

Immunohistochemical screening for ALK expression using the iAEP method in the present study revealed an immunohistochemical ALK signal in 12 (4.0%) of 302 NSCLCs but not in any of the 291 gastric carcinomas. The ALK signal was detected in the cytoplasm of the cancer cells in all of the ALK-positive NSCLCs. RT-PCR and subsequent sequencing analyses of RNA from the 10 RNA-available, ALK-positive NSCLCs revealed the EML4-ALK variant 1 in 3 cases, variant 2 in 3 cases, both variants 3a and 3b in 3 cases, and a novel variant consisting of a fusion between exon 14 of EML4 and a nucleotide within exon 20 of ALK in 1 case. These results suggest that the immunohistochemistry-based system is useful for screening NSCLCs for ALK fusions, and identification of a novel EML4-ALK variant would be helpful in diagnosing NSCLCs containing ALK fusions in the future.

The proportion of immunohistochemically ALK-positive NSCLCs in this study (4.0%) is almost the same as the proportions of NSCLCs containing ALK fusion transcripts reported in previous studies (4,6-18), and in the present study EML4-ALK variants were detected in all RNA-available, immunohistochemically ALK-positive NSCLCs. These results suggest that our immunohistochemical analysis was performed properly and that the iAEP method with 5A4 anti-ALK antibody is a useful diagnostic tool for screening for NSCLCs containing ALK fusion proteins.

The histopathological diagnosis of 10 of the 12 immunohistochemically ALK-positive NSCLCs and 9 of the 10 EML4-ALK-positive NSCLCs in this study was adenocarcinoma. The predominance of adenocarcinomas among EML4-ALK-positive NSCLCs is consistent with the results of previous studies (6,12). This finding is also consistent with the recent finding of the growth of hundreds of adenocarcinoma nodules in transgenic mice in which EML4-ALK mRNA was transcribed specifically in lung epithelial cells (21). In the present study the mean age of the patients with immunohistochemically ALK-positive NSCLCs was significantly lower than that of the patients with ALK-negative NSCLCs. Although the mechanism responsible for the age difference is unknown, early onset may be a characteristic of ALK fusion-positive NSCLCs.

A novel EML4-ALK variant was found in this study. In this novel variant, exon 14 of EML4 was connected to a 2-bp fragment that was in turn ligated to the nucleotide at position 53 of exon 20 of ALK. Notably, the connection in each of two EML4-ALK variants, 4 and 7, is known to be between exon 14 of EML4 and a nucleotide within exon 20 of ALK (11,13). In variant 4, exon 14 of EML4 is connected to an unidentified 11-bp cDNA fragment that in turn is ligated to the nucleotide at position 50 of exon 20 of ALK (11), while in variant 7, exon 14 of EML4 is connected to the nucleotide at position 13 of exon 20 of ALK (13). Thus, the variant identified in this study is the third variant with a connection between exon 14 of EML4 and a nucleotide within exon 20 of ALK. Connections located within, rather that at the 5' terminus of, exon 20 of ALK have also been reported in MSN-ALK and MYH9-ALK, both of which have been detected in anaplastic large-cell lymphoma (22,23). Since a systemic understanding of the ALK fusions is important to correctly diagnose NSCLCs containing ALK fusions, our identification of a novel EML4-ALK variant should contribute to establishing a practical and accurate diagnostic system in the future.

Since the intracellular region of ALK was used as the antigen to produce the 5A4 anti-ALK antibody used in this study, both EML4-ALK and wild-type ALK should have been detected by the antibody. Takeuchi *et al* attempted to determine whether both transcripts are expressed by quantitatively analyzing the amount of mRNA specific for wild-type ALK and ALK fusion transcript separately, and found that none of the EML4-ALK-positive tumors yielded a substantial amount of wild-type ALK mRNA (13). Thus, immunohistochemical staining with the 5A4 antibody using the iAEP method appears to detect ALK fusion proteins and not wild-type ALK in NSCLCs. Our results for detection of EML4-ALK variants in all of the RNA-available, immunohistochemically ALK-positive NSCLCs support this view.

Our immunohistochemical analysis did not detect ALK protein expression in any of the gastric carcinomas. This was the first search for ALK fusion proteins in gastric carcinomas, and the results clearly demonstrated the absence of ALK fusion in gastric carcinomas. Since previous RNA analyses showed no EML4-ALK transcripts in 96 gastric carcinomas (8) and 33 gastric carcinomas (11), our results are consistent. The only human carcinomas in which ALK fusions have ever been found are NSCLCs. However, since it is unknown whether ALK fusions are involved in the genesis and development of other types of carcinoma, it may be worth investigating various types of carcinomas for expression of ALK fusion proteins in the future.

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Imaging mass spectrometry of gastric carcinoma in formalin-fixed paraffin-embedded tissue microarray

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The popularity of imaging mass spectrometry (IMS) of tissue samples, which enables the direct scanning of tissue sections within a short time-period, has been considerably increasing in cancer proteomics. Most pathological specimens stored in medical institutes are formalin-fixed; thus, they had been regarded to be unsuitable for proteomic analyses, including IMS, until recently. Here, we report an easy-to-use screening method that enables the analysis of multiple samples in one experiment without extractions and purifications of proteins. We scanned, with an IMS technique, a tissue microarray (TMA) of formalin-fixed paraffin-embedded (FFPE) specimens. We detected a large amount of signals from trypsin-treated FFPE-TMA samples of gastric carcinoma tissues of different histological types. Of the signals detected, 54 were classified as signals specific to cancer with statistically significant differences between adenocarcinomas and normal tissues. We detected a total of 14 of the 54 signals as histological type-specific with the support of statistical analyses. Tandem MS revealed that a signal specific to poorly differentiated cancer tissue corresponded to histone H4. Finally, we verified the IMS-based finding by immunohistochemical analysis of more than 300 specimens spotted on TMAs; the immunoreactivity of histone H4 was remarkably strong in poorly differentiated cancer tissues. Thus, the application of IMS to FFPE-TMA can enable high-throughput analysis in cancer proteomics to aid in the understanding of molecular mechanisms underlying carcinogenesis, invasiveness, metastasis, and prognosis. Further, results obtained from the IMS of FFPE-TMA can be readily confirmed by commonly used immunohistochemical analyses. (*Cancer Sci* 2010; 101: 267–273)

Intensive genome-based surveys are performed on candidate biomarker transcripts relevant to cancer tissues by utilizing the advances in high-throughput microarrays.[1,2] Further, various single-nucleotide polymorphism (SNP) analyses have been performed to further understand cancer.[3,4] Recently, cancer genome resequencing has been increasingly performed to acquire specific genomic data.[5,6] To achieve the systematic understandings of cancer by systems biology, data from genome resequencing and the corresponding data from the transcriptomes should be combined with the individual metabolome and proteome data of the cancer patient.[7]

Several approaches for the investigation of global alterations in proteomics have emerged. Mass spectrometry (MS) is used in combination with 2D electrophoresis or liquid chromatography.[8,9] Further, protein microarrays offer a means of effective identification of cancer-specific protein alterations to researchers.[10]

Despite the existence of techniques for the global detection of cancer-specific alterations at the protein level, proteomic approaches continue to possess two major disadvantages. Under most circumstances, proteomic approaches only allow a limited number of samples to be analyzed in an experiment. Additionally, proteomic techniques are not adopted for the investigation of large amounts of archival specimens that are stored in hospitals and medical institutes. TMAs have been developed for the analysis of a large number of specimens by antibody labeling.[11] It allows high-throughput profiling of the molecular and pathological alterations in tissue specimens.

In recent years, IMS has emerged and developed dramatically in the field of proteomics and metabolomics.[12,13] IMS enables simultaneous analysis of thousands of proteins directly from a tissue sample without protein extraction and usage of target-specific reagents such as antibodies.[14,15]

In this study, we combine the TMA and IMS technique, and introduce a simple and easy-to-use protocol to detect, by a single experimental trial, cancer-specific or histological type-specific proteins. Further, we optimized the IMS procedure for the FFPE samples that are commonly used in hospitals and stored for long time.

Materials and Methods

Specimens. We chose gastric cancer to perform the study for the evaluation of our experimental paradigm. Gastric cancer is the fourth most frequent cancer and the second leading cause of cancer-related death in the world.[16] In Japanese hospitals, large amounts of gastric cancer specimens are stored and are, thus, readily available to perform studies.

Human gastric cancer tissues and adjacent normal tissues were provided by the Diagnostic Pathology Division, Hospital of Hamamatsu University School of Medicine, Shizuoka, Japan. The study was performed in accordance with the guidelines for pathological specimen handling, which was approved by the ethical committee of the Hamamatsu University School of Medicine. Histological classification was based on Japanese Classification of Gastric Carcinoma, 2nd English edition.[17] Further, each tissue did not contain non-tumor tissue, confirmed by two pathologists.

For IMS, we used the old specimens which had been fixed in 10% neutral formalin promptly after surgery and had been stored for up to 2 years in paraffin. Tissue blocks of three cancer tissues and one adjacent normal tissue were cored using tissue microarrayer type KIN (Azumaya, Tokyo, Japan). A cylinder, 3 mm in diameter, was taken and placed into the recipient block. Three cancer tissues and one non-tumor gastric mucosal tissue were aligned as shown in Fig. 1a.

Sample preparation. For analysis, the FFPE tissue microarray blocks were sliced into 10-μm-thick serial sections; further, for hematoxylin–eosin staining, these blocks were sliced into 1-μm-thick sections, using a microtome (Tissue-Tek, Feather Trustome; Sakura Finetek, Tokyo, Japan). The analysis samples were deposited onto indium-tin-oxide (ITO)-coated glass slides

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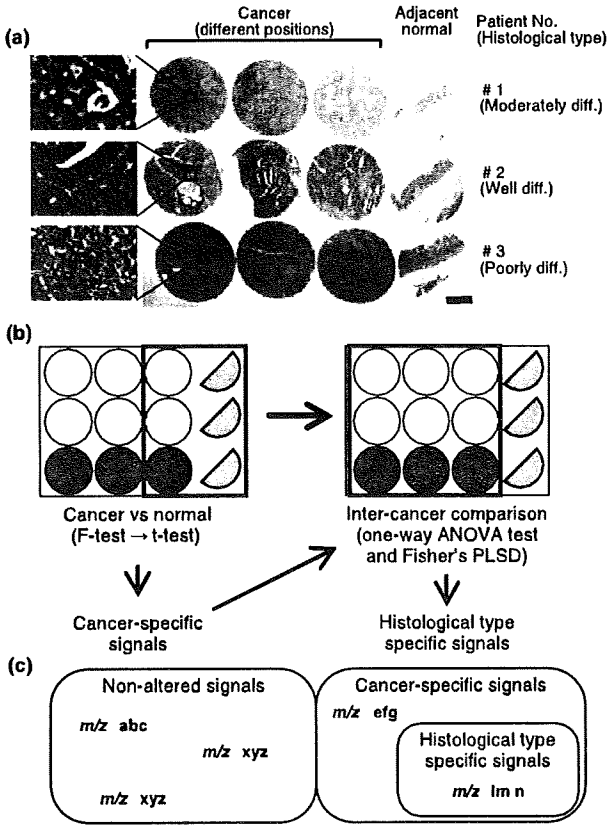

Fig. 1. Experimental paradigm design. (a) Formalin-fixed paraffin-embedded (FFPE) samples were cored with a 3-mm diameter needle and arranged in a line with three cancer tissues and one adjacent normal tissue. The histological type of the cancer of Patient 1 was moderately differentiated adenocarcinoma, that of Patient 2 was well-differentiated adenocarcinoma, and that of Patient 3 was poorly differentiated adenocarcinoma. Hematoxylin–eosin stain, ×10. Scale bar, 1 mm. Enclosed area corresponded to magnified microscopic image. Hematoxylin–eosin stain, ×400 (b) The schema of FFPE samples and the workflow of statistical analysis are shown. (c) The schema that categorizes the acquired signals is presented.

(Bruker Daltonics, Bremen, Germany), and the staining samples were loaded onto regular glass microscope slides by scooping the sections in a 50°C water bath, and then dried on an extender at 45°C. Paraffin was removed by 10-min immersion in xylene at 60°C. Subsequently, the slides were washed by stepwise immersions of 5-min duration each; this involved slide immersion in 100% ethanol twice, and once each in 90% ethanol, 80% ethanol, and 70% ethanol. After rehydration, these slides were incubated in a humid chamber at 55°C overnight.

Tryptic digestion. The sample slide was inserted into a slot on matrix-assisted laser desorption/ionization (MALDI) target plates affixed with conductive tape, and inserted into a chemical inkjet printer (CHIP-1000; Shimadzu, Kyoto, Japan). Trypsin solution was prepared by dissolving 20 μg of trypsin (Sigma, St. Louis, MO, USA) in 200 μL of 20-mM ammonium hydrogen carbonate (NH_4HCO_3). Trypsin microspotting was performed with CHIP-1000 in 5-nL droplets by five cycles of 1000 pl on each spot at a spatial interval of 250 μm. After spotting, MALDI target plates were incubated overnight at 37°C under high-humidity conditions.

Matrix deposition. The matrix solution was prepared by dissolving 50 mg of 2, 5-dihydroxybenzoic acid (DHB; Bruker Daltonics) in 1 mL of 70% methanol/0.1% trifluoroacetic acid. DHB is a widely used matrix for lower molecules including peptides.[18] A thin matrix layer was applied to the surface of the plates using a 0.2-mm nozzle caliber airbrush (Procon Boy FWA Platinum; Mr. Hobby, Tokyo, Japan). The spraying distance was maintained at 15 cm from the tissue surface. The total amount of the matrix solution on each slide was 2–3 mL. The spraying technique enabled full matrix coverage over the entire tissue surface and facilitated co-crystallization of matrix and bio-molecules. A desalting process such as ethanol wash was not performed, since the process does not significantly improve DHB-assisted imaging mass spectrometry (IMS) and NH_4HCO_3 is a highly volatile buffer.[18,19]

Direct analysis of tissue sections by MALDI mass spectrometry. Mass spectra were acquired using the QSTAR XL (Applied Biosystems, Foster City, CA, USA), a hybrid quadrupole/time-of-flight mass spectrometer equipped with an orthogonal MALDI source and a pulsed YAG laser that was operated at a repetition rate of 100 Hz, and a power modulator. Spectra were acquired in positive ion mode. Spectra were acquired in the range of m/z 500–2000. Representative mass spectra were acquired using random laser irradiation-sections. The number of laser shots was 150. An alignment of the mass spectra was performed to compare the datasets using SpecAlign software (http://physchem.ox.ac.uk/~jwong/specalign/). The peak intensity value of the spectra was normalized by dividing them with the total ion current (TIC) as previously described.[20,21]

Imaging of tissue section by mass spectrometry. IMS was performed using orthogonal MALDI (oMALDI) server software by defining a region of interest around the tissue slice. The mechanical resolution, which is the value that refers to the length of the stepwise movement of the laser beam on the sample stage, was 300 μm × 300 μm, and the accumulation time per spot was about 2 s. The acquired mass spectra were visualized using BioMap software (http://www.maldi-msi.org). Molecular images were captured using this software by applying baseline correction to the spectra and integrating these spectra over the peak of interest. Alignment of these mass spectra was performed using SpecAlign software.

Tissue protein identification. The quadrupole ion trap time-of-flight mass spectrometer, namely, AXIMA-QIT (Shimadzu), was used to perform MS/MS analysis. In the MS/MS operation, the data acquisition conditions (i.e. the laser power, collision energy, and the number of laser irradiations) were adjusted to obtain good-quality mass spectra with high intensity and signal-to-noise ratios (S/N) in the fragmented peaks. MS/MS spectra were processed using the Mascot search engine (http://www.matrixscience.com) using the National Center for Biotechnology Information (NCBI)/basic local alignment search tool (BLAST) protein database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with a taxonomy filter for humans, and the peptide and MS/MS tolerance at 0.3 Da. The search criteria was allowed to consider up to one missed cleavage and variable modifications including protein N-terminus acetylation, histidine/tryptophan oxidation, and methionine oxidation.

Statistical analysis. All statistical analyses were performed with StatView software version 5.0 (SAS Institute, Cary, NC, USA). First, statistical analyses were performed on adjoining cancer and normal tissue. The Student's t-test ($\alpha = 0.05$) was performed between peak intensity means of cancer and normal tissue samples on the basis of equal variance. Welch's test for unequal variance ($\alpha = 0.05$) was performed between peak intensity means of cancer and normal tissues. The corresponding P-value, i.e. P (T t), was reported as a measure of significant statistical variability between conditions.

We extracted signals that showed significantly higher intensity in cancer than in normal tissue in the form of cancer-specific

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peaks. To determine specific peaks related to the degree of differentiation, analysis of variance (ANOVA) and Fisher's protected least significant difference (PLSD) were performed as post-hoc tests on three histological types of three different cancer tissue samples. Fig. 1b shows the statistical workflow.

Immunohistochemical staining for histone H4. For immunohistochemistry (IHC), we used the formalin-fixed tissue microarray specimens containing a wide range of preservation time, up to 30 years after being embedded in paraffin. Each TMA was composed of 50 primary gastric tumors such as adenocarcinoma of various grades of differentiation. Three micrometer sections were cut from the TMA blocks. Immunostaining was performed by the Dako Autostainer System (Dako Japan, Tokyo, Japan) according to the manufacturer-recommended procedure. In brief, paraffin was removed by immersing the TMA slides in xylene for 5 min twice. Subsequently, the tissue sections were rehydrated by immersing the slides in 100% ethanol for 10 min twice, followed by two-time 10-min immersion in 95% ethanol. After rehydration, these slides were incubated at 96°C for 40 min in 10 mM sodium citrate buffer pH 6.0 and then cooled on the bench top for 20 min. Then, these sections were incubated in 3% hydrogen peroxide for 5 min and washed in Tris-buffered saline. A monoclonal antibody against histone H4 (L64C1; Cell Signaling Technology, Danvers, MA, USA) was used as the primary antibody at a dilution of 1:300. N-Histofine® Simple Stain MAX-PO (Multi) (Nichirei Biosciences, Tokyo, Japan) was used as the secondary antibody. After removing the secondary antibody, the sections were exposed to diaminobenzidine for 5 min, and then washed with distilled water. Counterstaining was performed with hematoxylin for 10 s.

IHC evaluation. The IHC evaluation was carried out in two independent ways by two of the authors (Y.M. and H.S.). An evaluation was performed by visual inspection, where IHC staining was classified into four ranks (0, negative; 1, slightly positive; 2, positive; 3, strongly positive) for common types of gastric carcinoma. Papillary adenocarcinoma was interpreted as well-differentiated adenocarcinoma and signet-ring cell carcinoma as poorly differentiated adenocarcinoma. Assignment of mucinous carcinoma category was made according to the other predominant elements. Special types of gastric carcinoma and other tumors were excluded. In total, 169 specimens were evaluated; they included 42 well-differentiated, 38 moderately differentiated, and 89 poorly differentiated adenocarcinomas. As described,[22] Steel–Dwass' test was performed by using free software available on a web site, MEPHAS (http://www.gen-info.osaka-u.ac.jp/testdocs/tomocom/). Another evaluation was carried out by quantifying the signal intensity of IHC staining with Scion image software version 4.0.3.2 (Scion, Frederick, MD, USA). We analyzed additional 170 specimens containing 43 well-differentiated, 40 moderately differentiated, and 87 poorly differentiated adenocarcinomas. The data were represented as the mean value of intensity ± SD. ANOVA and Fisher's PSLD were performed as post-hoc test among three histological types (well, moderately, and poorly differentiated).

Results

Experimental paradigm design. To statistically detect cancer-specific signals, we placed three cancer tissues and one normal tissue from three patients in a TMA. The histological type of cancer differed among the three patients (Fig. 1a). The sample from Patient 1 was moderately differentiated adenocarcinoma, that from Patient 2 was well-differentiated adenocarcinoma, and that from Patient 3 was poorly differentiated adenocarcinoma. We acquired tissue samples from three different regions of each patient for further statistical analysis to detect signals specific to cancer-differentiation status (Fig. 1a). During the first screening, we compared signals of three individual cancers with those of normal tissues (Fig. 1b). Thereafter, we screened the detected cancer-specific peaks by multiple comparisons of three different cancer regions belonging to three histological types (Fig. 1b). We categorize the obtained results on the basis of the statistical workflow in Fig. 1c.

Acquisition of mass spectra from FFPE-TMA samples. A previous report showed relatively weak signal intensities and low S/N ratio was obtained with FFPE tissue samples compared to freshly frozen ones.[23] We first examined if peptide signals could be sufficiently detected with FFPE-TMA. We employed chemical inkjet technology to equalize the quantity and the interval of trypsin solution application.[14] We detected vast quantities of signals that were sufficient to generate imaging data from FFPE-TMA samples. Figure 2 shows representative spectra obtained from three individual cancer tissues and normal tissues. The peaks obtained were mainly concentrated below m/z 2000 and could hardly be detected over m/z 2000. Thus, using SpecAlign, we performed signal-intensity normalization.

Detection of cancer specifically increased signals in IMS of digested FFPE tissue microarray. Subsequently, we performed IMS of FFPE-TMA on the samples and obtained mass spectra. We setup a spatial interval of 300 μm to prevent repeated laser irradiation, as the irradiated laser diameter was 200 μm. We completed the scanning of the TMA samples with 12 spots in approximately 1 h. We detected a total of 72 signals with FFPE-TMA samples. Fig. S1 shows the obtained array images. To perform statistical data analysis, we quantified the signal intensities of m/z peaks. The first statistical screening (Fig. 1b) revealed 54 signals, the intensity of which was detected to be significantly increased in cancer tissues (Fig. 3). We examined the reliability of this screening by performing two independent trials with sibling arrays. Forty of the 54 signals were detected in the two independent trials (Fig. 3). Figure 4(a) shows the representative array result of the signal significantly increased in cancer. In contrast, Fig. 4b shows that another signal has no significant difference between cancer and normal tissues, i.e. it shows an even distribution pattern.

Detection of histological type-specific increased signals in IMS of digested FFPE tissue microarray. We further analyzed the quantified signal intensities to examine whether such histological type-specific signals could be detected with our experimental paradigm. To this end, we compared signals detected in cancer tissues among the well-differentiated, moderately differentiated, and poorly differentiated tissues (Fig. 1b). To detect specific signals, we conducted statistical analyses with one-way ANOVA followed by Fisher's PLSD. Certain signals demonstrating a cancer-specific pattern appeared to demonstrate uneven signal intensities in different degrees of cancer differentiation (Fig. 3, Fig. 5a–c). Of the detected signals, peaks having m/z 537.2, 1168.4, 1387.6, 1475.8 were reproducibly detected in another experimental trial. Other signals were detected only once in two experimental trials. Owing to the two-step screening, it is probable that the detection of histological type-specific signals shows worse reproducibility than the simple detection of cancer-specific signals.

Identification of protein-specific increase in poorly differentiated cancer tissues by MS/MS analysis. We attempted to identify the signals that were specifically detected in poorly differentiated cancer tissue. We performed MS/MS analyses on the FFPE-TMA, and analyzed the resultant data with the Mascot search engine. We identified one signal with an m/z 1325.6 as histone H4 that is specific to poorly differentiated cancers (Fig. 6a), and identified a protein that demonstrated non-specific expression and had an m/z 976.4 corresponding to that of actin (Fig. 6b). Other peaks could not be detected due to their weak intensity.

IHC staining for histone H4 using another TMA specimen of larger numbers of the cases. Finally, we examined whether

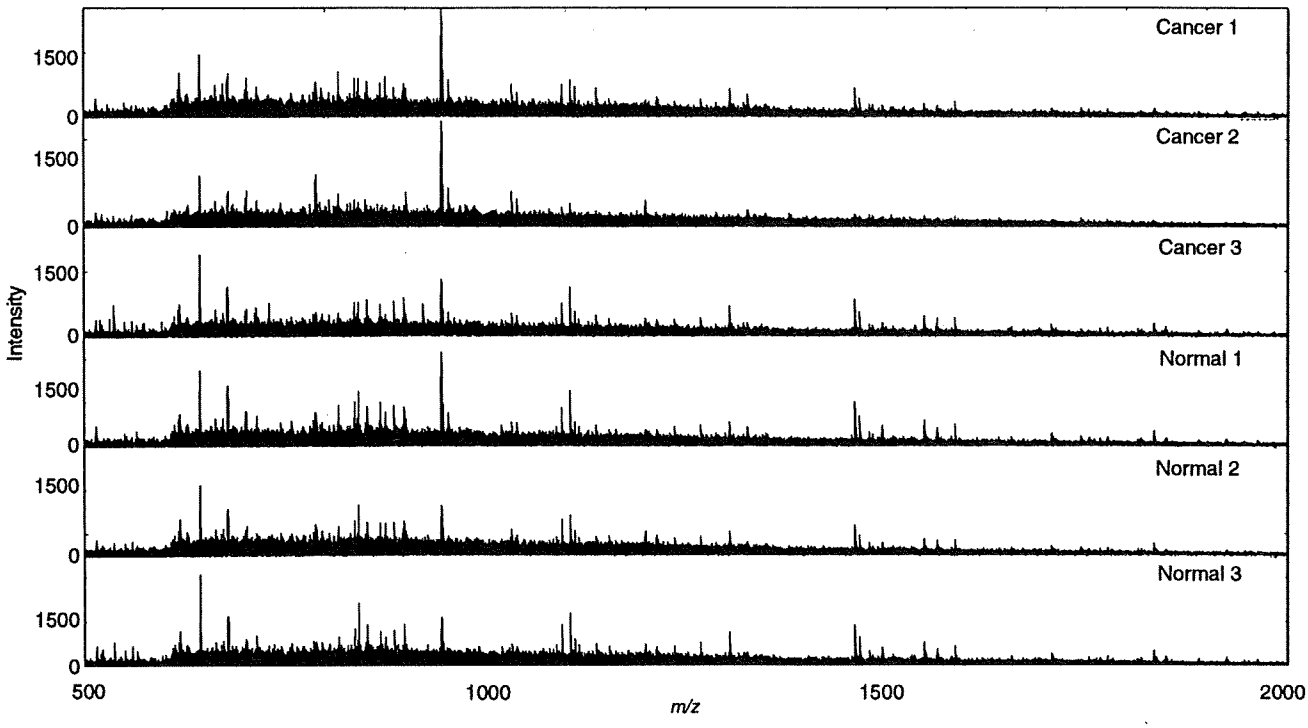

Fig. 2. Acquired mass spectra from formalin-fixed paraffin-embedded (FFPE) tissue microarray samples (TMA) by random laser irradiation. Acquired mass spectra from each TMA spot of adjoining cancer and normal tissue are shown as representative spectra. *m/z* refers to mass per charge ratio.

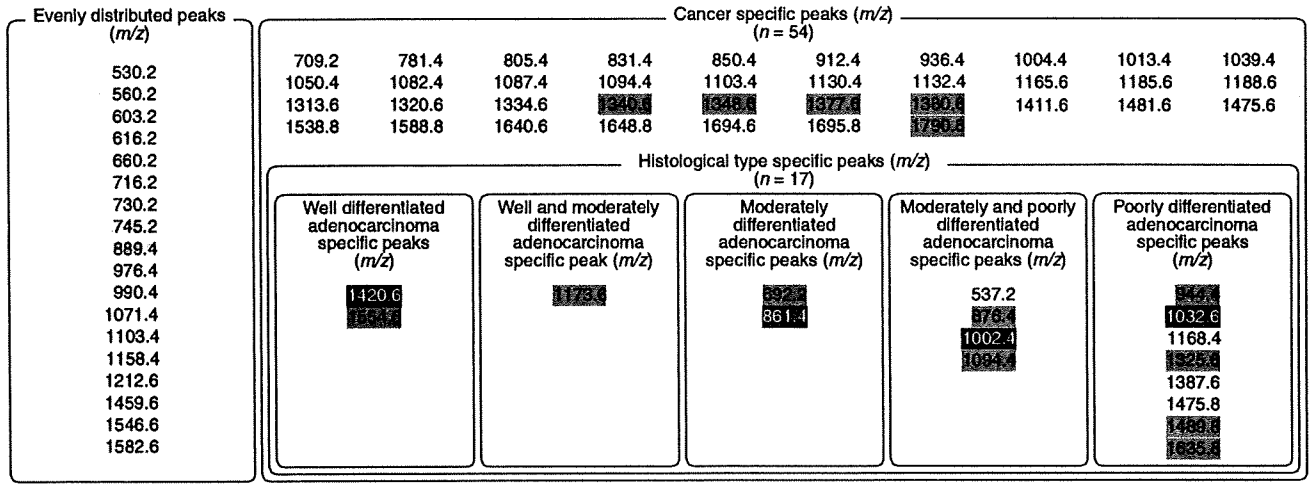

Fig. 3. The peak list acquired from mass spectra. The shaded values represent signals that showed significantly increased intensity in cancer tissues in one independent trial ($P < 0.05$). The white-on-black values represent signals that showed significant difference among three cancers in one independent trial ($P < 0.05$).

histone H4 was specifically strongly detected in poorly differentiated cancers. In total, we stained 400 gastric tumors including adenocarcinoma, squamous carcinoma, neuroendocrine carcinoma, metastatic carcinoma, malignant lymphoma, and adenoma. We excluded 61 specimens such as a special type of gastric carcinoma, malignant lymphoma, and benign lesion. We examined 339 gastric carcinomas composed of 85 well-differentiated carcinomas, 78 moderately differentiated carcinomas, and 176 poorly differentiated carcinomas. Figure 7(a) shows representative photomicrographs of each histological type. We evaluated the result of IHC in two approaches. First, we determined the staining appearance according to four ranks (0, negative; 1, slightly positive; 2, positive; 3, strongly positive). Slightly positive and positive staining reached a high rate in well- and moderately differentiated carcinoma. In contrast, poorly differentiated adenocarcinomas were categorized into much more positive staining such as rank 2 or rank 3 (Fig. 7b). Second, we also performed more quantitative analysis. We

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